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Application of probiotic bacteria for the improvement of sea bream (*Sparus aurata*) larval production

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ABSTRACT

The main object of this study was to evaluate the effect of two probiotic bacterial strains *Bacillus* sp. R2 and *Planococcus* sp. R11 and their mixture (1:1) on live food cultures (rotifers and *Artemia*) and study its effect on gilthead sea bream larvae until 40 day after hatching (DAH). Probiotics were supplemented in the larval rearing system in triplicates in different strategies, which starting the exogenous feeding, by introducing to live food through bio-encapsulation or in tank water or both. There was significance difference ($P<0.05$) in the survival and growth of *S.aurata* larvae that had probiotic achieved compared to control. Meanwhile total proteins, total lipids, fatty acid profiles were determined for live food during encapsulation and for the produced larvae after feeding. The results showed increasingly significant difference ($P<0.05$) towards R11 probiotic treatments especially in bioencapsulation among all other treatments against control. Protease activity showed significant increase ($P<0.05$) in sea bream enriched with *Planococcus* sp. R11 led to the highest value (8.95 ± 0.21 U/mg) of enzyme activity in comparison to control (1.3±0.03 U/mg). Otherwise, the digestive system histology of larvae supplemented with bacteria in the period from 3-20 DAH showed improving number of goblet cells (37± 7.5), as well as number and length of villi (22±2.5and 35±9µm, respectively). Similarly,in the second duration from 20-40 DAH the number of goblet cells (96±14.4)was also increased, as well as the number and length of villi (27±2.5 and 126 ±25 µm, respectively($P<0.05$)

INTRODUCTION

Aquaculture is the fastest growing food-producing sector in the world with the greatest potential to meet the growing demand for aquatic food (**Michael *et al*., 2014**). The unpredictable mortalities hamper the growth of aquaculture industry and many of which are caused by pathogenic microorganisms.

Egyptian aquaculture sector has significant and rapid development over the past four decades and ranks number ten worldwide and number two in tilapia production behind china and provides almost 79 percent of the country's fish needs (**Wally, 2016**).

Probiotic successes in human and animal feeding practice (**Giannenas et al., 2015**) and recently gained attention in aquaculture . Probiotics are microbial cells provided via the diet or rearing water that benefits the host fish (**Cerezuela et al., 2012**) and has beneficial effects (**Ramos et al., 2017)** in diseases control and competes with various environmental stressors as well as to promote the growth of the cultured organisms and manipulate the non-specific innate immunity among fishes, hence help them into resist many pathogenic agents and are actively used worldwide(**Ibrahem, 2015**). Probiotics can alter the intestinal morphology towards a more tolerant and less inflammatory mucosa (**Lazado & Caipang, 2014**) and provide nutrients, digestive enzymes (**Ray et al., 2012**) and enhance the absorptive surface area through longer villi (**Merrifield et al., 2010**) and denser microvilli (**Standen et al., 2016**).

Gilthead sea bream (*Sparus aurata*) is one of the most valuable cultured species in Mediterranean countries (**Suzer et al., 2008**). Its Latin name comes from the characteristic golden band between its eyes, live in marine waters as well as in the brackish waters of coastal lagoons. gilthead sea bream is one of the main components of Egyptian mariculture (**Rothius et al., 2013**). So,the aim of this work was to evaluate effect of two probiotic bacterial strains *Bacillus* sp. R2 and *Planococcus* sp. R11 and their mixture (1:1) on live food cultures (rotifer and *Artemia*) and study their effect on gilthead sea bream larvae until 40 day after hatching (DAH)

MATERIALS AND METHODS

Bacterial strains

Bacillus sp. R2 and *Planococcus* sp. R11, previously isolated and identified from gastrointestinal tract of sea bream (*Sparus aurata*), were used as potential probiotics. The two strains were preserved at -20°C in Luria-Bertani broth(LB; Difco) with 15% glycerol, prior to use.

Preparation of probiotic cultures

Bacillus sp. R2 and *Planococcus* sp. R11 selected as probiotic were cultured in nutrient broth prepared in sea water with salinity 28 ppt at 35°C for 48 h. The fresh cultures were harvested by centrifugation at 5000 rpm for 15 min, the supernatants were discarded and the pellets were washed twice in phosphate buffer saline pH 7.4 and finally suspended in PBS for application with density of $1x10^7$CFU/mL.

The time course study began with four applications (each in triplicate) and extended for 40 days after hatching (DAH) on sea bream larvae. The two examined probiotic strains *Bacillus* sp. R2 and *Planococcus* sp. R11 were used either directly to the rearing tank water, or enriched via live food , separately or mixed (1:1) at a final concentration of 1×10^7 CFU/mL .

Bio-encapsulation of live feed (Arığ *et al*., 2013)

Rotifer (*Brachionus plicatilis*)

Brachionus plicatilis were collected in 1500mL conical flask containing 1000 mL of sterile sea water (25 ppt) at a concentration of 800 ind/mL and incubated for 24h in cultures of probiotic bacteria at 1×10^7/mL. Rotifers were rinsed in sterile sea water (25ppt) in 40µm pore size net then used at ratio 10-15 ind/mL.

Brine shrimp (*Artemia salina*)

Artemia were collected after 24 h of hatching in 2500mL conical flask containing 2000 mL of sterilized sea water (28 ppt) at a concentration of 100 ind/mL and were incubated for 24 h in cultures of probiotic bacteria at 1×10^7/mL. *Artemia* were rinsed in sterile sea water (28ppt) in 100µm pore size net then fed at ratio 2-4 ind/mL.

Spawning and larval rearing

Adult and fully ripe *Sparus aurata* (5 males and 7 females with total length of 26 to 30 cm and total weight of 640 to 860 g were collected from Damietta Governrate in Egypt (E1-Ratoma fish farm, salinity 34 ppt). The fish were acclimatized for ten days in cylindrical tanks capacity 3 tonnes of sea water. The fish were fed with small live fish and crab about 2 to 4% of the fish weight twice daily at salinity 38 ± 2 ppt and temperature17 to 22°C. After twelve days the fish spawned naturally. Floating fertilized ova were collected in incubation tank till hatching and then Larviculture after acclimatization was conducted in green water recirculated system in triplicates. Larvae were stocked in 20 ind. l^{-1} densities at 150 L capacity cylindrical tanks .

Larval rearing parameters and protocols were carried out according to **Suzer *et al*., (2008).** The water in the tank was static during the first 2 days of the rearing period. From day 3 to 12, the tank water was partially replaced (25–75% daily) by draining through a 200 µm mesh size. The pH range was 8.3 ± 2, ammonia and nitrite did not exceed 0.012 mg^{-1}. Illumination was about 800 lux for 24 h, very gentle aeration was conducted.

Larval feeding regime

The feeding regime for *Sparus aurata* larvae started with mouth opening, newly hatched larvae (3 to 20 DAH) using *Brachionus plicatilis* at density 10–15 ind/mL (for sufficiently feeding of whole larvae) previously enriched with bacterial probiotics and green- water containing *Nannochloropsis* sp at a density of $1.5–2x10^5$ cells/mL. Between 10 and 30 DAH, *Artemia* nauplii (AF 480 INVE Aquaculture, Ghent, Belgium) was introduced at 4–7 ind/mL densities followed by *Artemia* metanauplii (EG, *Artemia* Systems SA, Ghent, Belgium) from 25 DAH until day 40 at 2–4 ind/mL.Both *Artemia* species were fed with probiotic bacteria and *Nannochloropsis* sp.

Growth of larvae

Density of rotifers was calculated per mL in control samples and in those fed with probiotic bacteria. Larvae were collected from each group and each tank by 7 days

interval until 40 DAH to measure growth expressed in total length, width and weight and at the end of experiment, specific growth rate was calculated by formula:

$$\mathbf{SGR_{(specific\ growth\ rate)} = 100\ (LnFBW\text{-}LnIBW)/\Delta T}$$

Where: IBW, FBW are initial and final body weight of fish (mg), and ΔT time interval (day)

Survival of larvae: at the end of experiments, larvae were directly counted and the percent of survival was determined using the following formula:

$$\text{Survival } \% = \frac{\text{Total number of survived larvae}}{\text{initial number of stocked larvae}} \text{x}100$$

To estimate total protein, total lipids and fatty acids profile, the larvae of sea bream were rinsed for a few seconds in fresh water, and transferred to individually pre-weighted vials and dried for a minimum of 48 h at 60 °C before weight determination.

Total protein

Total protein was extracted from samples of rotifer, *Artemia* and larval homogenate using 0.5 N NaOH according to the method described by **Rauch, (1981).** In a test tube 3-mL of 0.5 N NaOH were added to larval homogenate, shacked well and extracted at 80˚C in a water bath for 10 min with occasional stirring. The extract was quickly cooled at room temperature (using running cold water), centrifuged at 5000 rpm for 20 min and the supernatant was transferred to a graduated cylinder. Extraction was repeated two times with 0.5 N NaOH for 10 min at 80˚C. The combined supernatants were used for protein measurement.

Protein content was determined by the assay of **Bradford**, **(1976)** using bovine serum albumin as the standard. This was done by preparing a series of standard dilutions with 0.15 M NaCl to final concentrations of albumin (0; 0.2; 0.5 and 1 mg/mL) then 100 µL of each of the above dilutions were added to 5.0 mL of Coomassie BlueG-250 and mixed by vortex and absorbance at 595 nm was measured. Larval extract was treated at the same manner. Protein content was then calculated as mg/mL.

Total lipids

The lipids were extracted by the method of **Bligh & Dyer (1959)** and lipid content was determined gravimetrically as follows: the lipids of rotifer, *Artemia* and larval homogenate were extracted by the addition of 50 mL of chloroform and methanol mixture 2:1 (v/v) in a separating funnel. After setting, two layers were formed, the aqueous layer was discarded and the other layer (containing lipid) was washed with 0.4% $MgCl_2$ solution (100mL) followed by distilled water (100 mL) for several times to get rid of all the excess of $MgCl_2$. The chloroform layer containing the lipids was separated from the aqueous layer and dried over anhydrous Na_2SO_4 to get rid of water droplets then filtration took place. The obtained filtrate was evaporated using a rotary evaporator. Residue obtained represented the total lipids.

Preparation of fatty acids methyl ester (FAME)

The method adopted by **Radwan (1978)** was carried out**.** A sample of total lipids was transferred into screw cap vial, then 2mL benzene and 10mL 1% sulphuric acid in

absolute methanol were added. The vial was covered under a stream of nitrogen before heating in an oven at 90°C for 90min. After cooling the vial to room temperature, 10mL of distilled water were added to the cooled vial and the methyl ester in each vial was extracted with 5mL of petroleum ether for three times. The three petroleum ether extracts were combined and concentrated to a minimum volume using a stream of nitrogen then the fatty acid methyl esters were identified using gas liquid chromatography.

Determination of protease activity

Digestive tract of larvae administrated with bioencapsulated live feed with *Planococcus* sp. R11 and digestive tract of untreated larvae at 40(DAH) were homogenized in cold 50mM Tris HCl buffer pH 7.5 followed by centrifugation at 13.500 xg for 30 min at 4°C and the supernatant was used as enzyme preparation. Total protease activity was measured according to the method of **Walter (1984)** using casein as substrate. The enzymatic reaction mixtures consisted of 100 µL of enzymatic extract and 250 µL of 1% casein in 0.1M Tris-HCl buffer (pH 9) using 0.01 M $CaCl_2$,, incubated at 37°C for 10 minutes and stopped by adding 600 µL of 8% (w/v) trichloroacetic acid. The supernatant solution was read at 280 nm. One unit of total protease activity was equivalent to the amount of enzyme required to release 1µg of tyrosine/mL/min under standard assay conditions.

$$\text{Enzyme activity } (\text{Uml}^{-1}) = \frac{[\Delta\text{abs} \times \text{reaction final volume (mL)}]}{[\text{MEC} * \text{time (min)} * \text{extract volume (mL)}]}$$

Specific activity (Unit mg $prot^{-1}$) = Total activity/ soluble protein (mg)

where Δabs represents increase in absorbance, and MEC represents the molar extinction coefficient of tyrosine (0.005 mL/ µg/ cm).

Histological characteristics of digestive tract

A pool of three larvae from tank of larvea administrated with bioencapsulated live feed with *Planococcus* sp. R11 and tank of untreated larvae were collected at 20 (DAH) and 40 (DAH), the larvae were fixed in 10% neutral buffered formalin for histological analysis then dehydrated by passing through graded series of ethyl alcohol (70%, 80%, 90% and 100%) for clearing then embedded in paraffin wax and sectioned at 6-7µm after removing wax with xylene and hydration in ethanol series , sections were stained with hematoxylin and eosin (**Dimitroglou *et al.*, 2011**) and examined microscopically with Leica light microscope . The impacts of probiotic enrichment were monitored by light microscope in terms of Villus height (Vh, µm, measured from the tip to the base of villus), (number of villi and at 10 villi average number of goblet cells per villus was determined and the results were expressed as mean value.

Statistical analysis

Statistical tests were performed using IBM SPSS statistics 22. Data were represented as mean ± standard deviation of three replicates. Data were analyzed using overall one-way analysis of variance (ANOVA) and when differences observed were significant at $P \leq$

0.05, the means were compared by LSD test while Two-way Anova was used to compare groups and sub-groups and (P<0.05) was established as the level of significance.

RESULTS

Rotifers density

In this study rotifers density was calculated after 6 days of supplementation with either *Bacillus* sp.R2 or *Planococcus* sp. R11. Density of rotifers was significantly increase (P< 0.05) when enriched with *Planococcus* sp.R11($11^{b}\times 10^{2}\pm$ 1.5 rotifers/mL) and *Bacillus* sp.R2 ($6^{a}\times 10^{2}$ ±1.5 rotifers/mL) comparing to control ($4.35^{a}\times 10^{2}$ ± 0.55 rotifers/mL) as depicted in **Fig.1**.

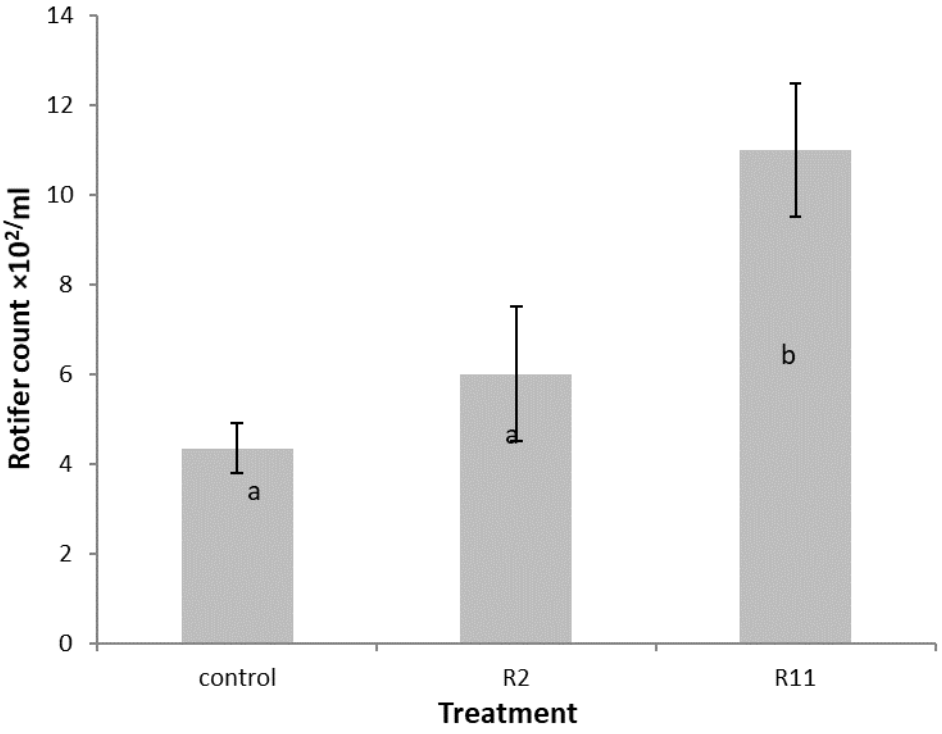

Fig.1 Rotifer count fed with *Bacillus* sp. R2 or *Planococcus* sp. R11 compared to control. Bars marked with different superscripts differ significantly with respect to each other (P<0.05).

Total protein

As shown in **Fig.2**, a significant increase in total protein (P <0.05) was observed in rotifers enrichment with *Planococcus* sp. R11 ($61.21^{c}\pm$ 1.066 mg/mL) followed by *Bacillus* sp. R2 (51.42^{b}±1.24 mg/mL) comparing to control (38.23^{a}±1.12 mg/mL).

With respect to *Artemia*, significant differences at P<0.05 in the total protein of treated *Artemia*. As in **Fig.3**, a higher value of total protein was recorded in *Artemia*

enriched with *Planococcus* sp. R11 (71.17^{c}±1.04mg/g) followed by those fed with *Bacillus* sp. R2 (49.64^{b}±1.52 mg/g) comparing to control (44.3^{a}±1.47 mg/g).

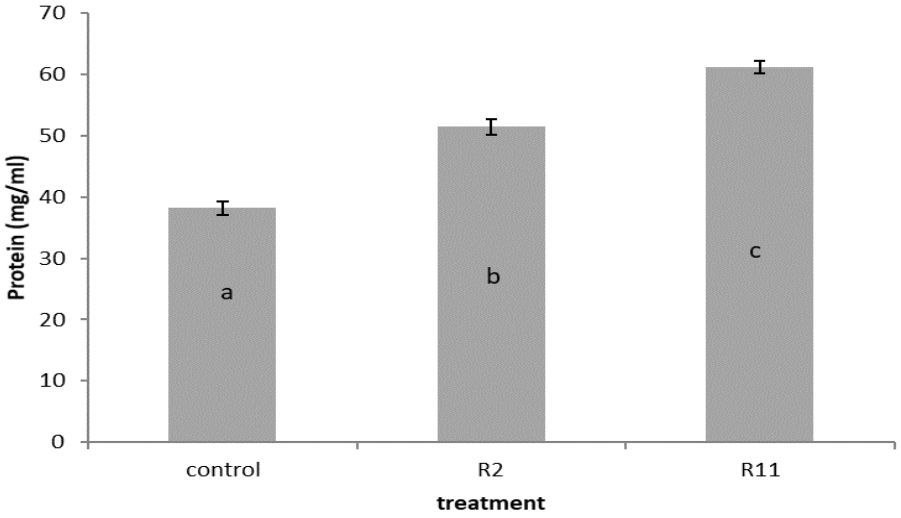

Fig.2 Rotifers' total protein (mg/mL) encapsulated with *Bacillus* sp. R2 or *Planococcus* sp. R11 compared to control. Bars expressed as mean± SD and marked with superscripts are significantly different with respect to each other (P<0.05).

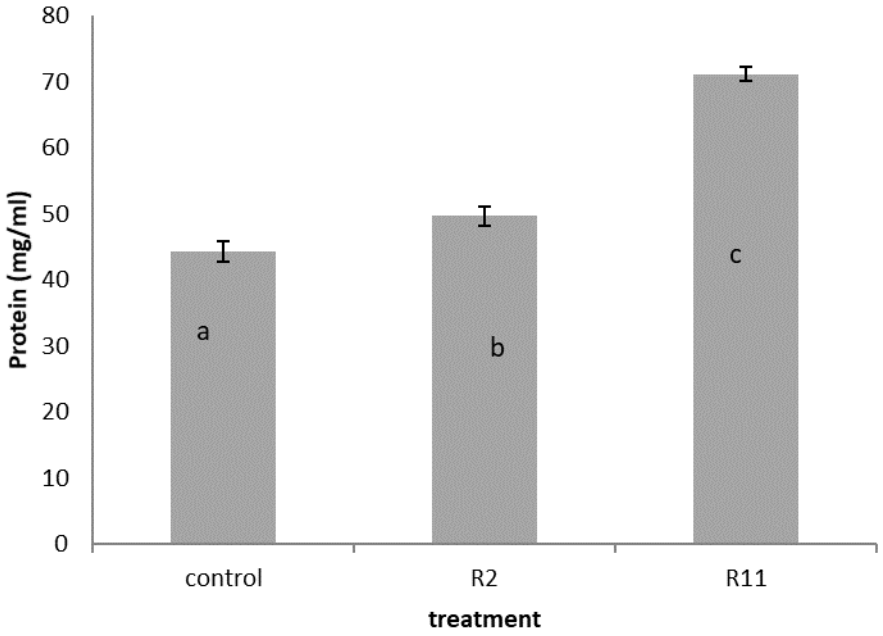

Fig.3 *Artemia* total protein (mg/mL) encapsulated with *Bacillus* sp. R2 and *Planococcus* sp. R11. Bars expressed as mean ± SD and marked with superscripts are significantly different with respect to each other (P<0.05).

Fatty acid profile

Most of the studies on the dietary effect of manipulation on growth and pigmentation had been focused on ω3 PUFA, probably due to their predominance in marine fish. Other studies had indicated the importance of ω6 PUFA on pigmentation, in particular ARA which is an essential fatty acid (EFA) for marine fish. eicosapentaenoic acid (EPA) 20: 5ω3, docosahexaenoic acid (DHA) 22: 6*ω*3 and arachidonic acid (ARA) 20: 4ω6 are effective for good growth and survival of *S. aurata* larvae

Composition of the fatty acids in untreated rotifer and treated rotifer with *Bacillus* sp. R2, *Planococcus* sp. R11and mixed of them is represented in **Table 1** the results showed that rotifers treated with *Planococcus* sp. R11 recorded higher total fatty acids (34.20 mg. g^{-1}). The higher production of total fatty acids due to rotifer treated with *Planococcus* sp. R11 has high percentage of PUFAs-ω3 (17.7%) and PUFAs-ω6 (5.6%) then rotifer treated with mixed has PUFAs-ω3 (14.24.0%) and PUFAs-ω6 (4.06%) and rotifer treated with *Bacillus* sp. R2 has PUFAs-ω3 (12.69%) and PUFAs-ω6 was (3.6%) while untreated rotifer has PUFAs-ω3 (11.2) and PUFAs-ω6 (3.02).

Fatty acids profile in untreated *Artemia* and treated *Artemia* with *Bacillus* sp. R2, *Planococcus* sp. R11 and mixture of them is represented in **Table 2** the results showed that *Artemia* treated with *Planococcus* sp. R11 recorded higher total fatty acids (26.05mg. g^{-1}). The higher production of total fatty acids due to *Artemia* treated with *Planococcus* sp. R11 has high percentage of PUFAs-ω3 (19.49%) and PUFAs-ω6 (5.6%) then *Artemia* treated with mixed of *Bacillus* sp. R2 and *Planococcus* sp. R11 has PUFAs-ω3 (16.54%) and PUFAs-ω6was (5.36). and *Artemia* treated with *Bacillus* sp. R2 has PUFAs-ω3 (12.6%) and PUFAs-ω6 was (4.8) while untreated *Artemia* has PUFAs-ω3 (13.6%) and PUFAs-ω6 was (4.9).

Table 1 Rotifer fatty acids profile (% of the total fatty acids) enriched with probionts compared with control

Fatty Acid	Untreated Rotifer	*Bacillus* sp. R2	*Planococcus* sp. R11	Probiont Mixture
Saturated fatty acid				
C6: 0	0.8	0.4	0.5	0.4
C8: 0	1.1	0.3	0.7	0.5
C12: 0	6.4	5.9	4.2	4.4
C13: 0	10.1	10.2	9.4	8.0
C14: 0	11.7	11.8	7.1	9.3
C15: 0	9.2	6.5	7.4	7.2
C16: 0	11.6	10.0	10.7	11.18
C17: 0	1.3	1.1	0.9	1.47
C18: 0	6.8	5.1	4.9	6.50
C20: 0	1.1	1.8	1.60	2.41
Monounsaturated FA				
C14: 1	6.3	6.3	5.4	6.60
C15: 1	9.47	9.5	9.0	8.70
C16: 1	2.1	1.7	2.0	2.08
C17: 1	0.0	1.0	1.0	0.83
C18: 1ω9c	5.5	9.5	9.9	10.32
Polyunsaturated FA				
C18: 2ω6	0.52	1.0	1.6	1.00
C18: 3ω3	0.61	1.2	4.1	1.35
C20: 4ω6	2.5	2.71	4.0	3.06
C20: 5ω3	3.4	3.49	5.0	4.09
C22: 2	2.3	2.5	2.0	2.0
C22: 6ω3	7.2	8.0	11.3	8.8
SFA	60.1	53.1	47.40	51.36
UFA	39.9	46.9	52.60	48.54
MUFA	23.37	28.0	27.3	28.53
PUFA	16.53	18.9	25.3	20.01
PUFAs-ω3	11.21	12.69	17.7	14.24
PUFAs-ω6	3.02	3.71	5.6	4.06
Total fatty acids (mg/g)	28.11	28.44	34.20	30.51

Table 2 . *Artemia* fatty acids profile (% of the total fatty acids) enriched with probionts compared with control

Fatty Acid	Untreated *Artemia*	*Bacillus* sp. R2	*Planococcus* sp. R11	Probionts mixture
Saturated fatty acid				
C8: 0	0.3	0.3	0.5	0.4
C10: 0	0.3	0.5	0.6	0.5
C12: 0	3.6	3.3	3.6	3.0
C13: 0	8.3	9.1	9.4	8.2
C14: 0	10.9	10.6	9.8	1.71
C15: 0	6.7	6.4	3.3	0.73
C16: 0	11.6	11.9	14.9	14.55
C17: 0	1.5	1.3	1.0	1.07
C18: 0	9.2	8.7	9.1	8.43
C20: 0	1.9	1.0	0.6	0.37
Monounsaturated FA				
C14: 1	8.5	8.3	7.0	7.42
C15: 1	1.5	8.3	0.4	0.5
C16: 1	2.9	1.5	1.6	2.42
C17: 1	2.8	1.8	2.1	2.92
C18: 1ω9c	6.6	9.1	13.0	13.34
C22: 1	1.3	0.71	0.93	0.85
Polyunsaturated FA				
C18: 2ω6	2.6	2.4	2.4	2.30
C18: 3ω3	3.1	1.20	2.3	3.2
C20: 4ω6	2.3	2.4	3.2	3.06
C20: 5ω3	2.6	3.40	5.19	5.14
C22: 2	3.7	3.3	3.5	3.5
C22: 6ω3	7.9	8.0	12.00	8.20
SFA	54.3	53.1	46.50	48.95
UFA	45.7	47.9	53.50	51.05
MUFA	23.6	27.2	25.03	26.65
PUFA	22.2	20.7	28.47	25.40
PUFAs-ω3	13.6	12.6	19.49	16.54
PUFAs-ω6	4.9	4.8	5.6	5.36
Total fatty acids (mg/g)	7.461	17.56	26.05	20.76

***In vivo* assays of probiotic bacteria candidates through feeding trial**

Effect of probiotic on larvae growth:

The two bacterial isolates *Bacillus* sp.R2 and *Planococcus* sp.R11 show potential probiotic effect by producing better Larval morphometric over untreated larval group as **.**

the growth parameters representing in total length (mm), weight (mg) and specific growth rate (% day^{-1}) were significantly increase ($p<0.05$) in probiotic administrated groups over untreated group of larvae.

As shown in **Figs. 4 and 5,** the best results on total length development and weight of larvaewere determined in treatment, Bioencapulation of *Planococcus* sp. R11 as 66.27±1.6 mg and 17.3^{ce}±1.15 mm, bioencapsulation of *Bacillus* sp. R2 as 57.87±2.57 mg and 16.3^{be}±1.52 mm as then treatment of mixture of *Bacillus* sp. R2 and *Planococcus* sp. R11 in bioencapsulation and rearing as 52.77±2.35 mg and 15.7^{cde}±0.57 mm comparing to control as 37.6±2.51 mg and 14.3^{ad}±1.52 mm.

There are significant difference ($P<0.05$) in specific growth rate (SGR) between experimental and control as in **Table 3.** *Planococcus* sp.R11 bioencapsulation show higher SGR (9.43 %day^{-1} ±0.4^{d}) then *Bacillus* sp.R2 bioencapsulation with rearing (8.56% day^{-1} ±0.4^{bce}) comparing to control group (6.85% day^{-1} ± 0.78^{a}).

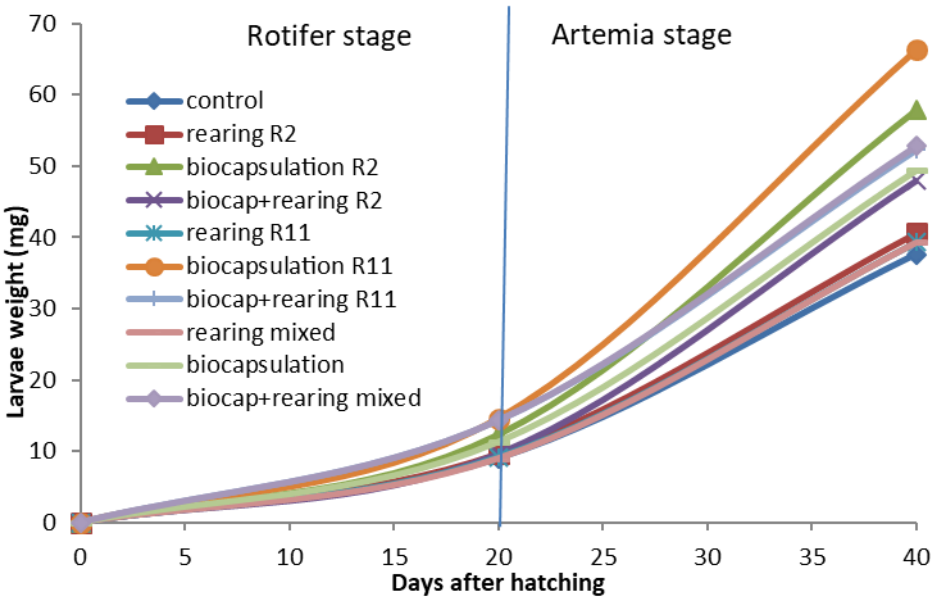

Fig.4 Growth of *S. aurata* larvae (mg) during the period from 3-40 DAH

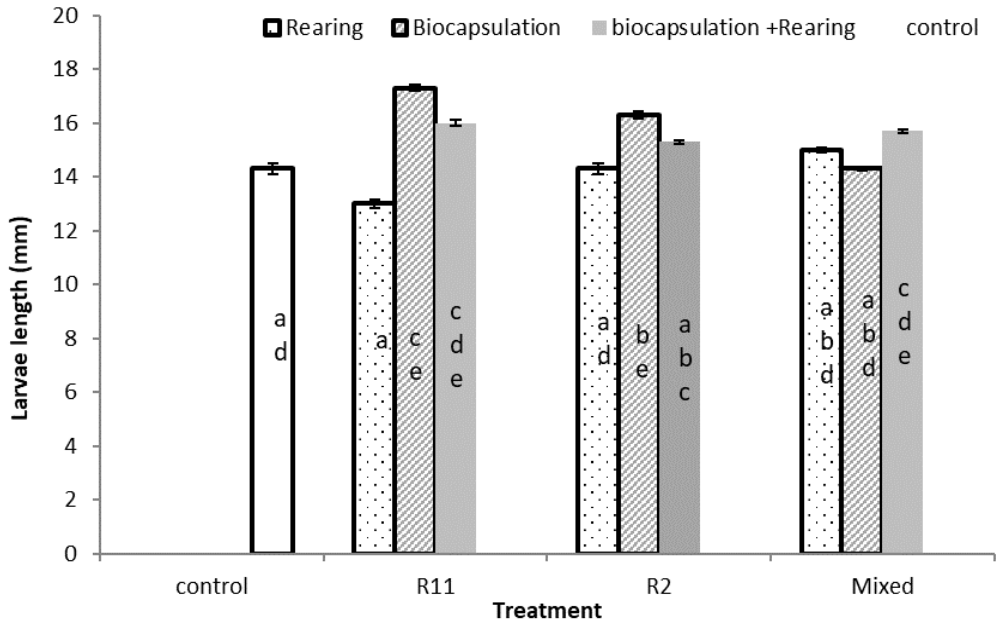

Fig.5 Total length(mm) of sea bream larvea during nine treatments. Bars are expressed as mean± SD. marked with different superscripts differ

Table 3 Specific growth rate of *S. aurata* larvae

Treatment	SGR (%day^{-1})
Control	6.85 ± 0.78^{a}
***Bacillus* sp. R2**	
Rearing	7.59 ± 0.52^{afh}
Bioencap	8.55 ± 0.51^{be}
Bioencap+rearing R2	8.56 ± 0.4^{bce}
***Planococcus* sp. R11**	
rearing	7.56 ± 0.51^{afh}
Bioencapsulation	9.43 ± 0.4^{d}
Bioencap+rearing	8.44 ± 0.45^{e}
Mixed of bacterial strains	
rearing mixed	7.3 ± 0.36^{a}
Bioencap mixed	8.39 ± 0.46^{bfe}
Bioencap+rearingMixed	8.38 ± 0.35^{bhe}

Each row with different superscripts are significant different (P<0.05) significantly with respect to each other (P<0.05).

Growth parameters (total length, weight and specific growth rate were increased significantly (Two-way ANOVA); $p<0.05$ (**Table 4**) with type of route of probiotic administration (rearing, bioencapsulation and rearing with bioencapsulation) and probiotic treated sea bream larvae and there were significant interaction between route of probiotic administration and type of probiotic in weight at 20 DAH and at 40 DAH and specific growth rate while there was insignificant interaction between route of probiotic administration and type of probiotic in total length.

Table 4 **Results of two ways analysis of variance on larval growth parameter (Total length, weight and specific growth rate). route of probiotic administration (rearing, bioencapsulation and rearing with bioencapsulation) and type of Probiotic (*Bacillus* sp. R2, *Planococcus* sp. R11 and mixed of *Bacillus* sp. R2 and *Planococcus* sp. R11) are fixed factor indicating effect ($p<0.05$)**

	F-value	F-value	P value
Total length	Probiotic	0.414	0.667
	Administration	5.381*	0.031
	Probiotic*Administration	2.571	0.69
Weight at 20DAH	Probiotic	6.477	0.007
	Administration	22.386*	0.001
	Probiotic*Administration	6.193*	0.002
Weight at 40DAH	Probiotic	13.24*	0.001
	Administration	111.08*	0.001
	Probiotic*Administration	18.39*	0.001
SGR	Probiotic	4.07*	0.021
	Administration	85.856*	0.001
	Probiotic*Administration	6.858*	0.001

Effect of Probiotic on survival rate

The survival rate was significantly ($P<0.05$) highest in larvae administrated with *Planococcus* sp. R11 in bioencapsulation of live food (88.61^{ed} % ±3.96), in bioencapsulation of live food and tank's water with *Planococcus* sp. R11 ($87.96\ ^{ef}$% ± 2.95) and larvae administrated with mixed in bioencapsulation ($87.6\ ^{abc}$±2.51) lowest in larvae administrated with *Bacillus* sp. R2 in tank water and live food (76.3^{cde} % ±1.13) comparing to untreated larvae (75.9^{a} % ±1.7) as **Fig6.**

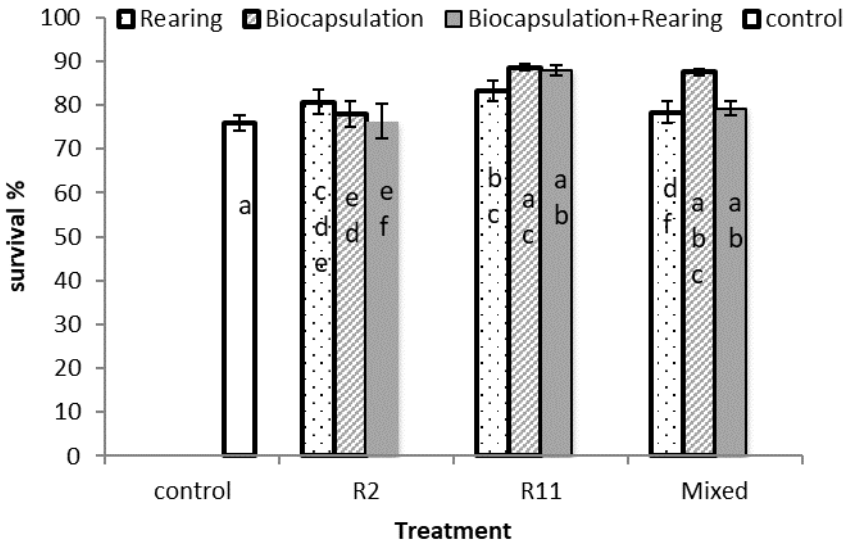

Fig.6 Survival percentage of sea bream larvae during nine treatments. Bars are expressed as mean ± SD. marked with different superscripts differ significantly with respect to each other (P<0.05).

Survival percentage was increased significantly (Two-way ANOVA); p<0.05 (**Table 5**) with type of route of probiotic administration (rearing, bioencapsulation and rearing with bioencapsulation) and probiotic treated sea bream larvae and there were significant interaction between route of probiotic administration and type of probiotic.

Table 5 Results of two ways analysis of variance on larval survival percentage after 40 day after hatching (DAH). route of probiotic administration (rearing, bioencapsulation and rearing with bioencapsulation) and type of Probiotic (*Bacillus* sp. R2, *Planococcus* sp. R11 and mixed of Bacillus sp. R2 and *Planococcus* sp. R11) are fixed factor indicating effect (p<0.05)

	Source of variation	F value	P value
Survival%	Probiotic type	14.163*	0.001
	Administration route	3.850*	0.039
	Probiotic *administration	5.499*	0.004

Effect of Probiotic on nutritional value of larvae

Total protein

There was significant differences at P<0.05 in the total protein. As **Fig.7**, total protein attained higher value in bioencapsulation *Planococcus* sp. R11 as 4.54^{d}±0.44mg/mL, in treatment of mixture of *Bacillus* sp. R2 and *Planococcus* sp. R11 in bioencapsulation and rearing as 4.36^{cdh}±0.125mg/mL, in treatment of *Planococcus* sp. R11 in bioencapsulation

and rearing as 4.01^{cde}±0.24mg/mL, mixture of *Bacillus* sp. R2 and *Planococcus* sp. R11 in bioencapsulation as 3.94^{cdf} ±0.3mg/mL and bioencapsulation of *Bacillus* sp. R2 as 3.92 ^{c}± 0.33mg/mL comparing to control as 2.97±0.46 ^{ah} mg/mL

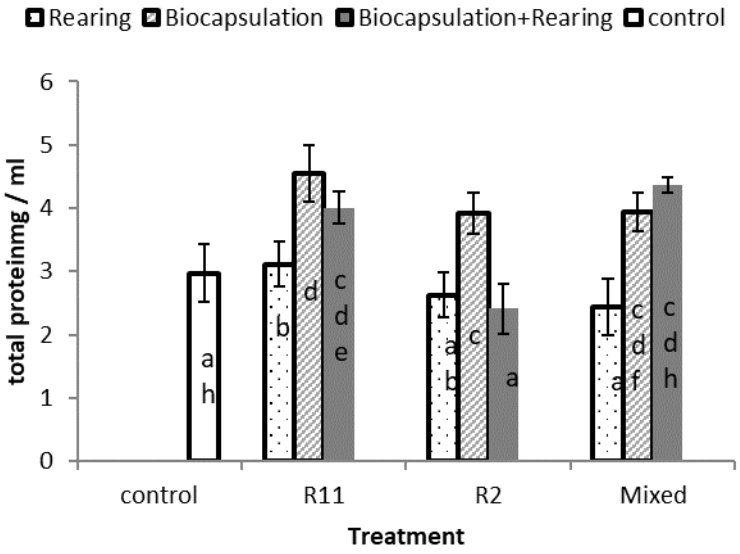

Fig.7 Total protein contents of sea bream larvae during nine treatments. Bars are expressed as mean ±SD. marked with different superscripts differ significantly with respect to each other (P<0.05).

Total protein was increased significantly (Two-way ANOVA); p<0.05 (**Table 6**) with type of route of probiotic administration (rearing, Bioencapsulation and rearing with bioencapsulation) and probiotic treated sea bream larvae and there was significant interaction between route of probiotic administration and type of probiotic.

Table 6 Results of two ways analysis of variance on larval total protein after 40 day after hatching (DAH). route of probiotic administration (rearing, bioencapsulation and rearing with bioencapsulation) and type of Probiotic (*Bacillus* sp. R2, *Planococcus* sp. R11 and mixed of Bacillus sp. R2 and *Planococcus* sp. R11) are fixed factor indicating effect (p<0.05)

	Source of variation	F value	P value
Total protein	Probiotic type	14.3338*	0.001
	Administration route	34.579*	0.001
	Probiotic *administration	8.008*	0.01

Total lipids

There was significant differences at P<0.05 in the total fatty acids as **Fig.8** total lipid attained higher value in R11 bioencapsulation as 127.4 ^{f} ±1.5mg/g, R2 bioencapsulation 114.77^{e} ±2.35mg/g, mixture of R2 and R11 in bioencapsulation 96.55 ^{hi} ± 1.5 mg/g and mixture of R2 and R11 in bioencapsulation and rearing as 94.37 ^{i} ±1.48 mg/g comparing to control as 91.85 ^{a} ±1.77mg/g.

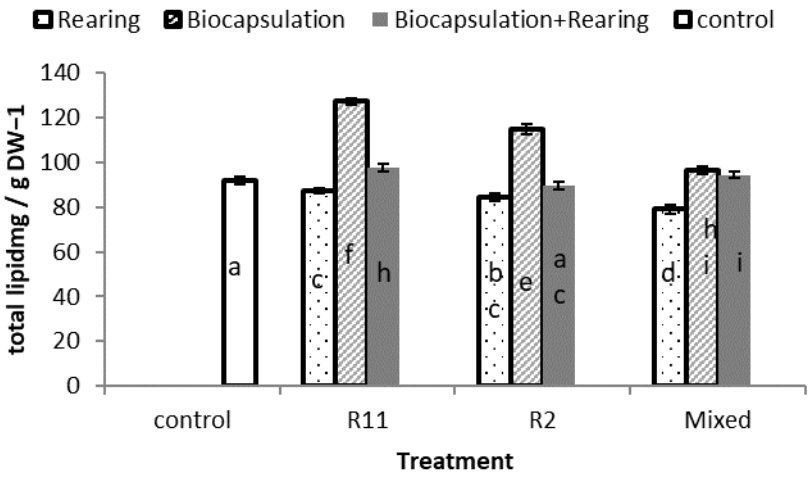

Fig.8 Total lipid contents of sea bream larvea under nine treatments. Bars are expressed as mean ± SD. marked with different superscripts differ significantly with respect to each other (P<0.05).

Total lipid was increased significantly (Two-way ANOVA); p<0.05 (**Table 7**) with type of route of probiotic administration (rearing, Bioencapsulation and rearing with bioencapsulation) and probiotic treated sea bream larvae and there was significant interaction between route of probiotic administration and type of probiotic.

Table. 7 Results of two ways analysis of variance on larval total lipid after 40 day after hatching (DAH). route of probiotic administration (rearing, bioencapsulation and rearing with bioencapsulation) and type of Probiotic (*Bacillus* sp. R2, *Planococcus* sp. R11 and mixed of *Bacillus* sp. R2 and *Planococcus* sp. R11) are fixed factor indicating effect (p<0.05)

	Source of variation	F value	P value
Total lipid	Probiotic type	60.980*	0.001
	Administration route	780.763*	0.001
	Probiotic *administration	417.851*	0.001

Fatty acids profile

Results in **Table 8** showed that the treatment of *Planococcus* sp. R11 in bioencapsulation had higher fatty acids composition (95.42 mg/g) then Mixed bioencapsulation as (90.73mg/g) and *Bacillus* sp. R2 bioencapsulation as (87.74mg/g) comparing to control as (69.85mg/g). PUFAS constituted high percentage of the total fatty acid in R11 bioencapsulation (69.8%) representing as PUFAs-ω3 as 63.8% and PUFAs-ω6 as (3.4%)

Effect of Probiotic on protease activity

The activity of protease was significant differences at P≤0.05as **Fig.9.** Protease activity attained highest value in *Planococcus* sp. R11 bioencapsulation as 8.95^{f} ± 0.21 U/mg comparing to control as1.3^{a}±0.03 U/mg.

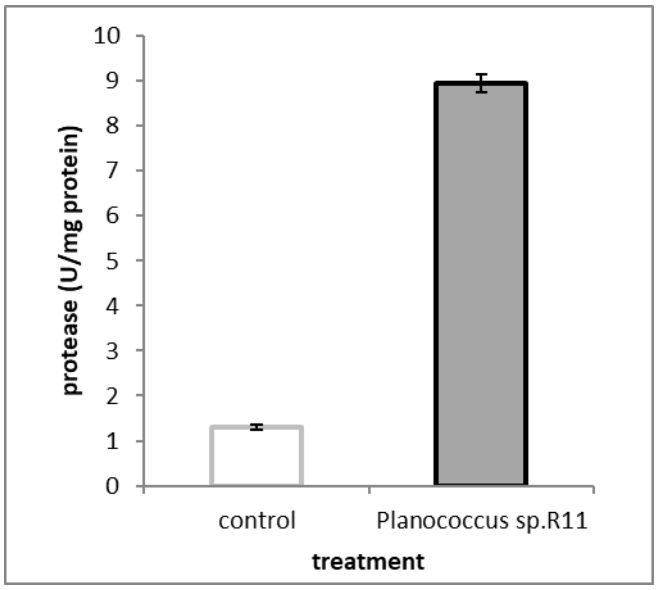

Fig.9 Proteses activity of sea bream larvea. Bars are expressed as mean ±SD.

Table 8 Fatty acids profile (% of the total fatty acid) detected in larval homogenates of sea bream at pH 8.3.

Fatty acids	*Bacillus* sp. R2				*Planococcus* sp. R11			Mixed		
Saturated FA	**Control**	**Rearing**	**Bio**	**Bio+ Rearing**	**Rearing R11**	**Bio R11**	**Bio+ Rearing R2**	**Rearing mixed**	**Mix Bio**	**Mix Bio+ Rearing**
C12: 0	3.4	0.0	1.5	1.3	2.3	0.0	0.0	0.0	0.0	0.0
C13: 0	6.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C14: 0	3.3	4.8	4.9	5.5	4.4	3.3	4.8	4.6	4.6	4.1
C15: 0	6.4	0.0	6.1	0.8	2.3	0.0	0.0	0.0	0.0	0.0
C16: 0	15.2	13.7	16.7	17.5	16.1	10.1	17.3	13.4	14.7	19.8
C17: 0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C18: 0	3.3	3.8	3.8	3.5	3.6	4.1	4.2	3.8	4.7	4.9
C20: 0	2.9	3.6	3.8	3.6	3.1	4.3	3.9	3.4	3.9	3.9
Mono unsaturated FA										
C16: 1	3.1	4.2	4.3	4.2	3.4	3.8	4.3	3.9	4.4	3.8
C17: 1	3.3	4.2	3.5	4.2	3.7	4.6	1.3	3.9	3.6	3.2
Polyunsaturated FA										
C18: 2ω6	1.2	1.9	1.2	1.8	1.5	2.1	1.8	1.7	2.2	2.3
C18: 3ω3	1.3	2.3	1.7	1.3	1.9	2.7	2.5	2.2	2.3	1.6
C20: 4ω6	0.7	1.3	0.7	0.6	0.8	1.6	1.3	1.2	1.1	1.3
C20: 5ω3	14.1	21.7	14.6	16.3	17.4	23.2	16.7	20.3	15.9	13.2
C22: 2	4.3	2.3	4.7	5.7	5.1	2.3	5.8	5.8	5.8	4.6
C22: 6ω3	30.3	36.2	32.5	33.7	34.4	37.9	36.1	35.8	36.8	37.3
SFA	41.7	22.3	36.8	32.2	31.8	21.8	30.2	25.2	27.9	32.7
UFA	58.3	74.1	63.2	67.8	68.2	78.2	69.8	74.8	72.1	67.3
MUFA	6.4	8.4	7.8	8.4	7.1	7.4	5.6	7.8	9	7
PUFA	51.9	69.3	55.4	59.4	61.1	69.8	64.2	67	64.1	60.3
PUFAs-ω3	45.7	60.2	39.8	45.3	53.7	63.8	55, 3	58.3	55	52.1
PUFAs-ω6	1.9	3.2	1.9	2.4	2.3	3.7	3.1	2.9	3.3	3.6
Totalfatty acids (mg/g)	69.85	65.41	87.74	67.33	69.28	95.42	74.58	62.54	90.73	72.25

Histological characteristics of digestive system

Supplementation of *Planococcus* sp.R11 through rotifer bioencapsulation at 20 DAH significantly increase (P<0.05) villi length and number of goblet cell but did not affect (P>0.05) on number of villi (**Table 9 and Fig. 10**). The(length, number of villi) and number of goblet cell were increased from 13±3 µm,20±3 and 20±2 in control to 35±9 µm, 22±2.5 and 37±7.5 in probiont bioencapsulation treatment respectively. Supplementation of *Planococcus* sp.R11 through *Artemia* bioencapsulation at 40 DAH significantly increase (P<0.05) in length, number of villi and number of goblet cell (**Table 9 and Fig. 11**). length and number of villi and number of goblet cell increased from 63±23 µm, 22±2.3 and 66±6 in control to 126±25 µm, 27±2.5 and 96±14.4 in probiont bioencapsulation treatment respectively.

Table 9 Length, number of villi and goblet cell's number in digestive tract of sea bream larvae treated with *Planococcus* sp. R11 bioencapsulation compared to control

Treatment	Villi length (µm)	Number of villi	Number of goblet cell per villi
Rotifer stage (3-20 DAH)			
Control	13±3	20±3	20±2
Treated larvae	35±9	22±2.5	37± 7.5
***Artemia* stage** (20-40 DAH)			
Control	63±23	22±2.3	66±6
Treated larvae	126 ±25	27±2.5	96±14.4

Each value is mean (±SD)

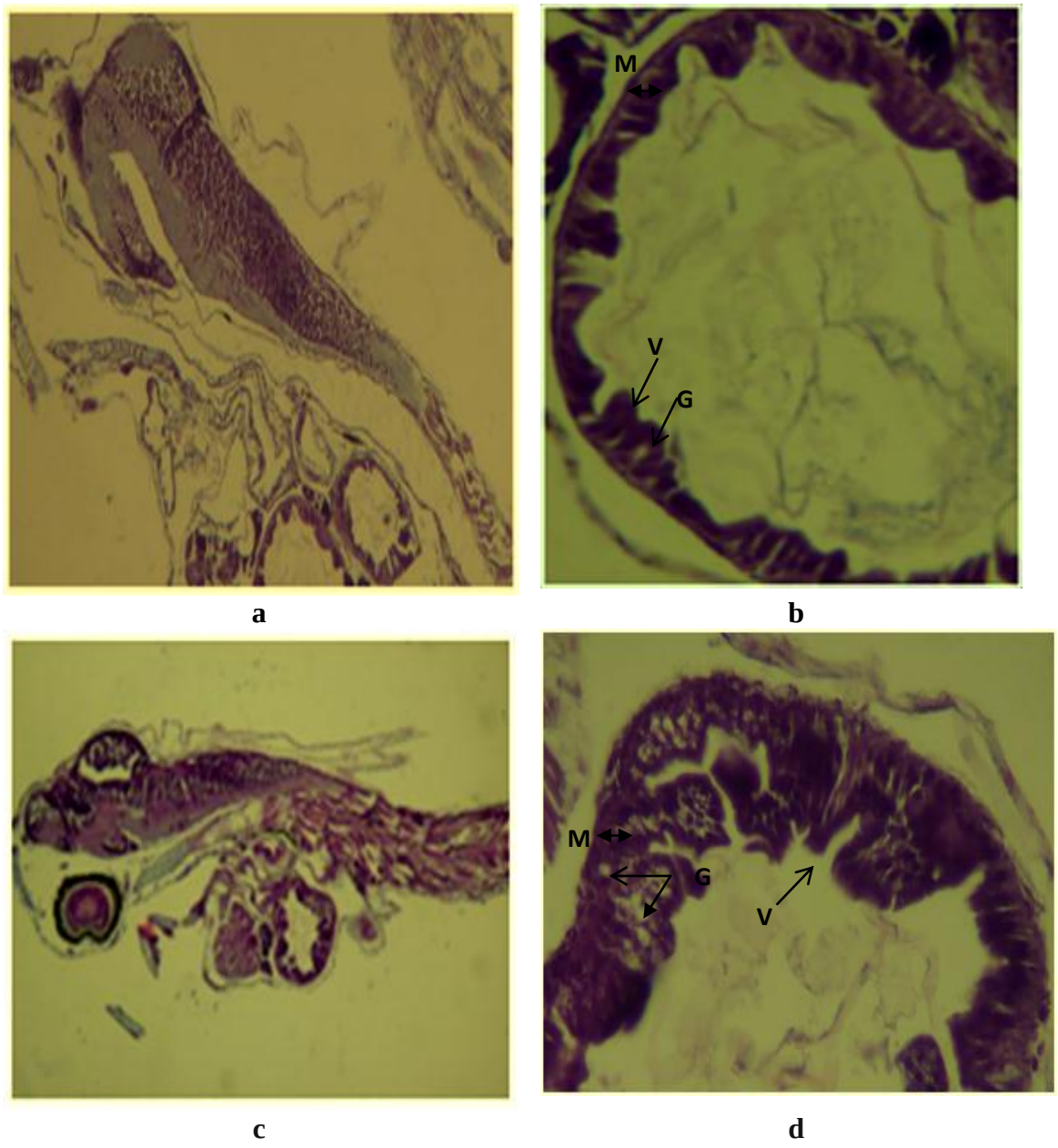

Fig.10 longitudinal section Showing digestive tract in gilthead sea bream larva at age 20 DAH fed on Rotifer (a) Control untreated larva (10x) (b) untreated larva (40x) (c) larvae treated with R11 bioencapsulation (10x) (d) larvae treated with R11 bioencapsulation (40x). M, mucosa; V, villi and G, goblet cell.

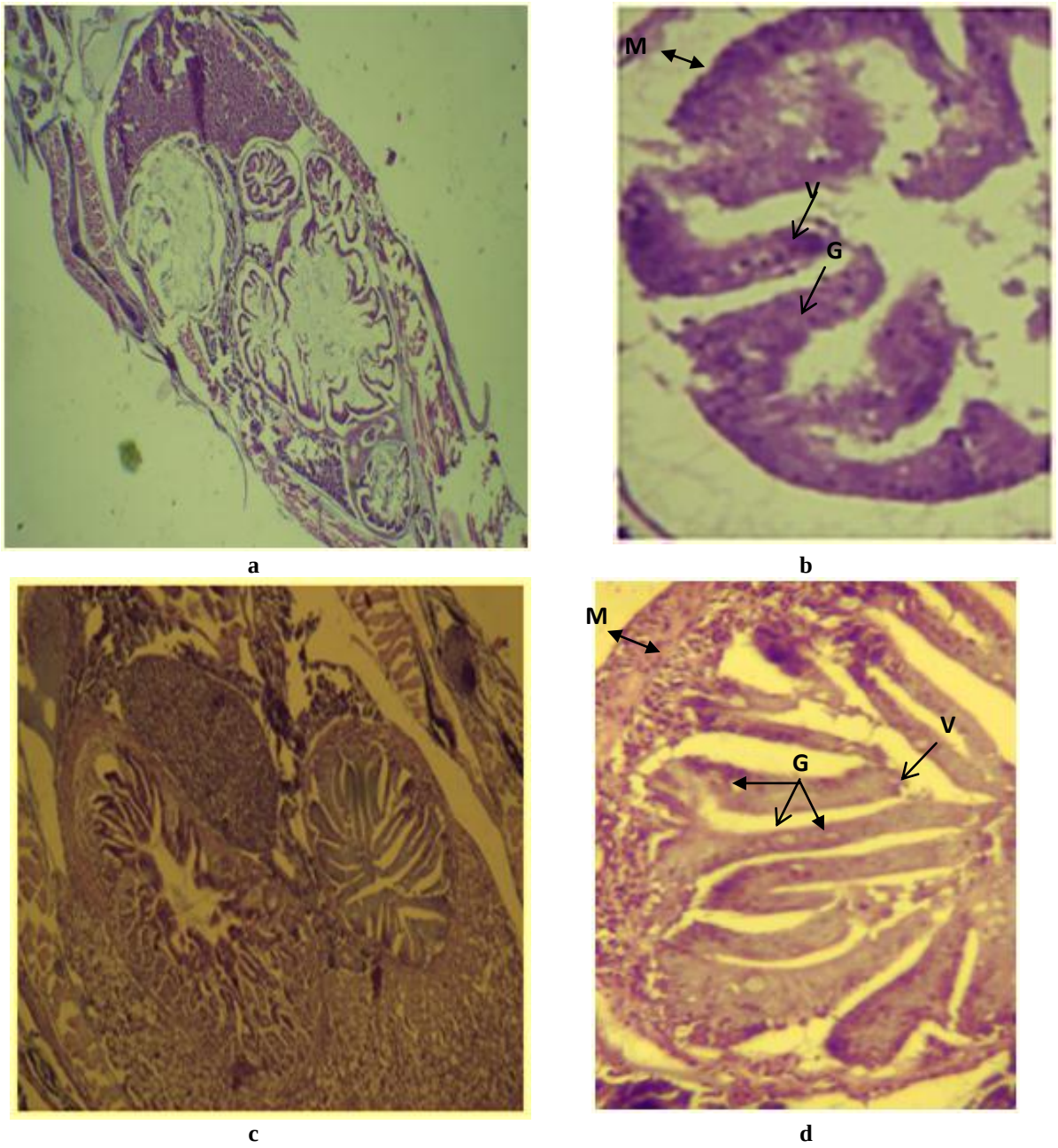

Fig.11 longitudinal section Showing digestive tract in gilthead sea bream larva at age 40DAH fed on *Artemia* (a) Control untreated larva (10x) (b) untreated larvae (40x) (c) larvae treated with R11 bioencapsulation (10x) (d) larvae treated with R11 bioencapsulation (40x). M, mucosa; V, villi and G, goblet cell.

DISCUSSION

Probiotics have the ability to control potential pathogens, and increase the growth rates and welfare of farmed aquatic animals, which has been demonstrated by several studies (**Zare *et al.*, 2017**). Manipulation of bacterial load present in rotifers and *Artemia* for fish feeding may constitute a valuable mechanism to increase survival rates and larval growth (**Gatesoupe, 1999; Robertson *et al.*, 2000**). The *in vivo* trials are required to validate the effectiveness of probiotic after getting *in vitro* trials results. This study aimed to examine the ability of all probiotic candidates on protein content and fatty acid profile of live food (rotifers and *Artemia*) and rotifer density and their effect on growth performances, survival, nutritional value, protease production in sea bream larvae for 40 days after hatching and histological characteristics of digestive system.

Rotifers (*Brachionus plicatilis)* and *Artemia* applied for fish feeding were enriched with *Bacillus* sp. R2 , *Planococcus* sp. R11 and mixture of them was significantly increased rotifers density and nutritional value expressed as protein and fatty acid contents compared to control receiving no probionts . This result coincides with **Leyton *et al.*, (2017)** who reported increase in the concentration of treated rotifers with *Pseudoalteromonas* sp, **Murillo & Villamil, (2011)** observed that the addition of *B. subtilis* to the rotifer culture water resulted in a significant increase in rotifer numbers, **(Hamsah *et al.*, 2017)** reported that the enrichment of *Artemia* sp. with the combination of *Pseudoalteromonas piscicida* 1Ub RfR and the prebiotic mannan-oligosaccharide improved protein content and **Khairy and El-sayed, (2012)** who reported higher production of total fatty acids in rotifer fed on *Tetraselmis chuii* grown on Erdschriber medium due to accumulation of poly unsaturated fatty acids.

The improvement of growth performance after probiotic feeding has been observed in tilapias receiving several probiont species (**Pirarat *et al.*, 2011; Standen *et al.*, 2016; Ramos *et al.*, 2017**). In the present study, the highest growth rate of sea bream larvae was obtained on *Planococcus* sp. R11 bioencapsulation . This is due to probiotics can enhance the activity of exogenous enzymes that can increase the digestibility of the feed, so that digestion of feed nutrients will increase and growth of shrimp will also increase(**Chandran *et al.*, 2014**). This was in line with high protein and lipid content comparing to control while treatment of *Planococcus* sp R11 and *Bacillus* sp. R2 bioencapsulation and rearing showed high fatty acids percentage than control.

The result showed that administration of bioencapsulation of *Planococcus* sp R11 also produced high survival on sea bream larvae. The higher survival rates of probiotics treated larvae than control were suspected due to probiotics could improve the immune system.The increasing survival in probiotic treatments could also occur due to antimicrobial compounds produced by probiotic bacteria can inhibit pathogenic bacteria that allegedly contained in the larvae or their living medium. Some of the previous studies also showed that the administration of probiotics could improve the survival of shrimp (**Widanarni *et al.*, 2010; Liu *et al.*,2010; Nurhayati *et al.*, 2015**).

Probiotics improve nutrient availability due to exogenous enzymes secreted into the host intestine or to endogenous enzymes available into the bacterial cells and released when they are lysed by the effect of the acidic environment of hosts' stomach (**Sánchez-Ortiz *et al*., 2015).**

During the early ontogeny of Sparids, digestive system where the probiotics could be worked effectively because Gram-positive bacteria especially members of the genus *Bacillus* could be secreted a wide range of exoenzymes (**Wu *et al*., 2012; Mohapatra et al., 2012).** 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 (**Suzer *et* al., 2008; Arığ *et al*., 2013**). The activity of protease of sea bream larvae at 40 (DAH) was significant differences at $P<0.05$. Protease activity attained highest value in *Planococcus* sp. R11 bioencapsulation comparing to control .

Histologically, the intestinal wall of gilthead sea bream consists in four layers from the lumen, (**Jorge, 2016**) which are: mucosa, sub-mucosa, muscularis (muscularis interna and externa) and serose. Mucosa: the epithelium is composed of a simple layer of high columnar cells, the enterocytes, among which lay goblet cells. The apical plasma membrane forms microvilli which constitute the brush border thus increasing the exchange surface between the lumen and enterocyte, submucosa, that is connective tissue layer with blood vessels, muscular that is divided into an inner circular and an external longitudinal layer, being responsible for movement of gut contents; formed by connective tissue delimited by a simple squamous peritoneal epithelium lymphatic aserosa tissue and nerve plexi, that supports the mucosa (**Ray & Ringø, 2014**)

Supplementation of *Planococcus* sp.R11 through rotifer bioencapsulation at 20 DAH significantly increase ($P<0.05$) villi length and number of goblet cell but did not affect ($P>0.05$) on number of villi. Supplementation of *Planococcus* sp.R11 through *Artemia* bioencapsulation at 40 DAH significantly increase ($P<0.05$) number of goblet , length and number of villi .

The role of the gut in nutrient digestion and absorption is well known in fish (**Grosell, et al., 2010**). The intestinal villi height, muscular layer thickness and the goblet cells count are good indicators of a healthy intestine (**Khojasteh, 2012**).

The goblet cells, present along the entire intestine, are responsible for the synthesis and secretion of the protective mucus layer that covers the epithelium surface. This mucus layer acts as a medium for protection, lubrication and transport between the luminal contents and the epithelial lining and it is an integral structural component of the intestine, demonstrated an increase in this cell population following yeast polysaccharide administration (**Zhu *et al*., 2012**).

In aquatic animals, intestinal villus height is regarded as a sign of absorption ability. Recent studies have demonstrated that ingredients such as *Lactobacillus rhamnosus*

(**Pirarat *et al*., 2006**) and polysaccharide yeast (**Zhu *et al*., 2012**) are able to increase villus height in different fish species**.** Another study reported a strong decrease in microvillus height provoked by the replacement of fish meal with soybean meal in fish diet (**Rombout *et al*., 2011**). **Dimitroglou *et* al., (2011)** found gilthead sea bream fed with mannanoligosaccharide resulted in higher fold height and absorption surface of the gut by measuring the length and density of the microvilli. **Merrifield *et al*.**, **(2010)** showed improvements in the morphology of the intestinal microvilli of the salmonides when fed probiotics. In *Oreochromis niloticus*, the increase in goblet cell count as an effect of commercial mixted species probiotic of probiotics treatment has been previously observed **(Standen *et al*., 2016). Elsabagh *et al*., (2018**) reported the increased intestinal absorptive area, with a subsequent increase in nutrient absorption and retention, and the enhanced goblet cells count highlight the observed improvement in growth performance, immune response and stress resistance in Nile tilapia.

CONCLUSION

The present work presents the importance of using probiotics in aquaculture to enhance growth and reduce mortality which is an important aspect in eco sustainability of aquaculture and prove that *Planococcus* sp.R11 can improve the growth and developement of sea bream larvae by increase the activity of potease and enhance the histology of digestive system by increase number of goblet cell ,number and length of intestine villi and this is the first report about application of *Planococcus* as potential probiotic in aquaculture.

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