

Protection against *Flavobacterium psychrophilum* Infection (Cold Water Disease) in Ayu Fish (*Plecoglossus altivelis*) by Oral Administration of Humus Extract

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ABSTRACT. Humic substances are formed during the decomposition of organic matter in humus, and are found in many natural environments in which organic materials and microorganisms have been present. In the present study, oral administration of humus extract to ayu fish (*Plecoglossus altivelis*) induced effective protection against experimental *Flavobacterium psychrophilum* infection (cold water disease). Mortality of fish and development of skin lesions, such as erosion and hemorrhages on the skin, gill cover or mouth, were significantly suppressed in fish treated with 10%, 5% or 1% humus extract adsorbed on dry pellets. Although *F. psychrophilum* was not re-isolated from gills and erosion lesions of the skin of dead fish, bacterial *gyrB* DNA could be amplified in these specimens from dead fish and surviving control fish using the polymerase chain reaction. The protective effect of the extract was not the results of direct killing of bacteria or antibiotic activity of the extract since no obvious reduction in the bacterial number was observed at 5 times to 5,000 times dilution of the humus extract having pH 5.45 to 7.40. These results clearly show that treating fish with humus extract is effective in preventing cold water disease.

KEY WORDS: Ayu fish, *Flavobacterium psychrophilum*, Humic substance, Humus extract.

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Cold water disease of fish is caused by the Gram-negative bacterium *Flavobacterium psychrophilum*. In Japan, local and farmed ayu fish (*Plecoglossus altivelis*) [2, 24], salmonid fish [23] and other freshwater fish are affected by cold water disease. This manifests in ayu fish as severe skin erosion and ulcers on the trunk and peduncle, and hemorrhages on the gill cover and lower jaws [12]. The disease is frequently fatal. These bacteria occur in almost all freshwater areas in Japan. Because of the serious losses of fish in rivers and fish farms, cold water disease is currently a serious disease economically. Furthermore, the appearance of antibiotic-resistant *F. psychrophilum* now causes serious problems since treatment with antimicrobial drugs is often ineffective in fish stocks [2, 3]. Vaccination is believed to be effective in principle against cold water disease, but no commercial vaccine has so far been available. Prophylactic approaches and stimulation of the defense system of fish by alternative approaches is therefore increasingly necessary.

Humic substances, such as humus, peat, sapropel and mumie, are formed during the decomposition of organic matter in humus. They can be found in many natural environments in which organic materials and microorganisms are or have been present [21]. Natural humification products have been used to develop pharmacologic agents that have diverse applications in medical practice [20, 22]. These have been successfully used as anti-viral and anti-inflammatory agents as a result of their local anti-inflammatory and analgesic properties and antimicrobial activity [15,

18, 19]. No measurable side effects have been observed [14, 19]. The potential of humic substances in the treatment of fish diseases has scarcely been investigated, although our previous experiment demonstrated a protective effect of humus extract against atypical *Aeromonas salmonicida* infection in carp (ulcer disease) [7], and humic substance suppresses the inflammatory responses of gills and fins in saprolegniasis in goldfish [10]. Here, we investigated the effect of oral administration of humus extract on the protection of ayu fish from cold water disease.

MATERIALS AND METHODS

Fish: Fry of the ayu fish that were free of *F. psychrophilum* were purchased from a commercial farm (Nisshin Marinetic Co., Tahara, Aichi, Japan). They were grown in 170 l plastic aquaria filled with dechlorinated and aerated tap water (passing through once at a flow rate of 40 l/hr; water temperature regulated to 16 to 18°C). The fish were fed a commercial dry pellet six times daily. A daily regimen was maintained of 15 hr of light followed by 9 hr dark. Absence of *F. psychrophilum* was confirmed by bacterial culture and the polymerase chain reaction (PCR) prior to the experiment.

Humus extract: Light brownish diatomaceous earth (humus) was collected below ground at depth 5 to 10 m in Aino-machi, Nagasaki Prefecture, Kyushu, Japan. Humus extract was prepared using water as follows [6, 7]. To the humus was added 6 volumes of dechlorinated water (v/w); the mixture was agitated every day for 30 days, and was then left to stand at 25 to 28°C for 4 months. The resulting supernatant was collected and filtered using a membrane filter (pore size: 25 µm). The resulting humus extract has pH2.4

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–2.8 and contains various minerals including Al, Ca, Mg, Na and Si. The extract contained 1,500 ppm of sulfate. No culturable bacteria were found in this extract. There were small amounts of protein and carbohydrate (0.7% of the total weight).

Administration of humus extract: The fish were divided into four groups (22 to 25 in each group) and acclimatized in 20 l aquaria (flow-through rate 20 l/hr, water temperature 16 to 18°C) which were aerated. Fish weighing 4.4 g (mean) were used for the experiments. Humus extract was sprinkled on the dry pellets to provide final concentrations of 10, 5 or 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 humus extract twice daily (total 2% of fish body weight per day) for 30 days prior to challenge by *F. psychrophilum*, and for the 21 days immediately after bacterial challenge. The concentrations of humus extract adopted in the present study were selected according to the effective concentrations obtained in the previous experiments [6–8]. Control fish were fed the dry pellet without humus extract.

Bacterial challenge: The fish were challenged with virulent *F. psychrophilum*. Strain SG990302 (donated by Shiga Prefectural Fisheries Experimental Station, Otsu, Japan) was cultured in cytophaga broth at 16°C for 7 days, with shaking. The fish were immersed in 9.0×10^5 cfu/ml for 120 min, and were observed for the next 21 days to determine survival, and formation of erosion and hemorrhagic lesions on the body surfaces. Bacteria were isolated by cultivation from gills and erosion lesions, and from visceral organs of dead fish. Similar procedures were performed in surviving fish.

PCR: A pair of primers specific for *gyrB* of *F. psychrophilum* was used in PCR to amplify a 1,017 bp DNA [4, 5]. The sequence of PSY-G1F and PSY-G1R are respectively 5'-TGCAGGAAATCTTACTACTCG-3' and 5'-GTTGCAATTACAATGTTGT-3'. PCR amplification was performed in a total reaction volume of 20 μ l using a PC708 Program Control System (Astec, Fukuoka, Japan). The reaction mixture contained 4 μ l of tissue homogenate, 0.2 nmol of each dNTP, 20 pmol of each primer and 1 unit of Taq DNA polymerase (Takara, Tokyo, Japan). The temperature profile used for the amplification was preheating at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 90 sec, and a final extension at 72°C for 5 min. A 10 μ l aliquot of the PCR reaction mixture was analyzed on 2% agarose gel electrophoresis, and DNA bands were then stained with ethidium bromide.

Bactericidal activity of humus extract: Ten-fold dilutions of humus extract were prepared using 0.15 M phosphate buffered saline (PBS, pH7.4) or deionized water, so as to obtain bacterial suspensions with differing pH. To 2 ml of each dilution, 20 μ l of precultured *F. psychrophilum* was added and incubated for 6 hr at 20°C. Ten-fold dilutions of each sample (20 μ l) were plated onto cytophaga agar, and the numbers of colonies developed was counted after incubation of the plates for 3 days at 20°C.

RESULTS

Protection after *F. psychrophilum* challenge: Figure 1 shows that administration to ayu fish of humus extract (10%, 5% or 1%) induced effective protection against

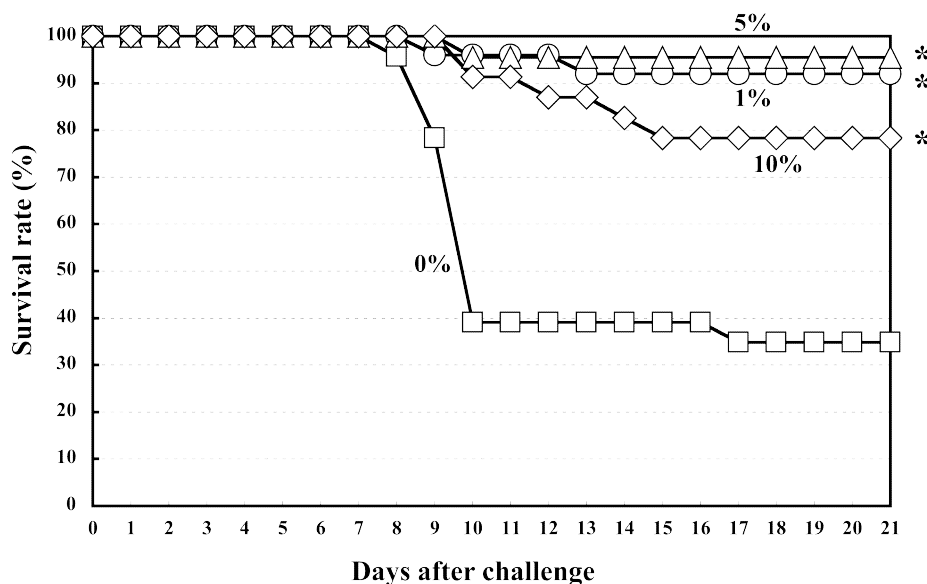


Fig. 1. Survival of ayu fish challenged with the SG990302 strain of *F. psychrophilum* after treatment with 10% (\diamond), 5% (\triangle), or 1% (\circ) humus extract. The extract was administered for 30 days orally as a dry pellet (total 2% of body weight per day) containing or without (\square) extract. Survival rates of fish in the humus-treated groups were significantly greater than in the control group (*, $P < 0.01$ by χ^2 test).

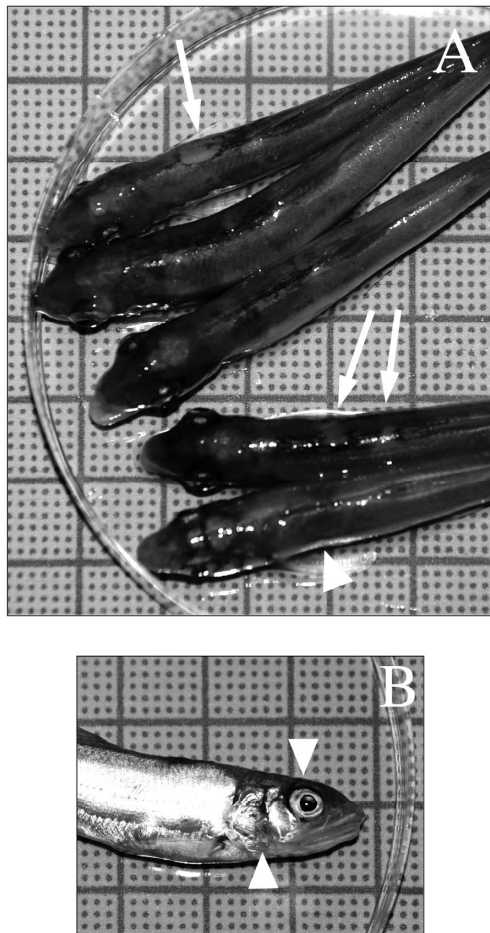


Fig. 2. Gross lesions in fish which died after *F. psychrophilum* challenge, showing erosion (arrow) and hemorrhage (arrow head) on the skin, gill cover and mouth (fish in the control group died 10 days after challenge).

experimental *F. psychrophilum* infection. Of the non-treated fish in the control group following challenge with *F. psychrophilum*, 65% of fish died within 18 days. In contrast, the survival rates of fish treated with 10%, 5% and 1% of humus extract were 78%, 95% and 92% ($P < 0.01$ compared to control fish by χ^2 test) respectively at 21 days after challenge.

Development of skin lesions: The control fish developed erosion and hemorrhages on their body surface and gill cover at 9 days after challenge (note erosion and hemorrhage on the skin (Fig. 2A), and hemorrhage on the gill cover and head (Fig. 2B). Gloss lesions were developed in 20 out of 23 of the control fish (87%) up to 21 days after the challenge. In contrast, development of skin lesions was suppressed significantly in the humus-treated fish by 4%, 5% and 22% in groups administered 1, 5 or 10% humus extract, respectively). Only the dead fish in humus-treated group showed these lesions, but no gloss lesion was observed in any surviving fish.

PCR: We undertook bacterial isolation from lesions, gills and visceral organs of dead fish and surviving fish (total: 33 fish). No *F. psychrophilum* was reisolated from these fish, but *Aeromonas hydrophila* was isolated from the body surface and gills of some of fish, verifying the bacterial population changes during progression of these skin lesions. With a pair of primers targeting the *gyrB* gene of *F. psychrophilum*, bacterial DNA was successfully amplified by PCR in gills from dead fish in the control (2/3) and humus-treated (3/3) groups, and also from surviving control fish (2/2) (Fig. 3). No bacterial DNA was amplified in gills of surviving fish in the humus-treated groups (0/6), however. Bacterial DNA was not amplified in specimens of the skin, kidney and liver of these fish. The size of the amplified DNA fragment was 1,017 bp, as expected [4, 5].

Anti-bacterial activity of humus extract: Figure 4 shows the results of the *in vitro* bactericidal test with the humus extract. The bactericidal activity was pH-dependent, since no obvious reduction in the bacterial number was observed when *F. psychrophilum* was diluted in PBS (5 times to 5,000 times dilution, having pH 5.45 to 7.40), whereas there was a marked decrease in the bacterial number when bacteria were diluted 5 to 500 times in deionized water with low pH of 2.50 to 4.45, indicating bactericidal activity in view of the acidity of the reaction mixture.

DISCUSSION

In previous studies we demonstrated the protective effect of humus extract against ulcer disease in carp (caused by atypical *Aeromonas salmonicida*) [7], experimental trypanosomiasis in mice [8], and anti-tumor effect in mice [6]. The humus extract showed its effect when administered orally to animals with food or drinking water. The present study clearly shows that the administration of humus extract adsorbed on dry pellets is effective in preventing cold water disease in ayu fish. We infer that the humus extract protect fish against *F. psychrophilum* challenge, as shown by reduced mortality, skin lesion formation and detection of bacterial DNA from fish. The survival rates of the humus-treated groups were significantly higher than in the untreated control fish group. The survival rate of fish receiving 10% of humus extract was lower than in groups treated with 5 or 1%, implying an optimal concentration for administration of the extract to animals as we reported previously [8]. In the present study, no *F. psychrophilum* was re-isolated from fish after the challenge, but *A. hydrophila* (rather than *F. psychrophilum*) was isolated from part of the fish, indicating changes of the bacterial population during the progression of skin lesions [7]. Amplification of bacterial DNA by PCR was nevertheless achieved in gills collected from dead fish and surviving control fish, showing the appropriateness of the test.

The main mode of action of the humus extract was not a direct killing effect of *F. psychrophilum*, since the humus extract did not show a bactericidal effect at neutral pH or pH greater than 5.45 (Fig. 4). The bacteria were killed only at

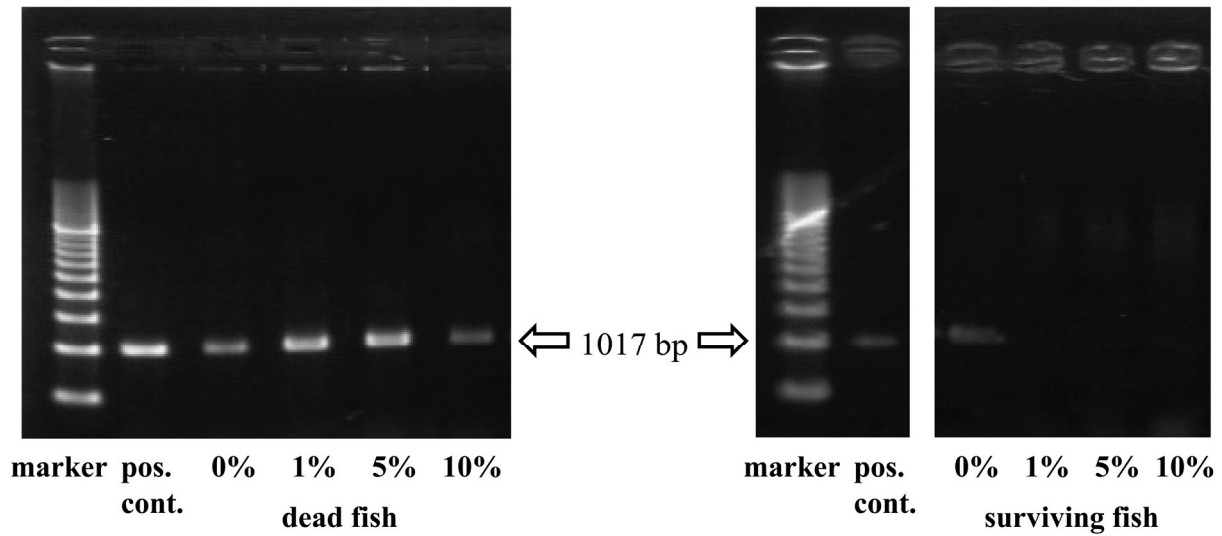


Fig. 3. Detection of *F. psychrophilum gyrB* DNA by PCR. Amplifications of DNA are shown in gills of dead and surviving fish treated with 0, 1, 5, 10% of humus extract. *F. psychrophilum* DNA was amplified only in specimens from dead fish and surviving control fish. Extracted *F. psychrophilum* DNA was used for positive control (pos. cont.). DNA size markers are also shown.

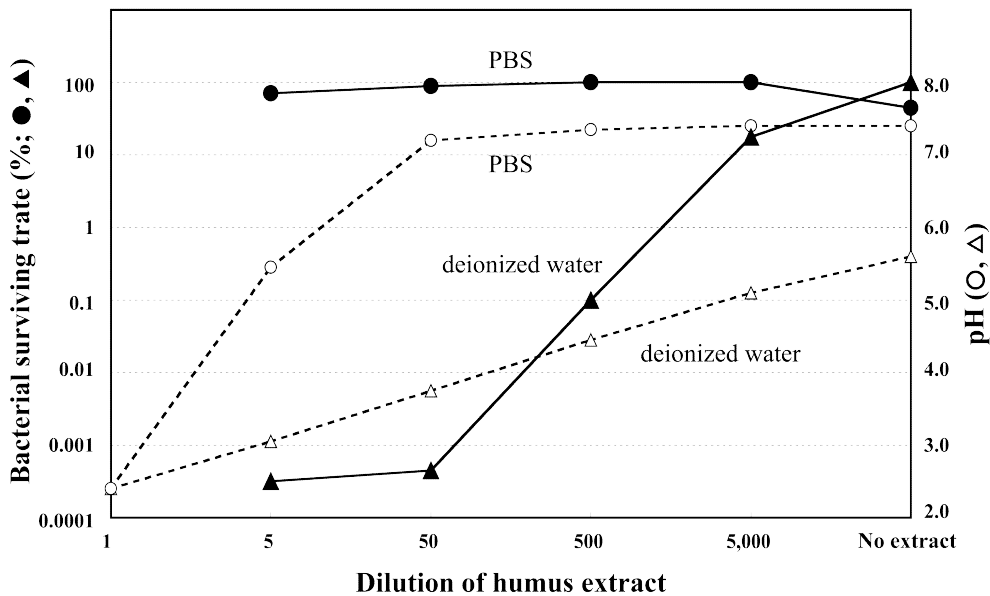


Fig. 4. Anti-bacterial activity of humus extract. Ten-fold dilutions of humus extract were prepared using 0.15 M PBS (—○—) or deionized water (—Δ—) so as to obtain bacterial suspensions with different pH. Each diluant was mixed with precultured *F. psychrophilum* and incubated for 6 hr at 20°C. The bacteria remaining were cultured on cytophaga agar for 3 days at 20°C (bacteria in humus extract diluted with 0.15 M PBS (—●—) or deionized water (—▲—)).

pH below 4.45. It follows that *F. psychrophilum* was killed as a result of the acidity of the humus extract, due to its high concentration. No inhibition of the growth of *Saprolegnia parasitica* by humic substances has been reported [9]. Our test also showed that several species of fish pathogenic bacteria (*A. salmonicida*, *A. hydrophila*, *Edwardsiella tarda*, *Lactococcus garvieae*, *Photobacterium damsela*,

Pseudomonas plecoglossicida and *Vibrio anguillarum*) were not killed by the incubation with humus extract having neutral pH (data not shown).

The mechanism by which the humus extract protects against *F. psychrophilum* infection is not clear at present, but it is likely that the physiological as well as immunological condition of the fish enhances after the administration of

the extract. Previous reports indicate that humic substances enhance host immune responses nonspecifically. We have reported that spleen cells of mice that received humus extract exhibited significant non-specific cytotoxic activity against L1210 mouse leukemia cells and enhanced production of interferon- γ [6]. Enhancement of the production of reactive oxygen species and nitric oxide in mouse peritoneal macrophages, and [^3H]thymidine uptake by splenic lymphocytes, were observed after treatment of mice with mumie [16]. Peat-based humic substance enhanced the proliferation of murine thymocytes stimulated by mitogens and prevented the immuno-suppressive effect of hydrocortisone [13]. Empirical evidence is currently accumulating that humic substances are taken up [1]. Once internalized, these substances can induce biotransformation enzymes and stress defense proteins, such as chaperons or heat shock protein 70 in fish and invertebrates [11, 17]. It is therefore reasonable to suppose that humic substances absorbed via the intestinal tract of the ayu fish affect host physiological conditions, such as innate immune responses, thereby conferring protection against *F. psychrophilum* infection. Further studies are necessary to determine how innate immune responses are induced in ayu fish after the treatment with humus extract.

No toxicity of humic substance has been reported [14, 19], since these are mostly carboxylic acids and aliphatic moiety, and ordinary physiological metabolites. Humic substances and/or humus extract can therefore be used as food additives for fish and in immunopotentiating materials in aquaculture. They should be applicable in fish farming for reducing the incidence of various infectious diseases by maintaining fish conditions favorable and healthy. Fundamental research is needed to identify the functional groups of the humic substances that are responsible for the effects observed.

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