

**Effect of lactic acid bacteria and the potential probiotic
Hafnia alvei on growth and survival rates of narrow clawed
crayfish (*Astacus leptodactylus* Esch., 1823)
stage II juveniles**

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Abstract

The aim of this study was to screen potential probiotic bacteria against *Aeromonas hydrophila* and determine the effects of antagonistic bacteria and a commercial product containing lactic acid bacteria on the survival and growth of stage II *Astacus leptodactylus* juveniles. For this purpose, a total of 110 bacterial strains were isolated from adult, stage II crayfish juveniles and rearing water screened for antagonistic activities against *A. hydrophila* with well diffusion agar assay. *Hafnia alvei* strain from stage II crayfish juveniles displayed the inhibition zone (10mm) against *A. hydrophila*. The experiment was conducted in a completely randomized design with four treatments for 60 days: (I) crayfish fed with live food without probiotics (control group); (II) crayfish fed with live food enriched with lactic acid bacteria (0.015 gL⁻¹); (III) crayfish fed with live food enriched with *Hafnia alvei* (10⁶ CFU mL⁻¹); (IV) crayfish fed with control diet and *H. alvei* added to rearing water (10⁶ CFU mL⁻¹). As a result of this study, lactic acid bacteria and *Hafnia alvei* applications did not positively affect growth and survival of stage II *A. leptodactylus* juveniles. In the future, studies on screening potential probiotic bacteria should be used *in vitro* and *in vivo* tests. In addition, it will be useful to investigate the lactic acid bacteria and *Bacillus* spp. from indigenous microflora of crayfish.

Keywords: *Astacus leptodactylus*, *Aeromonas hydrophila*, Lactic acid bacteria, *Hafnia alvei*, Growth, Survival

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Introduction

Astacids are considered valuable fishery resources, as there are no other commercially important species of Crustacea found in the fresh waters of Turkey. The only native crayfish species found in Turkey is *Astacus leptodactylus* Eschscholtz, 1823. Astacid culture is mainly based on semi-extensive systems in which stage II juveniles are stocked in natural earthen ponds or in artificial ponds. However, mortality of juveniles of Astacids is more than in warm water crayfish (e.g., cambarids) (Mazlum, 2007). One of the most important factors affecting survival and growth of these juveniles is nutrition. There have been a few studies describing nutrition (Cilbiz, 2011; Koca *et al.*, 2011), and feed additives (Mazlum *et al.*, 2011; Safari *et al.*, 2014; Safari *et al.*, 2015) for *A. leptodactylus*. There are a few studies on the effects of probiotic bacteria on growth and survival of fresh water crayfish (Didinen *et al.*, 2008; Sajedi-Raad *et al.*, 2010; Ambas *et al.*, 2013; Mona *et al.*, 2015). However, there is no published study on the effects of selected or commercial probiotic bacteria on growth and survival of stage II *A. leptodactylus* juveniles.

Lactic acid bacteria (LAB) are the most commonly applied probiotics in terrestrial animal nutrition, and their use as probiotics has been proposed for aquatic species (Gatesoupe, 1991; Ringo and Gatesoupe, 1998; Gatesoupe, 2002). Lactic acid bacteria are used as

probiotics in aquaculture because of their properties such as low or no virulence, outcompeting harmful bacteria by producing antimicrobial substances, adherence capacity to colonize the digestive tract and competing with pathogens for adhesion sites (Farzanfar, 2006; Mota *et al.*, 2006).

A. hydrophila is a pathogenic bacterium for freshwater crayfish (Jiravanichpaisal *et al.*, 2009). In addition, Wasow *et al.* (2004) reported that the incidence of plague in *A. leptodactylus* was accompanied by bacteremia caused by *Pseudomonas fluorescens*, *A. hydrophila* and *Aeromonas caviae*. However, there is no study looking for probiotic bacteria effective against this pathogen in *A. leptodactylus*.

The aim of this study was to isolate potential probiotic bacteria strains from healthy narrow clawed crayfish (*A. leptodactylus*) and rearing water based on antimicrobial activities against *A. hydrophila*. This study also evaluated the impact of the potential probiotic and a commercial probiotic containing lactic acid bacteria on the growth and survival of stage II *A. leptodactylus* juveniles.

Materials and methods

Isolation of potential probiotic bacteria

The samples were taken from hepatopancreas, gills and intestine of adult crayfish and the whole body of stage II crayfish juveniles; rearing water of adult and juvenile crayfish at

Suleyman Demirel University, Egirdir Fisheries Faculty, Aquaculture Unit. The samples were homogenized, serially diluted in phosphate buffered saline (PBS, Sigma), plated onto Tryptic Soy Agar (TSA, Merck) and Plate Count Agar (Merck) and incubated for 2–3 day at an ambient temperature of approximately 25 °C. Each isolate was stored at –80 °C in Tryptic Soy Broth (TSB, Merck) with 15% (v/v) glycerol.

Inhibitory activity of the isolates

All bacteria isolated from the samples were tested for antagonistic activity by a well diffusion agar assay (WDAA) against *A. hydrophila*. Target organisms were grown in 4mL TSB for 2 d at 25°C, and 10 µL of each culture was mixed into 10 mL of melted TSA (43.5–44°C). After solidifying and drying for 15–20 minutes, wells were punched (diameter, 3 mm) and 10 µL of a 2 day old potential probiotic bacterial culture (approx. 10^8 – 10^9 CFU mL⁻¹) grown in TSB at 25°C was added to wells in triplicates. Plates were incubated at 25°C for one day and observed for clearing zones around the wells. Strains causing clearing zones in the WDAA were tested once more in TSA to ensure that the antagonistic activity was stable after storage and sub-culture (Hjelm *et al.*, 2004).

Identification of potential probiotic bacterium

Identification of antagonistic bacterium was based on the following criteria: Gram reaction, catalase reaction (3%

H₂O₂), motility, oxidase reaction, ability to metabolize glucose by oxidation and/or fermentation in OF basal medium supplemented with 1.5% glucose, growth at 41°C in TSB, methyl red test, fermentation of carbohydrates (cellobiose, maltose, mannose, trehalose, xylose, raffinose, salicin, sorbitol, lactose). Additional tests were performed using API 20 E system (bioMérieux) (Holt *et al.*, 1994; Austin and Austin, 2007).

Pathogenicity test of potential probiotic bacteria on stage II crayfish juveniles

The juveniles were distributed into groups of 25 animals each in 10 L plastic troughs containing 5 L of ground water. Animals were challenged by immersion at the final concentration of 10^7 CFU mL⁻¹ with *H. alvei* for a period of 2 days. The experiment was carried out in triplicate and juvenile mortality was monitored for 14 days (Austin *et al.*, 1995).

Production of stage II A. leptodactylus juveniles for experiment

In this study for the production of stage II juvenile crayfish, females carrying fertilized eggs were obtained from Egirdir Lake. For adaptation, oviferous females were placed into circular fiberglass tanks (bottom area 1.5 m², volume 400 L) at a rate of 15 individuals per tank. The tank system was placed inside a room with natural photoperiod, and each tank was supplied with continuous aeration. A static system was used and 30% of the water in each tank was exchanged every

day. Water temperature inside the tank ranged from 17 to 20°C. Dissolved oxygen ranged from 5 to 7 mg L⁻¹, while the pH ranged from 7 to 7.6 during production for stage II juveniles.

Experimental groups

The experiment was conducted in a completely randomized design with four treatments for 60 days: (I) crayfish fed with live food without probiotics (control group); (II) crayfish fed with live food enriched with commercial probiotics (0.015 gL⁻¹); (III) crayfish fed with live food enriched with potential probiotics (10⁶ CFU mL⁻¹); (IV) crayfish fed with control diet and potential probiotics added to rearing water (10⁶ CFU mL⁻¹).

Live food culture and probiotic enrichment

Live food organisms (*Daphnia magna* and Chironomid larvae) were cultured in outdoor tanks (700 L). *Chlorella* sp. was introduced in culture tanks fertilized with horse manure (200 gL⁻¹). *Daphnia magna* and Chironomid larvae were grown in culture tanks containing *Chlorella* sp. culture at a cell density of 2x10⁵ cells mL⁻¹.

The probiotic (consisting of *Lactobacillus plantarum*, *L. delbrueckii* sub sp. *bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *Bifidobacterium bifidum*, *Streptococcus salivarius* subsp. *thermophilus*, *Enterococcus faecium*, *Aspergillus oryza*, *Candida pintolopesii* and Ascorbic acid) was used according to manufacturer's instructions.

The commercial probiotic and potential probiotic culture was added to live food (*Daphnia magna* and Chironomid larvae) tanks every day at the final concentrations of 0.015 gL⁻¹ and 10⁶ CFU mL⁻¹, respectively.

Experimental conditions

The experiment was performed using *A. leptodactylus* Stage II juvenile at the Aquaculture Department of Egirdir Fisheries Faculty, Suleyman Demirel University. A total of 450 juveniles (initial weight, carapax and total length were 0.035±0.003 g, 6.25±0.24 mm and 12.13±0.29 mm, respectively) were stocked into each fiberglass tank (3x0.5x0.5 m, bottom area 1.5 m²) filled with 300 L of ground water. The experiment groups were run in triplicates. Several bricks (27.5x17.5x13 cm) with 32 holes were placed in each tank as shelter. The tank system was housed inside a room with natural photoperiod and each tank was supplied with continuous aeration. A static system was used and 30% of the water in each tank was exchanged every day. The calcium concentration of water during the production of juveniles was 156 mgL⁻¹. Water temperature and dissolved oxygen ranged from 19 to 21°C, and 6 to 7 mgL⁻¹, respectively during the experiment. The juveniles were fed live food ad libitum every 24 h.

Measurements

The measurements of the carapax, total length, and weight of the animals were

performed both at the beginning (100 individuals) and at the end of the experiment (all individuals). The number of surviving juveniles in each tank was counted at the end of the experiment.

The specific growth rate (SGR, % day⁻¹) and survival rate were calculated as follows:

$$\text{SGR (\% day}^{-1}\text{)} = 100(\ln W_t - \ln W_0)/t,$$

where W_0 and W_t are the initial mean weight and final mean weights, and t is the time in days ($t=60$ day)

$$\text{Survival rate (SR, \%)} = [(\text{final number of crayfish}/\text{initial number of crayfish}) \times 100]$$

Statistical analysis

The final carapax and the total length, weight, specific growth rate, and survival rate of *A. leptodactylus* juveniles were analyzed with one way analysis of variance (ANOVA) to

determine if significant differences occurred among the probiotic treatments and control group. Duncan's multiple-range test was used to compare differences among individual means. The data were analyzed with SPSS 11.05. All statistical computations were performed at the probability level of $p < 0.05$.

Results

Antagonistic activities against *A. hydrophila* of 110 bacterial isolates were tested by the well diffusion agar assay. *H. alvei* strain isolated from stage II crayfish juveniles was displayed inhibition zone (10mm) against *A. hydrophila*. *H. alvei* didn't showed pathogenic effects on crayfish juveniles. Phenotypic characteristics of *Hafnia alvei* was given in Table 1.

Table 1: Phenotypic characterization of *Hafnia alvei* isolated from juvenile crayfish. Characteristics of *Hafnia alvei*

Gr staining	-	Acid production from:	
Oxidase	-	Glucose ^a	+
Catalase	+	Mannitol ^a	+
O/F Test	+/+	Inositol ^a	-
Motility	+	Rhamnose ^a	+
Growth at 41°C	+	Saccharose ^a	-
β-lactosidase(ONPG) ^a	+	Mellibiose ^a	-
Arginine dihydrolase ^a	-	Amygdalin ^a	-
Lysine decarboxylase ^a	+	Arabinose ^a	+
Ornithine decarboxylase ^a	+	Cellobiose	+
Production of H ₂ S ^a	-	Maltose	+
Urease ^a	-	Mannose	+
TDA ^a	-	Trehalose	+
Production of indole ^a	-	Xylose	+
VP ^a	+	Raffinose	-
Gelatinase ^a	-	Salicin	-
NO ₃ reduction ^a	+	Sorbitol	-
Methyl red	+	Lactose	-

^a Performed to API 20 E

The end of the experiment, final weights, total body lengths, specific growth rates and survival rates of crayfish juveniles was ranged 0.50-0.57 g, 26.49–28.3 mm, 3.02 - 3.08%, 18.32-26.04% in all groups, respectively. The survival rates of the groups treated with

H. alvei was found relatively high levels. However, there was no significant differences between survival and growth parameters of all groups ($p>0.05$) (Table 2).

Table 2: Growth parameters, survival and mortality rates of the crayfish in the end of experiment.

Parameters	Groups			
	I (Control)	II	III	IV
Total length (mm)	28.3±5.4	26.49±4	26.54±4.34	27.02±4.04
Carapax length (mm)	14.44±2.69	13.49±1.97	13.22±2.21	13.77±1.97
Final weight (g)	0.57±0.31	0.50±0.26	0.53±0.25	0.55±0.27
SGR (% day ⁻¹)	3.08±0.0028	3.02±0.0028	2.96±0.0021	3.06±0.056
Survival rate (%)	23.87±2.07	18.32±5.88	25.4±6.22	26.04±6.55

In each parameter was not shown non-significant difference between groups ($p>0.05$)

Discussion

In this study, *H. alvei* strain isolated from stage II *A. leptodactylus* juveniles showed the antagonistic property towards *A. hydrophila*. There is only one study on probiotic bacteria effective against bacterial pathogens in fresh water crayfish. Ambas *et al.* (2015) found that *Bacillus mycoides* A10 and *Shewanella* sp. A12 isolated from gastrointestinal tract (GIT) of *Cherax cainii* inhibited growth of pathogen *Vibrio mimicus* and *Vibrio cholerae*, respectively. In addition, a few reports about selection of potential probiotic bacterial strains in shrimp have been documented. Gullian *et al.* (2004) reported inhibitory effects of *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 isolated from the hepatopancreas of healthy wild shrimp (*Penaeus vannamei*) against *V. harveyi* (S2). Rattanachuy *et al.* (2007) noted *Pseudomonas* sp. isolated from water samples of intensive shrimp ponds showed high antagonistic activity

against shrimp *Litopenaeus vannamei* pathogen *V. harveyi*. Balcázar *et al.* (2007) demonstrated *in vitro* antagonistic effects of *V. alginolyticus* UTM 102, *B. subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03, and *P. aestumarina* SLV22 from the gastrointestinal tract of adult shrimp *L. vannamei* against the shrimp-pathogenic bacterium, *Vibrio parahaemolyticus* PS-017. Liu *et al.* (2014) also found that *Bacillus* sp. obtained from the gastrointestinal tract of healthy *L. vannamei* displayed antimicrobial activity against aquatic pathogens *A. hydrophila*, *A. sobria* and *A. caviae*.

One of the most important features of a probiotic is that it must not be pathogenic or toxic to its host (Kersarodi-Watson *et al.*, 2008). *H. alvei* did not cause any harmful effects to crayfish stage II juveniles upon challenge even at a dose of 10⁻⁷ cells mL⁻¹ introduced by immersion in this study. Capkin and Altinok (2009)

noted antagonistic effect of *Enterobacter cloacae* isolated from rainbow trout intestinal microflora against *Yersinia ruckeri* and it is safe for rainbow trout. Burbank *et al.* (2011), the two *Enterobacter* sp. (C6-6 and C6-8) also identified as non-pathogenic to rainbow trout, inhibit *Flavobacterium psychrophilum* *in vitro*.

In the present study, addition of *H. alvei* to rearing water and live food did not affect growth and survival of *A. leptodactylus* juveniles. Similarly, Ambas *et al.* (2013) also reported that dietary supplementation of *B. mycoides* (A10) and *Shewanella* sp. isolated and selected from *Cherax tenuimanus* had no significant impact on growth and survival of the marron. Rattanachauy *et al.* (2007) also noted that the addition of the potential probiotic *Pseudomonas* sp. to rearing water in shrimp did not affect growth in shrimp. In contrast, Balcázar *et al.* (2007), evaluated four bacterial strains isolated from the gastrointestinal tract of adult shrimp (*L.vannamei*), *V. alginolyticus* UTM 102, *B. subtilis* UTM 126, *R. gallaeciensis* SLV03, and *P. aestumarina* SLV22 for potential use as probiotics for shrimp. Feeding shrimp with diets containing all potential probiotics showed the best growth in comparison with the control groups. Gullian *et al.* (2004) also reported that shrimps *Penaeus vannamei* fed with probiotic (*Vibrio* P62, *Vibrio* P63 and *Bacillus* P64) showed significantly higher weight ($p < 0.05$) than the control group. In addition, use of dietary *S. haliotis* D4, *B. cereus* D7 and *A. bivalvium* D15

isolated from the gut of shrimp has improved growth of white shrimp (*L. vannamei*) (Hao *et al.*, 2014).

In this study, growth and survival of crayfish juveniles were not affected by feeding lactic acid bacteria enriched live food for 60 days. There is only one study on lactic acid bacteria in crayfish. Didinen *et al.* (2008) also reported that feeding lactic acid bacteria did not affect survival and growth in crayfish juveniles (average 15 g). However, the positive effects of lactic acid bacteria on growth of shrimp were noted in previous studies. Venkat *et al.* (2004) pointed out significantly higher growth performance in *Macrobrachium rosenbergii* post larvae fed *Artemia* bioencapsulated *L. sporogenes* over the control group. Similarly, Dash *et al.* (2014) indicated that *L. plantarum* can be used to improve growth in *M. rosenbergii*. Javadi *et al.* (2011) reported that the use of commercial probiotic containing different lactic acid bacteria in *P. indicus* led to a significant increase in growth parameters. Ajitha *et al.* (2004) noted that growth was superior in *Penaeus (Fenneropenaeus) indicus* fed lactic acid bacteria (*L. acidophilus*, *S. cremoris*, *L. bulgaricus* and *L. bulgaricus*) in comparison to the control group. Kongnum and Hongpattarakere (2012) also reported that use of *L. plantarum* supplemented diet in *Litopenaeus vannamei* improved relative growth rate (% RGR).

As a result of this study, *H. alvei* strain isolated from stage II narrow clawed crayfish juveniles displayed antimicrobial activity against crayfish

pathogen *A. hydrophila*. However, *H. alvei* and the commercial probiotic containing lactic acid bacteria have not shown positive effect on growth parameters and survival in stage II *A. leptodactylus* juveniles. In the future further studies should be used *in vitro* (tolerance to high bile and low pH conditions, hydrophobicity, antibiotic susceptibility) and *in vivo* [total haemocytes counts (THC), proportion of granular cells (GC) and determination of bacterial load in the haemolymph] tests. In addition, it will be useful to investigate the lactic acid bacteria and *Bacillus* species from indigenous microflora of narrow clawed crayfish in these studies.

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