Aquaculture 305 (2010) 59–65

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Improved gut environment of abalone *Haliotis gigantea* through *Pediococcus* sp. Ab1 treatment

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ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 13 April 2010
Accepted 14 April 2010

Keywords:
Abalone
Alginate lyase
Colonization
Gut microflora
Probiotics
Volatile short chain fatty acids

ABSTRACT

This study demonstrated that host-derived *Pediococcus* sp. Ab1, a potential probiotic, can colonize the gut of abalone (*Haliotis gigantea*) for 12 days from cessation of feeding with Ab1-supplemented commercial diet. The effective colonization by Ab1 of *H. gigantea* fed a supplemented diet led to the alteration of microbial populations, increased *in situ* alginate lyase activity, and increased volatile short chain fatty acids (VSCFAs) production in the abalone gut. *Vibrio halioticoli* clade dominated the gut microflora of abalone fed an Ab1-supplemented diet, while other *Vibrio* species were dominant in the abalone fed an unsupplemented commercial diet alone. The same species of bacteria were found in the gut of abalone fed diet with and without supplementation; however, the representation of the *V. halioticoli* clade was much higher in the gut of abalone fed the Ab1-supplemented diet compared to the gut of abalone fed the unsupplemented commercial diet. The alginate lyase activity and production of formate, a VSCFA, were also higher in the gut of abalone fed the Ab1-supplemented diet compared to the gut of abalone fed unsupplemented commercial diet. These results indicate that the host-derived *Pediococcus* sp. Ab1 has significant potential as an effective probiotic, especially in the improvement of the gut microflora and nutrition status in abalone aquaculture.
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1. Introduction

Abalones (*Haliotis*) mainly ingest algae, and they have a preference for brown algae consisting of polysaccharides like alginate and cellulose (Sawabe et al., 1995). Tanaka et al. (2003) reported that the dominant species in the gut of abalone *Haliotis discus hannai* were non-motile fermenters (NMF) like *Vibrio halioticoli* and *Vibrio* spp. El-Shanshoury et al. (1994) suggested that bacteria may enhance the digestive efficiency and, consequently, improve the growth rate of a host by supplying polysaccharolytic enzymes. Erasmus et al. (1997) suggested that enteric bacteria play an integral role in abalone nutrition by hydrolyzing complex polysaccharide components of macroalgae to simple polymers and smaller units that are rapidly assimilated by abalone. Sawabe et al. (2003) also demonstrated that *V. halioticoli* was abundant in the gut of three species of the Japanese abalone, *H. discus discus*, *H. diversicolor aquatilis*, and *H. diversicolor diversicolor*, as well as the South African abalone, *Haliotis midae*, and a turban shell, *Turbo cornutus*. It was also demonstrated that *V. halioticoli* plays an important role because they potentially contributed to the host's nutrition through alginate degradation and production of volatile short chain fatty acids (VSCFAs).

Recently, the use of probiotics has become popular not only in higher organisms like humans but also in aquaculture (Vine et al., 2006). Probiotics provide health benefits to hosts by stimulation of host immune responses and improvements in nutrition (Irianto and Austin, 2002). Lactic acid bacteria (LAB) are major probiotics in aquaculture. Some researchers reported that probiotic LAB can colonize the intestinal tracts of fish and crustaceans (Joborn et al., 1997; Panigrahi et al., 2005) and improve the survival and growth of the host species (Gildberg et al., 1997; Planas et al., 2004). There are many reports on probiotics for fish and crustaceans, but little research has been done on probiotics for abalone (Macey and Coyne, 2005, 2006; Doeschate and Coyne, 2008; Iehata et al., 2009). Studies of probiotics for abalone used gram-negative bacteria and/or yeast isolated from host gut or LAB isolated from terrestrial environments as probiotic candidates. However, there have been no studies using LAB isolated from gut of abalone as probiotic candidates. In this study, we investigated whether *Pediococcus* sp. strain Ab1 isolated from host gut can colonize the gut of the Japanese abalone (*Haliotis gigantea*). In addition, we evaluated whether Ab1 has a potential probiotic effect on the gut environment, such as alteration of gut microflora, increased alginate lyase activity, and increased production of VSCFAs.

2. Materials and methods

2.1. Microorganism and Culture Media

Pediococcus sp. strain Ab1 (Ab1) was isolated from the gastrointestinal tract of *H. gigantea* obtained from the Owase Farming Fishery Center (Owase, Mie, Japan). De Man–Rogosa–Sharpe (MRS) agar

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doi:10.1016/j.aquaculture.2010.04.012

medium [polypeptone 10.0 g, yeast extract 5.0 g, glucose 10.0 g, Tween 80 1.0 ml, K_2HPO_4 2.0 g, sodium acetate 5.0 g, $(NH_4)_2HC_6H_5O_7$ 2.0 g, $MgSO_47H_2O$ 0.2 g, $MnSO_4$ $5H_2O$ 0.05 g, agar 20 g, 50% artificial sea water (ASW) 1000 ml, pH 6.0–6.5] was used for the maintenance and enumeration of culturable LAB. MRS broth was used to subculture *Pediococcus* sp. Ab1. For the enumeration of culturable bacteria in the abalone gut and rearing water samples, Zobell 2216E agar medium [polypeptone 5.0 g, yeast extract 1.0 g, agar 15.0 g, 75% natural sea water (NSW) 1000 ml, pH 7.6] containing 0.5% sodium alginate (APY agar) was used (Sawabe et al., 1995). Zobell 2216E broth was used to subculture gut microbial samples.

2.2. Preparation of feed

Commercial abalone feed (Cosmo Business Support, Ehime, Japan) was used in this study. The Ab1-supplemented diet was constructed that commercial abalone feed immersed *Pediococcus* sp. Ab1 suspended in sterilized NSW to achieve a final concentration of 1.0×10^9 CFU/g-feed. Then the feed was dried and stored at -30 °C until needed within 1 week.

2.3. Microbiological analysis

2.3.1. Colonization experiment

A colonization experiment was conducted to determine whether *Pediococcus* sp. Ab1 is able to survive in the gut of *H. gigantea*. Abalones (9 months old) were obtained from the Owase Farming Fishery Center (Owase, Mie, Japan). Three abalones were randomly removed from the sample and immediately sacrificed (initial); the remaining animals were divided into two groups of 30 individuals and maintained in two separate, aerated acryl tanks at temperatures of 21 ± 0.5 °C. Animals in both tanks (30 per tank) were acclimated to the unsupplemented commercial feed for 10 days. Abalones were provided with fresh feed every second day after uneaten feed was removed from the tank, and the tanks were thoroughly cleaned once a week. After the 10-day acclimation, six weeks prior to the start of the experiment, animals in one tank were fed the Ab1-supplemented commercial feed, while the animals in the remaining tank were continually fed the commercial feed. Subsequently, animals in both tanks were fed the commercial feed on the following days: P = 2, 4, 6, 8, 10 and 12. On day 1 (P = 1 or control), three abalones were removed from each tank and immediately sacrificed. Thereafter, three animals were sacrificed from the probiotic treatment tank only on the following days: P = 2, 3, 5, and 12. Water samples from each rearing tank was collected in sterilized bottles on day 1 (P = 1 or control), while water samples was only collected from the Ab1-treatment tank on the following days: P = 2, 3, 5, and 12 (Fig. 1). Through the experimental periods, all abalones in each tank were kept in good condition and did not die.

2.3.2. Enumeration of culturable bacteria and culturable lactic acid bacteria (LAB) on MRS

The guts of individual abalone were aseptically excised and gut homogenates were prepared according to the method of Tanaka et al. (2003). For enumeration of culturable bacteria, serial tenfold dilutions (10^3 to 10^6) of gut homogenate and serial tenfold dilutions (10^0 to 10^3) of water samples were spread on APY agar medium and incubated at 20 °C for 7 days. For enumeration of culturable LAB, 10-fold serially diluted (10^1 to 10^3) gut homogenate and water samples from rearing tanks (0.1 ml) were spread on MRS agar medium and incubated at 25 °C for 2 days. All colonies on APY agar and white colonies on MRS agar were counted and expressed as CFU per gram of gut (wet weight) for homogenates or as CFU per milliliter for water samples. To validate that the cells in the white colonies on the MRS agar were indeed the *Pediococcus* sp. Ab1 that was initially added to the Ab1-supplemented diet, 16S rDNA RFLP (Restriction Fragment Length Polymorphism) analysis was carried out as described by Yanagida et al. (2005) and Chen et al. (2006). Three restriction enzymes, *Acc* II (CG/CG), *Hae* III (GG/CC) and *Msp* I (CC/GG), were used for grouping in our study.

2.3.3. Construction of gut microflora and biodiversity analysis

To determine abundance and species composition of culturable microorganisms, abalones were collected. Each gut sample was aseptically excised, homogenized, and spread on APY agar medium and incubated at 20 °C for 7 days. Thirty colonies were randomly selected and transferred into Zobell 2216E broth and incubated with shaking at 150 rpm until growth occurred. Thereafter, DNA from the each incubated culture was extracted using a phenol–chloroform method. The bacterial 16S rDNA primer pair 8F (5′-AGAGTTTGAT-CATGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) was used for PCR amplification of 16S rDNA from DNA using a thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA). The 16S rDNA PCR products were sequenced using the bacterial 16S rDNA 8F primer, the BigDye terminator cycle sequencing method, and an ABI 3100 sequencer (Applied Biosystems, Foster city, CA, USA). The 5′ region of 16S rDNA corresponding to bp 10–400 of the *Escherichia coli* 16S rDNA position was determined. Multiple alignments and

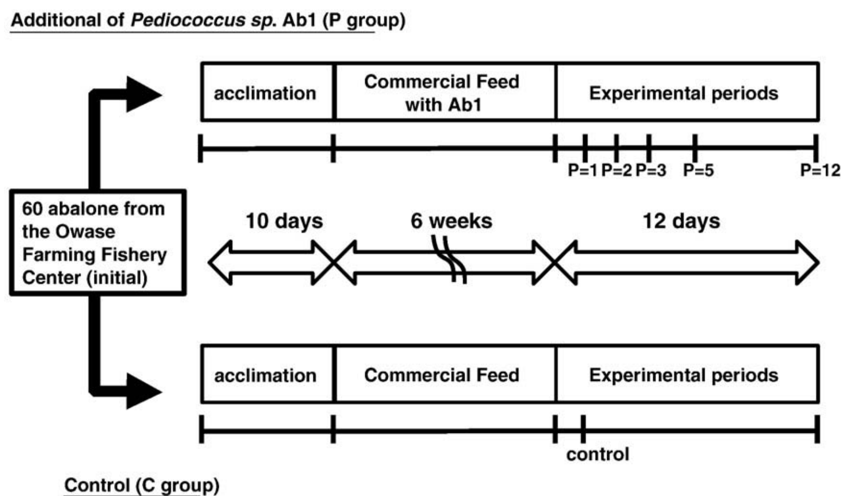

Fig. 1. Experimental design. The 30 abalones in each tank were maintained at 21 °C and fed a commercial diet throughout the experimental periods. After acclimation, P group only were fed a commercial diet with *Pediococcus* sp. Ab1 for 6 weeks. P = 1 means day 1 of P group after cessation of Ab1 supplemented diet for 6 weeks, whereas control means day 1 of C group corresponding to P = 1.

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