



Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG

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ABSTRACT

The use of lactic acid bacteria from human origins as a potential probiotic supplementation in aquaculture feed is now widely accepted. Here, we examined some of the properties and mechanisms of the action of *Lactobacillus rhamnosus* GG, originating from humans, on growth performance, gut mucosal immunity and humoral and cellular immune response in tilapia (*Oreochromis niloticus*). The results suggested that supplementation of *L. rhamnosus* gave an advantage in promoting the intestinal structure and the mucosal immunity of tilapia. Probiotic fish had a greater villous height in all parts of the intestines and, significantly, in the proximal and middle part. The population of intraepithelial lymphocytes was significantly higher in the probiotic group than in the control group in all parts of the intestines. The population of acidophilic granulocyte in the probiotic group was significantly higher at the proximal and distal parts when compared with the control group. The higher serum complement activity as well as the enhanced phagocytosis and killing ability of the head kidney leukocytes in the probiotic supplemented fish corresponded with the higher level of TNF alpha and IL-1 gene expression, suggesting that the induction of IL-1 and TNF alpha cytokines by *L. rhamnosus* served as an important regulator of gut associated immune systems.

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1. Introduction

Tilapia, a well-known freshwater fish, has been recognized as a food source for rural communities and industrial aquaculture ventures worldwide. Tilapia culture continues to show a high growth in output and has become the most important aquaculture crop this century (Roderick, 2000). A dramatic increase in production has led to tilapia being increasingly popular in major markets. Concern for food safety and the quality of imported products has now resulted in a high rate of scrutiny. For sustainable increase in fish production, probiotics derived from intestines of different kinds of animals are now widely focused. It is the alternative way to control the bacterial infectious diseases (Kesarcodi-Watson et al., 2008) and to respond to the limitations and adverse effects of antibiotics (Nayak, 2010). Among probiotic candidates, Lactobacilli have the longest history of use as probiotics and are still the most common ingredients of preparations intended for consumption by human beings and farm animals (Fuller, 1989; Gatesoupe, 1999). Various health effects for humans have been attributed to *Lactobacillus rhamnosus*, such as the prevention of acute diarrhea in children, the prevention of antibiotic-associated diarrhea and the

prevention and treatment of allergies, lowering cholesterol levels and the immune stimulation (Anuradha and Rajeshwari, 2005; Majamaa and Isolauri, 1997; Salminen et al., 1998). *L. rhamnosus* has been proven for its potential to control infection from *Aeromonas salmonicida*, *Vibrio anguillarum* and *Flavobacterium psychrophilum* in rainbow trouts and turbot (Nikoskelainen et al., 2001) and *Edwardsiella tarda* in tilapia (Pirarat et al., 2006). Therefore, supplementation of probiotics, of human origin, in aquaculture feed may not only promote an aquatic animal's health, but also benefit human health. The oral administration of probiotics activates the immune system in a non-specific way, providing resistance against a variety of pathogens. Many researchers were able to use the mRNA expression of cytokine genes, important mediators of the immune system and represent an essential part of the innate immune response in fish, as a tool for measuring immune responses against pathogenic bacteria (Low et al., 2003; Pierce et al., 2004; Panigrahi et al., 2007). However, information of the immune response to bacteria and the study of immune related cytokines in tilapias are very limited. While many *Lactobacillus* strains have been promoted as good probiotics for human or animal use, substantial supporting *in vitro* and *in vivo* data is available for only a few in aquatic animals. Biotherapeutic properties and mechanisms of action are too often unknown, and arguably too many studies are undertaken or contemplated with strains which

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have not been thoroughly characterized in these aspects. The present study was designed to investigate some properties and mechanisms of action relating to this possible biotherapeutic agent, *L. rhamnosus* GG, originating from humans on growth performance, gut mucosal immunity, humoral and cellular immune response in tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Fish and probiotic supplementation

Two hundred tilapias, *O. niloticus*, 30–50 g body weight, were allowed to acclimatize for 7 days and were randomly placed in two 700-l tanks (90 × 90 × 90 cm) (100 fish per tank). The water was kept at 29 °C, 5.0–5.8 mg/l of dissolved oxygen (DO) and 6.5–7.0 in pH throughout the experiment. The probiotic bacterium, *L. rhamnosus* GG (ATCC 53103), was cultured in MRS broth at 30 °C for 48 h, centrifuged and washed with sterile PBS three times. Bacterial pellets were measured in PBS and their densities were determined. Under sterile conditions, the bacteria were manually incorporated into commercial dry pellets (Lee Feed Mill Public Co. Ltd.: protein, not less than 25.00%; fat, not less than 3.00%; fiber, less than 8.00%; humidity, less than 12%) using plat counting on MRS agar at a rate of 10^{10} CFU/g in feed for probiotic group according to our previous data (Pirarat et al., 2006). Fish fed only commercial dry pellets served as a control. Fish were fed approximately 1.5% of body weight once a day. One month after feeding, fish were sampled for health parameters. The experiment was repeated three times at separate time points, with 200 tilapia per experiment.

2.2. Growth parameters

For the growth performance parameters, weight gain (%), specific growth rate and feed conversion ratio (FCR) were calculated using the following equations (Yanbo and Zirong, 2006):

$$\text{Weight gain (\%)} = 100 \times (\text{final mean body weight} - \text{initial mean body weight}) / \text{initial mean body weight}$$

$$\text{Specific growth rate} = [(\ln(\text{final body weight}) - \ln(\text{initial body weight})) / \text{days}] \times 100$$

$$\text{Feed conversion ratio} = \text{feed intake (g)} / \text{weight gain}$$

2.3. Measurement of villous height, intraepithelial lymphocyte (IEL), acidophilic granulocyte and mucous cell

After 30 days of feeding, three parts of the intestine, the proximal part (from after the pyloric part of the stomach to before the spiral part of the intestines), the middle part (the spiral part of the intestines) and distal part (from after spiral part of the intestines to 2 cm before anus), from these fish in the probiotic ($N = 6$) and control groups ($N = 6$) were collected and fixed in 10% buffered formalin. Fixed tissues were processed according to standard histological techniques and tissue sections were stained with hematoxylin and eosin (H&E). For the villous height measurement, 10 highest villi were selected per section. The villous length was measured from the villous tip to the bottom. An average of these 10 villi per section was expressed as the mean villous height for each section (Samanya and Yamauchi, 2002). The presence of IEL from the 10 highest villi which were selected to measure the villous height was given an arbitrary score for from 0 to 3 based on the frequency and population number: 0 – none, 1 – mild, 2 – moderate and 3 – marked IEL. For measurement of acidophilic granulocytes,

a number of cells in the lamina propria of the intestine were totally counted and compared between the probiotic and control fish (Gargiulo et al., 1998; Picchietti et al., 2009). For mucous cells, the fixed tissues were processed according to standard histological techniques and tissue sections were stained with a combination of Alcian blue (AB) and periodic acid-Schiff reagent (PAS) (Gargiulo et al., 1998). Then the 10 highest villi were selected and the positive mucous cells were averaged and scored on frequency and population number as 0 representing none, 1 representing 1–10, 2 representing 10–30 and 3 representing >30 cells per villus.

2.4. Immunological aspects

2.4.1. Preparation of head kidney leukocytes

Head kidneys and blood were collected from six fish in each group at 14 and 30 days. The head kidneys were rinsed with RPMI1640 and separated via a 100 µm metal mesh into RPMI1640 medium using a silicon tip to dislodge the leukocytes. Two milliliters of percoll solution (1.079 g/l) were added and spun at 350–400 g for 20 min at 4 °C. The leukocytes were obtained from the interface and washed twice with RPMI1640 (10% FBS). The number of leukocytes was counted using a hemocytometer with Trypan blue inclusion. The experiment was continued upon the survival of 95% of leukocytes for chemiluminescence assay. The blood samples were obtained by using syringes via the caudal vessels and these samples were centrifuged to make the sera for lysozyme and complement activity assay.

2.4.2. Phagocytosis assay

Zymosan (0.5 mg/ml) and leukocytes (1×10^6 cells/ml), each 300 µl were added, and incubated for 30 min at room temperature. After incubation, the contents were washed with RPMI1640 (10% FBS). The 200 µl of mixtures was spun and placed on a clean glass slide using cytospin. The slides were stained with Giemsa's stain and observed for phagocytic activity as the percentage of phagocytizing cells was quantified from 200 phagocytic cells under a microscope. The phagocytic index was calculated from the total number of ingested zymosan divided by the number of leukocytes ingesting at least one zymosan. The experiment was repeated three times and the values were analyzed statistically.

2.4.3. Chemiluminescence assay of leukocytes

The 1×10^7 cells/ml leukocytes used for this assay were incubated for 3 min at 28 °C, the contents were spun and the pellets were resuspended with 50 µl of RPMI1640. The CLA (2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazine-3-one) dependent chemiluminescence was assayed in a lumi-counter (Microtech Nichion NU2500, Japan) by mixing 50 µl leukocytes, 100 µl CLA, 300 µl PBS (–) and 50 µl phorbol 12-myristate 13-acetate (1 µg/ml). The peak value was recorded and tested for statistical analysis.

2.4.4. Serum lysozyme activity

Lysozyme activity in the serum was assayed according to the method of Demers and Bayne (1997) based on the lysis of the lysozyme sensitive gram positive bacterium, *Micrococcus lysodeikticus* (Sigma). Serum from six fish in each group was analyzed. The dilutions of hen egg white lysozyme (Sigma) ranging from 0 to 20 µg/ml (in 0.1 M phosphate citrate buffer, pH 5.8) along with the undiluted serum samples (25 µl) were placed into wells of a 96-well plate in triplicate. One hundred and seventy-five microliters of *M. lysodeikticus* suspension (75 mg/ml) prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured every 30 s for 5 min at 450 nm using a microplate reader.

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