Protection against Atypical Aeromonas salmonicida Infection in Carp (Cyprinus carpio L.) by Oral Administration of Probiotic Bacterial Culture

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Introduction
Atypical Aeromonas salmonicida is a fish pathogen that causes several diseases in freshwater fish such as goldfish (Carassius auratus L.) (6) and eel (Anguilla japonica) (2), and also seawater fish (5). Since 1996, A. salmonicida infection characterized by the formation of ulcers on the body surface and fins (ulcer disease) has become prevalent in colored carp (Cyprinus carpio koi L.) cultivation in Japan (4). Antibiotic treatment for the ulcer disease resulted in no effectiveness because of the development of drug-resistance bacteria. Thus, the use of chemotherapeutic agents as an infection control measure has become questionable because of the acquisition of antimicrobial resistance among pathogenic bacteria. Vaccination is believed to be effective in principle against the disease, but no effective vaccine has yet been developed for ulcer disease. Another alternatives to chemotherapy and vaccination in disease control could be the use of probiotics as biological control agents. Probiotics could be applicable to fish farming to reduce the incidence of various infectious diseases (1, 7). We therefore investigated the effect of oral administration of probiotic bacterial culture on protection of carp from A. salmonicida infection. The results clearly showed that treatment of fish with small amounts of killed probiotic bacterial culture was effective in preventing A. salmonicida disease.

Materials and Methods
Probiotic bacterial culture: Probiotic bacterial culture was prepared by cultivating bacterial mixture containing Lactobacillus sp., Bacillus sp., Pichia sp. and Saccharomyces sp. for 48 hours at 37°C in rice bran extract containing 5% dextrose. The culture was sterilized at 121°C for 15 min before administration to fish.

Fish: Aeromonas salmonicida-free common carp were purchased from a commercial farm. They were reared in 170 l plastic aquaria filled with dechlorinated tap water (passing through once, at a flow-through rate of 40 l/hr) and aerated. The fish were fed a commercial floating dry pellet twice daily. A daily regimen of 15 h of light followed by 9 h dark was maintained.

Administration of probiotic bacterial culture: Experiments were performed two times individually under different water temperatures, namely low (non-permissive for bacterial growth) and higher (permissive) temperature. Fish were divided in each group and acclimatized in 20 l aquaria (flow-through rate 11 l/hr) which were aerated. Fish weighing 32±5 g (first trial) and 30±4 g (second trial) were used for the experiments. Probiotic bacterial culture was sprinkled on the dry pellets to provide final concentrations of 5 to 0.1% of the dry weight, and was adsorbed into the pellets which were then dried in an incubator at 25°C. The fish were fed pellets containing probiotic bacterial culture twice daily (total 1% of the fish body weight per day) for 32 and 25 days in the first and second trials, respectively prior to challenge by A. salmonicida. Control fish were fed the dry pellet without probiotic bacterial culture or the pellet containing 5% culture medium.

Bacterial challenge: The fish were challenged with virulent atypical non-pigment producing A. salmonicida as reported previously (3). Strain T1031 donated by Niigata Prefectural Inland Water Fisheries Experimental Station, Nagaoka, Japan was cultured in heart infusion broth (Nissui Pharmaceutical Co., Tokyo, Japan) at 23°C for 5 days, with shaking. The fish were immersed at 6.7x10⁶ and 4.1x10⁶ cfu/ml of the bacteria for 60 min in each experiment, respectively. Water temperatures were 13°C and 18°C at challenge, respectively. Probiotic bacterial culture was administered for 33 and 22 consecutive days after the challenge in each experiment. Fish were observed to determine survival, and any formation of ulcers and hemorrhagic lesions on the skin. Bacterial isolation was performed by cultivation from hemorrhagic and ulcerative lesions, and from visceral organs of dead fish. This was also done in all surviving fish.

Lymphocyte activation: Mice spleen cells were used to study the ability of the probiotic culture in enhancing immune response. The spleens were cut into small pieces in Hanks Balanced Salt Solution (HBSS, pH 7.4; Nissui Pharm. Co.) containing antibiotics. Single cell suspensions were then prepared by passing the cells through stainless steel meshes. After centrifuging at 400 x g for 5 min at 4°C, the resulting cell pellets were mixed in 0.75% ammonium chloride dissolved in 0.15M Tris-HCl buffer (pH 7.6) in order to lyse the red blood cells. After treatment with the lysis buffer for 60 sec, the cells were washed twice with HBSS. Cell viability, as determined by the trypan blue dye exclusion test, was greater than 90%. Spleen cells (1x10⁵ cells/0.1 ml/well) were cultivated in RPMI 1640 (pH 7.4; Nissui Pharm. Co.) containing 10% fetal bovine serum and antibiotics at 37°C for 72 hrs in the presence of two-fold dilutions of the probiotic culture in microplates. Activation of spleen cells
was determined using a commercial kit (Premix WST-1 Cell Proliferation Assay System, Takara, Tokyo, Japan). For the control, Concanavalin A was added to the spleen cell cultures.

Results and Discussion

Fish mortality after *A. salmonicida* challenge and development of skin lesions: Table 1 shows that the administration to carp of 5% probiotic bacterial culture induced effective protection against experimental *A. salmonicida* infection in the first experiment (performed under low water temperature (13°C at challenge). Of the control group (5% medium or without probiotic culture), following challenge with *A. salmonicida*, majority of carp showed severe skin lesions such as prominent hemorrhages and ulcers, and 1 and 2 carp died in each control group, respectively. The control fish showed skin hemorrhages 6 days after challenge, and skin ulcers developed 9 days after challenge. In contrast, all fish survived treated with 5% of probiotic bacterial culture, and development of skin lesion was significantly reduced (restricted and mild hemorrhages in 3 fish) or completely suppressed (7 fish). Atypical *A. salmonicida* was isolated from hemorrhagic and ulcerative lesions of both dead and survived fish in control groups, but *Aeromonas hydrophila* and *Flavobacterium* sp. were also isolated from these fish, verifying bacterial population changes during the progression of skin lesions. No *A. salmonicida*, *A. hydrophila* or *Flavobacterium* was isolated from fish treated with 5% probiotic bacterial culture.

In the second experiment performed under higher water temperature (18°C at challenge), the protective effect by probiotic bacterial culture was attained in significant reduction of mortality of fish (Table 2). Of the non-treated carp in the control group, following challenge with *A. salmonicida*, 9 carp died within 20 days. In contrast, the survival rates of fish treated with 3%, 0.5% and 0.1% of probiotic bacterial culture were 90% (p<0.01 compared to control fish by χ² test), 40% (not significant) and 50% (p<0.05), respectively at 22 days after challenge. Gross lesions observed in control fish group were much more severe than those of the group of fish administered 3% probiotic bacterial culture.

*Lymphocyte activation*: Lymphocyte activation test showed that mice spleen cells were activated by the probiotic bacterial cultures dose-dependently which reaction was comparable to that of Concanavalin A. The probiotic bacterial cultures were separated on Sephacryl S-200 gel chromatography; two peaks of fractions were shown to activate spleen cells, eg. in high and low molecular weight fractions.

We infer that administration of small amount (5 to 0.1%) of killed probiotic bacterial culture protected carp against *A. salmonicida* challenge as shown by reduced mortality, extended survival, and only mild skin lesion formation. The mechanism by which probiotic bacterial culture protects against *A. salmonicida* infection is not clear at present. Though *Lactobacillus* is known to produce compounds that inhibit the growth of bacteria (7), the protective effect shown in the present study was not due to direct antimicrobial or antibiotic activity since probiotic culture used did not kill *A. salmonicida* in vitro. The protective effect was not due to specific antigenic stimulation of carp since the probiotic bacterial culture contained no antigenic substances those react specifically with anti-*A. salmonicida* antibody. It is likely that enhancement of innate protective responses would confer protection since mice lymphocytes were activated by the probiotic bacterial culture as shown in the present study. Bacterial components may cause a range of non-specific host immune responses. Bacterial metabolite or its degenerates, and components such as cell wall components or lipopolysaccharides etc. may be involved in anti-infectious response, therefore further studies are required to determine how innate immune responses are induced in carp after treatment with probiotic bacterial culture. Probiotic effects in aquaculture are not limited to control of infectious diseases, but also can improve the health of fish by improving water quality by modifying the microbial community composition on the water and sediment.

References

Table 1

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*Hemorrhagic lesions and ulcerations were categorized as no lesion (none), slight (+), mild (++), moderate (+++), severe (++++) and death (dead). **Number of fish.

Table 2

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