4種類の乳酸菌を投与したティラピアにおけるEdwardsiella tarda感染症に対するプロバイオティクス効果の差
Differences of Probiotic Effects on Edwardsiella tarda Challenged Nile Tilapia (Oreochromis niloticus) Fed with Four Lactobacillus Species

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Abstract: Lactobacillus species can be used as supplementary probiotics, although the mechanism of protection of host animals from pathogenic bacteria remains unknown. The protective abilities of four candidate probiotics (L. brevis, L. plantarum, L. salivarius and L. rhamnosus) were examined in vitro and in vivo systems. Zone of growth inhibition on the assay plates revealed that the extracellular products (ECP) and bacterial cells (BC) of some Lactobacillus species have a bacteriostatic effect on E. tarda. Serum obtained from fish fed with L. salivarius and L. rhamnosus in the 7th and 14th days post infection of E. tarda significantly inhibited the growth of E. tarda as compared to serum from fish fed with L. brevis and L. plantarum. Fish fed of L. plantarum, L. rhamnosus and L. salivarius showed low mortalities, while fish fed of L. brevis and control fish did high mortalities. Furthermore, histological examination revealed hyperplastic appearance of macrophage with cytoplasmic enlargement in the early period of E. tarda infection of fish fed fish of L. salivarius and L. rhamnosus and eventually the formation of pyogranuloma. These results implied that four Lactobacillus species have different effects to protect fish from E. tarda infection with increasing macrophage response and lower mortality of fish.

Key words: Histopathology; Lactobacillus; Probiotics; Tilapia

Bacterial diseases have been a major concern to the aquaculture industry and are typically controlled by several ways such as vaccination, administration of chemotherapeutic medicine and the delivery of various immunostimulants (Spanggord et al. 2001). Vaccination is increasingly used, but only for specific diseases. On the other hand, chemotherapy remains to be a means used in aquaculture, although it can be hazardous to humans and the environments and lead to the development of drug-resistant species (Amabile-Cuevas et al. 1995). The emergence of antibiotic-resistant bacteria has contributed to the concept of probiotics where it gained interest as a preventive measure to combat pathogenic bacteria causing aquaculture diseases (Iranto and Austin 2002; Brunt et al. 2007). Probiotics have been shown to lower the mortality of infected fish (Gildberg et al. 1998; Chang and Liu 2002; Iranto and Austin 2002; Pirarat et al. 2006). The immune responses of the host depend on the probiotic characteristics, such as their antigenic nature, dose and route of administration (Collado et al. 2000; Nikoskelainen et al. 2003; Romalde et al. 2005). The immune responses include increases in alternative complement activity (Pirarat et al. 2006), serum bactericidal activity, stimulation of inflammatory cytokine gene expression (Saeji et al. 2003; Tafalla et al. 2005; Fast et al. 2006), production of extracellular metabolites and adhesion of probiotics to the gastrointestinal tract (Vine et al. 2004). Lactic acid bacteria, such as Lactobacillus species, are generally considered to be harmless and have an advantage for human health as probiotics.

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Lactobacillus species have been shown to boost the immune system of fish (Nikoskelainen et al. 2003; Pirarat et al. 2006). Pathogenicity of Edwardsiella tarda causing edwardsiellosis, has been reported to cause large mortalities and losses in fish farming (Janda et al. 1991; Kusuda and Kawai 1998). Its virulence arises from its ability to invade and survive in the host cells and tissues (Srinivasa Rao et al. 2001) causing bacterial septicaemia (Saeji et al. 2003). In tilapia, probiotics appear to stimulate the activity of macrophages in head kidney which play a major role in the survival of fish challenged by E. tarda (Pirarat et al. 2006) and A. salmonicida (Dautremepuïts et al. 2006). However, it is unclear which factors give the highest resistance against E. tarda infection to host animal. This study aimed to examine which of the four Lactobacillus species links to lower mortality in the experimentally infected tilapia and to find the effects of probiotic species on the head kidney of tilapia by using histological techniques.

Materials and Methods

Experimental fish and diets

Tilapia, Oreochromis niloticus, were used and maintained in de-chlorinated water at 26°C ± 0.6°C. Fish were fed with commercial pellet at the rate of 1% of the biomass per day. Acclimatization was done for 2 weeks in a 61 l tank under the normal temperature of 25°C for tilapia before the experiment. Each tank contains 12 fish with the average weight of 60 g. One group served as control feeding with commercial food for the whole experimental period. The other 4 probiotic groups of fish were fed for two weeks with commercial pellet with the addition of 1 × 10^10 cfu/g of either Lactobacillus brevis JCM 1559, Lactobacillus plantarum JCM 1551, Lactobacillus salivarius subs salivarius JCM 1230, and Lactobacillus rhamnosus ATTC 53103 prior to be challenged with Edwardsiella tarda and continuously feeding with the same diet for another two weeks.

Extracellular products (ECP)

Extracellular products of bacteria were obtained by following the method of Villamil et al. (2002). Briefly, the four bacteria were grown overnight in DeMan, Ragosa and Sharpe (MRS) broth (Merck). The overnight cultures were centrifuged at 3000 × g for 15 minutes where supernatant was separated from the pellet and filtered through a sterilized membrane (0.45 µm-pore). The filtered supernatant was aliquoted in small volumes and stored at −80°C until use.

Antimicrobial activity of crude ECP and bacterial cells (BC)

Overnight cultures of Lactobacillus species were centrifuged to separate the supernatant and bacterial cell. The supernatant or the ECP was used in the assay as crude ECP and adjusted ECP (pH 7.0). ECP was adjusted to pH 7.0 to exclude the effect of low pH on the growth of E. tarda. On the other hand, BC were washed twice with PBS (−) and after washing, they were suspended again in PBS (−).

Agar well diffusion assay was utilized to detect the antimicrobial activities of candidate probiotics against E. tarda. Tryptic soy agar (TSA) (Difco) plates were overlaid with overnight culture of E. tarda and dried for 30 minutes. Holes were punched out of the agar by using metal borer (6 mm diameter) and then filled with 75 µl of crude ECP, adjusted ECP (pH7.0) and BC from Lactobacillus species. Inoculated plates were incubated for 24–48 h at 30°C and measure the zone of inhibition (in millimeter from the edge of the hole) as a product of antimicrobial activity.

Fish challenge test

Five experimental groups were set-up for the in vivo challenge test. One group was used as control, feeding with commercial feeds for the whole experimental period. Fish in each experimental treatment were injected with 0.5 ml suspension of E. tarda (1 × 10⁹ cfu/fish) via intraperitoneal. The cumulative mortality (%) of challenged fish was observed until the 14th days post infection (dpi). Head kidney
of the fish from each aquarium was randomly sampled at 1st, 7th and 14th dpi for histological observation. The cause of death was elucidated by isolation of the infecting organism from the liver of dead fish. Blood was withdrawn from caudal vein of the sampled fish and separated serum for the bactericidal activity. Mortality was expressed as percent of dead fish to initial fish number and evaluated statistically by Z-test.

**Serum bactericidal activity**

The bactericidal activity was measured by comparing the growth of *E. tarda* in serum from challenge control and probiotic-fed fish. Two control groups and 4 probiotic groups were included in the analysis: control (−) or challenge-free fish, control (+) or challenged fish without probiotics and the four challenged fish groups with probiotics feeding. Briefly, serum samples (50 μl) were placed in triplicate in each well of a 96-well plate. A suspension of overnight culture of *E. tarda* was added separately (50 μl of 1 x 10^6 cfu/ml), mixed and incubated for 6 h at 30°C. The plate was centrifuged for 10 mins at 200 × g, the supernatant was removed and a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) was added to each well. After 15 mins in the dark, the optical density (600 nm) of the viable bacteria was measured. Data were statistically analyzed by one way test analysis of variance (anova) among treatments and were further tested using Duncan's Multiple Range Test (DMRT).

**Histopathology**

Independent experimental set-up was done for the histopathology following the procedure as mentioned in the mortality test. Head kidney tissue was fixed in 15% phosphate-buffered formalin, dehydrated in ethanol series and embedded in paraffin. Sections (4–5 μm) were stained with haematoxylin and eosin (H and E). Histopathological scoring was based on the incidence and appearance of observable changes in head kidney of two fish.

### Results

**Inhibitory effects of ECP and BC**

Potential probiotic bacteria were assayed for their ability to inhibit the growth of *E. tarda*. Crude ECP and BC from *L. plantarum, L. rhamnosus* and *L. salivarius* clearly indicated the growth inhibitory activity against *E. tarda* (Table 1) while BC of *L. brevis* bacterial cells did not exhibit antagonistic effect. Contrary to the BC of *L. brevis*, crude ECP was able to inhibit the growth of *E. tarda*. Adjusted (pH 7.0) ECP did not show any inhibitory activity (data not shown).

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<th>BC</th>
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<tr>
<td>Control PBS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>L. brevis</em></td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>L. salivarius</em></td>
<td>+++</td>
<td>+++</td>
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<tr>
<td><em>L. rhamnosus</em></td>
<td>+++</td>
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Thickness of clear zone indicates in millimeter (mm) from the edge of the hole.

+++; 2–3 mm; ++; 1-2 mm; +, 1 mm; −, no inhibition.

PBS, Phosphate buffer saline; ECP, Extracellular product; BC, Bacterial cells.

**Serum bactericidal activity**

On the 7th and 14th dpi, serum of fish fed with *L. rhamnosus* and *L. salivarius* were significantly different as compared with those of control (+), *L. plantarum* and *L. brevis* at P<0.05 level (Fig. 1)

**Challenge test**

Challenged fish became anorexic, lethargic, and laid down at the bottom of the tanks as the effect of pathogenic bacteria set in. Death of the fish was observed on the 1st day in all groups except *L. rhamnosus*-fed fish. Eighty three percent of cumulative mortality occurred within the 7th dpi in the control group followed by *L. brevis* (50%), *L. plantarum* (33.3%), *L. salivarius* (33.3%) and the lowest average mortality was *L. rhamnosus*-fed fish group (16.6%) (Fig. 2). The control and *L. brevis* groups were classified as high mortality group and the *L. plantarum, L. salivarius* and *L. rhamnosus*-fed fish groups were...
Fig. 1. Bactericidal activity of tilapia serum fed with probiotics against *E. tarda* using MTT assay. Sampling periods were done on the 1st, 7th, and 14th days post infection. Error bars represent standard error of mean. The letters A, B, C denote data groups of one sampling period. Data within each group with the same superscripts are not significantly different at the *P* < 0.05 level. (*n* = 3).

Fig. 2. Cumulative mortalities of probiotic fish and control fish under *E. tarda* challenge trial for 2 weeks. The final mortality rate was significantly lower in the *L. brevis, L. plantarum, L. salivarius* and *L. rhamnosus* groups than in the control group. (significant difference among the groups, *P* < 0.05, *n* = 12). Control and *L. brevis* were further categorized as high mortality and *L. plantarum, L. salivarius* and *L. rhamnosus* as low mortality group. Symbol (*) denotes statistically significant differences (*P* < 0.05) with respect to the control group.

classified as low mortality group (Fig. 2).

**Histopathological observation**

Head kidney of the challenged fish revealed some marked responses (Fig. 3). The most noticeable effect was the appearance of macrophage aggregations situated in the hemato-poietic tissue. Aggregation of macrophages characterized by hyperplastic cytoplasmic enlargement was observed on the 1st dpi in all groups, especially in *L. salivarius*-fed fish (Table 2). Gradual accumulations of macrophages were beginning to form pyogranuloma on 1st dpi in *L. rhamnosus* and *L. salivarius*-fed
Fig. 3. Histopathological differences in tilapia head kidney as challenged intraperitoneally by *E. tarda*. Aggregation of macrophages in positive control was observed (A). Series of different hematopoietic cells in the control without *E. tarda* was noted (B). Early hyperplastic macrophages (arrow) in the 1st dpi of *L. brevis* (C) was observed and formation of pyogranulomas (arrow) in the head kidney of tilapia fed with *L. plantarum* was observed on the 7th dpi (D); *L. salivarius* (E) and *L. rhamnosus* 7th dpi (F) were observed to form hyperplastic macrophages and appeared to have cytoplasmic enlargement and eventually formation of pyogranulomas, respectively (arrow).

fish, although no pyogranuloma was observed in other groups (Table 2). Pyogranulomas were more noticeable in probiotic groups of fish fed with *L. plantarum, L. rhamnosus* and *L. salivarius* on the 7th dpi. The *L. salivarius*-fed fish was observed to be undergoing the healing or recovery process from infection as shown in 14th dpi. All treatments can be categorized into 2 main groups in connection to mortality and histopathological scores (Fig. 2 and Table 2). The high mortality group had lower pyogranuloma scores than the low mortality group.
Table 2. Average histopathological score of two Lactobacillus-fed tilapia head kidney challenged by Edwardsiella tarda showing hyperplastic cytoplasmic enlargement of macrophage and pyogranuloma

<table>
<thead>
<tr>
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<th>1st dpi</th>
<th>7th dpi</th>
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<tbody>
<tr>
<td></td>
<td>HCE</td>
<td>PG</td>
<td>HCE</td>
</tr>
<tr>
<td>Control (+)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control (+)</td>
<td>1.5</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>HM</td>
<td>L. brevis</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>2</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>L. salivarius</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>LM</td>
<td>L. rhamnosus</td>
<td>2</td>
<td>1.5</td>
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<td>2.5</td>
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Control (−), control fish without E. tarda; C (+), control fish challenged with E. tarda.

0, no remarkable changes; 1, mild; 2: moderate; 3, serious.
dpi, days post infection; HCE, hyperplastic macrophage with cytoplasmic enlargement; PG, pyogranuloma; HM, high mortality; LM, low mortality.

Discussion

The nature of probiotic is attributable to the different factors of defense action or mechanisms thereby killing the pathogens by adhesion property and competition for nutrients (Olsson et al. 1992), releasing inhibitory metabolites such as lactic acid, bacteriocin (Jack et al. 1995) and acting as immunostimulant (Pirarat et al. 2006; Panigrahi et al. 2007).

These defense mechanisms have been confirmed in probiotic-fed fish experimentally infected with pathogens. Gildberg et al. (1997) reported that probiotics decreased the mortality rate of Atlantic cod fry challenged with Vibrio anguillarum. Furthermore, Lactobacillus and Carnobacterium have been demonstrated to increase the resistance of turbot larvae against a pathogenic Vibrio sp. (Gatesoupe 1994). Moreover, serum components like complement factors (Nikoskelainen et al. 2002), lysozyme (Grinde 1989) and peroxidase (Rosaile et al. 2006) stimulate the immune system of the host and thereby protect the host against pathogenic bacteria (Spanggaard et al. 2001).

In our study, the four Lactobacillus species reduced the mortality of artificially infected tilapia. Lactobacillus-fed tilapia showed rapid and enhanced macrophage-pyogranuloma formation although to a variable degree. Immediate response of macrophages to infection was noticeable in head kidney as also observed by Pirarat et al. (2006). Although they are categorized as one genus, probiotic bacteria can be ranked according to their antagonistic effect. In fish, exogenous molecules can modulate macrophage activity and can also have both synergic and antagonistic effects on host cell (Novoa et al. 1996). Generally, probiotic bacteria are thought to kill pathogenic bacteria directly (Brunt et al. 2007). This was also evidenced in our results where ECP and BC bactericidal activity clearly inhibit the growth of E. tarda except for L. brevis by bacterial cells. Adjusted ECP (pH 7.0) did not prove inhibitory action compared to crude ECP, hence, low pH or acidity of the crude ECP confirmed antagonistic property on the growth of E. tarda (Table 1).

However, the probiotic bacterium did not come in contact with E. tarda in the host system because the former was incorporated in the diet and the latter was injected intraperitoneally. Serum may contain a variety of substances that can inhibit the growth of infectious microorganisms like the presence of lysozyme and complement which can boost the immune system (Pirarat et al. 2006) but the adverse result in serum bactericidal activity does not strongly support the mortality test.

Pirarat et al. (2006) reported that L. rhamnosus promotes rapid aggregation of macrophage in the head kidney which is responsible for the clearing of E. tarda during early stage of infection. In our study, the high mortality (control (+) and L. brevis groups) and low mortality group (L. plantarum, L. salivarius and L. rhamnosus-fed fish groups) corresponded to the appearance of hyperplastic cytoplasm enlargement and pyogranuloma as effects of challenging to fish. The four Lactobacillus species demonstrated the early response of hyperplastic appearance of macrophages with the cytoplasmic enlargement. The peak appearance of pyogranuloma occurred on about 7th dpi, which paralleled with the cumulative mortality rates of the low mortality group. This aggregation of macrophages shifted to pyogranuloma formation in all probiotic-fed fish, especially those fed with L. rhamnosus and L. salivarius as observed in the later stage when the fish are believed to...
be in the process of healing from infection. Our study revealed that the aggregation of macrophages and clearing of *E. tarda* and eventually appearance of pyogranuloma are general host reaction in *E. tarda* infection against pathogenic bacterium among *Lactobacillus*-fed group. This host response is thought to reduce the mortality where the key of immune reaction lies as the hyperplastic cytoplasm enlargement and pyogranuloma occurred more markedly in the low mortality group.

The results of ECP, BC and serum bactericidal activity in this study were limited. Serum components should also be validated to connect with the association of early immune response of the host on probiotic diet. Further studies are needed to examine whether inflammatory cytokines have a role in the manifestations of macrophage aggregation and formation of pyogranuloma to the infected site.

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**References**


4種類の乳酸菌を投与したティラピアにおける*Edwardsiella tarda*感染症に対する プロバイオティクス効果の差

Stephanie S. PIMENTEL・片桐孝之

乳酸菌が魚類の抗病性をいかに向上させるかは明らかにされていない。今回は4種類の乳酸菌*Lactobacillus brevis* (LB), *L. plantarum* (LP), *L. salivarius* (LS), *L. rhamnosus* (LR)の抗病性賦活能をin vitro, in vivoの両方から調べた。LBを除く他の3菌種は*E. tarda* (ET)の増殖を阻害した。4種類の菌を個々に14日間投与したティラピアにETを感染させて得た血清とETを混合し、殺菌作用を検討した。LS, LR区の魚の血清は殺菌作用が認められた。また、ET感染による死亡率は、菌の非投与区で83.3%だったが、4種の菌投与区では16.6 〜 50%であった。病理組織学的には、菌を投与した魚で頭髪マクロファージの肥大や肉芽腫の形成の早期化が顕著であった。その程度は死亡率の低下と関係しており、その差は個々の菌種で異なった。