J. Microbiol. Biotechnol. (2012), **22**(4), 526–529
http://dx.doi.org/10.4014/jmb.1108.08047
First published online February 14, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of a Probiotic Strain, *Enterococcus faecium*, on the Immune Responses of Olive Flounder (*Paralichthys olivaceus*)

Kim, Yu-Ri[1], Eun-Young Kim[1], Sun-young Choi[1], Muhammad Tofazzal Hossain[1], Ryunkyoung Oh[1], Won-Seok Heo[1], Jong Min Lee[1], Young Chai Cho[2], and In-Soo Kong[1]*

[1]*Department of Biotechnology, Pukyong National University, Busan 608-737, Korea*
[2]*Centre for Marine Biotechnology and Bioengineering Research, amBio Co. Ltd, Jinju, Gyeongsangnam-do, Korea*

Received: August 18, 2011 / Revised: November 24, 2011 / Accepted: November 27, 2011

The present study was aimed to investigate the effect of a probiotic, *Enterococcus faecium*, on the immune responses against infection with the marine fish pathogen *Lactococcus garvieae* in olive flounder (*Paralichthys olivaceus*). The immune responses were assessed by lysozyme activity, complement activity, protease activity, and expression of proinflammatory cytokines by RT-PCR. The lysozyme and complement activities were increased between 9 to 15 and 9 to 13 days, respectively, and antiprotease activity was slightly elevated after 5 days of probiotic treatment. The TNF-α and IL-1β expressions were observed from kidney and spleen. The results of this study reveal that *E. faecium* induces immune-responsible materials and protects olive flounder from lactococcosis.

Keywords: Probiotics, *Enterococcus faecium*, immune response, lactococcosis, olive flounder

Lactococcus garvieae is a Gram-positive bacterium that causes lactococcosis in cultured marine and fresh water fish species when water temperature increases over 16°C in the summer months. Lactococcosis is a kind of streptococcosis and causes important economic losses in aquaculture [24], and the most dominant strain is *L. garvieae* isolated from olive flounder (*Paralichthys olivaceus*) showing streptococcosis syndrome, which is one of the most important marine cultured fish in Korea [16]. Both young and adult fish become susceptible to lactococcosis under environmental stresses, and the mortality rate may rise over 50% during a period of 3–7 days of infection [15, 18].

Oral administration of antibiotics is commonly used to treat lactococcosis, but the appearance of antibiotic-resistant strains makes antibiotics use limited [23]. Instead, studies have been done on the immune enhancer against pathogenic bacteria as well as unspecified disease by increasing the self-defense capability and thus increasing the productivity of healthy fish by preventing mass mortality [2]. The immune enhancers improve the activity of nonspecific immune factors including phagocytic cells [10], natural killer cells [11], lysozyme [4], and complement activity [25].

Until now, many researches have been conducted on the application of lactic acid bacteria (LAB) as immune enhancers for the protection of fish from bacterial diseases [5, 18–20]. According to Gatesoupe [9], *Enterococcus faecium* can be used as a probiotic for controlling infection by *Edwardsiella tarda*, which is known to be a strong pathogen causing edwardsiellosis in marine organisms [5]. LAB have the ability to produce antimicrobial compounds as metabolites (*e.g.*, lactic acid, diacetyl, carbon dioxide, hydrogen peroxide, and bacteriocins) and thus inhibit intestinal harmful bacteria [5]. In addition, cell wall compounds of LAB induce cytokines of macrophagocyte [6] and activate complement [14]. *E. faecium* is a LAB and a normal intestinal microflora of human and some other animal species, including fish, with inhibitory effects against important enteropathogens [1]. *E. faecium* for manufacturing production as feed was provided from amBio Co. (Jinju, Korea).

The nonspecific immune responses, including lysozyme, complement, and antiprotease activities, are the first defense mechanism against pathogens in the fish body [17]. Lysozyme cuts the β-1,4 glycosidic bond in the peptidoglycan of the bacterial cell wall [9]. The complement stimulates the phagocytes to accumulate in the site of infection [3], and antiprotease inhibits the action of proteases of some pathogenic bacteria [22]. The cytokines are important signal molecules and play important roles in regulating immune response. Specifically, TNF-α and interleukin 1β

**Corresponding author*
Phone: +82 51 629 5865; Fax: +82 51 629 5863;
E-mail: iskong@pknu.ac.kr

(IL-1β) are involved in the inflammatory response of the innate immune system, and are the main components against bacterial pathogen in fish. Interleukin 6 (IL-6) plays important roles in regulating immune responses and inflammation [8]. In this study, we investigated the nonspecific immune response and expression of cytokines in olive flounder induced by *E. faecium* against *L. garvieae* infection.

For this, olive flounders collected from a farm located in Kijang, Busan were domesticated for more than 1 week. Apparently healthy flounder (average weight 33.4 ± 10 g, average height 15.4 ± 1.16 cm) was selected and 100 μl of ~10^9 *E. faecium* cells was intraperitoneally injected as a probiotic additive. To check the induction of immune response in olive flounder by probiotics against pathogenic bacteria, both probiotics-injected and control groups were intraperitoneally challenged with *L. garvieae* KCTC 3772 (10^7 CFU) from 1 to 15 days of post injection at 2-day intervals. After 24 h, blood was collected from 30 flounders per group for sera to measure the lysozyme, complement, and antiprotease activities, and organs (gill, liver, spleen, and kidney) were collected to determine the expression of cytokine genes by RT-PCR.

A turbidometric assay using lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, Co., MO, USA) was used to determine the lysozyme activity of sera collected from both probiotics-injected and non-injected fish [22]. The lysozyme activity of probiotics-injected olive flounder was 5–20 U/ml from 1 to 7 days, which was less than the control group (105 U/ml). However, the activity was increased to 115, 180, and 255 U/ml at 9, 11, and 13 days, respectively. In the 15-days-group, lysozyme activity decreased to 185 U/ml, maintaining the activity higher than the control group (Fig. 1). Pirarat *et al*. [20] used *Lactobacillus rhamnosus* in feed of tilapia to check the lysozyme activity against intraperitoneally injected *E. tarda* and found enhanced lysozyme activity as defensive power against *E. tarda*. *L. rhamnosus* as feed additive was also reported by Panigrahi *et al.* [19] for enhancement of nonspecific immune factors in rainbow trout. The results of the present study on lysozyme activity also support the findings mentioned above, except the probiotics administration route. This means that the probiotics supplemented with feed or as injection have beneficial effects on the lysozyme activity of the nonspecific immune system.

The complements of serum as chemotactic factors participate in bacteriolysis by influencing the phagocytes to the site of infection [3], and as active promoters in phagocytosis of fish pathogens through opsonization [7]. We followed the methods of Rao *et al*. [21] to determine the complement activity of serum collected from olive flounder challenged with *L. garvieae*. In Fig. 2, the complement activity was significantly higher on 9 (88.6%), 11 (98.6%), and 13 (87.6%) days after probiotics injection compared with the control group (72.9%). Lower complement activity was observed on 1, 3, 5, and 7 days compared with the control group (data not shown). According to Pirarat *et al*. [20], the complement activity was increased in tilapia supplemented with *L. rhamnosus* as probiotics for 2 weeks. Kim *et al*. [14] reported that the cellular component of *L. plantarum* induces complement system. In this study, *E. faecium*-injected olive flounder showed complement activity of more than 20% compared with the control group.

Antiprotease activity of serum was determined as described by Sharifuzzaman and Austin [22]. The percentage of trypsin inhibition was increased on 1 (88.1%), 3 (89.4%), 5 (91.2%), and 7 (89.5%) days after probiotics injection compared with the control group, 87.2% (Fig. 3). Total antiprotease activity of serum on 5 days increased, but not significantly. It has been also reported that there were no significant differences in antiprotease activity compared with the control group after administration of probiotics to cod and rainbow trout [13, 17].

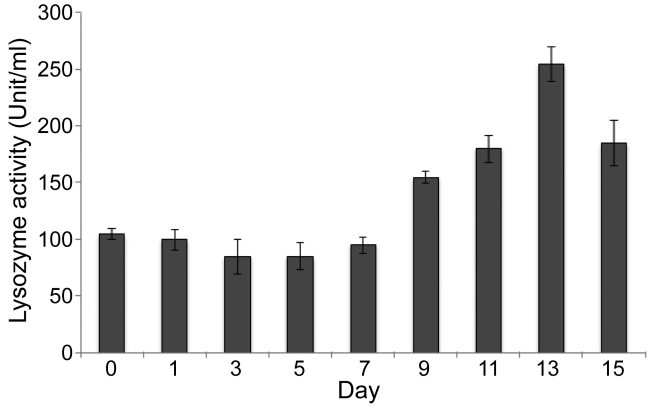

Fig. 1. Changes of lysozyme activity (U/ml) in serum of olive flounder, *P. olivaceus*, injected intraperitoneally with *E. faecium* (10^9 CFU).

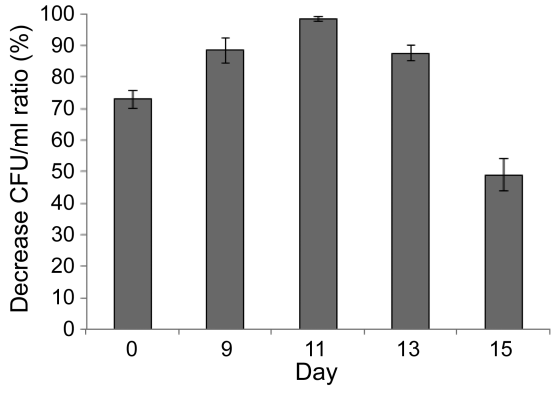

Fig. 2. Changes of complement activity of serum against *L. garvieae* in olive flounder, *P. olivaceus*, injected with *E. faecium* (10^9 CFU).

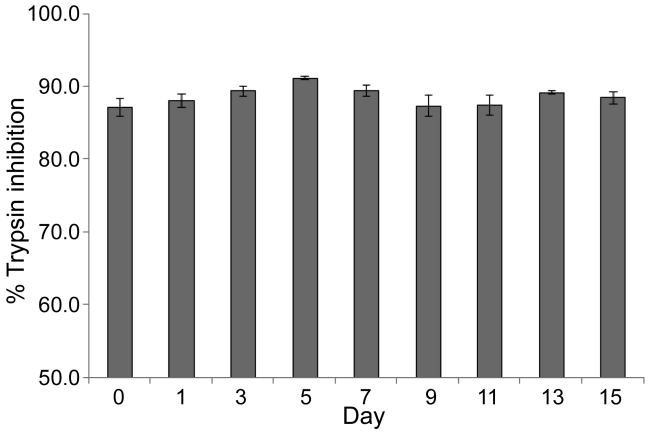

Fig. 3. Serum antiprotease activity in olive flounder injected with *E. faecium* (10^9 CFU).

To investigate the transcriptional levels of major cytokine genes using RT-PCR analysis, total RNA was isolated from the collected organs (gill, liver, spleen, kidney) by Tri-solution (BSK Bio Science, Korea). First-strand cDNA synthesis was carried out by a RevoScript Reverse Transcriptase PreMix Kit (iNtRON Biotechnology, INC., Korea). The TNF-α, IL-1β, and IL-6 as major cytokines and β-actin as positive control were examined by RT-PCR using the primers mentioned by Hong *et al*. [8]. The PCR conditions were also used according to Hong *et al*. [8]. The β-actin gene was expressed from all the organs. The cytokines IL-1β and TNF-α expression was detected in the spleen and kidney (Fig. 4), whereas no expression of cytokine genes was found in the gill and liver. In the spleen, TNF-α was expressed on 11, 13, and 15 days, and in kidney IL-1β was expressed on 13 days after probiotics injection. Kim and Austin [12] did not observe the expression of IL-1β, IL-8, TNF-α, and TGF-β in gut cells of rainbow trout supplemented with *Carnobacterium maltaromaticum* B26 and *C. divergens* B33, but observed the expression of IL-1β and TNF-α in head kidney. In this study, we also observed the expression of IL-1β and TNF-α in kidney of *E. faecium*-injected olive flounder (Fig. 4). This increase in amount of cytokines indicates the indirect activation of T-cell.

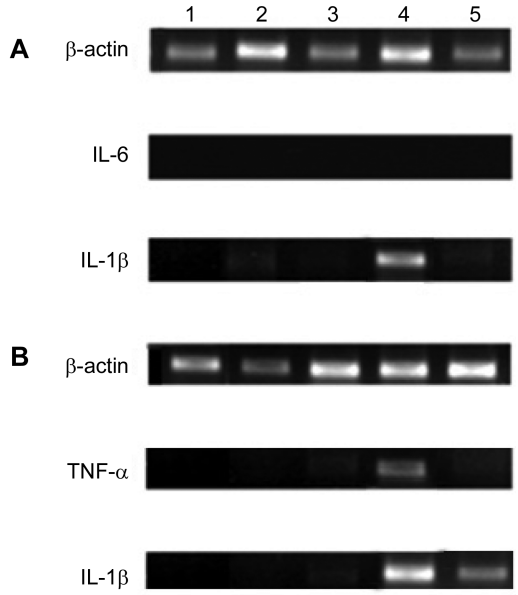

Fig. 4. Expression analysis of TNF-α, IL-1β, IL-6, and β-actin mRNA after injection with *E. faecium* in olive flounder.
Lanes 1–5: Control, 9, 11, 13, and 15 days after injecting *E. faecium*. (**A**) Expression of IL-1β mRNA in the flounder spleen tissue. (**B**) Expression of TNF-α and IL-1β mRNA in the flounder kidney tissue.

In conclustion, *E. faecium* as a probiotic induces immune responses in olive flounder and effectively controls the infection caused by *L. garvieae*. Further study is necessary on the use of *E. faecium* as a feed additive and the findings of this study will provide a fundamental reference.

Acknowledgment

This work (Grant No.00038153) was supported by the Business for Constructing Annex Research Centers for Companies Institute funded by the Korea Small and Medium Business Administration in 2010.

REFERENCES

1. Chang, C. I. and W. Y. Lin. 2002. An evaluation of two probiotic bacterial strains, *Enterococcus faecium* SF68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla anguilla* L. *J. Fish Dis*. **25:** 311–315.
2. Chen, D. and A. J. Ainsworth. 1992. Glucan administration potentiates immune defense mechanism of channel catfish, *Ictalurus punatatus* Rafinesque. *J. Fish Dis*. **15:** 295–304.
3. Ellis, A. E. 2001. Innate hose defense mechanisms of fish against viruses and bacteria. *Dev. Comp. Immunol*. **25:** 827–839.
4. Engstad, R. E., B. Robertsen, and E. Frivold. 1992. Yeast glucan induces increase in activity of lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood. *Fish Shellfish Immunol.* **2:** 298–297.
5. Gatesoupe, F. J. 1999. The use of probiotics in aquaculture. *Aquaculture* **180:** 147–165.
6. Haza, A. I., A. Zabala, and P. Morales. 2004. Protective effect and cytokine production of a *Lactobacillus plantarum* strain isolated from ewes' milk cheese. *Int. Dairy J.* **14:** 29–38.
7. Holland, M. C. H. and J. D. Lambris. 2002. The complement system in teleosts. *Fish Shellfish Immunol.* **12:** 399–420.
8. Hong, G. E., D. G. Kim, E. M. Park, B. H. Nam, Y. O. Kim, and I. S. Kong. 2009. Identification of *Vibrio anguillarum* outer membrane vesicles related to immunostimulation in the Japanese flounder, *Paralichthys olivaceus*. *Biosci. Biotechnol. Biochem*. **73:** 437–439.

9. Jollès, P. and J. Jollès. 1984. What is new in lysozyme research? Always a model system, todays as yesterday. *Mol. Cell. Biochem*. **63:** 156–189.
10. Jørgensen, J. B., H. Lunde, and B. Robertsen. 1993. Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **16:** 313–325.
11. Kajita, Y., M. Sakai, M. Kobayashi, and H. Kawaushi. 1992. Enhancement of non-specific cytotoxic activity of leucocytes in rainbow trout *Oncorhynchus mykiss* injected with growth hormone. *Fish Shellfish Immunol.* **2:** 155–157.
12. Kim, D. H. and B. Austin. 2006. Cytokine expression in leucocytes and gut cells of rainbow trout, *Oncorhynchus mykiss* Walbaum, induced by probiotics. *Vet. Immunol. Immunopathol*. **114:** 297–304.
13. Kim, D. H. and B. Austin. 2006. Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. *Fish Shellfish Immunol.* **21:** 513–524.
14. Kim, J. H., K. S. Shin, and H. Lee. 2002. Characterization and action mode of anti-complementary substance prepared from *Lactobacillus plantarum. Korean J. Food Sci. Technol.* **34**: 290–295.
15. Kim, J. S., R. Harikrishnan, M. C. Kim, C. Balasundaram, and M. S. Heo. 2010. Dietary administration of *Zooshikella* sp. enhance the innate immune response and disease resistance of *Paralichthys olivaceus* against *Streptococcus iniae*. *Fish Shellfish Immunol*. **29:** 104–110.
16. Lee, D. C., J. I. Lee, C. I. Park, and S. I. Park. 2001. The study on the causal agent of streptococcicosis (*Lactococcus garvieae*), isolated from cultured marine fishes. *J. Fish Pathol.* **14:** 71–80.
17. Magnadóttir, B. 2006. Innate immunity of fish (overview). *Fish Shellfish Immunol*. **20:** 137–151.
18. Min, E. Y., T. S. Kim, and J. C. Kang. 2010. Dietary effects of lactic acid bacteria on growth, hematological and immune response of grey mullet *Mugil cephalus*. *J. Fish Pathol*. **23:** 343–355.
19. Panigrahi, A., V. Kiron, T. Kobayashi, J. Puangkaew, S. Satoh, and H. Sugita. 2004. Immune response in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Vet. Immunol. Immunopathol*. **102:** 379–388.
20. Pirarat, N., T. Kobayashi, T. Katagiri, M. Maita, and M. Endo. 2006. Protective effects and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against experimental *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*). *Vet. Immunol. Immunopathol*. **113:** 339–347.
21. Rao, Y. V., B. K. Das, P. Jyotyrmayee, and R. Chakrabarti. 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.* **20:** 263–273.
22. Sharifuzzaman, S. M. and B. Austin. 2009. Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. *Fish Shellfish Immunol*. **27:** 440–445.
23. Smith, P., M. P. Hiney, and O. B. Samuelsen. 1994. Bacterial resistance to antimicrobial agents used in fish farming: A critical evaluation of method and meaning. *Annu. Rev. Fish Dis.* **4:** 273–313.
24. Vendrell, D., J. L. Balcázar, I. Ruiz-Zarzuela, I. de Blas, O. Gironés, and J. L. Múzquiz. 2006. *Lactococcus garvieae* in fish: A review. *Comp. Immunol. Microbiol.* **29:** 177–198.
25. Yano, T., R. E. P. Mangindaan, and H. Matsuyama. 1991. Enhancement of the resistance of carp *Cyprinus carpio* to experimental *Edwardsiella tarda* infection, by some β-1,3 glucans. *Nippon Suisan Gakkaishi* **55:** 1815–1819.