

Increased larval survival of *Seriola lalandi* using *Pseudoalteromonas* sp. as probiotics

Aumento de la sobrevivencia de larvas de *Seriola lalandi* usando *Pseudoalteromonas* sp. como probióticos

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Resumen.- La demanda mundial de alimentos aumenta el interés en el desarrollo de la acuicultura. En Chile se están haciendo esfuerzos significativos para desarrollar el cultivo de *Seriola lalandi*, debido a la alta demanda de su carne a nivel nacional e internacional. Sin embargo, esta especie plantea dificultades persistentes con respecto a la sobrevivencia de las larvas. El objetivo del presente estudio fue evaluar los efectos de la bacteria *Pseudoalteromonas* sp. como un suplemento probiótico en el cultivo de larvas de *S. lalandi*. Para esto, larvas de *S. lalandi* se alimentaron con rotíferos (*Brachionus rotundiformis* y *Brachionus plicatilis*) y *Artemia* sp. que se cultivaron previamente usando microalgas mezclado con *Pseudoalteromonas* sp. Rotíferos y artemias fueron buenos vectores de probióticos debido a que las larvas de *S. lalandi* alimentadas con suplemento probiótico presentaron mayor sobrevivencia y longitud que el control al final del experimento. Estos hallazgos ponen en evidencia que los probióticos de *Pseudoalteromonas* sp. son buenos candidatos para el uso en cultivos de larvas de *S. lalandi*. Sin embargo, se necesita más investigación en una escala de cultivo mayor para validar su uso a nivel comercial.

Palabras clave: *Pseudoalteromonas* sp., probióticos, alimentos vivos, *Seriola lalandi*, sobrevivencia

Abstract.- Global demand for food increases the interest in develop of aquaculture. Significant efforts are being made in Chile to develop *Seriola lalandi* culture, because of the high demand of this fish meat at national and international level. However, this species poses persisting difficulties regarding larval survival. The objective of the present study was to evaluate the effects of *Pseudoalteromonas* sp. bacteria as a probiotic supplement on the larval culturing of *S. lalandi*. For this, *S. lalandi* larvae were fed rotifers (*Brachionus rotundiformis* and *Brachionus plicatilis*) and *Artemia* sp. that were previously cultured using microalgae mixed with *Pseudoalteromonas* sp. Rotifers and Artemia were good probiotics vectors because *S. lalandi* larvae fed probiotic supplement showed higher survival that the control. These findings evidence that *Pseudoalteromonas* sp. are good probiotic candidates for use in *S. lalandi* larval cultures. However, is needed more research on major scale cultivation to validate their use commercially.

Key words: *Pseudoalteromonas* sp., probiotics, live food, *Seriola lalandi*, survival

INTRODUCTION

The fish *Seriola lalandi* (Valenciennes, 1833) has been recognized as having high potential for aquaculture in Chile (Fernández *et al.* 2015). The cultivation of this species in the larval stage has faced problems such as diseases (deformities) and mortalities. Survival obtained in the culture fluctuates between 1-2.5% (Avilés & Castelló 2004), this hindered the development of the industry. Among other variables, the main causes are attributed to nutritional deficiencies of the food, high density of the culture, and susceptibility to diseases in culture conditions. However, the incubation phase of this species must overcome this problem to ensure a sustainable production system in the future.

Probiotics can be defined as live microorganisms that orally administered can be beneficial to the host (Newaj-Fyzul & Austin 2015). Probiotic use in the aquaculture industry is currently seen as a sustainable and promising strategy not only in the treatment of diseases, but also in terms of fish nutrition, growth, and immunity (Lazado *et al.* 2014). Probiotics positively promote fish growth, reproduction, nutrient digestion, and stress tolerance, in addition to playing roles in inhibiting pathogens and bettering water quality, among other benefits (Martinez *et al.* 2012). In fish, Lobo *et al.* (2014a) suggest that proper nutrition during the first larval stages is important for successful cultivation, and that probiotic supplements contribute towards managing the stress of weaning.

Based on this information, the use of probiotic food supplements during the larval phase of *S. lalandi* could be a viable alternative for increasing survival rates and nutritional quality. Species within the bacterial from the genus *Pseudoalteromonas* present probiotic potential for different farmed organisms. For example, *Pseudoalteromonas* strains isolated from turbot (*Psetta maxima* L.) larvae are able to reduce the larval mortality of this fish species (Hjelm *et al.* 2004). Furthermore, *Pseudoalteromonas* sp. isolated from the intestinal tract of Atlantic cod (*Gadus morhua* L.) demonstrate antagonistic activities against the pathogen *Vibrio anguillarum* (Fjellheim *et al.* 2010). Research in sea cucumbers (*Apostichopus japonicus*) fed for 45 days with a diet supplemented by *Pseudoalteromonas* sp. FC228, evidenced improved digestive enzyme activity, thereby stimulating the immune response and providing greater resistance to *Vibrio splendidus* infection.

In fish feed, live rotifers and *Artemia* are good vectors for probiotics. For example, Jamali *et al.* (2015) evaluated the effects of adding the probiotics *Bacillus licheniformis* and *Bacillus subtilis* (at 1×10^6 CFU mL⁻¹) in the rotifer *Brachionus plicatilis* and *Artemia urmiana* cultures. Jamali *et al.* (2015) subsequently observed increased growth and higher survival rates *Litopenaeus vannamei* larvae, where individuals fed a probiotic supplemented diet obtained larger lengths, weights, and survival rates than the shrimp not probiotics fed. Likewise Garcés *et al.* (2015) found that encapsulating the *Lactobacillus pentosus* H16 probiotic in *Artemia franciscana* resulted in no deleterious effects for *A. franciscana* but did inhibit the pathogenic activity of *Vibrio alginolyticus* 03/8525.

Considering the beneficial potential of probiotics, the present study evaluated the survival of *S. lalandi* larvae fed with rotifers and *Artemia* previously given probiotic *Pseudoalteromonas* sp. bacteria. The objective of this research was to find a solution to the high rates of *S. lalandi* larval mortality under hatchery conditions, a situation that increases the production costs of this aquaculture candidate species.

MATERIALS AND METHODS

SELECTION OF MICROALGAE AND PROBIOTIC BACTERIA

The microalgae use for both live feed (rotifers) and larvae 'green water' was *Nannochloropsis gaditana* and the bacterium *Pseudoalteromonas* sp. (SLP1), previously isolated from the gonads of *S. lalandi* (Sayes *et al.* 2016). Both microorganisms were obtained from the strain collection of the laboratory, were selected due to its good fatty acid profile and easy management under culture conditions (microalgae) and, due to its probiotic

properties (bacteria), such as antibacterial effects against the pathogen *Yersinia ruckeri* and a lack of haemolytic, proteolytic, and lipolytic activities (Sayes *et al.* 2016).

EVALUATION OF ROTIFERS AS PROBIOTIC VECTORS

Photobioreactors (25 L) which were initially manufactured for benthic microalgae cultivation according to described in Silva-Aciaras & Riquelme (2008), they were conditioned for cultivating rotifers and they were equipped with air pumps and heaters, were filled with seawater filtered (0.2 µm), chlorinated (1 g L⁻¹) and neutralized with thiosulfate (3 g L⁻¹) previously. The photobioreactors were maintained at 28°C with 5.1 mg L⁻¹ dissolved oxygen and a pH 7. The rotifer *B. rotundiformis* used was obtained from strain collection of the laboratory. The experiment they were performed in triplicate and consisted in the **control** group contained 5 rotifers mL⁻¹, without probiotic bacteria and the **treatment** group contained 5 rotifers mL⁻¹, with *Pseudoalteromonas* sp. probiotic bacteria at a concentration of 1.0×10^3 cells mL⁻¹. Each photobioreactor contained an *N. gaditana* microalgae concentration of 7.0×10^6 cells mL⁻¹. Rotifers were counted daily, for 8 days to evaluate reproduction, and every 2 days, microalgae and bacteria were counted to ensure maintenance at the initially added concentrations. The counts were performed by normal light microscope (Olympus BX51, Model BX51TF).

To assess duplication of rotifer in larger volumes, two species of rotifers were tested *B. plicatilis* and *B. rotundiformis* in triplicate each. They were grown in bags of 500 liters (L) (LDPE sleeves, double background seal, transparent, 150 cm high x 180 cm wide. EROFLEX manufacturer), containing 450 L of culture medium (seawater chlorinated and neutralized with thiosulfate previously, filtered 0.5 microns/Last ultraviolet radiation, nitrate 0.3825 g L⁻¹, phosphate 0.03375 g L⁻¹, trace elements 0.2 mL L⁻¹, sodium bicarbonate 0.168 g L⁻¹), previously inoculated with *N. gaditana* (7.0×10^6 cells mL⁻¹). For control and treatment groups the initial inoculum was 10 rotifers mL⁻¹. The probiotic bacteria they were initially inoculated in the bags of the treatment to 1.0×10^3 cells mL⁻¹ after 7 days of initiated the rotifers culturing. Each one day, again the bacteria were inoculated a treatment at a concentration of 1.0×10^2 cells mL⁻¹ to keep concentration of probiotic bacteria. Prior to the administration of the probiotic bacteria, the amounts of microalgae, rotifers and bacteria in each bag, were quantified by normal light microscope (Olympus BX51, Model BX51TF).

EVALUATION OF ARTEMIA SP. AS A PROBIOTIC VECTOR

Artemia sp. cysts (40 g; Bio-Marine Inc., Hawthorne, CA, USA) were hydrated for an hour in freshwater. After this, the

cysts were incubated for 24 h in a photobioreactor (the same systems used for rotifers) with 25 L of seawater (seawater chlorinated and neutralized with thiosulfate previously, filtered 0.5 microns and was exposed ultraviolet radiation) and the following parameters: 28°C, pH 7, 6 mg L⁻¹ dissolved oxygen, constant aeration, and 2,000 lx. After of incubation, the photobioreactors contents were sieved (200-100 µm) to retain hatched nauplii. These were then moved to a clean photobioreactors (25 L) with 10 L of seawater with constant aeration. Experiments they were performed in triplicate and consisted in a control group (100 nauplii mL⁻¹ + 1.0 x 10⁴ cell mL⁻¹ *N. gaditana* microalgae + 0.4 g of Rotinor®/1,000,000 nauplii) and a treatment group (100 nauplii mL⁻¹ + 1.0 x 10⁴ cell mL⁻¹ *N. gaditana* + 1.0 x 10³ cell mL⁻¹ *Pseudoalteromonas* sp. probiotic bacteria + 0.4 g of Rotinor®/1,000,000 nauplii). After of 72 h of incubation was evaluated *Artemia* survival (%).

S. LALANDI LARVAE SURVIVAL

LARVAE OBTENTION

Experiments were performed at the fish culturing unit of the Universidad de Antofagasta, Chile. The broodstock eggs of the *S. lalandi* were recovered and placed into conic cylindrical tanks (250 L) with constant aeration, seawater flow, temperature of 21-23°C, pH 7 and dissolved oxygen 6 mg L⁻¹. The eggs were counted by volumetric method (number of eggs mL⁻¹). These tanks were incubated for approximately two days until larval hatching. The hatched larvae were kept for one additional day in the incubation tank before being counted. The number of larvae (larvae mL⁻¹) was obtained from average of three replicates, counting live larvae in 8 mL of samples coming from a volume of 20 L. Later placed in buckets with seawater (20 L), and transported to culture tanks.

EVALUATION OF SURVIVAL

The experiments were performed in triplicate using culture tanks (450 L) containing 300 L of filtered (0.45 µm) seawater with seawater to temperature 21-23°C, pH 7, dissolved oxygen 6 mg L⁻¹, aeration, flow and light constant. An initial inoculum concentration of 10,000 *S. lalandi* larvae/tank was used. The diet program consisted of two days of feeding with *B. rotundiformis* (2 rotifers mL⁻¹), followed by feeding *B. plicatilis* (5 rotifers mL⁻¹) until day 8. After, the rotifers were gradually replaced by nauplii *Artemia* sp. (8-16 nauplii mL⁻¹). For about 5 days it was fed with a mixture of rotifers and artemia. Later, they were fed only *Artemia* sp. until day 15 that ended the experiment. The experiments consisted of the following: **Control** (2 x 10⁵ cells mL⁻¹ of the microalgae *N. gaditana* + *B.*

rotundiformis/*B. plicatilis* rotifers + *Artemia* nauplii) and **Treatment** (2 x 10³ cells mL⁻¹ probiotic bacteria + 2 x 10⁵ cells mL⁻¹ of the microalgae *N. gaditana* + *B. rotundiformis* / *B. plicatilis* rotifer + *Artemia* nauplii).

LARVAE SAMPLING

After 15 days of culturing, the surviving larvae of *S. lalandi* were collected. In each pond the aeration was cut, this action causes that live larvae tend to rise to the surface, which were carefully collected from the water surface with a cup. These larvae were counted before being transitioned to a new water source. This process was repeated until collect the last larva of each tank. During larvae collection, 20 control and treatment larvae were sampled to measure standard length, which runs from the tip of the mouth to the last bone of the spine or urostylo.

STATISTICAL ANALYSIS

The data were checked for normality and homoscedasticity and analyzed by t-test. The data presented in percentages (survival of *Artemia* and larvae) were transformed into arcsin function before analysis. The analyses were performed using the GraphPad PRISM 5.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). To evaluate if the duplication of rotifers is related to the presence of probiotic bacteria in the culture, a comparison was made with unidirectional ANCOVA (Microsoft Excel 2010). Besides, the larval growth rate was estimated with the initial and final length of the larvae by adjusting the age-length relationship to a linear regression model, the variables were compared with one-way ANCOVA to establish whether the presence of probiotics significantly affects larval growth. Statistical significance was established at $P < 0.05$. Data are presented as values ± standard error of the mean.

RESULTS

EFFECTS OF PROBIOTIC BACTERIA ON ROTIFER REPRODUCTION

After 3 days of culture in photobioreactors, there was an increase in the concentration of treated rotifers with probiotic bacteria as compared to the control, reaching a concentration of 1.5 x 10⁷ rotifers mL⁻¹, at day 8 of culture (Fig. 1). However, no significant differences were observed between treatment and control (t-test= 1.639, $P = 0.1235$). From these results, it can be deduced that the presence of probiotic bacteria in the culture did not have deleterious effects on the rotifers, nevertheless, there was greater rotifer duplication in cultures with probiotic bacteria.

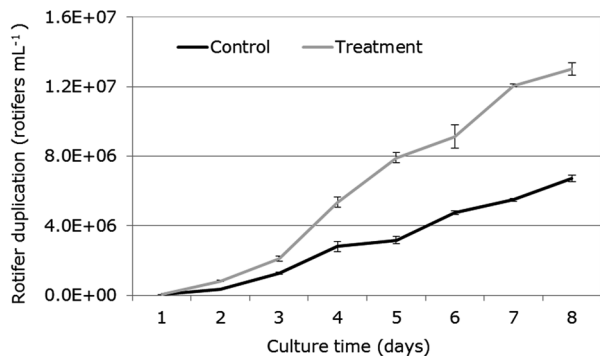


Figure 1. Growth of *B. rotundiformis* fed with *N. gaditana* microalgae in photobioreactors. Supplemented with probiotic bacteria (Treatment) and without probiotic bacteria (Control). Bars represent \pm standard error of the mean / Crecimiento de *B. rotundiformis* alimentado con microalgas *N. gaditana* en fotobiorreactores. Suplementado con bacterias probióticas (Tratamiento) y sin bacterias probióticas (Control). Las barras representan \pm error estándar de la media

After 5 days of culturing in bags of 450 L, there was an increment in both rotifer duplication, reaching concentrations of 1.47×10^8 rotifers mL^{-1} for *B. rotundiformis* and 1.49×10^8 rotifers mL^{-1} for *B. plicatilis* (Fig. 2), after an initial inoculation de 7.5×10^6 rotifers mL^{-1} . These results indicate the viability of culturing both rotifer species in 450 L volumes supplemented with probiotic bacteria. With the positive result of ANCOVA analysis of the bacteria-rotifers variables, *B. rotundiformis* (1.28) y *B. plicatilis* (2.37), it can be deduced that the presence of probiotic bacteria in the culture has a positive relation in the rotifer duplication.

ARTEMIA SP. SUPPLEMENTED WITH PROBIOTIC

Artemia sp. nauplius inoculated with bacterial probiotics presented higher survival (90%) than the control group (75%) after 72 h of incubation (Fig. 3). Statistical analyzes showed significant differences (t-test= 3,841, $P < 0.05$) of artemia treated with probiotics compared to controls.

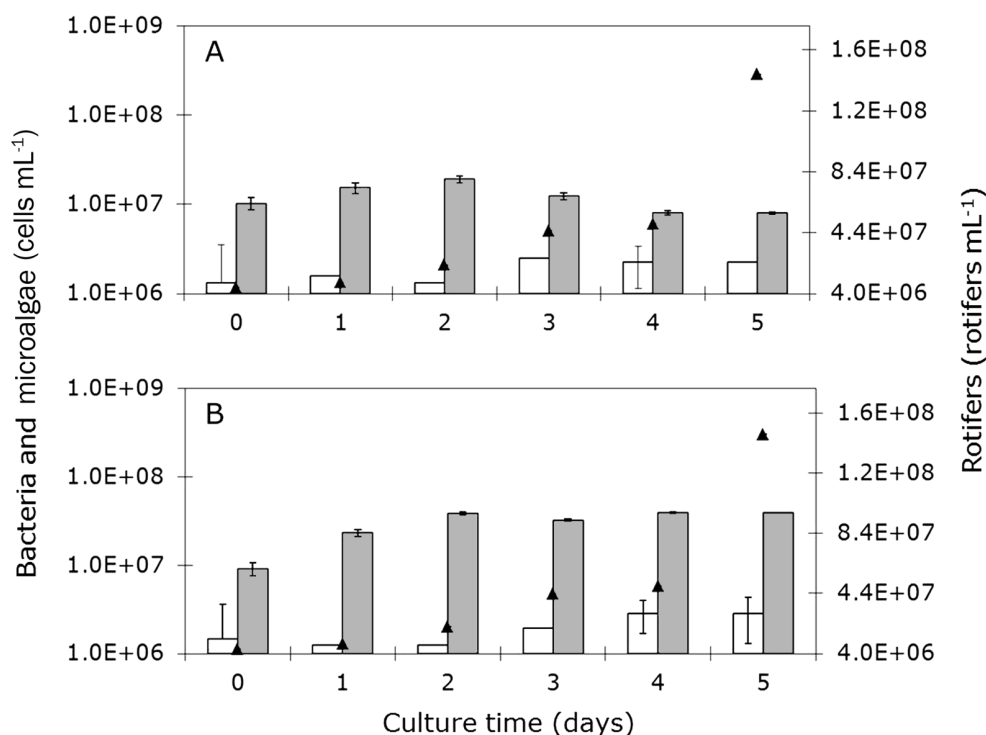


Figure 2. Reproduction of rotifers fed with microalgae and probiotic bacteria cultured in bags of 500 L. A) *B. rotundiformis*. B) *B. plicatilis*. White bars indicate bacterial; gray bars indicate microalgae and black triangles indicate rotifer. Bars represent \pm standard error of the mean / Reproducción de rotíferos alimentados con microalgas y bacterias probióticas cultivadas en bolsas de 500 L. A) *B. rotundiformis*. B) *B. plicatilis*. Las barras blancas indican bacterias; barras grises indican microalgas; y los triángulos negros indican rotíferos. Las barras representan \pm error estándar de la media

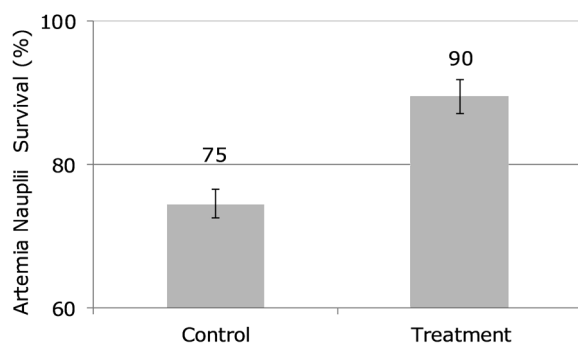


Figure 3. Survival of *Artemia* sp. nauplii. (Treatment) supplemented with probiotic bacteria and (Control) without probiotic bacteria. Bars represent \pm standard error of the mean / Sobrevivencia de nauplios de *Artemia* sp. (Tratamiento) suplementado con bacterias probióticas y (Control) sin bacterias probióticas. Las barras representan \pm error estándar de la media

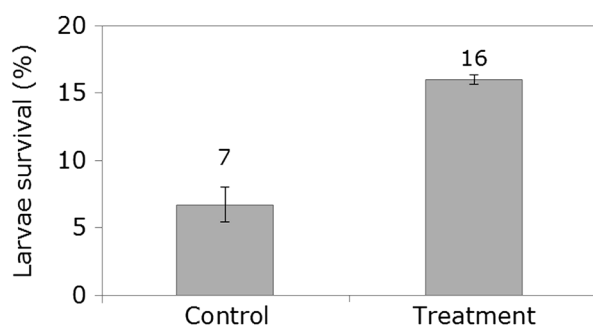


Figure 4. Evaluation of the survival of *S. lalandi* larvae fed with probiotics. Supplemented with probiotic bacteria (Treatment) and without probiotic bacteria (Control). Bars represent \pm standard error of the mean / Evaluación de la sobrevivencia de larvas de *S. lalandi* alimentadas con probióticos. Suplementado con bacterias probióticas (Tratamiento) y sin bacterias probióticas (Control). Las barras representan \pm error estándar de la media

S. LALANDI LARVAE SURVIVAL

The survival of *S. lalandi* larvae supplemented with probiotic was 16%, more than double that of control larvae with 7% survival (Fig. 4). The statistical analyzes showed significant differences (t-test= 5.093, $P < 0.05$) of the treatment with probiotic respect to the control.

At the end of the experiment (15 days), 20 larvae were sampled at random in treatment and control, and the standard length was measured. The obtained lengths were 7.5 mm in treatment and 4.4 mm in length in control. With the length data at the end of the experiment, plus the length measurement of the larvae at the start of the experiment, we estimated the growth rate for the study period and adjusted the data to a linear regression of the age-length relationships. The formulas obtained

were Treatment= $1.9 + 0.373x$ and Control= $1.9 + 0.167x$, indicating that growth rates for treatment and control were 0.373 and 0.167 mm day⁻¹, respectively. The analyses of ANCOVA were positive to variables treatment-control (1.32), age-treatment (7.92) and age-control (3.54). With the estimated daily lengths through linear regression, a t-test analysis was performed, obtaining that the larval survival in treatment was significantly greater than in the control (t-test= 3.179, $P < 0.05$); therefore, the presence of probiotics improve significantly larval growth and survival.

DISCUSSION

In recent years, the interest in using probiotics in the aquaculture industry has increased due to scientifically supported benefits. According to Martínez *et al.* (2012), there are various commercial strains of probiotics that contain one or more live microorganisms that can be used to improve the culturing of aquatic organisms, and these can be directly inoculated in the culture or mixed in the feed. The probiotics used in aquaculture must meet certain conditions to prevent any health problems in people. According, Martínez *et al.* (2012) the probiotics used in animal feed they are Carnobacterium, Alteromonas, Lactobacillus, Streptococcus, Streptomyces, Enterococcus, Micrococcus, Pseudomonas, *Roseobacter* sp., Saccharomyces, Phaffia, Vibrio, Carnobacterium, Lactococcus, Shewanella, Pediococcus and *Bacillus* spp. (Leyton & Riquelme 2010, Avella *et al.* 2012, Mohapatra *et al.* 2012).

In relation to the use of *Pseudoalteromonas* sp. as probiotics, it has been shown that *Pseudoalteromonas aliena* inhibits the pathogenic *Vibrio harveyi* (Morya *et al.* 2014). Furthermore, Longeon *et al.* (2004) identified the P-153 protein isolated from *Pseudoalteromonas* sp. X153 as an antibiotic capable of inhibiting bacterial strains of *Staphylococcus epidermidis*, *Propionibacterium acnes*, and *Propionibacterium granulosum*. Contributing to this research, the present study investigated the probiotic potential of *Pseudoalteromonas* sp. (SLP1), this strain has no haemolytic activity, which makes it a good candidate as probiotic (Sayes *et al.* 2016). Moreover, this bacterium was isolated directly from *S. lalandi*, further decreasing the risks of introducing exogenous probiotic strains that could alter the ecosystem. The obtained results revealed that the presence of *Pseudoalteromonas* sp. (SLP1) probiotic bacteria did not negatively affect rotifers or *Artemia* sp., even in the volume of 500 L of culture. The introduction of probiotic *Pseudoalteromonas* sp. into *S. lalandi* larvae, by using live vectors such as microalgae, rotifers, and *Artemia* sp. It seems a good alternative to introduce probiotics with life feed for directly delivering to the fish intestine, this allow develop its probiotic activity more efficiently.

Generally, probiotics have demonstrated promising results in relation to fish growth (Al-Dohail *et al.* 2009, Sáenz de Rodríguez *et al.* 2009). Specifically, probiotics improve food digestion and confer protection against harmful bacteria through mechanisms such as competitive exclusion via the production of organic acids, hydrogen peroxide, and various other compounds (Zhou *et al.* 2009, Rahiman *et al.* 2010, Abdullah *et al.* 2011, Youping *et al.* 2011, Lin *et al.* 2012, Tapia *et al.* 2012, Zhang *et al.* 2012). Additionally, Lobo *et al.* (2014b) reported that the incorporation of probiotic bacteria during the larval stage of the *Solea senegalensis* increased survival. The present results of survival evidenced that *S. lalandi* larvae treated with probiotic feed the survived is higher (16% survival) than those larvae without probiotics (7% survival). Similarly, those larvae treated with probiotics were longer (8 mm) than untreated larvae (4 mm). These results support that probiotics of *Pseudoalteromonas* sp. (SLP1) can positively affect survival rates and length in fish larvae of *S. lalandi*.

Martínez *et al.* (2012) argue that technological advances in food production and greater awareness about environmental protection will lead to better aquaculture practices that will ultimately decrease the overexploitation of organisms. One alternative for confronting the diseases present in aquaculture could be the use of non-virulent microorganisms isolated from the microbiota of farmed organisms. Further research is needed to identify if the probiotics present in the microbiota of *S. lalandi* larvae effectively colonize the digestive tract or if these bacteria are transitory. It is also important evaluate the use of these probiotics on an industrial scale of *S. lalandi* culturing, in addition to considering the economic feasibility of sustained probiotic application. The present results represent a significant advancement towards addressing these remaining doubts, contributing to the optimal larval culturing of *S. lalandi*.

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