The Efficiency of *Lactobacillus plantarum* in Diet for Juvenile Japanese Flounder *Paralichthys olivaceus* Reared in a Closed Recirculating System

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**Abstract:** A study was conducted to examine the effect of probiotics on the growth performance and survival of juvenile Japanese flounder, water quality, bacterial number and tolerance to stress. In the present study, *Lactobacillus plantarum* (LP) was orally given to juvenile Japanese flounder, *Paralichthys olivaceus* by feeding at two levels, 0.1% and 1.0% in the diet (the 0.1% LP group and 1.0% LP group, respectively), and fish were reared in a closed recirculating system for 50 days. The survival rate and growth performance of flounder, and water quality were not affected by oral administration of LP. By feeding a diet with LP, the number of *Lactobacillus* sp. in the rearing water and the intestine increased, particularly in the 1.0% LP group. In fish that received diets containing LP, the better tolerance to high temperature was observed significantly as compared to those receiving LP-free diet. This study suggested that LP orally administered could survive in the intestine of flounder and might be effective to enhance stress tolerance of *P. olivaceus*.

**Key words:** *Paralichthys olivaceus*; Probiotics; Stress; Closed recirculating system

Microbes have been used empirically to maintain the health of humans and livestock and have been applied as food preservatives (Bengmark 1998; Gatescope 1999). Tannock (1997) found that living microbial cells have the potential to improve the health of host animals, although this applied to humans but not aquatic animals. Probiotics have been applied to humans and terrestrial animals (Irianto and Austin 2002). In aquaculture, the application of probiotics is relatively recent; however, Yasuda and Taga already anticipated in 1980 that bacteria might be useable as food and as biological control agents against fish disease (Yasuda and Taga 1980).

In Japan, the Fisheries Agency (http://www.jfa.maff.go.jp/bouekianzen.htm) has permitted some kinds of antibiotics to be used in aquaculture for disease prevention. Recently, the usage of antibiotics has become limited because several pathogens have developed resistance to them (Miranda 2002). This gave rise to the use of probiotics as an alternative strategy for the prevention of infectious disease. In general, probiotics have been defined as a live microbial feed additive that benefits the host animal by improving the microbial condition of its gastrointestinal tract (Fuller 1989). Probiotic effects have been shown to increase disease resistance through the stimulation of immune responses, competition with pathogenic bacteria for limiting nutrients or adhesion points to the mucous, and the production of inhibitory substances (Vázquez et al. 2003). Recently, probiotics have included dead cells, which show no viability and do not contain metabolites, which function as immunostimulants and modify enzyme activity or microflora in...
the gastrointestinal tract.

Some studies have suggested that the potential benefits of probiotics in aquaculture include the recovery of water and sediment quality through, for example, the decomposition of organic matter and the reduction of nutrients in culture ponds (Quetroz and Boyd 1998; Boyd and Massaut 1999; Prab et al. 1999). Therefore, probiotics have been expected to be used as agents for water and sediment quality remediation in aquaculture. The fact that the microbial flora of the rearing water influence the gastrointestinal flora of the cultured organisms is widely known (Sugita et al. 1984; Olafsen et al. 2001).

As probiotics, lactic acid bacteria have been widely used to improve the health of humans and livestock (Irianto and Austin 2002). In fish, it has been confirmed that probiotics can enhance growth and improve survival (Gatescope et al. 1994; Byun et al. 1997; Riquelme et al. 1997; Rengpipat et al. 1998; Robertson et al. 2000). The Japanese flounder, *Paralichthys olivaceus*, is one of the highly valued species of cultured fish. This species is cultured on a large scale, and the production of it is increasing. However, the flounder market has been damaged by bacterial disease, such as vibriosis and Edwardsiellosis (Kanai et al. 1988; Yamanoi et al. 1998). Pathogenic bacteria are in some cases opportunistic, and fish can be easily infected when they are in stressful conditions. In aquaculture, fish were intensively cultured and accepted access feeding, which results in deterioration of the rearing environment. These culture conditions are stressful for fish, and increase the possibility of disease and infection (Snieszko 1974; Maule et al. 1989; Davis et al. 2002). Therefore, to achieve sustainable aquaculture, it is very important that fish have stress tolerance as well as disease resistance (immune response). It has been well established that stress affects the survival and growth of fish (Vijayan and Moon 1992).

The environmental law enacted by the Japanese Environmental Agency in 1993 requires more environmentally friendly aquaculture, particularly because un-eaten feeds and feces in cage cultures located in coastal areas are discharged into the environment (Maruyama et al. 1998). To control the effluent from aquaculture farms and thus promote environmentally friendly aquaculture, the use of a closed system was recently recommended (Maruyama and Suzuki 1998). The closed recirculating system has some advantages over the raceway and cage culture systems in that (i) the culture conditions are easy to control, and (ii) environmental events, such as typhoons and red tide blooms, have little effect on the culture. Furthermore, if probiotics are used in a recirculating aquaculture system, those introduced into the rearing system do not flow out of the system because the system is closed. Therefore, a closed recirculating system seems to be well suited for the application of probiotics in aquaculture.

Until now, there have been few studies of the application of probiotics to flounder culture, although Byun et al. (1997) reported that the oral administration of lactic acid bacteria enhanced the growth of adult flounder. Therefore, this study aims to investigate the effect of the oral administration of probiotic bacterium *Lactobacillus plantarum* on the growth and survival of the juvenile Japanese flounder, and on resistance to stress. The total viable count and the number of viable *Lactobacillus* sp. were monitored during a feeding trial to confirm the kinetics of *L. plantarum* in the rearing water and the intestinal tracts of the fish. Water quality in a closed recirculating system was monitored to confirm the efficacy of *L. plantarum* as a bioremediation agent.

**Materials and Methods**

**Experimental fish**

Four thousand juvenile Japanese flounders of 1.30 ± 0.10 g (mean ± standard deviation, \(n = 20\)) in weight were purchased from a commercial fish farm (Matsumoto Fisheries CO. LTD., Miyazaki, Japan) and transferred to the rearing facility in our department faculty. The fish were conditioned in a running water system for 1 week. One week before the start of the experiment, the fish were transferred into a closed recirculating system at a density of 15 fish per aquarium (30 l). Previously, natural seawater was filtered by a sand filter and used as the rearing water. During the conditioning period, the fish were fed the control diet...
Efficiency of *Lactobacillus plantarum* on Flounder (probiotics-free) twice daily at 0.5 – 1.0% of body weight. The recirculating system was equipped with four aquaria and a filter tank (100 l) consisting of a sand filter and a biological filter with plastic media. The water flow rate was 2.0 l per min.

**Experimental groups and diets**

As the probiotic, lyophilized *L. plantarum* (LP) cells were obtained from Morinaga Milk Industry CO., LTD., Kanagawa, Japan. Three treatments with two simultaneous replications were prepared: a) a control group fed the control diet (LP-free); b) a 0.1% LP group fed a diet containing 0.1% LP; and c) a 1.0% LP group fed a diet containing 1.0% LP. Each group was raised in a separate recirculating system to avoid inter-treatment contamination. The composition of the three test diets is shown in Table 1.

In the preparation of the diet containing LP, an adequate procedure based on preliminary experiment was employed. All powders were mixed well for 10 min. The sources of lipids, except the palm oil, were mixed with vitamins, and this mixture was added to the powder mixture. The probiotics were added to the palm oil at 40°C, and this was mixed well with the previous mixture of all ingredients for 10 min. Water was added at 40% to dry weight of the mixture and mixed well for 10 min to facilitate pelleting by a meat chopper (diameter: 1.2 – 1.5 mm). After pelleting, the diets were lyophilized until the moisture content was below 10%. For the preparation of the control diet, the same procedure was employed without the addition of LP. Test diets were stored at −20°C.

**Experimental design for rearing trial**

The experimental fish were fed twice daily at 0.5 – 1.0% per body weight and reared for 50 days at a water temperature of 24°C with a cooler. The water flow rate in the system was 2.0 l per min. Every 10 days fish survival was checked, and fish body weights were measured. At the same time, the rearing water and the fish intestine were collected for a bacterial count to be performed. Water was sampled with a polyethylene bottle every 5 days for water quality analysis and was stored at −20°C. After 50 days of rearing, all fish were removed from the recirculating system, and the system continued to circulate for 7 days without feeding. At 55, 56 and 57 days, the total viable count and the number of *Lactobacillus* sp. were enumerated as described below.

**Water quality analyses**

Urea-nitrogen was determined according to the method of Newell et al. (1967) NH₄-N, NO₂-N, NO₃-N and PO₄-P were determined using the method of Strickland and Parsons (1972). Total nitrogen (T-N) and total phosphorus (T-P) were determined according to the potassium persulfate oxidizing method. COD (chemical oxygen demand) was determined according to the potassium permanganate-iodine titration method (Japanese Industrial Standard, 1995). Dissolved inorganic nitrogen (DIN) was calculated as the sum of NH₄-N, NO₂-N and NO₃-N.

**Bacterial count**

For the enumeration of the total viable count in a water sample, 1/2 ZoBell 2216 agar medium

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**Table 1. Composition of test diets for growth trial (g/100 g)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>0.1% LP</th>
<th>1.0% LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBP¹</td>
<td>-</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Fish meal</td>
<td>41.6</td>
<td>41.6</td>
<td>41.6</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>α-starch</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Pollack liver oil</td>
<td>5.2</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Soybean lecithin</td>
<td>3.2</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Palm oil</td>
<td>2.1</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>b-3HUFA</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin mix²</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Mineral mix³</td>
<td>4.3</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>IMP</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Betain</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>α-cellulose</td>
<td>1.0</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>Berda</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

¹*Lactobacillus plantarum* lyophilized powder (Morinaga Milk Industry CO., LTD. Kanagawa, Japan)

²B-carotene; 17.92, Vitamin D3; 1.80, Menadione-NaHSO₃; 8.54, Atochromenol; 71.73, Thiamin-HNO₃; 10.75, Riboflavin; 35.77, Pyridoxine-HCl; 8.54, Cyanocobalamine; 0.01, Cellulose; 358.08, Biotin; 1.08, Inositol; 716.35, Nicotinic acid; 143.27, Ca-pantothenate; 50.16, Folic acid; 2.69, Choline chloride; 1464.50, P-aminobenzoic acid; 71.35, APM; 37.50 (mg/100 g-premix).

³MgSO₄; 512.64, Na₂HPO₄; 326.35, KH₂PO₄; 897.44, Ferric acid; 18.89, Calcium lactate; 1223.79, Al(OH)₃; 0.24, ZnSO₄; 12.95, CuSO₄; 0.38, MnSO₄; 2.99, Ca(IO₃)₂; 0.56, CoSO₄; 3.74 (mg/100 g-premix).
(polypeptone 5.0 g, yeast extract 1.0 g, ferric phosphate 0.1 g, agar 15.0 g, artificial seawater [ASW] 1000 ml, pH 7.6) was used. For the enumeration of the total viable count in the fish intestine, ZB-II agar medium (polypeptone 5.0 g, yeast extract 1.0 g, agar 15.0 g, 1/2 ASW 1000 ml, pH 7.6) was used. For the enumeration of viable Lactobacillus sp. (LBS count), modified LBS agar medium consisting of LBS agar medium (BBL, Becton, Dickinson, Sparks, MD) 84.0 g, Lab-Lemco powder (Oxoid, Basingstoke, England) 8.0 g, sodium acetate 15.0 g, glacial acetate 3.7 ml, and distilled water 1000 ml was used. The water sample (200 ml) was collected with a sterile glass bottle. For the bacterial count in the fish intestine, two fish were transferred from the rearing tank onto a clean bench. The surface of the fish body was sterilized with 70% ethanol. The whole fish intestine (from pyloric caeca to anus) was dissected out and washed with MITSUOKA buffer (KH2PO4 4.5 g, Na2HPO4 6.0 g, L-cystein 0.5 g, tween 80 0.5 g, DW 1000 ml). The intestine was homogenized in the same solution. Ten-fold serial dilutions were prepared from each sample with MITSUOKA buffer, and the aliquot (100 μl) was spread on each agar plate. In the case of the test diet, the sample was first powdered well and serially diluted. The agar plate inoculated with each dilution was incubated for 3 days at 25°C and 37°C for the total viable count and LBS count, respectively.

High temperature stress test

After a feeding trial lasting 50 days, the tolerance of fish to exposure to high temperature was examined. Five fish were starved for 24 hrs and transferred from each tank to filtered natural seawater in a glass aquarium (12 l). Beforehand, a preliminary experiment was conducted to confirm the time to death of P. olivaceus at various temperatures within an hour, and the results led to the use of a temperature of 34°C for the high-temperature stress test. The filtered natural seawater was used. The water temperature was maintained at 34°C with a thermostatically controlled glass heater based on a preliminary experiment. The pH, salinity and dissolved oxygen in seawater were 8.15 ± 0.03, 34 psu and 6.5 ± 0.1 mg/l, respectively. The time to death of the fish was recorded, and the time duration for 50% mortality was determined. This test was conducted in triplicate.

Statistical analysis

Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-t test. These analyses were conducted using SPSS Version. 10.0 (SPSS, Chicago, IL, USA). In the high-temperature stress test, the significant difference between groups was obtained using Student’s t-test. Significant difference was established at P < 0.05.

Results

Change of LBS count in a diet with LP stored at −20°C

Initially, the LBS count in the diet containing 1.0% LP was 1.0 × 10⁸/g-diet. It was stored at −20°C. One week later, the LBS count in the diet was 1.2 × 10⁸/g-diet. One month later, the number had decreased slightly to 8.1 × 10⁷/g-diet.

Effect of LP on the survival and growth performances of flounder

Table 2 shows the survival rate and growth performance after the 50-day rearing trial in which fish were fed with the control diet and the diets containing 0.1% or 1.0% LP. In the survival rate and all parameters of growth performance, no significant differences were observed among three groups after 50 days of rearing. Fifty days after rearing the survival rate was 75 – 82%, and the mean body weight was 8.99 – 9.29 g per individual in the three groups. WG, BWG, feed intake and FCE ranged from 7.59 – 7.89, 541 – 565, 9.9 – 10.2 and 76.2 – 78.1, respectively.

Effects of LP on water quality in a closed recirculating system

No obvious differences were observed among groups in the water quality parameters of the rearing water throughout the experiment (Fig. 1).
Efficiency of *Lactobacillus plantarum* on Flounder

**Effects of LP on the bacterial counts in the rearing water and the fish intestine**

Fig. 2 shows the results of the bacterial counts. In the total viable count in the rearing water, no difference was observed among the groups during the 50 days of the trial, although the count in the 1.0% LP group was higher than those in the control group and 0.1% LP group at 50 days. In the total viable count in the intestine sample, no differences were observed among the groups.

In the case of the rearing water, the LBS count was not detected in the control group. In Table 2, survival rate and growth performances of *P. olivaceus* were presented.

**Table 2. Survival rate and growth performances of *P. olivaceus***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival rate (%)</th>
<th>Mean BW¹ (g)</th>
<th>WG² (g)</th>
<th>BWG³ (%)</th>
<th>Feed intake (g)</th>
<th>FCE⁴ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>80.0 ± 7.7</td>
<td>1.41 ± 0.03</td>
<td>7.89 ± 0.57</td>
<td>565 ± 38</td>
<td>10.2 ± 0.4</td>
<td>77.6 ± 4.5</td>
</tr>
<tr>
<td>0.1% LP</td>
<td>75.0 ± 6.4</td>
<td>1.40 ± 0.03</td>
<td>7.59 ± 0.76</td>
<td>541 ± 56.8</td>
<td>9.9 ± 0.3</td>
<td>76.2 ± 5.1</td>
</tr>
<tr>
<td>1.0% LP</td>
<td>81.7 ± 6.4</td>
<td>1.40 ± 0.01</td>
<td>7.69 ± 0.33</td>
<td>549 ± 20</td>
<td>9.9 ± 0.4</td>
<td>78.1 ± 1.4</td>
</tr>
</tbody>
</table>

¹BW: body weight.
²WG: weight gain.
³BWG: (WG / initial body weight) x 100.
⁴FCE: (WG / feed intake) x 100 (n = 4).

**Fig. 1.** Change of water quality parameters in a closed recirculating system during 50 days of feeding trial with a control diet and a diet containing LP at 0.1% and 1.0% concentrations. ●: control group, □: 0.1% LP group, ▲: 1.0% LP group. Bars represent mean ± standard error (n = 2).
the 0.1% LP group and the 1.0% LP group, the LBS count was detected and was observed to increase throughout the rearing. At 50 days, in the case of the rearing water, the LBS count in the 1.0% LP group was higher than that in the 0.1% LP group. In the case of the intestine, the LBS count was not detected in the control group. From 10 days to 50 days, the LBS count in the intestine was detected in the 1.0% LP group. It rapidly increased to around $1.0 \times 10^4/g$ at 20 days and remained at that level until 50 days. In the 0.1% LP group, the LBS count in the intestine was observed at only 50 days, and that count was much lower than that in the 1.0% LP group.

**Change of bacterial count in rearing water after the feeding was stopped**

Fig. 3 shows the change in the bacteria count in the rearing water after 50 days of rearing and after the feeding was stopped. The total viable count in the rearing water was stable with some fluctuations in the 1.0% LP and 0.1% LP groups. The LBS count in the 1.0% LP group decreased slightly. In the case of the 0.1% LP group, the LBS count dramatically decreased and was not detected at 57 days.

**Effects of LP on the tolerance of flounder to high temperature**

In the high-temperature stress test, fish receiving the diet containing LP (the 1.0% LP group) showed significantly higher tolerance than the fish in the control group (Fig. 4).

**Discussion**

In this study, the result of the rearing trial showed that the survival rate and growth rate of flounder were not affected by the oral administration of LP (Table 2). This result was in disagreement with the findings of Byun (1997), who reported that the growth of flounder...
was enhanced by the oral administration of \textit{Lactobacillus} sp. One of the reasons for the disagreement of our finding with that of Byun (1997) was likely the difference in the bacterial strain used. Byun (1997) used the \textit{Lactobacillus} strain isolated from the intestine of the host fish, while we used the \textit{Lactobacillus} strain for mammals. In addition, Byun (1997) used adult flounder in contrast to juvenile flounder in our study, meaning that fish size used for the rearing trial was quite different in each study. Jeong et al. (2006) used three diets containing probiotic bacteria, \textit{Bacillus} polyfermenticus, \textit{B. licheniformis} or \textit{B. polyfermenticus} plus \textit{Saccharomyces cerevisiae}, to juvenile founder as well as a control diet without probiotics and confirmed that the feeding of probiotics did not enhance the fish growth. This result agreed with our result. It seems that the species of probiotic bacteria and the size of the fish are important factors in whether probiotics enhance the growth of fish.

Water quality is a very important factor in sustainable aquaculture. Therefore, sometimes probiotics are used to recover the water quality in culture ponds. Probiotic bacteria, genus \textit{Bacillus}, have been widely accepted as a bioremediation agent in aquaculture (Queiroz and Boyd 1998; Prabu et al. 1999; Corre et al. 2001). To our knowledge, there has been no report on the improvement of water quality in the culture ponds by means of lactic acid bacteria such as genus \textit{Lactobacillus}. In our study, the oral administration of LP did not affect the water quality parameters of the rearing water in a closed recirculating system (Fig. 1), indicating that LP has no potential to act as a bioremediation agent like \textit{Bacillus}.

In the high-temperature stress test, the time to death of fish fed with LP was longer in comparison with that in the control group (Fig. 4), which means that the oral administration of LP is effective in enhancing the stress tolerance of \textit{P. olivaceus}. Stress is well known as a major factor which increases the susceptibility of fish to disease (Maule et al. 1989). Stress inhibits antibody production by circulating lymphocytes in fish (Ellis 1981; Maule et al. 1987, 1989). Matsuyama et al. (2000) found that the host-defense system in tilapia was damaged by the addition of cortisol, which is known as a stress hormone, and cortisol administration inhibited the production of interleukin-like factors (Tripp et al. 1987). Maule and Schreck (1991) found that stress and cortisol altered the affinity of corticosteroid receptors in lymphocytes. Yokoyama et al. (2007) investigated the effect of lactoferrin (LF), which is known as an immunostimulant, on high-temperature stress in Japanese flounder. They reported that the stress tolerance of fish in high-temperature surroundings was improved by the feeding of a 1000 mg LF/kg diet. In addition, a significantly higher level of the heat shock protein 70 family (HSP70s) in the skin and livers of fish fed with LF was observed in comparison to that of the control group (0 mg LF), and this tendency was consistent with the result of the lethal stress test in their study. HSP is a molecular chaperone that binds to and prevents the aggregation of proteins, and has been known as a kind of stress protein (Elicker and Hutson 2007). Therefore HSP level is thought to reflect physicochemical stress.

It has been established that some components known as immunostimulants can stimulate the immune response and the stress response

\textbf{Fig. 4.} Time to 50% mortality of \textit{P. olivaceus} in the high temperature stress test (34°C). An asterisk shows significant difference ($P < 0.05$). Error bars represent means ± standard deviation ($n = 3$).
Yokoyama et al. (2007) suggested that oral administration of an immunostimulant affects not only immune responses but also the expression of HSP synthesis in fish under stressful conditions, which implies that the improvement of stress tolerance relates to immunostimulation in fish. The immunostimulatory effect of probiotics is well known. Some studies reported that the mortality of fish artificially infected with pathogens decreased due to the use of probiotics. In shrimp, those that received oral administration of probiotics showed higher survival in comparison with the control group after immersion in pathogens (Rengpipat et al. 1998). The feeding of a probiotic diet containing *L. rhamnosus* reduced the mortality of rainbow trout by furunculosis (Nikoskelainen et al. 2001). Atlantic cod *Gadus morhua* became more tolerant to *Vibrio anguillarum* by means of feeding with the lactic acid bacteria *Carnobacterium divergens* (Gildverg et al. 1998). Jeong et al. (2006) described that the oral administration of probiotics to juvenile flounder enhanced the non-specific immune response of the fish and their resistance against *Edwardsiella tarda*. The oral administration of LP may have potential as an immunostimulant. The effect of LP on resistance to disease should be further studied by conducting a pathogen challenge test.

The LBS count in the water and the intestine increased as a result of feeding the fish a diet containing LP, and the density of LP in the diet positively correlated with the LBS count in the water and the intestine (Fig. 2). After the feeding was stopped, the LBS count in the 0.1% LP group was dramatically decreased in the rearing water over the course of 7 days, while that in the 1.0% LP group decreased slightly (Fig. 3). Va’zwuez et al. (2003) suggested investigating the survival of lactic acid bacteria as a potential probiotic in seawater. Based on the action of the LBS count during the feeding trial and after the feeding was stopped, continuous feeding and a concentration of more than 1.0% LP (>10^8 CFU/g-feed) would be required to keep the high occupation of LP to a total viable count in the water and the intestine. In this study, a closed recirculating system was employed as the rearing method for fish. Some of the probiotics administered orally colonize on the intestinal wall, and others pass through the digestive tract and are expelled with feces. Olafsen (2001) found that fish larvae ingest bacteria by drinking water, resulting in the formation of an indigenous larval microflota. Therefore, even if some of the probiotics cannot adhere to the intestinal wall, it is thought that fish can repeatedly ingest the probiotic cells expelled by fish into the water by drinking the water in a closed recirculating system. In this manner, a closed recirculating system increases the opportunities for the colonization of probiotics on the intestinal wall and is adequate for the application of probiotics in aquaculture. Long-term monitoring of the adherence of LP to the intestinal wall should be done.

In general, probiotics administered orally colonize the intestinal wall and inhibit the growth of pathogenic bacteria, which results in a decrease in fish mortality due to disease. Previously, the authors confirmed that the LP used in this study showed strong antibacterial activity against a wide spectrum of fish pathogens, such as *V. anguillarum, V. harveyi, Vibrio spp.*, and *Edwardsiella tarda*, using the double layer agar plate method (data not shown). In our study, LP with viability was observed in the intestine of the flounder (Fig. 2). If pathogenic bacteria invade the intestines of flounder from the rearing water, there is a possibility that LP with antibacterial activities can kill or eliminate the invaders, which would reduce the risk of disease outbreaks. The interaction of LP and pathogens in the fish intestine should be further studied.

Some of the orally administered probiotics are killed and degraded by digestive enzymes in the gastrointestinal tracts of the fish, and the cell components are absorbed from the intestinal wall. Fish physiological functions, such as immune responses, might be influenced without the colonization of probiotics on the intestinal wall, meaning that the components of probiotic bacteria play the role of an immunostimulant. Murosaki et al. (1998) found that heat-killed *L. plantarum* could induce interleukin-12 in mice, which implied that the colonization of probiotic bacteria is not quite essential for the stimulation of immune responses. Otherwise,
some reports have indicated that live bacterial cells are more potent as probiotics than dead bacterial cells (De Simone et al. 1986; Panigrahi et al. 2005). Taoka et al. (2006) found that commercial probiotics consisting of live cells are superior as immunostimulants for Nile tilapia as compared to those that consist of dead cells. The effects of LP on the immune response of flounder should be further researched.

To our knowledge, this study was one of the first reports on the dietary administration of LP to juvenile Japanese flounder in a closed recirculating system. The results showed that LP could survive in the fish intestine and that the oral administration of LP could improve stress resistance.

Acknowledgements

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"Lactobacillus plantarum"の添加効果

田岡洋介・弓削寿哉・前田広人・越塩俊介

ヒラメの生残率、成長、飼育水質、細菌数および高水温ストレス耐性について調べた。
*Lactobacillus plantarum*（LP）を0.1％および1.0％の割合で試験飼料に添加し、ヒラメを経口投与して50日間閉鎖型循環水槽にて飼育を行った。LP投与によるヒラメの成長、生残および水質への影響は認められなかった。LPの経口投与に伴い、特に1.0％LP添加区において、飼育水およびヒラメ腸試料の*Lactobacillus* sp.数が増加した。無添加区と比較してLP添加区のヒラメでは、高温に対するストレス耐性が有意に増加した。以上の結果より、経口投与されたLPはヒラメ腸内で生残し、ヒラメのストレス耐性向上に有効であることが示された。