Effect of Continuous and Interval Administration of Peptidoglycan on Innate Immune Response and Disease Resistance in Japanese Flounder Paralichthys olivaceus

Jorge GALINDO-VILLEGAS1, *, Toshiro MASUMOTO2 and Hidetsuyo HOSOKAWA2

Abstract: Peptidoglycan (PG), derived from Brevibacterium lactofermentum, is known to enhance innate immune response and disease resistance. The present study investigated the effect of the interval dietary administration of PG on the innate immune response and the disease resistance against Edwardsiella tarda in Japanese flounder. Continuous and interval dietary administration at 2.5% body weight per day of supplemental 0.3% PG in dry diet were tested following one of four patterns for 40 days: control group (only control diet), 10/10 group (PG diet/control diet, every 10 days), 15/15 group (PG diet/control diet, every 15 days) or daily group (only PG diet). Innate immune response (serum lysozyme activity, chemotaxis and intracellular respiratory burst) were evaluated after 7, 10, 15, 20, 25, 30 and 40 days. Furthermore, three bacteria challenges were conducted on days 10, 25 and 40. Significant enhancements in serum and leucocytes activities were observed along the trial. Chemotaxis was enhanced in all sampling day in most PG-administrated groups. The enhanced disease resistance was positively correlated with the enhancement of innate immune response in all PG-treated groups after 10 days and only in both 10/10 and 15/15 groups on days 25 and 40. These findings suggest that interval administration of PG persist the enhanced disease resistance of Japanese flounder against E. tarda infection through cyclical activation of the immune system, while daily administration for more than 15 days has inert effect in disease resistance.

Key words: Paralichthys olivaceus; Disease resistance; Interval administration; Peptidoglycan

Immunostimulants can increase resistance to infectious diseases, not by promoting the specific immune response, but by enhancing non-specific defense mechanisms. The use of immunostimulants is an effective mean of increasing the immunocompetence and disease resistance in fish. Research on fish immunostimulants such as lactoferrin, glucans and chitin has been recently initiated and many agents are currently being used as immunostimulant in the aquaculture industry (Sakai 1999).

The conserved bacterial structure of peptidoglycan (PG), used widely in Japan, works excellently as an immunostimulant. PG prepared from Brevibacterium lactofermentum increased phagocytosis in yellowtail (Seriola quinqueradiata) and resistance to Enterococcus seriola infection (Itami et al. 1996). The efficacy of PG was also demonstrated against vibriosis in rainbow trout (Oncorhynchus mykiss) (Matsuo and Miyazono 1993) and yellow-head baculovirus infection in black tiger shrimp (Penaeus monodon) (Boonyaratpalin et al. 1995). Also in our previous study, oral administration of PG diet (0.3% PG in diet) enhanced innate immune response (chemotaxis, phagocytic activity and respiratory burst activity of leucocytes and serum lysozyme activity) and disease resistance against Edwardsiella tarda in Japanese flounder (Paralichthys olivaceus) (Galindo-Villegas et al. 2006).
in press). However, previous studies showed that long-term administration of PG diet tended to have a negative effect on disease resistance (Matsuo and Miyazono 1993). Frequent PG administration as immunostimulant has a high impact on fish disease resistance and/or the innate immunity, which might result in exhausting immune system. The persistence of PG effects may be determined by the period of dietary administration in fish. Some immunostimulant substances are known to stimulate different components on the immune system via control of dosage and duration of feeding, which confer disease resistance against most common fish pathogens (Sealey and Gatlin III 2001).

The aim of this study was to investigate the effect by the continuous or interval dietary administration of PG on the innate immune response and the disease resistance against *E. tarda* in Japanese flounder.

**Material and Methods**

**Fish**

Five hundred and ten juvenile Japanese flounder (*P. olivaceus*), with an average weight of 55 g, were obtained from Sakamoto Fish Farm Tosa, Japan. No occurrence of edwardsiellosis has been observed in the farm from where the fish were obtained. Fish were divided randomly into four duplicated 1000 l tanks with running seawater. The tanks were artificially oxygenated, with a flow rate of 1800 l/h under natural photoperiod at 24°C.

**Feeding**

To acclimatize fish to the experimental condition and achieve similar physiological condition, all groups were fed to satiation for seven days with the same control diet as described in Table 1. PG isolated from *B. lactofermentum* (PG-AQUA) was purchased from Ajinomoto (Tokyo, Japan). According to our previous observations, the experimental diet (PG diet) was prepared by adding PG-AQUA (3 g/kg diet) to basal diet in place of equal amount of α-cellulose (Galindo-Villegas et al. in press). To dry mixtures of control diet or PG diet, 40% water was added to allow extrusion with a food grinder. Subsequently, pellets were cut into suitable size in regard to fish mouth.

Fish in each tank with its replica were fed with one of the following regimes: control group (only control diet), 10/10 group (PG diet/control diet every10 days), 15/15 group (PG diet/control diet every15 days) or daily group (only PG diet). Fish were fed the diets twice a day at the rate of 2.5% biomass/day for 40 days. In each tank, total biomass was determined only at the beginning of the trial to avoid handling stress. Daily feeding ratio was adjusted after each sampling.

**Analytical methods**

Blood and head-kidney cells were randomly sampled from ten fish of each tank, which were kept 24 hours without feeding before sampling after 7, 10, 15, 20, 25, 30 and 40 days of dietary treatment. Blood was obtained from the caudal vessel of individual fish after anaesthetization with 200 ppm MS-222 (Tricaine methanesulfonate) (Sigma, USA). Serum samples were obtained from whole blood collected using non-heparinised syringes and allowed to clot for 1 h at room temperature and for 6 h in the cold (4°C) before its centrifugal separation. Samples were preserved at −80°C prior to analysis.

After completely drawing the blood, the anterior kidney of each fish was aseptically

<table>
<thead>
<tr>
<th>Table 1. Composition of the experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients</td>
</tr>
<tr>
<td>Brown fish meal</td>
</tr>
<tr>
<td>Grill meal</td>
</tr>
<tr>
<td>Blood meal</td>
</tr>
<tr>
<td>Vitamin mix</td>
</tr>
<tr>
<td>Mineral mix</td>
</tr>
<tr>
<td>CMC - Na</td>
</tr>
<tr>
<td>α - Cellulose</td>
</tr>
<tr>
<td>PG</td>
</tr>
</tbody>
</table>

1Supplied (g/kg diet): Thiamine hydrochloride (0.022), riboflavin (0.022), pyridoxine hydrochloride (0.023), nicotinic acid (0.096), phptothenic acid (0.072), inositol (0.6), biotin (0.0014), folic acid (0.024), choline chloride (3.0), cyanocobalamin (0.0004), ascorbic acid (0.112), palmitate (0.011) and tocopherol acetate (0.119).
2Supplied (g/kg diet): KH2PO4 (4.12), Ca(H2PO4)2 • H2O (6.18), Ca-lactate (2.82), Fe-fumaric acid (1.6), ZnSO4 • 7H2O (0.1765), MnSO4 • 4H2O (0.081), CuSO4 • 5H2O (0.0155), CoCl2 • 6H2O (0.0005) and KIO3 (0.0015).
3Carboxymethyl cellulose sodium salt (Wako Pure Chemical Industries, Ltd., Japan).
4Peptidoglycan PG-AQUA, (Ajinomoto, Japan).
removed and immersed in ice-cold RPMI-1640 medium (Sigma, USA) supplemented with 10.3 ml/l penicillin-streptomycin-L-glutamine (Sigma, USA), 0.1% heparin (Sigma, USA) and 2% fetal bovine serum (ICN, USA). Cell suspension was obtained by forcing fragments of the organ through a 100 µm nylon cell strainer (BD Falcon, USA). Cell suspensions were centrifuged on 51% discontinuous Percoll (Sigma, USA) density gradients. Leucocytes were recovered and adjusted to 2 × 10⁶ cells/ml RPMI-supplemented media. Cell viability was greater than 98%, as determined by the trypan blue exclusion test.

**Chemotaxis assay**

The chemotaxis activity of head-kidney leucocytes was examined as described by Ninomiya et al. (1995). Briefly, two hundred microliters of Zymosan-activated normal Japanese flounder serum was placed in the lower well of Blind Well Chambers (Nucleopore Co., USA) and used as the chemotactic agent. Nucleopore filters (5 µm) were placed onto the wells. After fixing on the screws, 200 µl of the head kidney cell suspension was added. Chambers were incubated at 25°C for 3 h. Thereafter, filters were washed, removed from chambers and stained with Giemsa’s solution (Merck, Germany). Migrated leucocytes were counted in 30 optical fields under a microscope BX-40 (Olympus, Japan).

**Superoxide anion production**

Respiratory burst activity from head kidney leucocytes was determined by the detection of extra cellular O₂⁻ produced following reduction of ferricytochrome C (Cyt C) as described by Secombes (1990). One hundred microliters of head-kidney leucocytes suspension in Hanks’ balanced salt solution, phenol red-free, containing 2 × 10⁷ cells/ml was seeded into triplicate wells (per sample) of a 96-well plate (Nunc, USA). Leucocytes were allowed to adhere for two hours at 25°C and non-adherent cells were removed by carefully washing with Hanks’ solution. Following this procedure, 100 µl of Hanks’ solution containing 1 µg/ml phorbol myristate acetate (PMA) and 2 µg/ml Cyt C were added to each culture well and was incubated at room temperature for 60 min. Cyt C solution alone or containing PMA and Super Oxide Dismutase (SOD) without cells were used as blanks. After incubation, Cyt C reduction optical density was read at 550 nm with a microplate reader model 550 (Bio-Rad, USA).

**Lysozyme activity**

Serum lysozyme activity was determined by the turbidimetric method based on the lysis of *Micrococcus lysodeikticus* (Sigma, USA) as described by Ellis (1990) and modified by Villamil et al. (2003). Fifty microliters of undiluted serum were plated in triplicate into each well of a 96-well microtitler plate and 150 µl of *M. lysodeikticus* suspension in 0.1 M phosphate citrate buffer, (pH 6.2), was added to each well. After rapid mixing at 37°C, the change in turbidity was measured every 30 s for 5 min at 450 nm using a micro plate reader model 550 (Bio-Rad, USA). A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min. Lysozyme units present in sera were obtained from a standard curve made with hen egg white lysozyme (Sigma, USA).

**Experimental infection**

After 10, 25 and 40 days of feeding trial, bacterial challenges were performed by intraperitoneal injection with 0.1 ml of a 3 × 10⁶ cfu/ml E. tarda strain EF-1 suspended in saline obtained from the Fish Disease Laboratory, Faculty of Agriculture, Kochi University. Duplicate groups of 10 fish in each 400 l tanks were supplied with running sea water (average temperature of 22.5°C) at a flow rate of approximately 10 l/min. Dead fish were removed twice a day from the tanks and survivors recorded for 15 days. Kidney samples of resulting mortalities were examined by the API 20E identification kit (Biomerieux, France) to verify the cause of death. For each treatment, the relative per cent survival (RPS) was calculated with the formula of De Baulny et al. (1996). RPS = ((Mortality (%) of untreated control – Mortality (%) of treated group)/(Mortality (%) of untreated control)) (100).
**Statistical analysis**

Mean values of all parameters were subjected to one-way ANOVA followed by the Tukey-Kramer test to determine significant differences. Comparisons were made at the 5% probability level. SAS software (Ver. 2.0, SAS Institute Inc., USA) was used for the analysis.

**Results**

**Chemotaxis activity**

PG diet enhanced Japanese flounder head-kidney leukocyte migration when compared to control diet in most of the assayed times. A significant difference was observed in all groups fed PG diet on days 7, 10 and 15. A constant enhancement was observed in the 10/10 group during the trial. On day 40, both the 10/10 and 15/15 groups presented significant enhancement when compared to the control, but no differences were detected between those two groups. Between days 20 to 30, daily and control groups were not significantly different as did it on day 40 (Fig. 1).

**Respiratory burst**

The respiratory burst activity of Japanese flounder head-kidney leukocytes isolated from all groups fed PG diet presented similar activity on days 7, 15 and 20. On day 10, 10/10 and 15/15 groups showed significant difference to the control, but not to daily group. Response on days 25 and 30 was significantly enhanced in the 10/10 group as the 15/15 group on day 40 (Fig. 2).

**Lysozyme activity**

Serum lysozyme activity was positively affected in all groups fed PG diet from day 20. Significant enhancement was detected first in the daily group from day 20. All groups fed PG diet showed significant increased activity from day 25 to day 40. The highest activity was observed on day 30 for the 15/15 group and day 40 for the 10/10 group (Fig. 3).

**Disease resistance**

On days 10, 25 and 40, fish were challenged by peritoneal injection with the pathogenic bacteria *E. tarda* and mortalities were monitored for 15 days. In the first challenge (day 10), all groups fed PG diet finished with a higher survival rate compared to the control group. In the second challenge (day 25), the 10/10 group presented the highest survival rate. In the last challenge (day 40), both 10/10 and 15/15 groups indicated high survival rate compared to control and daily groups (Fig. 4). Relative percent survival for the three groups fed PG diet were (56:47:51), (60:40:34) and (44:67:11) in day 10, 25 and 40,
Effect of Peptidoglycan by Interval Administration

Fig. 2. Respiratory burst activity in head kidney-leucocytes of Japanese flounder fed supplemented PG under three different administration protocols or control diet for 40 days. Data are represented as mean values ± SD (n = 10). Sub-indexes over the bar graph denote significant differences (Tukey-Kramer, P < 0.05).

Fig. 3. Effect in serum lysozyme activity in head kidney leucocytes of Japanese flounder fed supplemented PG under three different administration protocols or control diet for 40 days. Data are represented as mean values ± SD (n = 10). Sub-indexes over the bar graph denote significant differences (Tukey-Kramer, P < 0.05).

Fig. 4. Changes in survival rate of Japanese flounder fed supplemented PG under three different administration protocols or control diet. On day 10, 25 and 40, ten fish per treatment in duplication were challenged against the pathogenic bacteria *E. tarda* strain EF-1. Data are expressed as total percent survival for each treatment after 15 days of challenge.

respectively. In addition, all resulting mortalities were confirmed to be due *E. tarda* infection.

**Discussion**

The use of immunostimulants in some marine fish species has been promoted as a way to increase disease resistance (Sakai 1999; Galindo-Villegas and Hosokawa 2005). Immunostimulants enhance the humoral and cellular innate immune system, which increases disease resistance (Yano 1996; Secombes 1996; Ellis 2001; Shoemaker 2001).

Administration of immunostimulants has been
mostly done with laboratory methods such as injection (mostly as vaccine adjuvants), immersion, intragastric or intestinal administration. However, such methods are not suitable for large scale fish production systems (Sakai 1999; Raa 2000; Sealey and Gatlin III, 2001; Galindo-Villegas and Hosokawa 2004). Oral administration of immunostimulants is useful for large-scale aquaculture. The effect of immunostimulants is likely to be of short duration (Anderson 1992; Sakai 1999). The length of enhancement of disease resistance by immunostimulants using the oral administration is unclear (Sakai 1999; Sealy and Gatlin III 2001). In our previous study using several dietary substances, innate immunity was enhanced at day 10, but was decreased at day 20 by oral administration of PG (unpublished data). Thus, in this study, it was examined the interval oral PG administration to maximize the positive PG effect.

The PG effects were evaluated by measuring innate immunity and observing disease resistance to E. tarda in this study. The major role of immunostimulant is to prevent fish from diseases. Oral administration of PG in Japanese flounder showed obvious positive effect in disease resistance except in daily group on day 40. The disease resistance of 10/10 and 15/15 groups persisted higher survival rate compared to that of control group during the trial. The survival rate of daily group started to decrease from day 25 and showed almost same ratio as that of control group on day 40, which suggests that the long-term administration of PG is not effective on disease resistance. In the study with yellowtail, fish were challenged by immersing them in Vibrio anguillarum suspension after 28 or 56 days of oral PG administration (Matsuo and Miyazono 1993). A significantly higher survival rate compared to that of control was observed on only day 28. On day 56, no significant difference in the survival rates was observed between the PG-administration group and the control group. Also using glucans (polysaccharide similar to PG) as immunostimulant, negative effects on the resistance against pasteurellosis were found by the long-term administration [two cycles of test diet (2 weeks) and control diet (1 week)] in gilthead seabream (Sparus aurata) (Couso et al. 2003). Rainbow trout fed with high doses of glucans were more susceptible to infection by Flexibacter columnaris (Jeney et al. 1998). The intraperitoneally injection with high doses of glucan to Atlantic salmon (Salmo salar) sometimes caused higher mortalities (Robertsen et al. 1990). High doses and/or long-term administration of immunostimulants seemed to have negative or no effect as observed in our study.

Jeney et al. (1998) and Robertsen et al. (1990) suggested that the phagocytic cells become overloaded with glucan particles, decreasing their phagocytic capacity to engulf bacteria, which resulted in the decrease of disease resistance. High doses of glucans stimulated the respiratory burst of fish leucocytes in vitro, but the cells sometimes became exhausted and did not respond to a second stimulation with PMA (Castro et al. 1999). In this study, phagocytes and respiratory burst activity of leucocytes and serum lysozyme activity were measured in humoral and cellular innate immune system. The innate immune response showed similar pattern with the results of survival rate on each day. In the first bacteria challenge (day 10), chemotaxis and lysozyme activities were well reflected in the survival rate. The survival rate in the second bacterial challenge (day 25) was well correlated with respiratory burst and lysozyme activities. In the third bacteria challenge, chemotaxis was well coincident with the survival rates. Those observations suggest that the disease resistance in Japanese flounder was conferred by stimulating innate immune system.

The innate immunity in the 10/10 and 15/15 groups were higher than the control in most sampling days. The innate immune system in daily group might be exhausted from day 20, which might cause the inert effect of PG.

In conclusion, these findings indicate that the diet supplemented with 0.3% PG has positive effect on disease resistance but needs the appropriate feeding interval to exert its effects. The 10 or 15 days of interval in oral PG administration persisted high disease resistance through the cyclical activation of the innate
immune system in Japanese flounder.

References


ベブチドグリカン添加飼料の給餌間隔がヒラメの自然免疫と抗病性に及ぼす効果

Jorge GALINDO-VILEGAS・益本俊郎・細川秀毅

Brevibacterium lactofermentum 由来のベブチドグリカン（PG）は自然免疫応答と抗性を向上させることが知られている。本研究では給餌間隔が自然免疫応答と Edwardsiella tarda に対する抗病性向上能に及ぼす影響について調べた。試験飼料に PG を0.3％で添加した飼料または無添加飼料を用いて試験魚に以下の4つの給与間隔で40日間給与した：対照群（無添加のみ）、10/10群（添加/無添加を10日毎）、15/15群（添加/無添加を15日毎）、添加のみ。試験期間中の7, 10, 15, 20, 25, 30および40日にリソチーム活性、遊走能、活性酸素産生能を測定した。さらに10, 25, 40日に攻撃試験も実施した。PG の給与により血清リソチーム活性と活性酸素産生能が投与期間に伴い有意に亢進し、遊走能は7日後から試験終了時までほとんどの群で有意な上昇を示した。10/10群と15/15群では25日と40日に実施した攻撃試験の結果と自然免疫応答の結果との間に正の相関関係があった。添加飼料のみを給餌した群では、40日後の生存率が対照群とほとんど変わらなかった。以上の結果からヒラメでは PG の給与間隔により自然免疫を周期的に亢進することでエドワジラ症に対する抵抗力を維持させることが明らかとなり、15日以上の PG の連続給与は抗病性を低下すると考えられた。