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# Effect of Enriched *Artemia parthenogenetica* with Probiotic (*Bacillus spp.*) on Growth, Survival, Fecal Production and Nitrogenous Excretion in Rainbow Trout (*Oncorhynchus mykiss*) Larvae

**Jamali H[1*], Tafi AA[1], Jafaryan H[2] and Patimar R[2]**

[1]*Department of Fishery, Urmia University, Urmia, Iran*

[2]*Department of Fishery, Gonbad University, Gonbad, Iran*

[*]**Corresponding author:** Hadi Jamali, Department of Fishery, Urmia University, Urmia, Iran, Tel: 021-66568883; E-mail: saeed.jamali11@gmail.com

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## Abstract

This study was carried out to evaluate the effect of Probiotic (*Bacillus spp.)* on growth and survival rate and nitrogenous excretion in Rainbow Trout (*Oncorhynchus mykiss*) larvae. Rainbow Trout larvae were fed on *Artemia parthenogenetica nauplii* enriched at suspension of five probiotic bacilli at doses of 0, $1\times10^8$, $2\times10^8$ and $3\times10^8$ (CFU $l^{-1}$).The feeding level was 30 percent of total biomass per day. The daily ration was divided into four feeding portions. The end all the trout larvae were sampled for biometry and growth determination. Feces were collected twice a day by pipetting and ammonia and urea contents in each tank were determined. Results showed that, with the inoculating of probiotic Bacilli in suspension of broth, trout larvae survival and growth parameters generally increased. Fish larvae fed with enriched *Artemia nauplii* at dose of $2\times10^8$ CFU $l^{-1}$ in suspension of broth had significantly higher survival and growth parameters than the control group ($p<0.05$). Ammonia excretion, urea excretion and fecal production decreased in experimental treatments ($p<0.05$). Then, our results confirm the potential of probiotic bacillus had promoted effects on enhancement of survival rate and growth performance in experimental treatments and probiotics can reduce ammonia and urea excretion in Rainbow trout larvae.

**Keywords:** Bacillus; Rainbow trout larvae; Bio-enrichment; *Artemia parthenogenetica*; Ammonia

## Introduction

Rainbow trout culture is economically important in Iran and bacterial infectious disease in trout farming seems to be the major reason for decreasing the production level in some farms. Success and failure of fish culture programs are determined by early life stage conditions [1]. On the other hand, the occurrence of sub-clinical infections under farming conditions probably lead into reduced growth and increased mortality [2]. Probiotics are a cultured product or live microbial feed supplement, which beneficially affects the host by improving its intestinal balance and health of the host [3]. Most studies with probiotics conducted to date in fish have been undertaken with microbial strains isolated and selected from aquatic environments. There are a wide range of microalgae (*Tetraselmis*), yeast (*Debaryomyces, Phaffia* and *Saccharomyces*), gram positive (*Bacillus, Lactococcus, Micrococcus, Carnobacterium*, *Enterococcus, Lactobacillus, Streptcoccus, Weisslla*) and gram negative bacteria (*Aeromonas*, *Alteromonas, Photorhodobacterium, Pseudomonas* and *Vibrio*) that have been evaluated as a probiotic in aquaculture [4]. Probiotics in aquaculture have been shown to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of host species and enhancement of nutrition of host species through the production of supplemental digestive enzymes [5]. Because Bacillus bacteria secrete many exoenzymes [6], these bacteria have been used widely as putative probiotics. Some works have been done to evaluate competitive exclusion of potential probiotics on rainbow trout [7-9].

| Composition | *A.parthenogenetica* | *O.mykiss* |
|---|---|---|
| Dry matter | 11.76 | 17.04 |
| Crude protein | 39.09 | 72.05 |
| Crude lipid | 17.86 | 12.88 |
| Ash | 10.25 | 11.22 |
| Gross energy | 4592 | 4480 |

Table 1: Body chemical composition (g $g^{-1}$ d.W.) and energy content (kJ $g^{-1}$ d.W.) of *Oncorhynchus mykiss* larvae and *Artemia parthenogenetica* at beginning of the experiment.

Stimulation of immune system in rainbow trout with several candidate probiotics has also been evaluated by some researchers [7,10-12]. The present study examined the effects of probiotic *Bacillus* spp. on growth and survival in *Oncorhynchus mykiss* larvae, when the *Bacillus* spp. were bioencapsulated within *Artemia parthenogenetica.*

## Material and Methods

### Preparing of probiotic Bacillus

The probiotic Bacillus was prepared from the commercial product Protexin aquatic (Iran-Nikotak), which is a blend of five Bacillus species. The blend of probiotic Bacilli (*Bacillus licheniformis, B. subtilis, B. polymixa, B. laterosporus* and *B. circulans*) from suspension of spores with special media were provided. Three concentrations of bacterial suspensions, $1\times10^8$, $2\times10^8$ and $3\times10^8$

bacteria per liter (CFU $l^{-1}$) were provided by Protexin Co. and the colony forming unit (CFU) of probiotic Bacilli were tested by microbial culture in Tryptic Soy Agar (TSA) [13].

### *Artemia parthenogenetica* removal and bioencapsulation

The *Artemia parthenogenetica* had been collected from the Lake Maharloo. The *Artemia parthenogenetica* were bioencapsulated in three doses of bacterial suspensions for 10 h at 29 ± 1ºC, in glass con with 1 liter of seawater (30 $gl^{-1}$ salinity) at a density of 2.0 g $l^{-1}$ with constant illumination and oxygenated through by setting air pump [14]. The bioencapsulated *nauplii* were used as a vector to carry probiotic bacillus to digestive system of *Oncorhynchus mykiss* larvae. The *Artemia parthenogenetica* at a density of 2 g live *Artemia* $litter^{-1}$ was held in a broth suspension with *Bacillus licheniformis, B. subtilis, B. polymixa, B. laterosporus* and *B. circulans* at densities of $1\times10^8$, $2\times10^8$ and $3\times10^8$ bacteria per liter for 10 hours.

### Experimental design

This experiment was conducted in a completely randomized design with four treatments (three probiotic levels and a control), and three replicates per treatment for a total of twelve fiberglass tanks (each with a capacity of 10 liters). Larvae of *Oncorhynchus mykiss* (initial weight: 176 mg) were obtained from Hatchery of Zamiry center, Golestan, Iran. The density of fish larvae in per tank were 40 fish. Rainbow Trout larvae in control and experimental treatments were fed 30 percent of their body weight for 4 times a day (6.00, 12.00, 18.00 and 22.00).The control treatment was fed unbioencapsulated *Artemia parthenogenetica*. Water quality parameters of input water to rearing system were monitored each week throughout the experimental. The water temperature was 14 to 16°C, pH was 7.85 ± 0.26 and water oxygen level was maintained above 7.65 ± 0.55 mg $l^{-1}$ during the experiment an electrical air pump.

Feces were collected twice a day by pipetting, oven dried at 70°C, weighed, at the beginning and end of which period, water was sampled, and ammonia and urea contents in each tank were determined by the method of Chaney and Marbach [15] and converted into energy by multiplying by 24.83 kJ $g^{-1}$ for ammonia and 23.03 kJ $g^{-1}$ for urea [16]. Potential loss of nitrogenous compounds through bacterial action or diffusion was quantified by setting controls without fish and the value was used to adjust determined nitrogenous excretion.

### Sample collection

The fish were weighed individually at the beginning and at the end of the experiment. Before distributing fish to the experimental tanks (in the beginning of exogenous feeding), 10 fish were sampled from the holding tank for biometry. In the termination of experiment, 30 larvae from each tank were sampled and the final weight and length of body were measured.

### Chemical analysis

Moisture contents of fish and feed were determined after oven-drying to constant weight at 105°C for diet and 70°C for fish [17]. Protein contents of fish and *Artemia* were measured by the Kjeldahl method using an auto Kjeldahl system. Lipid contents of fish and *Artemia* were measured by ether extraction. Ash contents of fish and *Artemia* were determined by a muffle furnace at 550°C for 8-10 h. Gross energy contents of fish and *Artemia* were measured by oxygen calorimetric bomb [18]. For each variable, at least duplicate samples were determined and the mean of duplicate determination was taken as the result when the relative deviation was less than 2%.

| | Dry matter | Crude protein | Crude lipid | Ash | Gross energy |
|---|---|---|---|---|---|
| Control | 17.75 ± 1.00$^b$ | 72.90 ± 0.31$^c$ | 13.64 ± 0.58$^a$ | 12.82 ± 0.52$^a$ | 4565 ± 46.59$^a$ |
| $1\times10^8$ | 19.27 ± 1.59$^b$ | 74.05 ± 0.21$^b$ | 13.45 ± 0.23$^a$ | 12.18 ± 0.94$^a$ | 4483 ± 76.86$^a$ |
| $2\times10^8$ | 19.86 ± 2.94$^b$ | 75.39 ± 0.39$^a$ | 12.49 ± 0.32$^b$ | 11.98 ± 1.08$^a$ | 4402 ± 56.42$^b$ |
| $3\times10^8$ | 23.57 ± 1.40$^a$ | 75.66 ± 0.46$^a$ | 11.94 ± 0.37$^b$ | 11.68 ± 0.61$^a$ | 4389 ± 29.87$^b$ |

Table 2: Body chemical composition (g $g^{-1}$ d.W.) and energy content (kJ $g^{-1}$ d.W.) of larvae Rainbow Trout at different Treatment. (Values (mean ± SD) in the same row with different letters are significantly different ($p<0.05$)).

### Statistical analysis

All data are presented as Mean ± Standard deviation (SD). Data was transformed where necessary and statistical analysis was conducted using SPSS statistics version 15 for windows (SPSS Inc., Chicago, Il, USA) and accepted at the $p<0.05$ level. Data were analyzed using a one-way ANOVA. Significant differences between control and treatment groups were determined using post-hoc Fisher's Duncan test.

## Results

### Body chemical composition

The contents of Dry matter, protein, lipid, ash and energy in the body of Rainbow Trout at different treatments are listed in Table 2. A positive correlation to treatments was observed for the protein and Dry matter, and a negative correlation for lipid, ash contents and energy contents.

| Y | a | b | n | $R^2$ | P |
|---|---|---|---|---|---|
| D.M | 17.4 | 0.18 | 12 | 0.9 | <0.01 |
| C.P | 73.1 | 0.096 | 12 | 0.9 | <0.01 |
| C.L | 13.8 | -0.06 | 12 | 1 | <0.01 |
| Ash | 12.7 | -0.04 | 12 | 0.9 | <0.01 |
| G.E | 4551 | -6.1 | 12 | 0.9 | <0.01 |

**Table 3:** Coefficients of the regression equation (Y=a+bRL) relating to Dry matter (g $g^{-1}$ d.W.), Crude protein (g $g^{-1}$ d.W.), Crude lipid (g $g^{-1}$ d.W.), Ash (g $g^{-1}$ d.W.) and Gross energy (kJ $g^{-1}$ d.W.) to Probiotic cells for larvae Rainbow Trout.

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## Nitrogenous excretion and fecal production

Ammonia excretion, Urea excretion and fecal production decreased in experimental treatments when larvae were fed by bioencapsulated *Artemia parthenogenetica* (Table 5). The higher rate of Nitrogenous excretion and fecal production were observed in control treatment ($p<0.05$). One-way ANOVA showed that ammonia excretion, Urea excretion and fecal production was affected significantly by probiotic *bacillus* and least rate observed in $2\times10^8$ treatment (ammonia excretion: 51.32, $p<0.05$; Urea excretion: 12.83, $p<0.05$; fecal production: 2.23, $p<0.05$).

## Final weight, Survival, Specific growth rate and feed conversion efficiency

Final weight, Survival, Specific Growth Rate in Wet Weight (SGRw), Dry Weight (SGRd), Protein (SGRp) and Energy (SGRe) of larvae Rainbow Trout increased in experimental treatments (Figure 1, 2 and Table 4). ANOVA showed that Final weight, Survival, Specific growth rate was affected significantly by probiotic bacillus and higher rate observed in $2\times10^8$ and $3\times10^8$ (CFU $l^{-1}$) treatment (SGRw: 6.35, $p<0.05$; SGRd: 7.57, $p<0.05$; SGRp: 6.95, $p<0.05$; SGRe: 6.23, $p<0.05$).

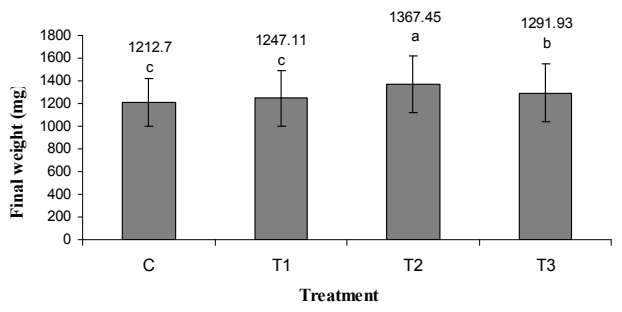


Figure 1: Final weight of Rainbow Trout larvae in experimental treatments (C, Control; T1, $1\times10^8$; T2, $2\times10^8$; T3, $3\times10^8$ $CFUL^{-1}$).

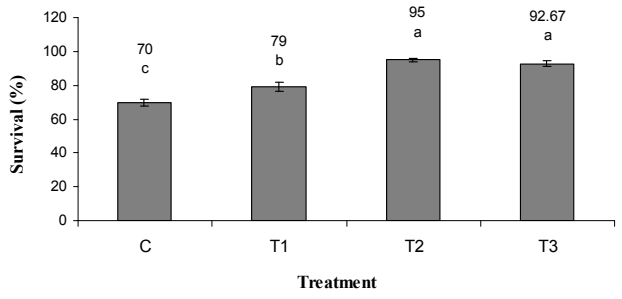


Figure 2: Survival of Rainbow Trout larvae in experimental treatments (C, Control; T1, $1\times10^8$; T2, $2\times10^8$; T3, $3\times10^8$ $CFUL^{-1}$).

Feed conversion efficiency in wet weight, dry weight, protein and energy of larvae Rainbow Trout also increased in experimental treatments and were highest at $2\times10^8$ and $3\times10^8$ (CFU $l^{-1}$) treatment (Table 4).

## Discussion

Final weight, Survival and Specific growth rate was affected significantly by probiotic *bacillus* and higher rate observed in $2\times10^8$ $CFUL^{-1}$ treatment. The advantages of using probiotics in fish aquaculture were recently reviewed by Nayak [19] and Qi et al. [20]. The main beneficial effects of probiotic use in fish aquaculture are growth performance improvement [21,22] and disease control through immunity enhancement [19,20,23] and pathogens exclusion [20]. The bacteria could also have improved digestive activity via synthesis of vitamins and cofactors or via enzymatic improvement [4]. Among probiotics, *Bacillus* strains have become more and more popular and widely used in fish aquaculture [19,20,24]. In the use of live prey, *Artemia nauplii* are widely recognized as the best natural storable live feed available and are extensively used in marine finfish and crustacean hatcheries throughout the world because of their nutritional and operational advantages [25,26] and have been used as a vector for the carrying of different materials, including probiotics [27].

| | Treatment | | | |
|---|---|---|---|---|
| | **Control** | **$1\times10^8$** | **$2\times10^8$** | **$3\times10^8$** |
| [1]SGRw | $5.98 \pm 0.57^c$ | $6.05 \pm 0.62^{bc}$ | $6.35 \pm 0.57^a$ | $6.17 \pm 0.62^b$ |
| [2]SGRp | $6.47 \pm 0.57^c$ | $6.60 \pm 0.63^c$ | $6.95 \pm 0.56^a$ | $6.78 \pm 0.57^b$ |
| [3]SGRd | $6.50 \pm 0.57^d$ | $6.83 \pm 0.62^c$ | $7.22 \pm 0.56^b$ | $7.57 \pm 0.57^a$ |
| [4]SGRe | $5.97 \pm 0.57^b$ | $5.99 \pm 0.63^b$ | $6.23 \pm 0.57^a$ | $6.04 \pm 0.57^b$ |
| [5]FCEw | $71.63 \pm 2.27^c$ | $73.66 \pm 4.58b^c$ | $80.77 \pm 4.76^a$ | $76.31 \pm 5.17^b$ |
| [6]FCEp | $117.03 \pm 12.90^c$ | $122.98 \pm 17.63^c$ | $139.22 \pm 18.48^a$ | $131.14 \pm 19.37^b$ |
| [7]FCEd | $94.81 \pm 8.53^d$ | $107.41 \pm 8.53^c$ | $123.11 \pm 14.94^b$ | $139.65 \pm 20.41^a$ |
| [8]FCEe | $61.58 \pm 4.23^b$ | $62.98 \pm 3.98^b$ | $67.06 \pm 4.15^a$ | $62.58 \pm 4.50^b$ |
| [9]HIS | $1.24 \pm 0.34^a$ | $1.43 \pm 0.44^a$ | $1.36 \pm 0.49^a$ | $1.35 \pm 0.45^a$ |
| [10]VIS | $9.69 \pm 1.85^a$ | $9.42 \pm 1.54^{ab}$ | $9.38 \pm 1.59^{ab}$ | $8.74 \pm 1.75^b$ |

Table 4: Specific growth rate in wet weight (SGRw, % day−1), dry weight (SGRd, % day−1), protein (SGRp, % day−1) and energy (SGRe, % day−1) and feed conversation efficiency in wet weight (FCEw, %), dry weight (FCEd, %), protein (FCEp, %) and energy (FCEe, %) of larvae Rainbow Trout at different Treatment (% per day). (Values (mean ± SD) in the same row with different letters are significantly different ($p<0.05$)).

[1]SGRw=100×[ln(final body weight)−ln(initial body weight)] / days of the experiment.

[2]SGRp=100×[ln(final body weight×final protein content)−ln(initial body weight×initial protein content)] / days of the experiment.

[3]SGRd=100×[ln(final body weight×final dry matter content) −ln(initial body weight×initial dry matter content)] / days of the experiment.

[4]SGRe=100×[ln(final body weight×final energy content)−ln(initial body weight×initial energy content)] / days of the experiment.

[5]FCEw=100×(final body weight−initial body weight) / feed intake.

[6]FCEp=100×[(final body weight×final protein content)−(initial body weight×initial protein content)] / (feed intake×protein content).

[7]FCEd=100×[(final body weight×final dry matter content)−(initial body weight×initial dry matter content)] / (feed intake×dry matter content).

[8]FCEe=100×[(final body weight×final energy content)−(initial body weight×initial energy content)] / (feed intake×energy content).

[9]Hepatosomatic index (HSI) = (Liver weigh/body weight) ×100.

[10]Viserosomatic index (VSI) = (Viseral weight /body weight) ×100.

| | Treatment | | | |
|---|---|---|---|---|
| | **C** | **1×10$^8$** | **2×10$^8$** | **3×108** |
| CN(Consumed nitrogen, mg N kg$^-$ day$^-$) | 778 | 778 | 778 | 778 |
| EI(Energy intake, KJ Kg$^-$ day$^-$) | 2388 | 2388 | 2388 | 2388 |
| Ammonia-nitrogen(mg NH$^3$-N Kg$^-$ day$^-$) | 74.32 ± 2.28$^a$ | 67.10 ± 5.1$^{ab}$ | 51.32 ± 0.00$^b$ | 54.91 ± 4.3$^b$ |
| Urea-nitrogen(mg Urea-N Kg$^-$ day$^-$ | 18.58 ± 0.57$^a$ | 16.78 ± 2.75$^{ab}$ | 12.83 ± 0.00$^b$ | 13.73 ± 1.07$^b$ |
| Total nitrogen(mg N Kg$^-$ day$^-$) | 92.9 ± 2.5$^a$ | 83.88 ± 8.01$^{ab}$ | 64.15 ± 0.00$^b$ | 68.63 ± 5.39$^b$ |
| Waste Energy- Ammonia | 1845 ± 56.74$^a$ | 1666 ± 27.39$^{ab}$ | 1247 ± 15.10$^b$ | 1363 ± 10.71$^b$ |
| Waste Energy- Urea | 427.91 ± 13.15$^a$ | 386.35 ± 63.53$^{ab}$ | 295.48 ± 11.10$^b$ | 316.12 ± 24.13$^b$ |
| Retained fecal | 3.46 ± 0.06$^a$ | 2.87 ± 0.03$^b$ | 2.23 ± 0.04$^d$ | 2.67 ± 0.04$^c$ |
| Total Waste Energy (%) | 89.01$^a$ | 80.35a$^b$ | 60.40$^b$ | 65.75$^b$ |

**Table 5:** Nitrogenous excretion (u, mg $g^{-1}$ $d^{-1}$) and faecal production (f, mg $g^{-1}$ $d^{-1}$ ) of larvae Rainbow Trout at different treatments. (Values (mean ± SD) in the same row with different letters are significantly different ($p<0.05$)).

In this study, *Artemia parthenogenetica* were used as a vector to carry the probiotic *Bacillus* to the digestive tract of Rainbow Trout larvae. The probiotics in this experiment promoted the feeding and growth parameters in Rainbow Trout larvae in experimental treatments in comparison to control treatment. Effects of commercial probiotic on aquaculture has been investigated by researchers, and some of this research has not shown any positive effects on growth parameters or survival rate or any promising result on the cultural condition. For instance, Shariff et al. [28] found that treatment of *Penaeus monodon* with a commercial Bacillus probiotic did not significantly increase survival. These results disagree with our findings.

Results of all the probiotic treatments showed better growth performance and feeding parameters than the control. The beneficial effect of probiotic *Bacillus* sp. on the feeding efficiency of Rainbow Trout larvae was completely observed. The results indicated that the probiotic *bacillus* had significantly effects on the growth and feeding parameters in experimental treatments. The better body weight and SGR for weight, protein and energy were obtained in experimental treatments also better Feed conversion efficiency in wet weight, dry weight, protein and energy of larvae Rainbow Trout were obtained in experimental treatments. Similar finding were observed by Gatesoupe [29] in using *Bacillus toyoi* on turbot (*Scophthalmus maximus*), Swain et al. [30] in Indian carps that improved the growth factors and feeding performance and Ghosh et al. [31] on the Rohu. Bagheri et al. [32] found that supplementation of trout starter diet with the proper density of commercial Bacillus probiotic could be beneficial for growth and survival of rainbow trout fry. This finding agrees with our results. Ghosh et al. [1] indicated that the *B.circulans*, *B. subtilis* and B. pamilus, isolated from the gut of Rohu, have extracellular protease, amylase, and cellulose and play an important role in the nutrition of Rohu fingerlings. The photosynthetic bacteria and *Bacillus* sp. (isolated from the pond of common carp) was used in diet of common carp (*Cyprinus carpio*) by Yanbo and Zirong [33]. The results indicated that this probiotics increased growth parameters and digestive enzyme activities. Gram-positive bacteria, including members of the genus Bacillus, secret a wide range of exoenzymes [6], which might have supplied digestive enzymes and certain essential nutrients to promote better growth. *Bacillus subtilis* and *B. leicheniformis* can break down proteins and carbohydrates [34,35]. So it can be suggested that administration of Bacillus bacteria to trout fry results in enhanced digestion of food and improved growth [32].

There were significant ($p<0.05$) differences in dry matter, crude protein and crude lipid of *O. mykiss* between experimental treatments and control. Crude lipid and Gross energy were decreased in experimental treatments while crude protein was increased. Results showed that crude lipid and moisture of Rainbow Trout decreased in experimental treatments after feeding with probiotic while crude protein were increased [32,36]. El-dakar [37] reported that use of a dietary probiotic/prebiotic on Spinefoot rabbitfish (*Siganus rivulatus*) Although significant differences in moisture level and crude protein proportion are apparent among treatments, they do not follow a trend and thus suggest that variations among results are probably due to

inherent variation associated with using wild undomesticated gene stock in research. However, results suggest a positive correlation between NEM supplementation and lipid concentration of the carcass.

Ammonia excretion, urea excretion and fecal production decreased in experimental treatments when larvae were fed by bioencapsulated *Artemia parthenogenetica*. Faramarzi [38] showed that ammonia and urea excretion were decreased in experimental treatments by inclusion of the probiotic bacilli in comparison with control treatments in *Acipenser percicus* larvae. Ammonia excretion rates are directly related to dietary nitrogen and protein intake in teleosts [39,40]. Increasing the dietary level of non-protein, digestible energy increases nitrogen retention by decreasing nitrogen losses [41,42]. The decrease in NH3-N concentration in this study appears to be the result of increased incorporation of ammonia into microbial protein, and may be the direct result of stimulated microbial activity [43]. Lashkarboloki [44] showed that enrichment of daphnia with *Saccharomyces cerevisiae* extract (Amax) probiotics can reduce ammonia and urea excretion and also cause increase protein retention in *Acipenser percicus* larvae body notably. The results of these studies showed that the blend of bacterial probiotics can increase the growth and feeding efficiency and also decrease ammonia and urea excretion in fish of experimental treatments in comparison of control.

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