The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Published under auspices of

The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB),

University of Hawai'i at Mānoa Library

&

University of Hawai'i at Mānoa Aquaculture Program

in association with

AquacultureHub

http://www.aquaculturehub.org

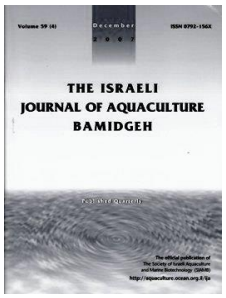
ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH - Kibbutz Ein Hamifratz, Mobile Post 25210, ISRAEL

Phone: + 972 52 3965809

http://siamb.org.il



Effects of Enzyme-Producing Probiotic Bacteria Isolated from the Gastrointestinal Tract of Trout on the Growth Performance, Survival, and Digestive Enzyme Activity of Rainbow Trout Fry (*Oncorhynchus mykiss*)

Seval Bahadır Koca*[1], Nalan Özgür Yigit[1], Behire Işıl Didinen[1], Secil Metin[1], Halit Bayrak[2], Ertan Emek Onuk[3], İlter İlhan[4], Hasan Eralp[1], İbrahim Diler[1]

[1]Egirdir Fisheries Faculty, Suleyman Demirel University, Isparta, Turkey; [2]Water Institute, Suleyman Demirel University, Isparta, Turkey; [3] Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey; [4] Department of Medical Biochemistry, Suleyman Demirel University, Isparta, Turkey

(Received 13.2.2015, Accepted 2.4.2015)

Key words: enzyme-producing bacteria, probiotic, rainbow trout fry, growth, enzyme activity

Abstract

In this study, we investigated the effects of enzyme-producing probiotic bacteria isolated from the gastrointestinal tract of rainbow trout on the growth performance, feed conversion ratio, and digestive enzyme activity, of fry (*Oncorhynchus mykiss*). Three isolates (G8/2013, T7/2013 and U5/2013) of candidate bacteria elicited the highest protease, lipase, and amylase activities, respectively. Isolates were identified as *Aeromonas* sp., *Bacillus* sp. and *Citrobacter braakii* by morphological, physiological, biochemical characterizations as well as 16S rRNA gene sequence analysis. The fry basal diet was supplemented with probiotics at varying concentrations; G8 group, *Aeromonas* sp. 1.72 x 10^8 CFU/g; U5 group, *Bacillus* sp. 3.01 x 10^8 CFU/g; T7 group, *C. braakii* 2.96 x 10^8 CFU/g and a mixed group (same bacterial concentrations), and control group (no bacteria). The rainbow trout fry were fed ad libitum in triplicate treatments with supplemented and non-supplemented probiotic diets for 70-days. The total bacterial count in the intestine was significantly higher in the mixed group (30^{th} and 50^{th} days) and U5 group (50^{th} and 70^{th} days) compared with the control group. However, there was no significant difference in weight gain, specific growth rate (SGR), feed conversion ratio (FCR), nutrient digestibility, or digestive enzyme activity among the groups.

* Corresponding author. e-mail: sevalkoca@sdu.edu.tr

Introduction

The bacterial flora in the gastrointestinal (GI) tract of fish contain various enzymatic potentials capable of producing proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes. These enzymes are important for digestion of proteins, carbohydrates, cellulose, lipids and chitin (Bairagi et al., 2002; Gutowska, et al., 2004). During the larval stage and before active feeding, the total bacterial load in the GI tract is low (Reid et al., 2009). The initial colonization process of microbial composition in the GI tract of fish affects the developmental stage of fish, gut structure, and is affected by the surrounding environment such as ambient water temperature, rearing and farming conditions. In addition, feed and feeding conditions considerably influence the composition in the GI tract of fish (Uchii et al., 2006; Martin-Antonio et al., 2007) during the larval stage (Reid et al., 2009; Nayak, 2010). Larvae are potentially susceptible to GI microbiota associated disorders, since they start feeding when the digestive tract is not yet fully developed (Timmermans, 1987). Therefore, probiotic treatments are particularly desirable during the larval stages (Gatesoupe, 1999; Lara-Flores, 2011). The enzyme producing microbiota can be used as probiotic supplements in the fish diets (Bairagi et al., 2002). The presence of high concentrations of Aeromonas in the GI tract can play an important role in digestion secreting proteases (Pemberton et al., 1997).

There are reports of microbial enzyme production in the GI tract of fish (Bairagi et al., 2002; Mondal et al., 2008; Ray et al., 2010; Ray et al., 2012). Reports have not however focused on the effects of a specific enzyme producing microbiota from the GI tract of fish on growth performances and digestive enzyme activities. This study attempts to investigate the effects of probiotic bacteria isolated from the GI tract of fish on growth performance and digestive enzyme activity in rainbow trout fry.

Materials and Methods

Isolation of candidate probiotic bacteria. 83 healthy rainbow trout ((*Oncorhynchus mykiss (*mean weight 133.54± 4.39 g) were used for bacterial isolation. The fish were starved for 24 hours before sampling. After sedation whole intestines of the fish were removed aseptically and homogenized with phosphate-buffered saline (PBS). The homogenate was used as inoculum. Spread plate method was carried out in triplicate at 22 ^{o}C for 24 hours on nutrient agar plates with starch (1%), on peptone-gelatin enriched nutrient agar, and tributyrin agar for screening amylase, protease, and lipase activity of bacteria, respectively. Nutrient agar culture plates with starch (1%) were then flooded with 1% lugol's iodine solution. Amylase producers showed a clear zone surrounding their colony. The appearance of a clear zone around the colony on peptone-gelatin enriched nutrient agar after flooding the plate with 15% $HgCl_2$ indicated the presence of proteolytic activity. Lipase producers immediately showed a clear zone (Bairagi et al., 2002). Lipase catalyzes the hydrolysis of lipids. Three bacterial strains (G8/2013, T7/2013 and U5/2013) were selected for incorporation into the experimental diets because of their excellent protease, lipase, and amylase producing capacities.

Qualitative extracellular enzyme activity of candidate probiotics. Qualitative extracellular enzyme activity was assessed based on the measurement of a clear zone (halo) around the colony as follows: + (low, 4-6 mm halo diameter), ++ (moderate, 7-9 mm halo diameter), +++ (high, >10 mm halo diameter) (Ray et al.,2010).

Bacterial identification:

Phenotypic characterization. Selected bacteria were identified with Gram staining, catalase reaction (3 % H_2O_2), motility in TSB, oxsidase reaction, and oxidation-fermentation of glucose and API 20 NE rapid identification systems (bioMérieux SA, Marcy l'Etoile, France). The cultures were frozen at -80 ^{o}C in trypticase-soy broth (TSB, Merck) with 15 % (v/v) glycerol.

Molecular identification; DNA extraction, PCR amplification, and sequencing.

16S rRNA sequence analyses of isolates were performed by a commercial sequencing company (Refgen Ankara, Turkey). Bacterial genomic DNAs for PCR assays were isolated the DNeasy Blood and Tissue kit (Qiagen, GmbH) according to the manufacturer's instructions. PCR amplification of the 16S rRNA genes was performed using the universal primers 27f (AGAGTTTGATC(A:C)TGGCTCAG) and 1492r(TACGG(C:T)TACCTTGTTACGACTT) (Weisbergh et al.,1990). The PCR reaction mixture containing 1xPCR Buffer, 1.25 mM of MgCl2, 0.2 mM each dNTP, 2.5 U Taq polymerase (Fermentas), 50 pmol of each primer,

and 2 µl template DNA in DEPC-treated water, was made up to a final volume of 50 µl. Amplification was initiated with a denaturation step at 95 °C for 5 min, followed by 30 cycles each consisting of DNA denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min, with a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gel (Prona), stained with ethidium bromide (Sigma) and visualized with UV illumination. In consequence amplification of 16S rRNA gene, the amplicons obtained were almost 1,500 bp in length.

16S rRNA genes amplification products of bacterial strains were purified and sequenced using the same PCR primers in an ABI Prism 310 Genetic Analyzer. Purified PCR products were subjected to DNA sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems). 16S rDNA sequences of isolates were examined for nucleotide-nucleotide matches in the BLAST database at the NCBI homepage (Anonymous, 2014) to establish strain identity.

Pathogenicity of candidate probiotics. Candidate probiotics were grown to log phase in 20 mL of TSB at 22 °C and subsequently harvested by centrifugation at 1600 g for 15 min at 15 °C. The supernatant was poured off and the pellet was re-suspended with PBS. Subsamples were taken to determine CFU/mL using the drop plate method (Chen et al., 2003), resulting in actual concentration 10^7 CFU/mL (Irianto and Austin 2002; Burbank et al., 2012). Duplicate groups of 15 fish (mean weight 3 g) were injected intraperitoneally (IP) with 25µl of each candidate probiotic bacteria resulting in doses of approximately $2.5x10^5$ CFU/fish. Control groups were injected with 25µl PBS.

Diet preparation and analysis. Formulation and chemical proximate composition of the experimental diets is given in Table 1. The moisture, crude protein, crude fiber and ash contents of the experimental diets, feces samples and body composition were determined according to standard AOAC methods (AOAC, 2000). The total lipids of all samples were determined by the chloroform-methanol extraction method (Bligh and Dyer 1959).

Table 1. Formulation and chemical proximate composition of experimental diets (%)

Ingredients	*Treatments*				
	Cont.	Mix.	U5	G8	T7
Fish meal	35.00	35.00	35.00	35.00	35.00
Soybean meal	30.00	30.00	30.00	30.00	30.00
Full fat soybean	6.90	6.90	6.90	6.90	6.90
Corn meal	16.00	16.00	16.00	16.00	16.00
Fish oil	11.00	11.00	11.00	11.00	11.00
Vit-Min *	0.60	0.60	0.60	0.60	0.60
Chromic oxide	0.50	0.50	0.50	0.50	0.50
Chemical analysis					
Crude protein	54.07	54.56	54.27	54.91	55.02
Crude lipid	14.27	14.10	14.39	14.15	14.26
Crude cellulose	1.99	2.10	2.05	2.19	2.11
Digestible energy (kcal/kg)	4000	4000	4000	4000	4000

Cont., Control; Mix, *Bacillus* sp.+ *Aeromonas* sp.+*Citrobacter braakii*; U5, *Bacillus* sp.; G8, *Aeromonas* sp.; T7, *Citrobacter braakii*

*Provided the following per kg diet: 20,000 IU vitamin A; 2,400 IU vitamin D3; 12 mg vitamin E; 12 mg vitamin K3; 20 mg vitamin B1; 30 mg vitamin B2; 2 mg Niacin; 50 mg Cal.D. Pantothenate; 20 vitamin B6; 0.05 mg vitamin B12; 0.5 mg D-Biotin; 6 mg folic acid; 200 mg vitamin C; 300 mg inositol, Mn; 118 mg as manganese sulfate; Zn, 375 mg as zinc oxide; Cu, 15 mg copper as copper sulfate; Co, 10 mg as cobaltous carbonate; I, 13.75 mg as potassium iodate; Se, 0.50 mg as sodium selenite; Mg, 10 mg as magnesium oxide.

The different treatment groups of fish were fed with diets supplemented with candidate probiotic bacteria at a concentration of 10^8 bacteria/g of diet (Irianto and Austin, 2002). Addition of candidate probiotic bacteria to the diet was achieved by growing the bacteria to log phase in 15 mL TSB at 22 °C for 24 h. The 250 mL culture was centrifuged at 1600 g for 15 min at 15 °C, the media supernatant was poured off and the pellet was re-suspended in 250 mL PBS (Chen et al., 2003; Burbank et al., 2012). The isolates having the highest protease, amylase, and lipase activities were added to diet formulation as

probiotics in four forms, respectively; G8 $1.72x10^8$ CFU/g; U5, $3.01x10^8$ CFU/g; T7, $2.96x10^8$ CFU/g, and a mixture of all the bacteria at the same concentrations. The control group was fed a diet without bacteria. The experimental diets were air-dried in a drying cabinet using an air blower at 20 °C until moisture levels were around 10%. After air-drying, the diets were broken up and sieved into pellets of appropriate size (1mm) and stored at +4 °C for 15 days.

Experimental animal and culture conditions. Rainbow trout fry (mean weight 0.14 g) were obtained from a fish farm in Isparta, Turkey. Fish were distributed randomly into 15 circular fiberglass tanks (water volume 0.08 m^3). There were three replicates per treatment, and 200 fish per replicate. The rainbow trout fry were fed with trial diets beginning with the initial feeding to facilitate bacterial colonization. Fish were fed five times daily, at 8:00, 11:00, 14:00, 17:00 and 20:00 hours ad libitum for a 70 day feeding trial. Every day, each tank was cleaned and fish feces removed. Tanks were supplied with ground water (flow rate: 45 L/ min). Temperature, dissolved oxygen level, and pH of tank water were recorded (11±1 ^{0}C, 6 mg/L and 6.8, respectively).

Monitoring of bacteria. Total bacteria counts (TBC) in the intestine of fish were determined on 30th, 50th, and 70th day of the experiment. The intestines were removed using a sterile technique then homogenized and diluted serially with PBS. Total counts of bacteria were determined by plating on Plate Count Agar (Merck) for 48 h at 30°C. *Citrobacter braakii, Aeromonas* sp. and *Bacillus* sp. were counted on EMB Agar (Eosin Methylene-blue Lactose Sucrose Agar, Merck), AA (Aeromonas Agar, Lab-167) and on Nutrient agar with polymyxin B for 72 h at 22 °C, respectively.

Digestibility study. Fish feces were collected for digestibility analysis at the end of the feeding trial. The last feeding was given at 09:00 and the uneaten feed and feces were siphoned out. Thereafter feces were collected for digestibility analysis at 15:00. The fecal material of each treatment was strained through a fine-mesh-size net kept at -20°C, oven dried at 50°C for 48 h, and submitted to chromic oxide and nutrients analysis (Lim et al., 2001).

Assay of enzyme activity. On the 0th, 30th, and 70th days of the trials, samples were removed for enzymatic analysis. Fish were starved for 24 h before sampling. Whole fish were used for enzymatic analysis. Three fish from each tank were homogenized and pooled as one unit. Fish samples were washed with cold distilled water, immediately frozen, and stored at -80 ^{0}C before use. The supernatant of each sample was assayed in triplicate. Total soluble protein was measured according to the Bradford method (1976). Total protease activity was assayed according to a method modified from Anson (1938) using casein (Merck) as the substrate and causing a reaction with a folin reagent. Alfa amylase and lipase levels were measured spectrophotometrically using a Beckman Coulter biochemical autoanalyser (Beckman Coulter, ABD, Serial number: 2012010171) with compatible test kits according to kinetic reaction principle.

Statistical analyses. One-way ANOVA was used to analyze experimental data between the treatments. All the data were analyzed using SPSS for Windows. Duncan's multiple range test was used to determine the mean differences between the treatments (p= 0.05).

Results

Qualitative extracellular enzyme activity of candidate probiotics. Enzymatic activities of candidate probiotics are given in the Table 2. The highest protease, amylase, and lipase activities were determined in G8, U5 and T7 strains, respectively.

Table 2. Enzymatic activities of candidate probiotics

Enzymatic activities	*G8*	*T7*	*U5*
Amylase	+	+	+++
Protease	+++	+	+
Lipase	+	+++	+

+++ (high, >10 mm halo diameter); ++ (moderate, 7-9 mm halo diameter); + (low, 4-6 mm halo diameter)

Bacterial identification. Based on their morphological, physiological, and sequence data, three bacterial isolates, G8, U5 and T7 were identified as *Aeromonas* sp., *Bacillus* sp. and *C. braakii*, respectively. Phenotypic characteristics of the candidate probiotic bacteria are

summarized in Table 3. Their 16S rRNA gene sequences were deposited in GenBank with the accession numbers KJ531386, KJ531388 and KJ531387 respectively.

Table 3. Phenotypic characteristics of candidate probiotics

	G8	T7	U5
Gr staining	- bacil	- bacil	+ bacil
Motility	+	+	-
Oxidase	+	-	-
Catalase	+	+	+
O/F Test	F	F	-/-
Nitrate reduction[a]	+	+	+
Indole production[a]	-	-	-
Glucose acidification[a]	+	+	-
Arginine dihydrolase[a]	+	-	-
Urea hydrolysis[a]	-	-	-
Esculin hydrolysis[a]	+	-	+
Gelatin hydrolysis[a]	+	-	+
p-Nitropyhenyl-ßD	+	+	-
Glucose assimilation[a]	+	+	+
Arabinose assimilation[a]	+	+	+
Mannose assimilation[a]	+	+	+
Mannitol assimilation[a]	+	+	+
N-Acetyl Glusomine[a]	+	+	+
Maltose Glusomine[a]	+	+	+
Gluconate Glusomine[a]	+	+	-
Caprate Glusomine[a]	+	-	-
Adipate Glusomine[a]	-	-	-
Malate Glusomine[a]	+	+	+
Citrate Glusomine[a]	-	+	-
Phenyl acetate Glusomine[a] -		-	-

[a]: performed API 20 NE, F: fermentative

Pathogenicity of candidate probiotics. No mortality was observed in any fish from the potential probiotics groups and control group. In addition, bacterial growth from fish was not observed in any of the challenge groups.

Intestinal colonization. Total bacteria counts in GI tract of rainbow trout fed the experimental diets are presented in Table 4. TBC in the mixed group was found to be higher than the control group at 30th and 50th days of the experiment (P<0.05). TBC in the U5 group was higher than the control group at 50th and 70th days (P<0.05). TBC in the G8 group was lower than control at 50th day (P<0.05). T7 group was lower than control at 50th and 70th days (P<0.05).

Table 4. Bacteria counts in intestines tracts of rainbow trout fry (x10^4 CFU g^{-1})

Treatments	*Experiment Days*		
TBC	30	50	70
PCA agar			
Cont.	12.20±0.24^{Ba}	40.00±4.04^{Ac}	26.96±2.09^{Bb}
Mix.	84.66±12.4^{Ab}	82.45±11.16^{Ab}	27.01±1.18^{Bb}
U5	2.27±0.20^{Ca}	185.07±21.75^{Ba}	258.67±12.92^{Aa}
G8	6.48±1.82^{Ba}	2.07±0.44^{Bd}	32.85±6.58^{Ab}
T7	1.32±0.20^{Aa}	0.23±0.03^{Ad}	0.19±0.07^{Ac}
EMB agar			
Cont.	0.12±0.01^{Aa}	0.18±6.47^{Aa}	0.15±1.03^{Aa}
Mix	0.19±0.06^{Aa}	0.25±0.77^{Aa}	0.23±0.13^{Aa}
T7	0.14±0.03^{Aa}	0.26±0.08 ^{Aa}	0.12±0.18 ^{Aa}
Nutrient agar			
Cont.	0.13±0.01^{Bb}	0.79±0.05^{Ac}	1.03±0.11^{Ab}
Mix	0.58±0.32 ^{Ba}	2.26±0.31^{Ab}	0.54±0.21^{Bb}
U5	0.27±0.01^{Cb}	6.59±0.37^{Ba}	24.04±7.66^{Aa}

Small letters are vertical statistical, big letters are horizontal statistical. Different superscript letters in the same row indicated significant difference (P< 0.05).

Bacterial counts in the intestinal tract of rainbow trout fry on EMB Agar, and Nutrient agar with polymyxin B are presented in Table 5. Intestinal colonization of Bacillus sp. appeared on the 50th day and continued on the 70th day of the experiment in nutrient agar ($P<0.05$). *Aeromonas* sp. and *C.braakii* did not colonize the intestines of the rainbow trout fry.

Growth performance and nutrient digestibility. There were no differences in terms of final weight, weight gain, SGR, and FCR between probiotic groups and the control ($P>0.05$). There were no significant differences in apparent digestibility coefficient of dry matter, lipid and protein between the treatment groups ($P>0.05$) (Table 5).

Table 5. Growth performance and apparent digestibility coefficients of trout fry fed experimental diet.

	Treatments				
Growth performance	Cont.	Mix.	U5	G8	T7
Initial mean weight(g)	0.14±0.01	0.13±0.01	0.14±0.01	0.14±0.01	0.13±0.01
Final mean weight(g)	2.78±0.10^{ab}	2.93±0.08^{a}	2.90±0.08^{a}	2.58±0.09^{b}	2.96±0.04^{a}
Weight gain (g)	2.65±0.10^{ab}	2.81±0.08^{a}	2.76±0.08^{a}	2.44±0.09^{b}	2.83±0.05^{a}
SGR (%)	3.87±0.04	3.95±0.05	3.88±0.06	3.74±0.12	3.96±0.03
FCR	1.16±0.12	1.20±0.12	1.27±0.05	1.27±0.18	1.02±0.06
Survival rate (%)	72.33±1.36	69.00±1.15	70.50±1.15	70.33±1.69	71.33±1.96
Apparent digestibility coefficients					
Crude protein	88.93±0.34	89.25±0.34	88.68±0.29	88.33±0.63	88.81±0.31
Crude lipid	89.32±0.56	88.76±0.68	89.93±0.51	90.46±0.63	90.54±0.65
Dry matter	76.93±0.45	75.68±0.13	75.92±0.24	76.08±1.00	75.40±0.32
Ash	39.71±0.63	36.25±1.55	35.89±1.53	39.09±1.24	33.10±1.75

Different superscript letters in the same row indicate significant difference ($P < 0.05$).
Weight gain (g) = (final body weight (g) – initial body weight (g).
Specific growth rate (SGR) (%) = [(ln final BW)-(ln initial BW)/days]x 100
The feed conversion rate (FCR) = feed given (g) / fish weight gain (g).
Survival rate (%) = (fish number at the end of experiment/ fish number at the beginning of experiment) x 100
Apparent digestibility coefficients (ADC) (%) =100-[100x (Cr_2O_3 in diet (%)/Cr_2O_3 in feces (%)) x (nutrient in feces (%)/ nutrient in diet (%)]

Digestive enzyme activity. Digestive enzyme activity in trout fry fed experimental diets are given in Table 6. There were no significant differences in α-amylase, protease, and lipase activities, between probiotic groups and the control on the 30th and 70th day ($P>0.05$) (Table 6).

Table 6. Protease, α-amylase and lipase activities (Unit/mg protein) in trout fry fed experimental diet

		Treatments				
	Days	*Cont.*	*Mix.*	*U5*	*G8*	*T7*
Protease	0	0.095±0.005	0.095±0.005	0.095±0.005	0.095±0.005	0.095±0.005
	30	0.301±0.057	0.308±0.049	0.303±0.098	0.498±0.130	0.292±0.082
	70	8.069±0.928	6.622±1.299	6.105±0.672	8.576±0.556	7.796±0.605
α-Amylase	0	0.013±0.001	0.013±0.001	0.013±0.001	0.013±0.001	0.013±0.001
	30	0.103±0.007	0.082±0.004	0.102±0.002	0.131±0.027	0.083±0.019
	70	0.946±0.166	1.324±0.367	0.562±0.279	0.788±0.068	0.832±0.191
Lipase	0	0.011±0.001	0.011±0.001	0.011±0.001	0.011±0.001	0.011±0.001
	30	0.021±0.002	0.017±0.002	0.023±0.002	0.029±0.006	0.020±0.003
	70	0.151±0.049	1.112±0.023	0.098±0.039	0.143±0.014	0.152±0.019

Discussion

Intestinal Colonization. In the present study, *Aeromonas* sp. and *C.braakii* did not colonize the intestines of rainbow trout fry. However, intestinal colonization of *Bacillus* sp. in U5 group was seen at the 50th and 70th days. Similarly, high intestinal colonization capability of Bacillus genus has been reported in many studies on fish and crustacea (Wang and Xu 2006; Ziaei-Nejad et al., 2006; El-Haroun et al., 2006; Wang, 2007; Bagheri et al.,

2008; Far et al., 2009; Ai et al., 2011; He et al., 2011; Faramarzi et al., 2011; Boonthai et al., 2011; Adineh et al., 2013; Wu et al., 2013). The count of *Bacillus* in the intestine of rainbow trout fry was higher than in the control group (Bagheri et al., 2008). High numbers of Bacillus were found in the gut of *O. niloticus* fed with *B. Amyloliquefaciens (*Ridha and Azad, 2012). In the present study, TBC in the mixed group was higher than in the control group at 30^{th} and 50^{th} days of the experiment. The reduction of TBC in the mixed group at day 70 may have been caused by the suppression of *Bacillus* sp. by other bacteria in the microflora.

Growth performance. In the current study, the growth performance of trout fry fed with bacteria suplemented diets (in combination or alone) was similar to the control group. Similarly, no significant improvement in weight gain or specific growth rate between groups when *B. subtilis, B. licheniformis* and *Enterococcus faecium* were used individually or in combination was observed (Merrifield et al. 2010). Similar results were seen in tilapia fed with *B. amyloliquefaciens and Lactobacillus sp.* (Ridha and Azad 2011). In contrast, diets supplemented with probiotics (in combination or singly with photosynthetic bacteria and *Bacillus* sp.) showed significantly better results than control diet in growth performance of common carp (Wang and Xu 2006). Higher growth rate was observed in rohita fingerlings fed a combination of *B. subtilis*, *Lactococcus lactis* and *Saccharomyces cerevisiae* in equal proportions (Mohapatra et al. 2012). Better growth and feed conversion ratio was found in rainbow trout (0.7g) fed a commercial product containing five species of *Bacillus* ($2x10^5$ CFU/g) (Adineh et al. (2013). The discrepancy between this study with the present one is possibly due to differences among the strains of Bacillus and lower supplementation levels of bacteria to the diet. The addition of *Bacillus* spp. at five different levels to rainbow trout fry (0.12g) diets showed better growth rate and feed conversion ratio than the control (Bagheri et al. 2008). *Ctenopharyngodon idella* (Wu et al., 2012) and koi carp (He et al., 2011) fed with diets supplemented with *B. subtilis* reported higher growth and lower FCR. These differences may derive from use of different fish species and strains.

Nutrient digestibility. In this study, protein, lipid and dry matter digestibility did not change with supplementation of bacteria to rainbow trout fry diets. In contrast, Mohapatra et al.(2012) found higher nutrient digestibility in rohita fingerlings fed a combination of three probiotics (*B.subtilis, S. cerevisiae* and *L. lactis*). Better digestibility was found in *C.idella* fed with the addition of *B. coagulans* probiotic (Wang 2011). The differences between these results may be due to different fish and bacteria species used in these studies.

Digestive enzyme activities. In the present study, enzyme activity among groups was not affected by supplementing probiotics to diets. In contrast, protease, amylase, and lipase activity in *C. idella* fed diets supplemented with *B. subtilis* was higher than in the control (Wu et al., 2012). Higher protease, amylase, and lipase activities were found when *Bacillus* sp was added to carp diets (Wang and Xu, 2006). There was higher protease and lipase activity in *Labeo rohita* fed with combinations of *B. subtilis, Lactococcus lactis* and *Saccharomyces cerevisiae (*Mohapatra et al. 2012). Better protease, amylase and cellulase activities were obtained in *Ctenopharygodon idella* fed a diet containing *B. coagulans (*Wang, 2011). The variability of these results may be due to the differences between fish species and bacteria strains used in these studies.

The results of this study show that the addition of *Citrobacter braakii, Aeromonas* sp. and *Bacillus* sp. to rainbow trout fry diet had no significant effect on growth performance, nutrient digestibility, and enzyme activities. Generally, the reason for the different results in the present study and previous studies is the use of different fish species. This is due to the fact that each species has different feeding habits and gastrointestinal tracts. Other bacterial strains as enzyme-producing probiotics for rainbow trout should be tried in future studies.

Acknowledgements

This study was supported by Suleyman Demirel University, Scientific Research Project Grant (SDUBAP- 2622-M-10).

References

Adineh H., Jafaryan H., Sahandi J. and M. Alizadeh, 2013. Effect of *Bacillus spp.* probiotic on growth and feeding performance of rainbow trout (*Oncorhynchus mykiss*) larvae. *Bulg. J. Vet. Med.* 16: 29−36.

Ai Q., Xu H., Mai K., Xu W., Wang J. and W. Zhang, 2011. Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*. *Aquaculture* 317: 55–161.

Anonymous, 2013. http://www.ncbi.nlm.nih.gov/BLAST

Anson M.L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* 22: 79-89.

AOAC 2000. Official Methods of Analysis, 16th edn. Association of Official Analytical Chemists. Arlington, VA, USA.

Bagheri T., Hedayati S.A., Yavari V., Alizade M. and A. Farzanfar, 2008. Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. *Turk. J. Fish. Aquat. Sci.* 8: 43-48.

Bairagi A., Ghosh K.S., Sen S.K. and A.K. Ray, 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquacult. Int.* 10: 109–121.

Bligh E.G. and W.J. Dyer, 1959. Rapid Method of total lipid extraction and purification. *Can. J. Biochem. Phys.* 37: 911-917.

Boonthai T., Vuthiphandchai V. and S. Nimrat, 2011. Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaeus monodon*). *Aquacult. Nutr.* 17: 634-644.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Burbank D.R., LaPatra S.E., Fornshell G., and K.D. Cain, 2012. Isolation of bacterial probiotic candidates from the gastrointestinal tract of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and screening for inhibitory activity against *Flavobacterium psychrophilum*. *J. Fish. Dis.* 35: 809-816.

Chen C.Y., Nace G.W. and P.L. Irwin, 2003. A 6×6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes* and *Escherichia coli*. *J. Microbiol. Meth.* 55: 475-479.

El-Haroun E.R., Goda A.S. and M.A. Kabir Chowdhury, 2006. Effect of dietary probiotic Biogens supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Aquac. Res.* 37: 1473-1480.

Far H.Z., Saad C.R.B., Daud H.M., Harmin S.A. and S. Shakibazadeh, 2009. Effect of *Bacillus subtilis* on the growth and survival rate of shrimp (*Litopenaeus vannamei*). *Afr. J. Biotechnol.* 8:14.

Faramarzi M., Jafaryan H., Patimar R., Iranshahi F., Boloki M.L., Farahi A., Kiaalvandi S., Ghamsary M. and N.M. Makhtoumi, 2011. The effects of different concentrations of probiotic *Bacillus spp.* and different bioencapsulation times on growth performance and survival rate of persian sturgeon (*Acipencer persicus*) larvae. *World. J. Fish. Mar. Sci.* 3: 145-150.

Gatesoupe F.J., 1999. The use of probiotics in aquaculture. *Aquaculture* 180: 147-165.

Gutowska M.A., Drazen J.C. and B.H. Robison, 2004. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 139: 351-358.

He S., Zhou Z., Meng K., Zhao H., Yao B., Ringø E. and I. Yoon, 2011. Effects of dietary antibiotic growth promoter and *Saccharomyces cerevisiae* fermentation product on production, intestinal bacterial community, and nonspecific immunity of hybrid tilapia (*Oreochromis niloticus* female× *Oreochromis aureus* male). *J. Anim. Sci.* 89: 84-92.

Irianto A. and B. Austin, 2002. Probiotics in aquaculture. *J. Fish. Dis* 25: 633-642.

Lara-Flores M., 2011. The use of probiotic in aquaculture: an overview. *International Research J. Microbiol.* 2: 471-478.

Lim H.A., Ng W.K., Lim S.L. and C.O. Ibrahim, 2001. Contamination of palm kernel meal with *Aspergillus flavus* affects its nutritive value in pelleted feed for tilapia. *Oreochromis mossambicus. Aquac. Res.* 32: 895-906.

Martin-Antonio B., Manchado M., Infante C., Zerolo R., Labella A., Alonso C. and J.J. Borrego, 2007. Intestinal microbiota variation in Senegalese sole (*Solea senegalensis*) under different feeding regimes. *Aquac. Res.* 38: 1213-1222.

Merrifield D.L., Dimitroglou A., Bradley G., Baker R.T.M. and S.J. Davies, 2010. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) I. effects on growth performance, feed utilization, intestinal microbiota and related health criteria. *Aquacult. Nut.* 16: 504-510.

Mohapatra S., Chakraborty T., Prusty A.K., Das P. and K. Paniprasad, 2012. Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibity and retention, digestive enzyme activities and intestinal microflora. *Aquacult. Nut.* 18: 1-11.

Mondal S., Roy T., Sen S.K. and A.K. Ray, 2008. Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. *Acta. Ichthyol. Piscat.* 38: 1-8.

Nayak S.K., 2010. Role of gastrointestinal microbiota in fish. *Aquac. Res*. 41: 1553-1573.

Pemberton J.M., Kidd S.P. and R. Schmidt, 1997. Secreted enzymes of *Aeromonas*. *Fems. Microbiol. Lett.* 152: 1-10.

Ray A.K., Roy T., Mondal S. and E. Ringø, 2010. Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. *Aquac. Res.* 41: 1462–1469

Ray A.K., Ghosh K. and E. Ringø, 2012. Enzyme-producing bacteria isolated from fish gut: a review. *Aquacult. Nut.* 18: 465-492.

Reid H.I., Treasurer J.W., Adam B. and T.H. Birkbeck, 2009. Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae. *Aquaculture* 288: 36-43.

Ridha M.T. and I.S. Azad, 2011. Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative probiotic bacteria. *Aquac. Res.* 43: 843-852.

Timmermans L.P.M., 1987. Early development and differentiation in fish. *Sarsia* 72: 331-339.

Uchii K., Matsui K., Yonekura R., Tani K., Kenzaka T., Nasu M. and Z.I. Kawabata, 2006. Genetic and physiological characterization of the intestinal bacterial microbiota of bluegill (*Lepomis macrochirus*) with three different feeding habits. *Microbial. Ecol.* 51: 277-284.

Wang Y. and Z. Xu, 2006. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Anim. Feed. Sci. Tech.* 127: 283-292.

Wang Y., 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture* 269: 259–264.

Wang Y., 2011. Use of probiotics *Bacillus coagulans*, *Rhodopseudomonas palustris* and *Lactobacillus acidophilus* as growth promoters in grass carp (*Ctenopharyngodon idella*) fingerlings. *Aquacult. Nut.* 17: 372-378.

Wu Z.X., Feng X., Xie L.L., Peng X.Y., Yuan J. and X.X. Chen, 2012. Effect of probiotic *Bacillus subtilis* Ch9 for grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844), on growth performance, digestive enzyme activities and intestinal microflora. *J. Appl. Ichthyol.* 28: 721–727.

Wu S.G., Tian J.Y., Gatesoupe F.J., Li W.X., Zou H., Yang B.J. and G.T. Wang, 2013. Intestinal microbiota of gibel carp (*Carassius auratus gibelio*) and its origin as revealed by 454 pyrosequencing. *World J Microb Biot.* 29: 1585-1595.

Ziaei-Nejad S., Rezaei M.H., Takami G.A., Lovett D.L., Mirvaghefi A.R. and M. Shakouri, 2006. The effect of *Bacillus spp.* bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* 252: 516- 524.