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Original article

Enrichment of probiotic *Lactococcus lactis* strain K-C2 in *Artemia* sp.

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Introduction

In intensive aquaculture, the growth and proliferation of microbes including pathogenic bacteria in the rearing habitat are accelerated by excess feeding and excretion of inorganic nitrogen and carcass [1]. For sustainable aquaculture, microbial control is very important to prevent the fish mortality by disease. Generally, antibiotics have been used to cure bacterial disease in aquaculture, but the usage is regulated because of the occurrence of resistant bacteria to drugs.

Probiotics are defined as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" by Fuller [2]. In the field of aquaculture, the research on probiotics as alternative agent to antibiotics for disease prevention have been performed all over the world [3]. Probiotics are orally administered to aquatic animals by feeding or directly supplemented to the rearing water [4-5]. However, these trials were performed in limited experimental conditions, for example, short rearing period or limited growth stage of cultured animal such as larval stage or adult stage. Therefore, we focused the comprehensive probiotic treatment in the aquaculture production process. In short, we considered that continuous probiotic treatment from feed organisms (rotifer and *Artemia*), larval stage and to adult fish is more effective for sustainable aquaculture.

As a first step trial, this study was carried out to enrich the probiotics to a feed organism, *Artemia*. In this study, probiotic candidate, *Lactococcus lactis* strain K-C2 found in our laboratory based on the screening test was added to *Artemia* culture, and the enrichment level of probiotics in *Artemia* was evaluated.

Materials and methods

Probiotic strain and observation of cell morphology by scanning electron microscope (SEM)
In this study, *L. lactis* strain K-C2 (accession No. LC212968) was used as probiotics. The cells for SEM observation were prepared according to standard process of ethanol-dehydration and lyophilization with t-butyl alcohol in a lyophilizer (ES-2030, Hitachi, Tokyo, Japan). A small amount of the lyophilized cells was mounted directly on an aluminum SEM stub with carbon conductive tape. The morphology of lyophilized cells were observed with an SEM (SU3500, Hitachi, Tokyo, Japan).

Antagonistic activity test
Three strains of fish pathogens, *Edwardsiella tarda* were used. These strains were cultured in BTH (Bacto Todd Hewitt, BD Bacto™, USA) broth for 24 h at 28°C. The antagonistic activity was assayed according to the method described by Geis et al. [6] with slight modification. Strain K-C2 and pathogens were incubated overnight in 5 ml MRS broth and 5 ml BTH broth, respectively, at 28°C. Aliquot of strain K-C2 suspensions (5 µl at 1×10^8 CFU/ml) were spotted onto an MRS agar plate, and the plates were kept on the clean bench for 1 h to completely allow the spots to attach to the surface of the agar plates. The overnight cultured *E. tarda* strains were washed with saline solution once and suspended in 0.1 ml of saline solution again. The cell suspension (0.1 ml) of *E. tarda* was mixed with 5 ml of BTH medium containing 0.5% (w/v) agar at a final concentration of 1×10^7 CFU/ml and overlaid on the MRS agar plates spotted with strain K-C2. The plates were incubated at 28°C for 72 h, and the presence of the inhibition zones was checked.

Enrichment experiment
Artemia sp. commercial eggs of *Artemia* were purchased from Pacific Trading Co. Ltd. (Fukuoka, Japan). Eggs were hatched in an artificial seawater (SEALIFE, Nihonkaisui Co., Ltd., Tokyo, Japan) under the vigorous aeration and light irradiation. The *Artemia* after 4 days of hatching was introduced to 80 ml of artificial seawater in a flask (26 individuals/ml). Strain K-C2 was added to the seawater containing *Artemia* at 3×10^8 CFU/ml. As a control group, *Artemia* was cultured without addition of strain K-C2. As feeds, *Chlorella* suspensions (Chlorella industry Co., Ltd.,

Tokyo, Japan) were fed at 1×10^6 CFU/ml in both group every 24 hrs. After 72 hrs, *Artemia* was harvested by plankton net and washed by sterile artificial seawater and homogenized with a pestle in a mortar. The homogenates was serially diluted with sterile saline solution, and each dilutions were inoculated on a GYP agar plate medium containing calcium carbonate. The plates were incubated at 28°C for 4 days and viable colonies which degraded the calcium carbonate in the plate were counted to calculate colony forming unit (CFU).

Detection of Lactococcus lactics by colony PCR with specific primers

In the randomly selected colonies, colony PCR was carried out using *Lactococcus* specific primer set (Forward primer, LacF 5'-GTA CTT GTA CCG ACT GGA T-3' and reverse primer, LacreR 5'-GGG ATC ATC TTT GAG TGA T 3') [7,8]. A mixture (total volume: 20 µl) contained 2 µl of 10 × ExTaq buffer, 0.4 µl deoxynucleoside triphosphate (dNTP) (10 mM), 0.2 µl of forward and reverse primers (1 µM in final concentration) and 0.1 µl of ExTaq DNA polymerase (5 U/µl) included in the Takara ExTaq kit (Takara Bio Inc., Japan). The PCR amplification was performed following cycle conditions were 94°C, 40 s; 58°C, 40 s; 72°C, 1 min (35 cycles). The PCR products were visualized by electrophoresis (100 V for 25 min) with a 1.5% (w/v) agarose gel in 1× TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA) and staining with SYBR® Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific Inc., USA). The sizes of the PCR products were estimated using standard DNA ladder makers (Shinkouseiki, Co., Ltd., Fukuoka, Japan).

Results and discussion

The cell morphology of strain KC-2 was ovoid shape (Fig. 1).

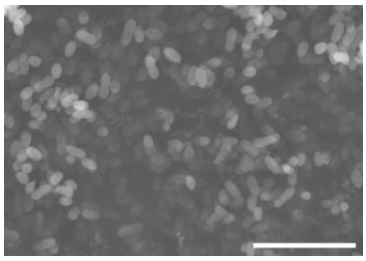

Fig. 1. Morphological observation of strain K-C2 with a SEM (scale bar, 5 µl)

Strain K-C2 showed antagonistic activity against 3 strains of *E. tarda* (Fig. 2).

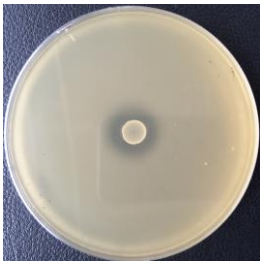

Fig. 2. Detection of antagonistic activity of strain K-C2 against *E. tarda* strain HDK1 by double-layer agar method.

In enrichment experiment, the selected colonies due to degradation of calcium carbonate were estimated as *L. lactis* by PCR with specific primers (Fig. 3). In strain K-C2-added group, the specific single band (ca. 160 bp) were detected. In control group, these bands were not detected. The number of *L. lactis* in *Artemia* was calculated to 4.2×10^3 CFU/ 100 individuals.

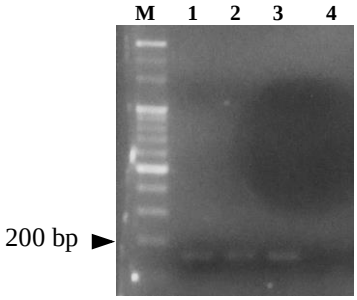

Fig. 1. PCR amplification products using the *L. lactis* species-specific primer LacF and LacreR and electrophoresis. Lanes: M, size marker (100 bp DNA ladder); 1, 2, 3, PCR products from colonies in K-C2 added-group; 4, PCR products from colonies in control.

The number of strain K-C2 detected in *Artemia* was significantly lower than estimated one. More efficient enrichment procedure should be investigated by arranging dose of K-C2 cells or exposure time.

Defoirdt et al. (2006) reported that the addition of short-chain fatty acids known as inhibitory substances to microbial growth reduced the mortality of *Artemia* by *Vibrio campbellii* [8]. By enrichment of strain K-C2 in *Artemia*, the proliferation of pathogens such as *E. tarda* via *Artemia* to larval fish would be inhibited in fish seedling production.

This study indicated that probiotic *L. lactis* strain K-C2 could be incorporated in *Artemia* by exposure in seawater and survived.

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