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# Effects of dietary supplementation of intestinal autochthonous bacteria on the innate immunity and disease resistance of grass carp (*Ctenopharyngodon idellus*)

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ABSTRACT

Intestinal microorganisms of aquatic animals have a crucial impact on the host and some may have the potential to be used as probiotics. In this work, three strains of autochthonous bacteria were isolated from the intestinal tract of the healthy grass carp (*Ctenopharyngodon idellus*). The effects of single or combined use of dietary *Shewanella xiamenensis* A-1, *S. xiamenensis* A-2, and *Aeromonas veronii* A-7 were assessed on innate immunity and disease resistance of grass carp. The grass carp were fed 28 days by five experimental diets: i) one control diet with no probiotics, ii) three diets supplemented with $1 \times 10^8$ cell $g^{-1}$ of intestinal bacteria A-1 (group A1), A-2 (group A2) and A-7 (group B), and iii) mixed diet (group MIX) comprising of bacteria A 1, A-2 and A 7 dosed at $1 \times 10^8$ cell $g^{-1}$, at a ratio of 4:2:1, which was set according to their quantity ratio in our study. At the end of the 28-day feeding experiment, the survival of grass carp against *Aeromonas hydrophila* was challenged for 14 days. Results showed that responses of several non-specific immune parameters of groups fed probiotics were enhanced; i.e. all probiotic groups led to significant enhancement of both respiratory burst, phagocytic and lysozyme activities than that of control activity. The complement C3, total serum proteins, albumin and globulin levels were also significantly improved on day 14 of feeding regime with probiotics compared to the control. Moreover, the expression of four immune-related genes (IL-8, IL-1β, lysozyme-C, and TNF-α genes) was significantly up-regulated in grass carp fed probiotic diets compared to control group on days 7, 14, and 28. The cumulative mortality of grass carp, experimentally challenged with *A. hydrophila*, was reduced in groups fed probiotics, i.e. A-1 = 46.67%, A-2 = 33.33%, B = 53.33% and mixed = 26.67%, respectively, than that of controls (80%) after 14 days of post-infection. Overall, the results of this study indicated the potential of using the three isolated autochthonous bacteria (single or combined) to improve immunity and disease resistance of grass carp.



## 1. Introduction

Grass carp (*Ctenopharyngodon idellus*) is one of the most important freshwater species in China, and it has been introduced into more than 100 countries (Song et al., 2009). In recent years, owing to the rapid development of intensification farming model, bacterial diseases have arisen and resulted in high economic losses. Antibiotics have been selected as traditional disease control strategy for decades in aquaculture. However, long term use of the antibiotics leads to many negative impacts such as drug residues and drug resistance (Bachère, 2000; Nomoto, 2005). The drawbacks of antibiotics drive to find effective alternative means for the control of bacteria infections, as well as improving the quality of the aquatic animals. In the past decennium, probiotics have received a great deal of attention to replace antibiotics in aquaculture (Hao et al., 2014; Newaj-Fyzul et al., 2014).

Probiotics are originally defined as the live organisms which contribute to the intestinal microbial balance (Fuller, 1987). According to the currently adopted definition by the Food and Agricultural Organization/World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO Food Agriculture Organization of the United Nations, 2001). The beneficial effects of probiotics on fish survival, growth and feed conversion, immune response, and disease resistance in aquaculture have been widely recognized (Gatesoupe, 2007; Kesarcodi-Watson et al., 2008; Merrifield et al., 2010; Nayak, 2010). Now most of commercial probiotics used for terrestrial animals such as *Lactobacillus* spp. and *Bacillus* spp. are being used in aquaculture (Nayak, 2010); nevertheless, the safety and efficiency of these exogenous probiotics are still questionable in aquaculture (Cerezuela et al., 2012).

An ideal probiotic, irrespective of its source, should be able to colonize, establish and multiply in the host gut (Nayak, 2010). There is a general consensus that probiotics selected from autochthonous origin may have a greater ability of competing with resident microbes and

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becoming predominant and persisting in the enteric environment (Carnevali et al., 2004; Cerezuela et al., 2012; Goldin and Gorbach, 1992). Moreover, intestinal microorganisms of aquatic animals have a crucial impact on the host (Balcázar et al., 2006; Wu et al., 2012a), and therefore, the intestinal microorganisms may have the potential to be used as probiotics. The intestinal microbial community of grass carp was mainly composed of the genus of *Aeromonas*, *Vibrio*, *Pseudomonas* and *Shewanella*, as reported by previous studies (Huang et al., 2009; Li et al., 2014). This was consistent with our previous study, which perhaps could provide a valuable probiotic source for grass carp. In recent works in our laboratory, some probiotics isolated directly from the gut of aquatic animals were found with property of enhancing host immunity and inhibiting harmful bacteria (e.g. *Shewanella japonica*, *Aeromonas veronii*, *Shewanella colwelliana*, *Shewanella haliotis*, and *Aeromonas bivalvium*) (Chi et al., 2014a, 2014b; Hao et al., 2014; Jiang et al., 2013).

Taking into account the above considerations, the aims of the present study are: i) to isolate the autochthonous gut bacteria from intestinal tract of grass carp and, ii) to study the effects of single or combined use of dietary autochthonous gut bacteria on immune responses and resistance to disease of grass carp.

## 2. Materials and methods

### 2.1. Fish

Three hundred and sixty grass carp, weighing 35 g ± 5 g, were obtained from Shaanxi Xinmin fish seed field in Heyang (Shaanxi, China). The fish had been examined before the trial to ensure that they were healthy, i.e. i) the selected fish come from the same standardized pond where the fish were fed carefully; ii) there was no abnormal color change of body from the selected fish; iii) did not find death of fish and moreover ingestion of fish was normal during period of acclimatization. The selected fish were randomly divided into 15 fiberglass tanks (200 l) at 24–25 °C and acclimatized to laboratory conditions (light–dark conditions: 16 h/8 h; dissolved oxygen: 5.64 ± 0.87 mg $l^{-1}$; pH: 7.65 ± 0.45; nitrites: 0.015 ± 0.004 mg $l^{-1}$; ammonia: 0.130 ± 0.024 mg $l^{-1}$) for 28 days before the experiment. Fish were fed a basal diet (Table 1) twice (9:00 and 17:00) everyday at 2% of the body weight (BW). In order to keep the water quality, 30% water in each tank was changed with aerated tap water every day.

**Table 1**
Ingredient and chemical proximate composition of basic diet (% as feed).

| Ingredient | % |
|---|---|
| Soybean meal[a] | 36 |
| Fish meal[b] | 25 |
| Wheat meal | 20 |
| Corn meal | 15 |
| Soybean oil | 2 |
| α-Starch | 1 |
| Mineral and vitamin mixture[c] | 1 |
| *Proximate analysis* | |
| Crude protein | 25.9 |
| Crude lipid | 4.6 |
| Crude fiber | 5.8 |
| Ash | 10.7 |

[a] Soybean meal, obtained from Shaanxi Huaqin Feed Group Co. Ltd., Shaanxi, China.
[b] Fish meal, obtained from Shaanxi Huaqin Feed Group Co. Ltd., Shaanxi, China.
[c] Every 250 g of mineral–vitamin mixture provided vitamin A, 500,000 IU; vitamin $D_3$, 100,000 IU; vitamin $B_1$, 7 g; vitamin $B_2$, 20 g; vitamin $B_6$, 6 g; vitamin $B_{12}$, 80 mg; vitamin E, 30 g; vitamin $K_3$, 6 g; vitamin C, 50 g; pantothenate, 15 g; niacin, 65 g; folic acid, 3 g; inositol, 65 g; biotin, 150 mg.

### 2.2. Strain selection

The autochthonous gut bacteria were isolated from the gut of healthy grass carp according to the procedure of Chi et al. (2014a) with slight modifications. Firstly, twenty fish were randomly selected and divided into two groups (10 fish/group). During the experiment (28 days), the feeding group was fed the basal diet (2% of BW) twice every day, and the other group was not fed. No mortality or symptoms of disease were examined in these two groups during this period. After 28 days, three fish were randomly selected from each group and euthanized with MS-222 (Geruien, Jinan, Shandong, China). The intestines of each fish were collected, cut open and rinsed with sterilized 0.85% fish physiological saline to remove the intestinal contents. Afterwards, half a gram of each gut was weighed and homogenized separately in 5 ml of sterile 0.85% fish physiological saline. The intestinal tract homogenates were serially diluted using a nine-fold sterile (v/v) 0.85% fish physiological saline and 100 μl of aliquots was separately spread on nutrient agar plates (NAP) (Lovley and Phillips, 1988). After incubating at 25 °C for 48 h, the bacterial colonies were counted and characterized based on colony morphology, gram staining (Wang et al., 2011). Thirty colonies were randomly picked (to pick many different phenotypes) from every sample, and identified by 16S rDNA gene sequencing as described by Weisburg et al. (1991). Three strains were selected after hemolytic and injection assays. Further characterization of three autochthonous gut bacteria was achieved using 16S rDNA gene (Weisburg et al., 1991) by blast analysis: comparing with the sequences in the GenBank nucleotide database and constructing phylogenetic tree. Finally, stock cultures were kept in liquid nutrient broth (NB) containing sterile 20% glycerol at −70 °C.

### 2.3. Safety of the isolated bacteria

#### 2.3.1. Hemolytic assays

The hemolysis test was applied to exclude potential pathogens (Luis-Villaseñor et al., 2011). Hemolysis of the isolated bacterial strains was analyzed on sheep blood agar plates, and the results were determined, as described by Luis-Villaseñor et al. (2011) as: α-hemolysis results in slight destruction of hemocytes and erythrocytes with a green circle around the bacterial colonies, β-hemolysis leads to a clean hemolysis zone around colonies, and γ-hemolysis causes no change on the agar plates around the colonies.

#### 2.3.2. Injection assays

The safety of the selected isolates was evaluated in hemolytic assays and showed no probable pathogenic. The grass carp were intraperitoneally injected with 0.1 ml of saline (0.85% NaCl) containing $1 \times 10^8$ cells $ml^{-1}$ selected bacteria, while grass carp in the control group were intraperitoneally injected with 0.1 ml of sterile saline (0.85% NaCl) (Abd El-Rhman et al., 2009). 30% water in each tank was renewed on a daily basis with aerated tap water. Daily observation was recorded for disease incidence and mortality during a two-week experimental period.

### 2.4. Bacteria preparation

Three strains A-1, A-2, and A-7 that indicated no potential pathogenicity after hemolytic and injection assays were used for diet making, and inoculated in NB with an incubator shaker at 28 °C for 2 days. The cells were collected by centrifugation at 3000 ×*g* for 10 min at 4 °C and washed twice with sterilized 0.85% NaCl solution. The sterilized 0.85% NaCl solution was used to adjust the bacteria density to be ~$10^8$ cell $ml^{-1}$ with hemocytometer slide which was used to count the density of bacteria under a light microscope (Olympus BX41, Tokyo, Japan) at 10 × 40 magnification. The adjusted bacterial suspension was added into basal feed to obtain a final bacteria concentration of $1.0 \times 10^8$ cell $g^{-1}$, using the method described by Jiang et al. (2013).