International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Probiotic Effect of *Lactobacillus*, *Leuconostoc* and *Candida* Strains against *Vibrio Alginolyticus* Infection in *Dicentrarchus Labrax* Larvae

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Abstract: ***The present investigation was carried to evaluate the potential probiotic effect on Dicentrarchus labrax by using single or mixture probiotic bacteria and yeast. Four probiotic strains were successfully isolated from the live prey produced by the National Institute of Science and Technology of the Sea INSTM (Monastir Tunisia). Lactobacillus plantarum and Candida famata isolated respectively from artemia and rotifer, Leuconostoc mesenteroides was isolated from fish intestine and Lactobacillus paraplantarum has been isolated from rotifers. The identified strains were studied for their effect on fish growth in the form of probiotics. Feed application of probiotic strains was studied and showed that their application culture improved significantly growth and protection against the pathogenic Vibrio alginolyticus and improved weight and linear growth of Dicentrarchus labrax. On the basis of these results, it is suggested that the tested strains show remarkable possible for use as a probiotic for the breeding of fish, particularly with regard to improving the survival and growth of Dicentrarchus labrax larvae and their ability to inhibit the pathogen in vivo and in vitro conditions.***

Key words: Bacteria, yeast, probiotic, rotifer, Artemia, *Dicentrarchis Labrax.*

1. Introduction

Fish infections are one of the major evils in the fish farm industry. The use of antibiotics to treat bacterial infection and avoid fish mortality in aquaculture is fitting limited as pathogens develop resistance to the drugs [1-2].

Further, favorable bacterial flora are killed or inhibited by orally administered antibiotics, leading to efforts to find different disease avoidance methods such as the use of nonpathogenic bacteria as probiotic biocontrol agents. [Fuller (1987) [3] defined probiotics as "a live microbial adjunct which has a beneficial effect on the host by modifying the host associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment"]. Probiotics could enhance intestinal health, including competition for limited nutrients, inhibition of the epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, the production of antimicrobial substances and/or the stimulation of mucosal immunity. Several studies demonstrated that probiotics enhance growth performance, survival, immunity, and disease resistance [4-5].

It has been generally known that probiotics represent a possible implement in the reduction in water or live food can be a good strategy to provide of mortalities in the raise of aquatic organisms [6]

The search for choice to antibiotic therapy has turned to the use of probiotics during the early larval stages. Several studies have reported encouraging results in the application of probiotics in aquaculture [7-8] and application of probiotics to fish aviculture in particular has yielded to positive effects, mainly in survival and growth rates [9-10].

The present study was undertaken to isolate probiotic strains from microalgae (*Picochlorum sp.*), rotifer (*Brachionus plicatilis*), artemia (*Artémia salina*) and fish intestines (*Chelon labrosus*). We screen them by *in vitro* testing of their antagonism to pathogens and attachment to substrates. In addition, we studied the effect of probiotic bacterial strains isolated against pathogenicity of *Vibrio alginolyticus* on *Dicentrarchus labrax.*

2. Materials and Methods

2.1 Isolation of Probiotic Strains

The strains were isolated from the culture of rotifer (*Brachionus plicatilis*), Artemia (*Artemia salina*) and also from fish intestine (*Chelon labrosus*). The samples were obtained from National Institute of Science and Technology of the Sea (Monastir Tunisia), 1 ml of each sample was incubated in 9 ml of Sabouraud and MRS (Man Rigosa and Sharp) broth seawater (salinity 34 g l^{-1} and pH 7.99) then incubated at 37°C and 44°C. After 24 hours of incubation, 10 µl from each broth were transplanted on Sabouraud agar and MRS (seawater agar). All petri plates were incubated at 37°C and 44°C for 24 to 72 h. To purify, the colonies isolated and differentiated by morphology and their color have been transplanted twice on the same environments. Stock cultures were frozen at -80°C with 20% (vol/vol) glycerol.

2.2 Molecular Cracterization of the Isolated Strains

Total DNA was extracted from the four strains according to the method of Sambrook et al (1989) [11] and stored at -20 °C. The two primers used were: for *Lactobacillus* IDL04F 5'-AGGGTGAAGTCGTAACAAGTAGCC-3'and IDL03R 5'-CCACCTTCCTCCGGTTTGTCA-3' [12], for *Leuconostoc* LeuF 5'-CGAAAGGTGCTTGCACCTTTCAAG-3' and LeuR 5'-TTTGTCTCCGAAGAGAACA-3' [13] and for *Candida* ITS86 5'-GTG AAT CAT CGA ATC TTT GAA C-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3' [14]. Amplification was carried out using a reaction volume of 50 µl, including 2 µl DNA, 0.2 mmol of each deoxynucleoside triphosphate, dATP, dGTP, dCTP and dDTP, 1× buffer solution, 1.5 mMMgCl2, 1.0mM of each primer (IDL04F and IDL03R, Leu F and LeuR, ITS86 and ITS4) and 1.0 unit of Taq DNA polymerase (Promega,USA). The mixture was subjected to 35 amplification cycles (PTC 100 thermal cycler (Biorad)) by applying the following parameters : denaturing at 94°C for 2 min, denaturing at 94°C for 20s , hybridization at 51° C for 40s, extension at 68°C for 30s and final extension at 68 °C for 7 min. The yeast mixture was subjected to a program of 30 cycles, denaturing at 94° C for one minute, annealing at 55°C for one minute, and elongation at 72°C. These three steps were preceded by an initial hybridization step at 94°C for 5 minutes and ends with a final extension at 72°C for 7 minutes.

2.3 Well Diffusion Agar Assay (WDAA)

Potential probiotic strains were tested for their antagonistic activity using the well diffusion agar assay (WDAA) [15] against three target strains: *Vibrio alginolyticus* isolated from infected fish, described previously [16], *Salmonella typhimurium* ATCC1408, *Escherchia coli* ATCC35218 and *Pseudomonas aeruginosa* ATCC 27853. The pathogenic bacteria were grown in 10 ml of nutrient broth and cultured for 24 hours on nutrient agar at 30°C. The common colonies from pure culture were suspended in 10 ml of physiological medium and well mixed during 5 min. One ml was spread over the agar plates. Potential probiotic strains were cultured in 10 ml nutrient broth for 24 hours, 100 µl of the supernatant were introduced into the wells of the MH agar medium and incubated for a period of 24 h at 30°C. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well.

2.4 Adhesion Assay

Adherence to surfaces, especially in intestinal mucus is an important parameter for probiotic bacteria once colonizing the gastrointestinal tract. Biofilm assemblage by probiotic strains was indomitable by means of a semi-quantitative adherence assay on 96-well tissue culture plates. Strains were grown in Trypticase Soy broth supplemented with 1% (w/v) NaCl (TSB 1%, Pronadisa, Spain). After incubation at 37°C, optical density at 595 nm (OD595) of the bacteria was measured. An overnight culture, grown in TSB 1% at 30°C, was diluted to 1:100 in TSB supplement with 2% (w/v) glucose. A total of 200 l of cell suspension was transferred to an Ubottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Wells with sterile TSB unaided provided as controls. The plates were incubated at 37°C for 24 h. The cultures were removed and the wells were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non-adherent cells and dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 l of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 l of sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 595 nm (OD595) using an automated Multiskan reader (GIO. DE VITA E C, Rome, Italy). Adhesion ability was interpreted as high (OD595 ≥ 1), fair (0.1 ≤ OD595 < 1), or slight (OD595 < 0.1) [17].

2.5 Pathogenicity/Toxicity of the Probiotics on *Dicentrarchus Labrax Larvae*

2.5.1 Inoculation of Yeast and Bacterial Suspensions

Yeast and bacterial suspension (10^6 CFU ml^{-1}) is obtained by adjusting the optical density of a wavelength 600 nm from a young bacterial culture from 18 to 24 h grown on MRS, Sabouraud and nutrient agar prepared with seawater .The enumeration of every strain is made in various optical densities according to the protocol of Hoben and Somasegaran (1982) [18].

2.5.2 Rotifer Culture

The rotifers *Brachionus plicatilis* (200 rotifers ml^{-1}) were cultivated on seaweed *Picochlorum sp.* (2 × 10^6 cells ml^{-1}) and DHA-Protein Selco (INVE) following the instructions provided by the manufacturer for 24 h in tanks of 200 liters at 24°C. Rotifers are then filtered through a sieve (60 µm), washed and transferred (200 rotifers ml-1) in buckets of 5 l containing sea water and probiotic bacteria and yeast (1 × 10^6 CFU) ml-1. Rotifers have been maintained in enrichment with the suspension bacterial for 3 h and filtered, washed to sea water and distributed to *Dicentrarchus labrax* larvae at the rate of 3 to 5 rotifers ml^{-1}. *Brachionus plicatilis* is obtained from the Institute of Aquaculture of the Hellenic Center for Marine Research However *Picochlorum sp.* has been isolated at the National Institute of sciences and technology of the Sea (Monastir Tunisia).

2.5.3 Breeding of *Dicentrarchus Labrax Larvae*

Dicentrarchus labrax larvae coming from the hatchery of the National Institute of Science and Technology (Monastir Tunisia) were transferred on day 1 (30 to 35 larvae / l) in cylindrical-conical tanks 60 litters. The temperature was maintained at 18°C, water was aerated (oxygen saturation N90 %) and light intensity is provided by daylight fluorescent lamps on the surface of larval reservoirs adjusted into 3.5 µE s-1 m-2. Day 3 to day 12 to assess the effect of microorganisms isolated from larvae of fish as well as their ability to remove pathogenic *Vibrio alginolyticus* in vivo, different tests has been made: Test 1: the larva have been fueled by rotifers enriched by alga and DHA-Protein Selco (control T), Test 2: the larva have been fueled by rotifers enriched by alga, DHA-Protein Selco and added the pathogenic *Vibrio alginolyticus* in water at the rate of 1 × 10^6 CFU ml^{-1} (L + V) day 9; tests 3, 4, 5 and 6 have been made with the four strains *plantarum* (L + L3),

paraplantarum (L + L20), *mesenteroides* (L + L27) and *Candida famata* (A + C), Test 7: the larvae were fed by rotifers enriched with mixture of three strains: *Lactobacillus plantarum, Lactobacillus paraplantarum* and *Leuconostoc mesenteroides* (L + K) and tests 8, 9.10, 11 and 12 trials were carried out using the four strains a beneficial effect in the presence of the pathogen *Vibrio alginolyticus* (L + V + L3), (L + V + L20), (L + V + L27),(L+V+C) and (L + V + K) day 9. All tests were conducted in triplicate.

2.5.4 Microbiological Analysis

The samples from larvae, rotifer and water were taken under aseptic conditions during the breeding. Ten larvae and 400 rotifers were separated by using a filter of 250-µm and 30 µm. The Larvae and the rotifer were washed with sterile seawater and homogenized. Processed samples were diluted in series in seawater, plated on MRS Agar (MRS, Difco 2216-212185) and incubated for 3 days at 37 °C in the dark. Plates with 30–300 colonies were counted. For Vibrionaceae counting, appropriate dilutions were replica-plated on to TCBS (Cultimed 413817), incubated one day at 37°C and colonies were counted.

2.5.5 Survival, Linear and Weight Growth of Larvae

For growth study, 30 larvae were sampled in each tank every day. The samples were anesthetized with ice cold sea water and then fixed with formaldehyde (4% in phosphate buffered solution pH 7.4) and kept refrigerated until analysis at the end of the experiment. Photographs of the fish were taken using a digital camera (Nikon Coolpix 4500) and measurements were carried out later using image analysis software Image J 1.29. An object micrometer was photographed with each set of photos to avoid errors due to the auto-focus of the camera. Drained weights of fixed larvae were measured immediately after photographing using a precision balance.

2.6 Statistical Analysis

The differences between final survivals, growth and weights of the larvae were tested by covariance's ANCOVA and with Duncan's test using the Stat View software (version 5.0, SAS Institute Inc.).

3. Results

3.1 Identification of Bacterial Strains and Yeast

The PCR amplification of DNA extracted from pure cultures of bacteria and yeast gives bands with a 107 pb moleculaure size and corresponds to the *paraplantarum* (isolated in rotifers and the Artemia), 318 pb that identifies the *mesenteroides* strain (found in the intestine of fish) and 428 pb for *plantarum* (isolated the Artemia and rotifers). For strain *famata* (isolated from rotifers and the Artemia), after the specific amplification of the intergenic sequences ITS2 of genus Candida, a band of size 92 pb has been obtained and which confirms the affiliation of this strain to this kind (Fig.1.).

3.2 Antagonism Assay and Adherence

The isolated strains showed stability in their antagonistic ability, as their inhibiting effect against pathogens (*Vibrio alginolyticus* ATCC17749, *Salmonella typhimurium ATCC1408, Escherchia coli ATCC35218, Pseudomonas aeruginosa* ATCC 27853), with inhibitory zones measuring about 11,5–16,5 mm in diameter (Table 1).

All isolated strains were exanimate for their adherence to polystyrene microplates. The results showed fairly adherent, with values ranging between 0.189 and 0.603 at 595 nm (Table2).

3.3 Pathogenicity/Toxicity of Probiotics on *Dicentrarchus Labrax Larvae*

3.3.1 The Number of Ingested Bacteria

A simple addition of *Vibrio alginolyticus* in water maintained a constant concentration in values around 10^5 to 10^6 CFU ml^{-1} from day 9 to day 11 and it was the predominant bacteria in water. Thereafter, the concentration of *Vibrio* decreased constantly reaching 10^4 CFU ml^{-1} at day 12. *Vibrio alginolyticus* was detected in larvae from the day 9 in concentration of 10^2 CFU $larvae^{-1}$. The level of *Vibrionaceae* in water and larvae during the experimental period were similar in all treatments. The rotifers which are enriched by algae (*Picochlorum sp.)* and probiotic bacteria (L3, L20, L27 and K) have a concentration between 3×10^4 and 5×10^4 CFU ml^{-1}. The bacterial load in larvae feeding on enriched rotifers with probiotics was between 10^2 and 10^2 (Table 3).

3.3.2 Larval Survival Rate

The comparison of the larval survival rate of *Dicentrarchus labrax* with the tested strains (L + L3); (L + L20); (L + L27) (L + C) and the control treatment (larvae fed with rotifers enriched by Culture Selco), showed non-significant differences ($P > 0.05$). Indeed, the addition of enriched rotifers with isolated bacteria (L3, L20, L27, C) provided a survival rate higher than 70% (figure 1a). These strains have also the ability to inhibit the pathogen *V.alginolyticus* because during these folowing treatments: (L + V + L3); (L + V + L20) ;(L+V+L27); (L + V + C), and (L + V + K) larvae have a survival rate which varies between 40% and 60%. The difference is significant ($P < 0.05$) by comparison between treatments where we have used the pathogen. However, a significant difference was noted also in comparison with the treatment (L + V), where the survival rate almost vanishes at the end of treatment ($P < 0.05$) (figure 1b). We noted that the survival rate in the tests or larvae were fed by rotifers treated with probiotic strains and the pathogenic Vibrio improve significantly compared to the treatment only with the pathogen (L + V) ($P < 0.05$) (Fig.2.).

3.3.3 Linear Growth of Larvae

Linear growth of *Dicentrarchus labrax* larvae fed by enriched rotifers with isolated bacteria and yeast showed a significant difference ($P < 0.05$), indeed the feeding larvae with enriched rotifers with three strains of the bacteria (*Lactobacillus palantarum*, *Lactobacillus paraplantarum*, *leuconostoc mesenteroides)*, showed the strongest growth with a height of 7±0.023 mm end of the experiment

(J12)also a significant improvement in the linear growth was obtained by comparison with the treatment of control over the experience where we got a size of 5.33 mm at J12 (Fig. 3.).

3.3.4 Weight Growth of Larvae

At the end of the treatment, the ponderal growth of sea bass (*Dicentrarchus labrax*) larvae nourished with enriched rotifers by the isolated bacteria and yeast, showed a significant improvement ($P < 0.05$) compared to the witness and the most important weight is obtained in larvae fed by enriched rotifers with three strains at the same time (L3 + L20 + L27) with 0.785 mg (J12). The larvae fed with rotifers which enriched only by Culture Selco grew less important than the other plans and they were able to reach a weight of 0.42 mg at the end of the experiment (Fig.4.). There is no significant difference between the five treatments ($p < 0.05$).

4. Discussion and Conclusion

Since the first application of probiotics in aquaculture, a growing number of scientific papers have dealt with this subject and have demonstrated the validity of their use to control potential pathogens and to increase the survival rates and welfare of reared fish larvae [19-20-21]. A method gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria [22]. Larvae of marine fish usually do not feed on artificial diets during the early stages of development and require live food. Thus feeding with living organisms offers the interesting possibility of using carriers that allow the bacterial community of the host to be modified through the addition of probiotics [23].

The present study provided evidences on the effects of probiotic treatment both on welfare and growth in European sea bass, one of the most important farmed species for the Tunisian and European ichthyic market. For this purpose, the bacterial strains and yeast probiotic was isolated from rotifer and artemia (*Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Candida fama*). Leuconostoc mesenteroides was isolated from adult *Chelon labrosus* intestine. On diffusion agar plates, the strains showed a zone of clearance against *Vibrio alginolyticus*, *Salmonella*, *Esherchia coli* and *Pseudomonas aeruginosa*. This effect can be associated to the aptitude of probiotic strains found to produce antibacterial compounds which are active against a wide range of gram-positive and gram-negative bacteria [24-25].

With the aim to revealing the aptitude of probiotic bacteria to control the pathogenic Vibrio in *vitro* and in *vivo* conditions in the larvae of *Dicentrarchus labrax*. The strains administered live as probiotic using as living carriers *Brachionus plicatilis* [26-27]. During the present study, it was observed that rotifers did accumulate probiotic bacteria after short incubations in bacterial suspensions and they were still present in rotifers 24 hours after transfer. Talpur et al. described similar results when incubated rotifers with probiotics. Rotifers with this latent can be use as specific vector to carry any kind of probiotic matters or antibiotics. It means that this organism could be used for bio-vaccination for diseases curing and controlling.

Results from microbial analysis evidenced that, in groups treated with strains probiotic, intestinal population was composed of the majority by *Lactobacillus palntarum*, *Lactobacilllus paraplantarum*, *Leuconostoc mesenteroides* and *Candida famata*, confirming that the technique of probiotic administration using zooplankton as carrier was effective [26-27].

The results of the pathogenicity and toxicity assay of the tested strains and their effect on larval showed that these strains were able to enhance *Dicentrarchus labrax* resistance to pathogenic Vibrio. This result confirms the potential of these strains to be considered as potential probiotic candidates for larvae. We have also demonstrated that a mixture of *Lactobacillus plantarum, Lactobacillus paraplantarum and Leuconostoc mésenteroides* strains can suppress pathogenic Vibrio both in vivo and in vitro.
The presence of probiotic bacteria and yeast reduced mortality but not *V. alginolyticus* counts. The strains might act by reducing the pathogenicity of Vibrio rather than diminishing the numbers of Vibrio. Therefore could perform the antagonistic effect at specific sites, and therefore improve survival of larvae [28-29-30-31].

After 12-day feeding trials, we found that seabass fry fed the probiotic strains had significantly greater weights and lengths ($P<0.05$) than control and the other treatment fish.

The use of *Candida famata*, *lactobacillus plantarum*, *lactobacillus paraplantarum* and *Leuconostoc mesenteroides* has been shown to be safe in the hatchery live food environment and it fulfills the requirements of a probiotic, although, clearly, much remains to be done to optimise the quantity and frequency of addition of strains, in which case greater benefits should be expected.

5. Future Scope

It is hoped that more studies are required in order to clarify the exact contribution of potential candidates probiotics used in this study an improvement of larvae *Dicentrarchus labrax* performance and protection against pathogens using host gene – expression and overcome disease in aquaculture farms.

6. Acknowledgements

The authors would sincerely like to express their thanks to Prof. Amina Bakhrouf for his technical help and for providing many valuable suggestions and comments during the experimental period. Particular thanks are addressed to hatchery staff of the INSTM for their help and technical assistance. The authors greatly acknowledge the reviewers for their helpful comments.

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Table 1: Inhibitory effect of potential probiotic strains measured by mm. *Candida famata* (C), *Leuconostoc mésenteroides* (L27), *Lactobacillus paraplantarum* (L20), *Lactobacillus plantarum* (L3)

Strains	*L3*	*L20*	*L27*	*C*
Vibrio alginolyticus ATCC17749	13.5±0.70	13±1.41	14±00	12.5±0.5
Salmonella typhimurium ATCC1408	12.5±0.70	13±00	13±1.41	12±00
Escherchia coli ATCC35218	14±00	11.5±0.70	11.5±0.70	13.5±0.98
Pseudomonas aeruginosa ATCC 27853	15.5±1.41	17±00	16.5±0.70	15±0.98

Table 2: Biochemical identification of the strains studied and semi quantitative study of adhesion.

Strains	*Adhesion to polystyrene DO (595±SD)*
Lactobacillus plantarum	0.234±0.19
Lactobacillus paraplantarum	0.189±0.22
Leuconostoc mesenteroides	0.216±013
Candida famata	0.603±0.15

Table 3: Number of culturbable bacteria by fish and water (day12) growing and MRS and TCBS media

	In the water			Larvae		
Treatment	MRS	SABAURAUD	TCBS	MRS	SABAURAUD	TCBS
L+V	N.D	N.D	1.10^6	N.D	N.D	310^2
L+V+L3	$5.\ 10^1\pm$	N.D	2.10^5	5.10^2	N.D	2.10^2
L+V+L20	$1.10^1\pm$	N.D	3.10^5	1.10^2	N.D	2.10^2
L+V+L27	$2.10^1\pm$	N.D	4.10^6	2.10^2	N.D	1.10^2
L+V+C	N.D	3.10^1	6.10^6	N.D	5.10^2	4.10^2
L+V+K	$4.10^1\pm$	N.D	5.10^5	4.10^2	N.D	1.10^2
L+L3	$5.10^1\pm$	N.D	N.D	5.10^3	N.D	N.D
L+L20	$5.10^1\pm$	N.D	N.D	5.10^3	N.D	N.D
L+L27	$3.10^1\pm$	N.D	N.D	3.10^3	N.D	N.D
L+K	$4.10^1\pm$	N.D	N.D	4.10^3	N.D	N.D
L+C	N.D	6.10^2	N.D	N.D	6.10^3	N.D

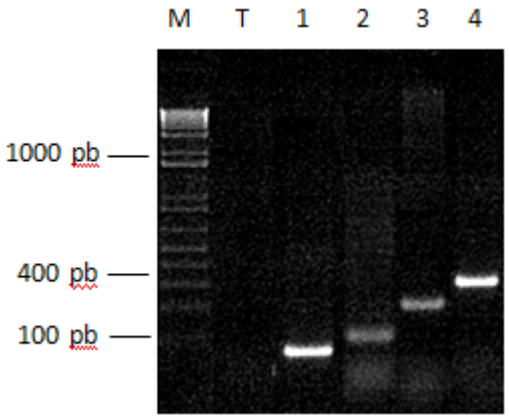
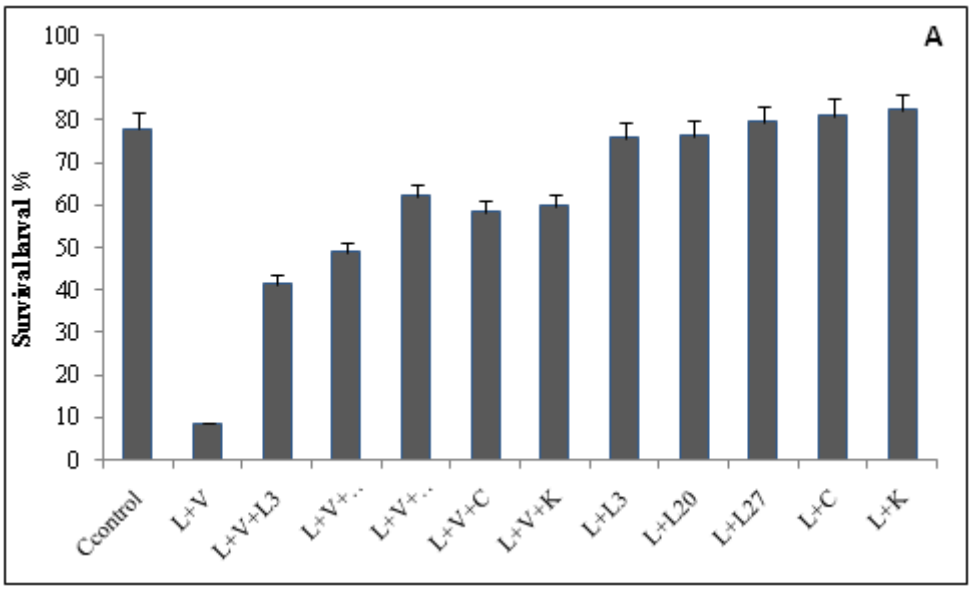
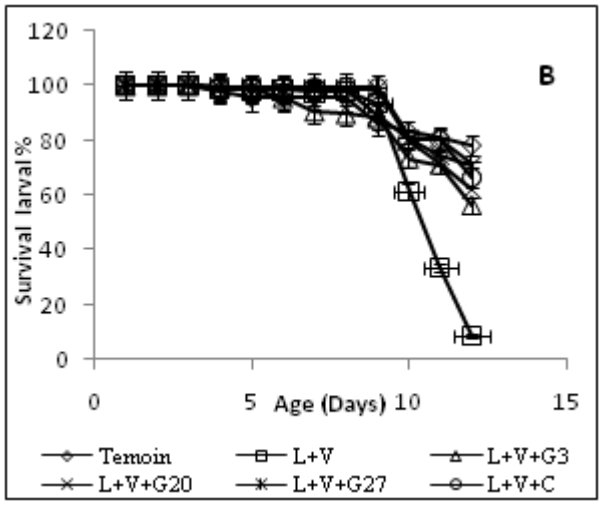
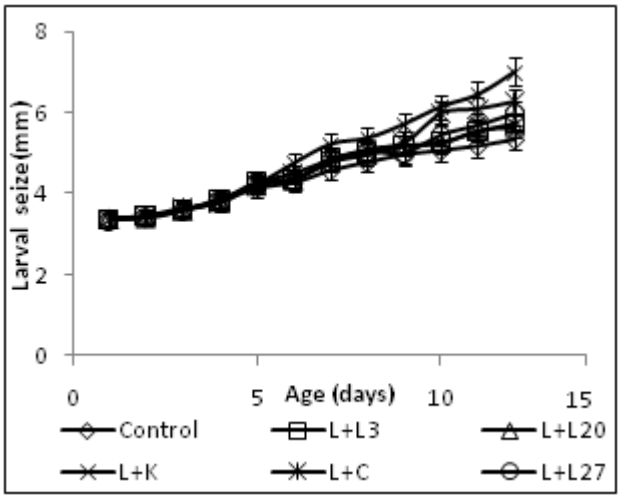


Figure 1. Overview of the patterns obtained after PCR agarose gel electrophoresis (1.5%), Lane 1 *Candida famata* (C), Lane 2 *Leuconostoc mésenteroides* (L27), Lane 3 *Lactobacillus paraplantarum* (L20), Lane 4 *Lactobacillus plantarum* (L3) , M : molecular size marker (100 bp to 1500 bp). T: negative control.







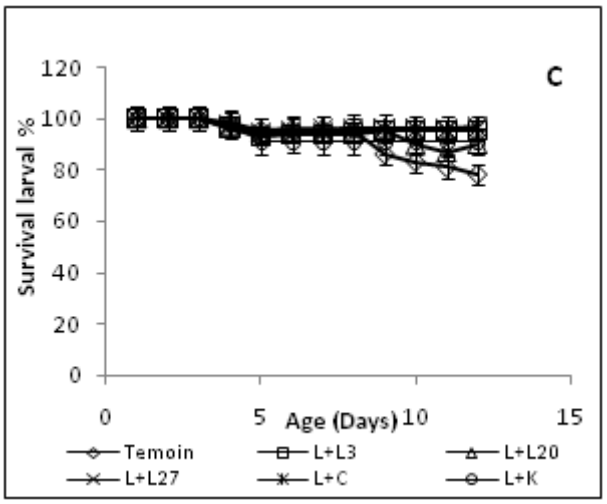


Figure 2: A. Survival of larval *Dicentrarchus labrax* at the end of treatment (Day 12), figure 2.B and fig.2.C: percentage of daily survival of larvae in the presence and absence of pathogenic L: larval *Dicentrarchus labrax*; V: *Vibrio alginolyticus*; L3: *Lactobacillus plantarum*; L20: *Lactobacillus paraplantarum*; L27: *Leuconostoc mesenteroides*; C: *Candida famta* and K : L3 + L20 + L27.

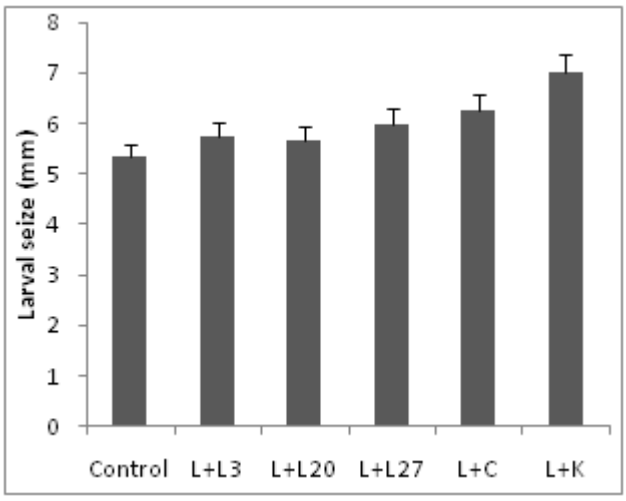


Figure 3: Linear growth of *Dicentrarchus labrax* larvae (day 1 to day 12); B: Linear growth of larval *Dicentrarchus labrax* at the end of treatment (Day 12); L: larva Dicentrarchus *labrax*; V: *Vibrio alginolyticus*; L3: *Lactobacillus plantarum*; L20: *Lactobacillus paraplantarum*; L27: *Leuconostoc mesenteroides*; C: *Candida famta* and K : L3 + L20 + L27. Mean ± error deviation (n = 30)

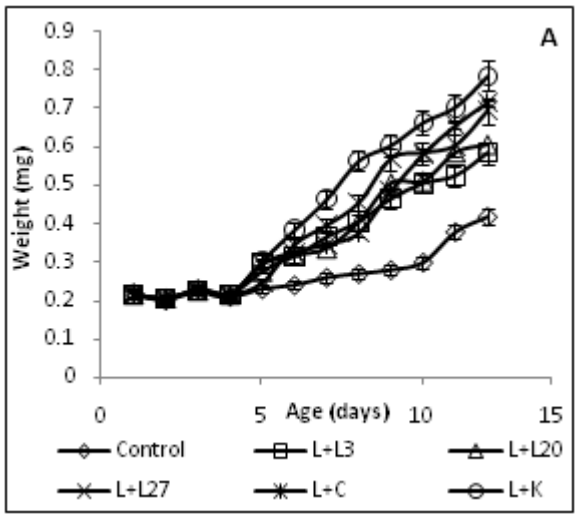


Figure 4 (A): Daily weight growth of *Dicentrarchus labrax* larvae (day 1 to day 12);

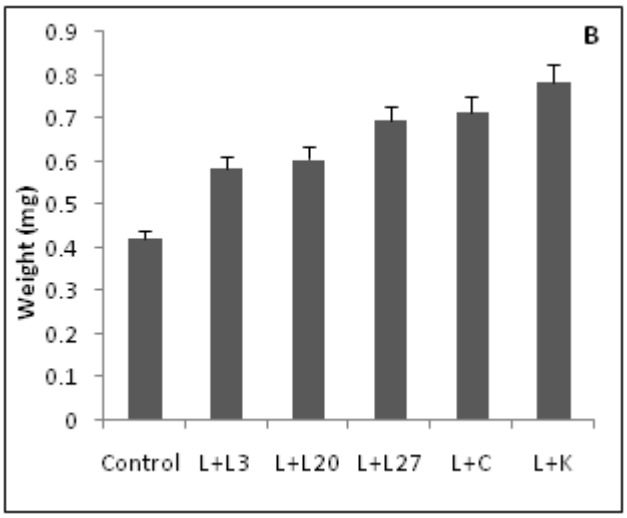


Figure 4 (B): Growth of larval weight of *Dicentrarchus labrax* at the end of treatment (Day 12); L: larval; V: *Vibrio alginolyticus*; L3: *Lactobacillus plantarum*; L20: *Lactobacillus paraplantarum*; L27: *Leuconostoc mesenteroides*; C: *Candida famta* and K: L3 + L20 + L27. Mean ± error deviation (n = 30)