

Improvement of water quality with probiotics inclusion during simulated transport of yellowfin seabream (*Acanthopagrus latus*) larvae

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Research Article

Keywords: Yellowfin seabream, Probiotics, Transportation stress, Bacteria, Water quality

Posted Date: November 7th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-2228929/v1>

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Abstract

The effects of using a commercial probiotic mixture (PM) (*Bacillus licheniformis*, *Bacillus subtilis*, *Pedococcus acidilactici* and *Lactobacillus acidophilus* with a total count of 10^7 CFU/g) on water quality, bacterial population and the survival of *Acanthopagrus latus* larvae were evaluated during a 24-hour simulated transportation experiment. The one-day-old larvae were transported using purified seawater (as control) and purified seawater supplemented with 3 g of PM (as PM48 treatment). For the PM48, 3 g of PM was added to 1 L of purified seawater and after 30 min, mixed with 200L of purified seawater 48h prior to the main experiment. The obtained results showed that the use of probiotics led to an increase in larval survival up to 93.3%. Statistically, difference was found between control and PM48 at the initial sampling in terms of NO₂. The pH of the control treatment showed a significant decrease at the end of the experiment. The investigation of a total number of bacteria and the total number of *Vibrio* spp. in water and fish larvae showed that within 48 hours, the probiotics became the dominated population in PM48 treatment. After 24 hours, water samples and larvae of control treatment showed a significant increase in bacterial load. In general, the obtained results showed that the use of probiotics played a significant role in maintaining water quality chemically and bacterially and caused more survival of larvae during transportation.

1. Introduction

There has been a growing demand for aquaculture products as generally known as healthy food with growing human knowledge and population. According to food and agriculture organization (FAO), further expansion of aquaculture by 32% accounting for 109 million tons of global aquaculture production in 2030 (FAO, 2020). However, natural resources have been under over-exploitation leading to a steadily decrease global fisheries production. Sustainable development of aquaculture can significantly reduce the pressure on the natural stocks of marine fish which requires aquaculture to diversify cultured species and use the marine environment for large-scale fish production.

Despite the progress made in mariculture, some challenging issues such as low survival rate, growth, and low quality at the larval stage, which is the same for most marine fish species. For many cultivated species, larval survival on an industrial scale it is usually between 10 and 15 percent and this is the index improper breeding conditions (Vadstein et al., 1993; Vadstein et al., 2018). Several techniques have been studied to tackle these issues using reticulating aquaculture systems (RAS), the addition of probiotics into rearing water and diet (live and manufactured aquafeeds) (Benetti et al., 2008; Newaj-Fyzul et al., 2014) in order to control water quality and reduce the population of opportunistic bacteria, UV and O₃ (Attramadal et al., 2012; Vadstein et al., 2018) treatments.

The key limiting factor in the transportation of fish is density in the transport packages, which reduces the quality of water due to the accumulation of excreted metabolites of fish (Lim et al., 2003). Suggested methods to reduce ammonia and other water chemical factors such as NO₂ and NH₄ during fish transportation include lowering the temperature during transportation, starving the fish before

transportation, and using anesthetics during transportation (Turner & Bower, 1982; Lim et al., 2003). On the other hand, these strategies can be stressful, and after transportation, handling stress would cause double stress resulting in high mortality. These strategies seem applicable for juveniles and adult fish and are not suitable for small one-day-old larvae of marine fish. The addition of probiotics into water suggests a less invasive strategy to maintain water quality and ensure higher survival rates.

The main chemical reactions that may occur during fish transportation include consumption of dissolved oxygen resulting in dropping oxygen level, the accumulation of carbon dioxide (CO₂) due to respiration, decreasing pH value due to the increase of CO₂, and increasing ammonia due to excretion. According to the literature, as the transportation time increases, the amount of ammonia in the water increases, resulting in the increase of opportunistic bacterial communities and depletion of oxygen (Hossain et al., 2021). High concentrations of CO₂ can be problematic for fish by disrupting the fish's oxygen supply system. The gaseous CO₂ formed in the water becomes a weak acid and the non-toxic bicarbonate ions enter the fish blood which may cause harmful consequences by acidifying fish blood (Berka, 1986; Bhuiyan et al., 2022). Extensive research studies have reported a wide range beneficial effects of using probiotics such as stimulation of the innate immune system, controlling opportunistic bacterial communities and disease, improving water quality, improving larval survival, protecting fish against *Vibrio* spp. is, reducing larval mortality after transportation and stress (Vine et al., 2006; Benetti et al., 2008; Gomes et al., 2009; Zink et al., 2011; Verschuere et al., 2000; Sorroza et al., 2012; Touraki et al., 2012).

Yellowfin seabream (*Acanthopagrus latus*) is a carnivorous species of the Sparidae family with commercial importance in the coastal countries of the Persian Gulf and the Sea of Oman. This fish is an appropriate candidate for aquaculture due to its good economic value, easy reproduction and spawning in captivity, high resistance to salinity fluctuations and environmental temperature (Karimi et al., 2012) and higher larval survival compared to other cultivated species in the region. Many studies have been conducted on the reproduction and rearing of *A. latus* larvae, however, no study is available regarding its larval transportation. Sometimes research breeding centers need to transport one-day-old larvae over different distances, and considering the fact that maintaining the survival and quality of larvae during transportation has a direct relationship with chemical factors of carrier water supply (DO, NH₄, CO₂) and bacterial load, it is therefore important appropriate methods are developed to maintain water quality and ensure maximum larval survival. Thus, the present study was carried out to evaluate the potential effects of using a mixture of commercial probiotics to maintain water quality while transporting *A. latus* larvae.

2. Material And Method

The Bio-Clar® product containing five different bacteria including *Bacillus licheniformis*, *Bacillus subtilis*, *Pedococcus acidilactici* and *Lactobacillus acidophilus* in the form of powder (10⁷ CFU/g) was purchased from Biodep ® Company.

2.1. Larvae Supply And Experiment Design

This experiment was conducted to simulate the transportation of one-day-old *A. latus* larvae in Marine Fish Reproduction Center (Bandar Imam, Iran) according to a previous study by Zink et al. 2011. The larvae used in this experiment were obtained from the same center by naturally spawning broodstocks at ambient temperature. Briefly, the fertilized eggs were separated and incubated in 300 L fiberglass tanks at 24°C for 24h. The volumetric method was used to determine the density of newly hatched larvae. The experiment was designed with two treatments in triplicate including a control treatment with no supplementation and PM48 where carrier water was supplied with a mixture of probiotics. For PM48 treatment, according to the manufacturer's instructions, 3 g of Bio-Clar® was added to 1 L of seawater and after 30 minutes mixed with 200 L purified (10 µm filtration) and distilled (UV treatment) seawater 48h prior to transportation.

2.2. Transportation And Water Quality Parameters

The larvae were transported using polyethylene bags with stock density of 900 larvae in each bag containing 10 L of control purified seawater (control) or purified seawater containing PM as described above (PM48). The larvae were allowed in the bags for 24h to simulate a 24h-transportation condition. Prior to transportation, water quality parameters including pH, NO₂, NH₄, NH₃, and dissolved oxygen, salinity, temperature and bacterial load were measured in both treatments. NH₄ and NO₂ were measured based on ASTM D1426 and Standard Method 4500-NO₂-B methods respectively. Then the remaining bag's capacity was filled with O₂ gas and secured with another bag layer to avoid any possible damage to the bags and leak of O₂ and water and put in a Styrofoam cooler box. The boxes were kept at room temperature (26°C) for 24h and were shaken every once in a while for 10 seconds to simulate transportation conditions. After 24h, the same parameters were measured in addition to measuring larval survival rate. Bacterial load was measured in both water and larvae in which after measuring survival rate, all larvae were collected using a 40 µm mesh size net and used for counting the bacterial colonies.

2.3. Bacterial Count

To count the total number of bacteria and the total number of *Vibrio* spp. in the water, serial dilutions were first prepared from the water sample, and then 100 µl of each dilution was added to 10 cm plates containing tryptic soy agar medium TSA (adjusted with the desired salinity). To count the total *Vibrio* spp., TCBS medium (adjusted with the desired salinity) was used. The plates were incubated at 37°C for 24 to 48 h. After that, plates with 30 and 300 colonies were counted and multiplied by the dilution factor and results were reported as the number of bacteria in ml of water (CFU/ml).

For the larvae, first the sampled larvae were washed with sterile seawater, homogenized, and serially diluted in PBS. In order to count the total number of bacteria, 100 µl of each dilution was inoculated in a plate containing TSA medium (adjusted to the salinity of the sampled area). In order to count the total

number of *Vibrio*, 100 µl of each dilution was inoculated with a sterile sampler on TCBS (adjusted to the salinity of the sampled area. The plates were then incubated at 37°C for 24 to 48 h, then the plates with 30 to 300 colonies were counted, and the results were reported as the number of bacteria in 1 gram of larvae (CFU/g).

2.4. Statistical Analysis

All measurements were carried out in triplicate and results are reported as mean \pm SD. The initial and final water quality parameters were analyzed using One-Sample T-Test followed by Independent-Sample T Test to compare means. Total bacterial count and the number of *Vibrio* spp. were analyzed using One-way ANOVA followed by Tukey HSD test to indicate significant differences at 0.05 confidence level. The data was processed and analyzed using SPSS 18.0 software (version 18.0 for Windows, Chicago, IL, USA).

3. Results

3.1. Survival rate

The survival rate in both control and PM48 treatments was 85% however PM48 showed a significantly higher survival rate when compared to control ($P = 0.004$) (Table 1).

Table 1

Physiochemical parameters of water in shipping bags and survival of yellowtail seabream larvae during a simulated shipping trial for 24h.

Time point	Temperature (°C)	Salinity (ppt)	DO (mg/L)	pH	No ₂ ⁻ (mg/L)	NH ₄ (mg/L)	NH ₃ (mg/L)	SR (%)
Initial-Control	23.30 \pm 1.00	40.70 \pm 1.00	6.55 \pm 0.20	8.54 \pm 0.20*	£0.82 \pm 0.20	0.20 \pm 0.20	0.018 \pm 0.01	-
Initial-PM48	23.80 \pm 1.00	41.00 \pm 1.00	6.50 \pm 0.20	8.47 \pm 0.20	£0.04 \pm 0.01	0.19 \pm 0.20	0.17 \pm 0.00	-
Final-Control	24.16 \pm 0.28	40.60 \pm 0.20	7.24 \pm 0.44	7.93 \pm 0.18*	0.45 \pm 0.31	0.21 \pm 0.00	0.02 \pm 0.00	£89.85 \pm 0.67
Final-PM48	24.43 \pm 0.05	40.80 \pm 0.15	8.10 \pm 1.49	8.00 \pm 0.18	0.04 \pm 0.01	0.19 \pm 0.02	0.18 \pm 0.00	£93.30 \pm 0.75
Mean (\pm SD) temperature, salinity, dissolved oxygen (DO), pH, Nitrite (No ₂ ⁻), Ammonia (NH ₃), Ammonium (NH ₄), and Survival rate (SR). Date represent Mean \pm SD of three measurements. Within the same column, values sharing the same symbols are significantly different in terms of independent (left) and pair sample (right) T-test. ($P < 0.05$).								

3.2. Water Quality Parameters

No significant differences were observed between control and PM48 in terms of DO, temperature, salinity, NH₃, NH₄ and pH ($P \geq 0.05$), while in the primary PM48 water sample, the amount of NO₂ was significantly decreased ($P = 0.003$) (Table 1). No significant difference was observed between the initial sample and the final sample of the control treatment after 24 hours in terms of DO, NO₂, NH₃, NH₄, salinity and temperature ($P \geq 0.05$) however pH showed a significant decrease ($P = 0.03$) (Table 1). No significant difference was observed between the initial and final samples of PM48 treatment after 24 hours in terms of DO, NO₂, NH₃, NH₄, pH, salinity and temperature ($P \geq 0.05$) (Table 1). After 24h, the final samples of control and PM48 treatments showed no significant differences in terms of all measured physiochemical parameters.

3.3. Bacterial Count

The total number of bacteria and the total number of *Vibrio* spp. in larvae and water were measured, and the results are represented in Table 2. According to Table 2, a significant difference was observed in the total number of larval bacteria among the treatments ($P \geq 0.05$), so that the total number of bacteria in the control treatment showed an increasing trend after 24 hours from the start of the experiment, and in the PM48 treatment, this amount showed a decreasing trend (Table 2). The total number of *Vibrio* spp. primary larvae showed a significant difference from the control and PM48 groups ($p \geq 0.05$) and in the control and PM48 treatments, the total number of *Vibrio* spp. larvae showed an increasing trend compared to the original sample, but with a significant difference not ($p \geq 0.05$) (Table 2).

Table 2

Total number of bacteria in larvae and total number of *Vibrio* spp. in larvae of yellowtail seabream larvae during a simulated shipping trial for 24h.

Time point	Treatment	Log total bacteria (CFU/g)	Log total <i>Vibrio</i> spp. (CFU/g)
Initial	Control	6.44 ± 0.00003 ^b	3.03 ± 0.079 ^a
Final	Control	6.78 ± 0.00001 ^a	4.33 ± 0.004 ^{bc}
Final	PM48	6.43 ± 0.00003 ^c	4.29 ± 0.004 ^{bc}
Within a column, values sharing the same letter are significantly different ($p < 0.05$). Data represent Mean ± SD of three measurements.			

The total number of water bacteria in the control treatment showed a significant increase after 24 h from the start of the experiment ($P = 0.000$) (Table 3), and the total number of bacteria in the PM48 treatment showed a significant decrease after 24 hours from the start of the experiment ($P = 0.000$) (Table 3). Comparing the total number of water bacteria in the initial and final samples of the control treatment and PM48, a significant difference was observed between the treatments ($P = 0.000$) (Table 3). The total number of *Vibrio* spp. in the water of the control treatment increased significantly after 24 hours from the

start of the experiment ($P = 0.001$) (Table 3), and the total number of *Vibrio* spp. in the PM48 treatment was significantly decreased after 24 hours from the start of the experiment. ($P = 0.002$) (Table 3). In the initial comparison between the control treatment and PM48, the total number of *Vibrio* spp. had a significant difference ($P = 0.02$) (Table 3), also after 24 hours from the start of the experiment, a significant difference was observed between the treatments ($P = 0.000$) (Table 3).

Table 3

Total number of bacteria in water and total number of *Vibrio* spp. in water of yellowtail seabream larvae during a simulated shipping trial for 24h.

Time point	Treatment	Log total bacteria (CFU/l)	Log total <i>Vibrio</i> spp. (CFU/l)
Initial	Control	$£5.38 \pm 0.0003^*$	$£3.44 \pm 0.031^*$
Initial	PM48	$£5.94 \pm 0.0001¥$	$£3.52 \pm 0.025¥$
Final	Control	$§ 5.44 \pm 0.0003^*$	$§ 3.69 \pm 0.017^*$
Final	PM48	$§ 5.78 \pm 0.0001¥$	$§ 3.34 \pm 0.039¥$
Date represent Mean \pm SD of three measurements. Within a column, values sharing the same symbols are significantly different ($p < 0.05$). Different symbols in each column show significant differences between the two groups in terms of independent (left) and pair sample (right) T-test.			

Discussion

This study was conducted to investigate the benefits of using a mixture of probiotics to improve the survival rate, water quality parameters and the amount of bacteria in the transportation water of one-day-old yellowfin *Acanthopagrus latus* larvae. The present results showed a significant difference in larval survival between control and PM48 treatments. Other researchers have reported similar findings (Gomes et al., 2009, *Paracheirodon axelrodi*; Talpur et al. 2013, *Portunus pelagicus*; Tarnecki et al., 2019, *Centropomus undecimalis*) indicating the highest survival rate when probiotics were used. Based on the water quality parameters, it is suggested that adding the mixture of probiotics into the transportation after 48h prior, might help maintaining and improve the physiochemical parameters resulting in minor stress and thus increasing the larvae survival after 24h transportation.

DO level is one of the limiting factors for stocking density of fish for long-distance transportation (Hosseini et al., 2021). The final DO level in both treatments did not show a significant difference; however DO in the PM48 treatment showed an increasing trend. The reason for the increase in the DO levels of both treatments could be due to the use of pure oxygen to fill the bags while the increase in the final DO level in PM48 treatment may be due to the low number of dead larvae compared to the control treatment and the effect of probiotics on reducing larval stress, which results in a decrease in oxygen consumption. Similar to our results, Zink et al (2011) also reported an increase in DO in their probiotic treatment. During the transportation of fish in packages, oxygen is usually not considered a limiting factor because there is

enough oxygen under pressure inside the bags. Lack of oxygen is felt when the density of fish inside the bags is higher than usual, or dead fish become a suitable substrate for the growth of unwanted bacteria and result in lack of oxygen inside the bags (Berka, 1986).

Generally, the amount of ammonia increases during fish transportation due to protein catabolism, bacterial decomposition of organic matter, excretion by fish, and decayed algae (Hossein et al., 2021). In the present experiment, the ammonia level remained constant in PM48 while a slight increase was observed in the control treatment. Considering the fact that 0.1 mg of NH_4 requires merely 4.3 mg of oxygen to oxidize, high concentrations of NH_4 and NH_3 can greatly affect the amount of dissolved oxygen in water (Golombieski et al., 2003).

There was a significant difference in NO_2 level in the initial water samples of control and PM48 treatments (Table 1) and it showed that treatment of transporting water 48h prior reduces the NO_2 . On the other hand, the presence of probiotics in the water maintained the NO_2 level stable during the test period, which could reduce stress in the larvae. The results of Turner & Bower (1982) showed that the use of nitrifying bacteria and different culture media for them is associated with the reduction of nitrite during fish transportation. The results of the study by Talpur et al. (2013) showed that the use of probiotics in the water of *Portunus pelagicus* larva breeding tanks reduced the amount of NO_2 and improved larval survival.

The pH level is a limiting factor for fish during transportation because ammonia and CO_2 are directly related to the pH level of water (Berka, 1986). The pH level in both final samples of the treatments shows a decrease, but the control treatment after 24h from the start of the experiment showed a significant decrease compared to the initial sample (Table 1), consistent with our results other researcher have reported a decreasing trend of pH during fish transportation (Benetti et al., 2007; Colburn et al., 2008; Zink et al., 2011; Stuart et al., 2013; Hossain et al., 2021; Bhuiyan et al., 2022) however contrary to our results Zink et al., 2011 observed a decrease pH in probiotic group.

The decrease in pH in the control treatment indicates the metabolic activities of the larvae and the increase in the number of bacteria in the larvae transfer packages (Clot & Orwicz, 1991; Bhuiyan et al., 2022). There are confined conditions and low efficiency of carbon dioxide removal that lead to higher carbon dioxide level and a decrease in pH level. At high levels of CO_2 and low pH, the oxygen-carrying capacity of fish blood is significantly reduced through the Bohr effect, so even if the oxygen level is within the acceptable range, the mortality will increase (Lim et al., 2003). Furthermore, NH_4 is more toxic in low-pH seawater due to higher permeability which could be a potential reason for the mortalities observed in control treatment. In addition, in seawater, with the decrease in pH due to the increase in permeability, the tolerance to NH_4 toxicity decreases (Although decreasing pH results in lower NH_3 concentrations, lower pH also decreases ammonia toxicity tolerance, and in seawater NH_4 remains toxic due to the enhanced permeability of fishes to this ion) (Zink et al., 2011), this can be one of the causes increase the mortality of larvae in the control group.

Newly hatched fish larvae do not have a developed gut and bacterial colonies in the intestinal tract, and on their skin or gills. Therefore, the primary bacterial flora of newly hatched larvae is closely related to the surrounding environment, thus the dominant type of bacteria in the aquatic environment plays a high role in the larval survival rate (Cahill, 1990; Verschuer et al., 2000; Tarnecki et al., 2019).

In the present experiment, the total bacterial count and total number of *Vibrio* spp. has been measured before and after transportation. Since the water from PM48 treatment was inoculated 48h prior to transportation thus showed a greater load of bacteria as opposed to the control group before transportation which indicates that the addition of probiotics into the water could grow well enough to limit the opportunistic and pathogenic bacterial communities.

Table 2 shows that in larvae, the total bacterial count and the total *Vibrio* spp. in the PM48 treatment were significantly decreased when compared to the initial sample and the control treatment. In addition, the total bacterial count in the water at the end of the experiment showed a decreasing trend in the PM48 treatment (Table 3). It has been suggested that some microbial populations may release chemicals with a bacteriostatic effect on other microbial populations (Verschuer et al., 2000). The results of the studies of Skjermo et al (1997) showed the proliferation of opportunistic bacteria in the breeding water of newly hatched larvae of Atlantic halibut (*Hippoglossus hippoglossus*), while this amount was much lower in the breeding water enriched with bacteria and thus showed a higher survival rate.

In the control treatment, the amount of the total bacterial count and the total number of *Vibrio* spp. showed an increasing trend in water and larvae. Ofelio et al, (2021) showed that the use of probiotic *Lactobacillus rhamnosus* (Strains: IMC 501) can reduce pathogenic bacteria of the Vibrionaceae family in *Artemia*. This is however, Hossein et al, (2021) have reported a significant increase in bacterial count in the transport water of Climbing perch (*Anabas testudineus*) where no probiotics were used in their experiment. This increase in the number of bacterial colonies could be one of the reasons for the decrease in DO and increase in NH₄ in the control treatment (Power & Nagy, 1999; Garcia-Ochoa et al., 2010).

Conclusion

The present findings suggest that the addition of probiotic mixture in water for transportation of day-old larvae of *A. latus* can significantly increase survival rate and mildly improve water chemical parameters and decrease total bacterial count and *Vibrio* spp. It is therefore concluded that using the mixture of probiotics 48h prior to transportation is an effective approach to minimize larval mortality and increase the chance of larvae survival after usual 24h transportation period. Therefore, further study is required for evaluation of larvae performance after transportation as well as implementation of molecular techniques to accurately monitor bacterial community dynamics.

Declarations

Acknowledgement

We are grateful to the director and staff of Nasr Zarin Shrimp Company, Khouzestan, Iran.

Authors' contributions

All persons listed as authors have read and contributed to preparing the manuscript as given below:

Vahid Morshedi, Mojtaba Najafabadi, Mehdi Dashtebzorg and Reza Gamoori carried out fish maintenance and sample collection

Naser Agh carried out fatty acid profile analysis

Vahid Morshedi, Reza Gamoori and Ghasem Rashidian wrote the article

Vahid Morshedi, and Reza Gamoori the experimental design and statistical analyses.

Mina Ahangarzadeh and Yaghob Mohammadi carried out microbial analyses

Ethics approval

This study was carried out by the principle of the Basel Declaration and recommendations of the Faculty of Veterinary Medicine at the University of Tabriz, the FVM.REC.1396.939. The protocol was approved by the FVM.REC.1396.939.

Conflicts of interest/Competing interests

The authors declare that there is no conflict of interest regarding the present data and manuscript.

Availability of data and material (data transparency)

The supporting data is accessible from the corresponding author upon reasonable request.

Consent to participate

The authors declare that they have every consent to participate.

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