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Inhibition ability of probiotic, *Lactococcus lactis*, against *A. hydrophila* and study of its immunostimulatory effect in tilapia (*Oreochromis niloticus*)

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Abstract

The present study was designed to investigate the inhibition ability of probiotic, *Lactococcus lactis* RQ516, against *A. hydrophila in vitro* and its immunostimulatory effect in tilapia, *Oreochromis niloticus* as growth promoter. Six tanks were used (T-1 and control treated with and without RQ516, respectively) and three replicates per treatment. The inhibition ability assay *in vitro* showed that the minimum diameter of the inhibition zone ($P < 0.05$) was 7.43 ± 0.47 mm at 6 h and the final diameter at 24 h was 14.77 ± 1.17 mm. After 40 days, probiotic treatment as water additives significantly improved the final weight and daily weight gain (DWG) ($P < 0.05$). A significant increase ($P < 0.05$) in the total protein and globulin concentration of tilapia blood serum could be found in T-1 compared with the control. These immune responses were generally more pronounced with probiotic-treated tilapia. As for immune responses, the higher respiratory burst activity (RBA), lysozyme content (LC), myeloperoxidase (MPO) and superoxide dismutase (SOD) activities were observed in T-1 ($P < 0.05$) than the control. It indicated that probiotic *Lactococcus lactis* RQ516 was beneficial for tilapia in terms of increasing final weight, DWG and the concentrations of serum protein and globulin and enhancing immune responses.

Keywords: Probiotic; *Lactococcus lactis*; Tilapia; *Oreochromis niloticus*; immune response

1. Introduction

Fish diseases are major problem for the fish farming industry and among those bacterial infections are considered to be a major cause of mortality in fish (Gomez-Gil *et al.*, 2000). The motile aeromonads, especially *A. hydrophila*, affects a wide variety of freshwater fish species and occasionally marine fish (Chu and Lu, 2005). Thus, many measures have been tried to improve production levels such as the routine use of antibiotics. However, the excessive and inappropriate use of antibiotics has resulted in the presence of resistant strains of bacteria in fish culture (Nomoto, 2005). In addition, there are environmental problems associated with the antibiotics (Wang and Xu, 2004). Therefore, the need for alternatives is increasing and the contribution of probiotics may be considerable.

With increasing demand for environment friendly aquaculture, the use of probiotics in aquaculture is now widely accepted (Gatesoupe, 1999; Verschuere *et al.*, 2000; Wang *et al.*, 2005; Vine *et al.*, 2006; Wang and Xu, 2006; Kesarcodi-Watson *et al.*, 2008). Probiotic bacteria have a possible competition with pathogens (Gatesoupe, 1997) or the hypothetical stimulation of the immune system, as the activation of macrophage (Spanggaard *et al.*, 2001; Wang *et al.*, 2008). Lactic acid bacteria (LAB) are great producers of bacteriocins and organic acids (lactic and acetic acids) which have inhibitory effects *in vitro* on the growth of some pathogens in fish (Planas *et al.*, 2004) and these antimicrobial substances have provided these organisms with a competitive advantage over other microorganisms to be used as probiotics (Salminen *et al.*, 1998). A considerable interest in the use of LAB as probiotics for improving disease resistance, growth of fish and in enhancing fish immune response has been developed (Ringø and Gatesoupe, 1998; Balcázar *et al.*, 2007; Balcázar *et al.*, 2008).

However, there has been little study of the use of LAB as water additives in tilapia for growth performance and immune response. The present study therefore was designed to investigate the inhibition ability of LAB, *Lactococcus lactis* RQ516, against *A. hydrophila in vitro* and its immunostimulatory effect in tilapia, *Oreochromis niloticus* as growth promoter. For the immune parameter assays, C3, C4, lysozyme, IgA, IgG and IgM level were used as indicators.

2. Materials and Methods

2.1 Bacteria

The LAB strain, *Lactococcus lactis* RQ516, previously isolated from fresh milk (Zhou *et al.*, 2005) and was grown in DeMan, Rogosa, and Sharpe (MRS) broth (Oxoid, UK). Stock culture of probiotic was stored at -70 ^{o}C (Forma 702, Thermo, USA) in powdered skimmed milk suspension with 25% glycerol prior to use (Cabo *et al.*, 1999). Pathogen strain, *A. hydrophila* (ATCC 14715) was grown in tryptic soy agar (TSA, Oxoid, UK) according to Popovic *et al.* (2000). They were cultured in the laboratory and checked routinely for purity based on their morphological and biochemical characteristics during this investigation (Bergey's Manual of Systematic Bacteriology, 1989).

2.2 Growth inhibition of A. hydrophila in vitro

In order to assess the growth inhibition of *A. hydrophila in vitro*, *Lactococcus lactis* RQ516 was grown in MRS broth at 30 ^{o}C for 24 h. After incubation, bacteria were harvested by centrifugation (2000 × *g*), washed twice with phosphate buffered saline (PBS, pH 7.2, Sangon, China), and resuspended in the same buffer. The inhibition assay was performed using well-diffusion method as described by Nikoskelainen *et al.* (2001) and Hai *et al.* (2007). The cultures of the *A. hydrophila* were prepared by pouring 1 ml of the inoculum (10^7 cfu ml^{-1}) onto TSA plates to completely cover the surface of the agar. The 2.5-mm diameter wells were punched into the agar using sterile pipette tips, which were cut to obtain a 2.5-mm diameter bore. Four wells were made in each agar plate. Ten microliters of prepared *Lactococcus lactis* RQ516 (10^7 cfu ml^{-1}) was carefully pipetted into each well. The diameter of the inhibition zones around the wells were recorded in millimeters after incubating the plates for 6, 12, 18 and 24 h at 30 ^{o}C. These assays were carried out in triplicate and independently repeated at least three times.

2.3 Fish and experimental design

Tilapias (*Oreochromis niloticus*) were obtained from the Yueteng Fish Hatchery of Zhejiang, China. The fish were fed with a standard commercial feed (37.87% crude protein, 5.42% crude fat, 10.22% crude ash and 7.25% moisture by wet weight) at a rate of 3% of the biomass per day. The fish had not been vaccinated nor exposed to fish diseases and were deemed specific pathogen free. The fish were acclimated for 2 weeks in tanks before the start of the trial. After the acclimation period the health fish with similar body weight (6.84 ± 0.15) were selected from them and were divided randomly into six 200 l tanks, each containing 30 fish. The tanks were supplied with running fresh water filtered through a special cotton filter (flow rate: 1 l min^{-1}), then passed successively through a tungsten heater. All fish were maintained at 26 ± 1 ^{o}C with a 30 % water change every day and a 12 h dark/12 h light photoperiod during the entire trial. Dissolved oxygen level was maintained above 6.0 mg l^{-1} by setting the air pump (ADP-2200, Jinlai Pump Factory, China).

Three tanks served as T-1 and the prepared probiotic *Lactococcus lactis* RQ516 was supplemented in tanks water as water additives at a final concentration of 1.0×10^7 cfu ml^{-1} every four days, and the other three tanks were added without any probiotic and treated as the control. The trial was carried out for 40 days, after which all the fish were collected, counted and weight. Fish survival was also determined by counting the individuals in each tank. The daily weight gain (g d^{-1}) (DWG) was calculated as: (final weight – initial weight) / 40 (g d^{-1}).

2.4 Sampling and analytical methods

At the end of experiment, fishes were sampled randomly from each tank and the blood were taken from the caudal vein of anaesthetized fishes (MS-222, Ethyl 3-aminobenzoate methanesulfonate, Tricaine; Sigma; 1:2500) by sterile syringe containing anticoagulant solution. The blood samples were used for determining respiratory burst activity (RBA) by nitroblue tetrazolium (NBT, Sigma) assay following the method of Choudhury *et al.* (2005). The blood (50 µl) was placed into the wells of 96-well plate and incubated at 30 °C for 60 min to allow adhesion of cells. Then the supernatant was removed and the wells were washed three times in PBS (Phosphate Buffered Saline, pH 7.2). After washing, 50 µl of 0.2% NBT was added and incubated for a further 60 min. The cells were then fixed with 100% methanol for 3 min and washed three times with 30% methanol. The plates were air-dried and 60 µl 2 N potassium hydroxide and 70µl dimethyl sulphoxide were added to each well. The absorbance (OD) was read at 540 nm in a plate reader (Thermo Multiskan MK3).

Blood samples were withdrawn from caudal vein in the remaining anaesthetized fish into Eppendorf tubes without anticoagulant in syringe and the tubes were kept at 4 °C for 10 h and then centrifuged at 3000 *g* for 15 min and the supernatant serum was collected. The serum was stored immediately at -80°C for further biochemical analyses. The different serum samples collected earlier were analyzed for total protein using the method of Bradford (1976) and albumin content according to Doumas *et al.* (1971). Furthermore, globulin content (substracting albumin from total protein) and albumin/globulin (A/G) ratio were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) based on the methods described in the instructions. Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Ransod kit (Randox, Crumlin, UK). One unit of SOD was defined as the amount required inhibiting the rate of xanthine reduction by 50 %. The specific activity was expressed as SOD U ml^{-1} (Biagini *et al.*, 1995).

Myeloperoxidase (MPO) activity in blood serum was measured according to Kumari and Sahoo (2006) with a slight modification. Serum samples (10 µl) were diluted ten times with HBSS (Hanks Balance Saline Solution, Ca^{2+} and Mg^{2+} free) in

96-well plates, and then freshly prepared substrate of MPO, 35μl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 were added and incubated at 30 °C for 30 min to allow the colour change reaction progress completely. Then the colour-reaction was stopped by adding 35 μl of 4 M sulphuric acid (H_2SO_4) and the optical density was read at 450nm in a plate reader (Thermo Multiskan MK3). Standard samples without serum were also analyzed. A unit of MPO activity was defined as 1 μmol of H_2O_2 was reduced by every liter serum at 30°C.

A turbidometric assay utilizing lyophilized *Micrococcus flavus NCIMB8166* was used to determine lysozyme content in serum according to Parry et al (1965) with a slight modification. Lyophilized *M. flavus* cells were re-suspended in 2.0 ml of 0.05M phosphate buffer (pH 6.2) at a concentration of 0.25 mg ml^{-1} and incubated at 30 °C for 5 min, after which 200 μl of serum was added to 2.0 ml of the suspension and the transmittance was measured after 5-second and 125-second at 540 nm (Thermo Multiskan MK3). The lysozyme content was calculated as follows: (Amount of sample causing a decrease in transmittance / Amount of the standard causing a decrease in absorbance) × Standard concentration.

2.5 Statistical analysis

Statistical analysis was performed using analyses of variance (One-way ANOVA) and Student's *t*-test to determine differences between treatments (levels of significance are expressed as $P < 0.05$). The Kruskal-Wallis test was used before the ANOVA analysis. All analyses were performed using the Statistical Analysis System (SAS computer software, North Carolina, USA) program.

3. Results

3.1 Inhibition ability assay in vitro

Table 1. The inhibition ability of probiotic, *Lactococcus lactis* RQ516, against pathogen strain *A. hydrophila* in vitro after incubated the plates for 6, 12, 18 and 24 h.

Time (h)	6	12	18	24
Inhibition zone (mm)	7.43 ± 0.47 a	12.77 ± 0.75 b	14.67 ± 1.15 b	14.77 ± 1.17 b

Results were presented as means ± S.D.
Means in the same row with different superscript were significantly different ($P < 0.05$).

Data on the inhibition ability assay of probiotic, *Lactococcus lactis* RQ516, against pathogen strain *A. hydrophila in vitro* were shown in Table 1. The minimum diameter of the inhibition zone ($P < 0.05$) was observed at 6 h and was 7.43 ± 0.47 mm. However, there was no significant difference ($P > 0.05$) among the inhibition zone diameter after incubating the plates for 12, 18 and 24 h. Though not significant, there was an increase in inhibition zone diameter with the incubation time. At the end of this assay, the final diameter of the inhibition zone against *A. hydrophila in vitro* was 14.77 ± 1.17 mm.

3.2 Growth performance

Table 2. Growth performance and survival rate of tilapia treated with (T-1) or without (control) probiotic, *Lactococcus lactis* RQ516 in tanks water as water additives.

Group/Treatment	Control	T-1
Initial weight (g)	6.92 ± 0.16 a	6.79 ± 0.16 a
Final weight (g)	28.47 ± 2.07 a	37.68 ± 2.13 b
DWG (g d^{-1})	0.54 ± 0.05 a	0.77 ± 0.05 b
Survival rate (%)	100	100

DWG, daily weight gain.
Results were presented as means ± S.D. of triplicate observations.
Means in the same row with different superscript were significantly different ($P < 0.05$).

The results of tilapia growth performance and survival rate were presented in Table 2. No significant differences ($P > 0.05$) were shown in initial weight between T-1 and the control. However, after 40 days, probiotic treatment as water additives significantly improved the final weight and DWG ($P < 0.05$). Furthermore, no deleterious effect or mortality was encountered during the course of the experiment and the survival rate of tilapia both in T-1 and control was 100%.

3.3 Biochemical analysis and Immune response

Table 3. Effect of probiotic, *Lactococcus lactis* RQ516 on total protein, albumin, globulin and A/G ratio of tilapia at the end of 40 days culture.

Group/Treatment	Control	T-1
Total protein (g/L)	41.57 ± 3.35 a	56.80 ± 7.77 b
Albumin (g/L)	3.92 ± 0.41 a	4.33 ± 0.22 a
Globulin (g/L)	38.14 ± 4.02 a	52.48 ± 5.68 b
A/G ratio	0.10 ± 0.01 a	0.08 ± 0.01 a

Results were presented as means ± S.D. of triplicate observations.
Means in the same row with different superscript were significantly different ($P < 0.05$).

The effect of probiotic addition on biochemical parameters of the tilapia blood serum was also assessed (Table 3). A remarkable increase ($P < 0.05$) in the total protein and globulin concentration of tilapia blood serum could be found in T-1 treated with probiotic, *Lactococcus lactis* RQ516 (56.80 ± 7.77 g L^{-1} and 52.48 ± 5.68 g L^{-1}, respectively) compared with the control (41.57 ± 3.35 g L^{-1} and 38.14 ± 4.02 g L^{-1}, respectively). As for albumin content and A/G ratio, there was not significantly different ($P > 0.05$). However, the probiotics RQ516 group exhibited higher albumin value than the control.

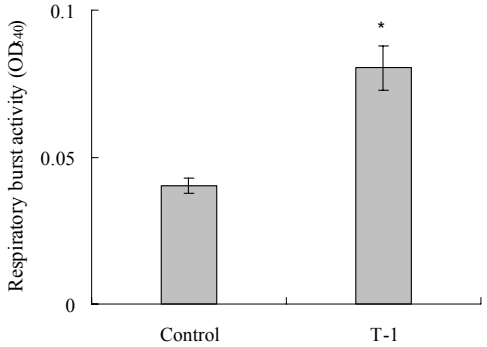

Fig. 1. Effect of probiotic, *Lactococcus lactis* RQ516, on respiratory burst activities (RBA) of tilapia at the end of 40 days culture. Means with asterisk superscript were significantly different ($P < 0.05$).

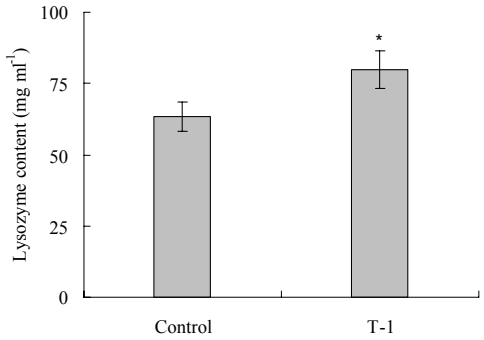

Fig. 2. Effect of probiotic, *Lactococcus lactis* RQ516, on lysozyme content (LC) of tilapia blood serum at the end of 40 days culture. Means with asterisk superscript were significantly different ($P < 0.05$).

These immune responses were generally more pronounced with probiotic-treated tilapia. The higher respiratory burst activity (RBA) was obtained in the group receiving the probiotic, *Lactococcus lactis* RQ516 ($P < 0.05$) than the control group (Fig. 1). Significant difference ($P < 0.05$) also occurred between T-1 and control in lysozyme content (Fig. 2). The activities of the MPO and SOD in the probiotic treated groups (T-1) (254.33 ± 21.73 U ml^{-1} and 74.02 ± 5.06 U ml^{-1}, respectively) were significantly

higher ($P < 0.05$) than the control (75.33 ± 6.51 U ml^{-1} and 47.45 ± 4.98 U ml^{-1}, respectively).

4. Discussion

Nowadays, LAB was widely used in combination with other probiotic bacteria such as *Bifidobacterium* to manufacture fermented dairy products (Zhou *et al.*, 2005) and the evaluation of LAB for their use as probiotics in farm animals was still increasing (Guerra *et al.*, 2007). When looking at probiotics intended for an aquatic usage it was important to consider certain influencing factors that were fundamentally different from terrestrial based probiotics. Thus, Moriarty (1998) proposed to extend the definition of probiotics in aquaculture to microbial ''water additives''. Here, for the first time, an enhancement of final weight, DWG and immune responses (RBA, LC, MPO and SOD activities) of the tilapia, *Oreochromis niloticus* were as the results of supplemented the tanks water with LAB, *Lactococcus lactis* RQ516 as probiotic.

In the present research, *Lactococcus lactis* RQ516 examined inhibited the growth of *A. hydrophila in vitro*. This finding was supported by Ivanova *et al.* (1993) who notice antimicrobial activity in heterotrophic bacteria. Similarly, Balcázar *et al.* (2007) had reported the inhibition of *Lactococcus lactis* culture supernatants against the fish pathogenic strains, *A. salmonicida* and *Y. Rukeri in vitro*. Although, the inhibitory mechanism of the interaction was not characterized in this study, previous studies had suggested that the inhibitory effects of LAB might be due to either individual or joint production of organic acids, hydrogen peroxide, or bacteriocins (Klaenhammer, 1993; Vandenbergh, 1993; Ringø and Gatesoupe, 1998; Villamil *et al.*, 2003a; Vázquez *et al.*, 2005). Moreover, a nisin-like bacteriocins produced by *Lactococcus lactis* RQ516 was active against a wide range of gram-positive organism including many spoilage bacteria and some pathogens (Lewus *et al.*, 1991; Enserink, 1999; Zhou *et al.*, 2005).

Harzevili *et al.* (1998) investigated the effect of the probiotic *Lactococcus lactis* AR21 added to tank water on the growth performance of rotifers, resulting in enhanced growth of the rotifers. A similar finding was also obtained by Planas *et al.* (2004), who investigated the effect of terrestrial lactic acid bacteria on the growth of the rotifer, *Brachionus plicatilis* and showed that *Lactococcus casei*, *Pediococcus acidilactici* and *Lactobacillus lactis* promoted significantly the growth rates of rotifer. Our findings showed the similar result and probiotic, *Lactococcus lactis* RQ516 increased final weight and DWG. A mixture of *Lactobacillus* spp. isolated from chicken gastrointestinal tracts had improved the survival rates of juvenile *P. monodon* when fed these strains for 100 days (Phianphak *et al.*, 1999). Currently, the use of *Lactococcus lactis* AR21 in tilapia had shown inconsistent results. In contrast, no significant survival increases were detected and the survival rate of T-1 and the control was 100% after 40 days culture in this study. This result might be explained by the culture days and species of experimental animals used in this study in contrast to theirs. In addition, it might be associated with our good experimental conditions and appropriate water quality to tilapia during the whole trial.

Serum proteins were divided into two major groups, albumin and globulins. Serum albumin and globulin values in fish treated with different immunostimulants were always higher than the control (Choudhury *et al.*, 2005). The results indicated higher ($P < 0.05$) total protein and globulin concentrations of tilapia blood serum were obtained in T-1 treated with *Lactococcus lactis* RQ516 as water additives compared with that of the control in the present study. This was in agreement with the previous studies which showed that serum protein and globulin in fish treated with different immunostimulants, i.e. β-glucan and yeast RNA, were always higher than the control (Choudhury *et al.*, 2005; Misra *et al.*, 2006).

The stimulation of the immune system of terrestrial animals by lactic acid bacteria had already been demonstrated (Perdigon *et al.*, 1995; Fuller and Perdigón, 2000; Nousiainen *et al.*, 2004; Matsuzaki *et al.*, 2007; Fortun-Lamothe and Boullier, 2007). These aspects deserved further attention in fish, especially to develop immunostimulants against bacteria and viruses. Villamil et al (2003b) evaluated the immunomodulatory effects of lactic acid bacteria in turbot (*Scophthalmus maximus*) and found that *L. lactis* significantly increased the immune functions including macrophage chemiluminescent response and the serum nitric oxide concentration after 7 days of daily oral administration. In our study, the presence of *Lactococcus lactis* RQ516 indicated that it could increase tilapia immune responses such as RBA, LC, MPO and SOD. These results suggested that the increased SGR might be attributed to improved immune responses.

It is generally accepted that fish phagocytes after activation are able to generate superoxide anion (O_2^-) and its reactive derivatives (i.e. hydrogen peroxide and hydroxyl radicals) during a period of intense oxygen consumption, called the respiratory burst (Secombes, 1996). These reactive oxygen species are considered to be toxic for fish bacterial pathogens (Itou *et al.*, 1996). Hence, it is evident that increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993). It had been demonstrated that administration of a lactic acid bacterium *Lactobacillus rhamnosus* (strain ATCC 53103) at a level of ~10^5 cfu g^{-1} feed, stimulated the respiratory burst in rainbow trout (*Oncorhynchus mykiss*) (Nikoskelainen *et al.*, 2003). In the present study, similar results were observed and the higher RBA was registered in T-1 supplemented with *Lactococcus lactis* RQ516 in tanks water.

In a conclusion, the probiotic, *Lactococcus lactis* RQ516 was beneficial for tilapia in terms of increasing growth performance and the concentrations of serum protein and globulin and enhancing immune responses. The use of *Lactococcus lactis* RQ516 in tilapia and other fish is an important management tool, but its efficiency depends on understanding the nature of competition between species or strains.

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