

Effect of *Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus* on growth performance, gut microbial flora and digestive enzymes activities in *Tor grypus* (Karaman, 1971)

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Abstract

In this study, two main probiotics (*Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) were isolated from the intestine of *Tor grypus* and their effects on growth performance, gut microbial flora and digestive enzymes activities (i.e., α -amylase, lipase, trypsin, chymotrypsin, and alkaline phosphatase) of *Tor grypus* (Karaman, 1971) were determined. Juveniles of *T. grypus* (n=480, weighing 45 ± 10 g) were randomly divided into 4 equal groups (with 3 replications) and fed on diets containing 5×10^7 cfu g⁻¹ of *L. plantarum* (G1), *L. bulgaricus* (G2), *L. casei* (G3) and a control diet (without probiotic) for 60 continuous days. Results showed that probiotic supplementations had generally significant effects on growth performance ($p < 0.05$). The G2 group had the best effect on specific growth rate (SGR) and feed utilization efficiency in *T. grypus*. The digestive enzyme activities, on day 30 after feeding, significantly increased in G1 when compared with those in the control group ($p < 0.05$). Significant changes in bacterial intestinal flora were observed in all probiotic groups compared with the control ($p < 0.05$). Our results indicated that supplementation of isolated bacteria from the intestine of *T. grypus* (i.e., G2) can efficiently improve growth performance, intestinal microbiota and some digestive enzyme activities in juvenile *T. grypus*. Therefore, it can be isolated and used as a growth enhancer just like the commercial probiotics.

Keywords: *Tor grypus*, Intestine lactobacilli, Growth performance, Digestive enzyme activity, Microbial flora.

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Introduction

Significant increase in production and optimizing profitability is one of the most important goals of modern aquaculture (Denev *et al.*, 2009). During the past decades, the aquaculture industry developed due to the use of various fish species, application of intensive culture systems, and improved metabolic assimilation of dietary nutrients. The concept of functional feeds formulated by probiotics is a novel idea to the aquaculture industry. The application of probiotics as an alternative to therapeutics in aquaculture is not so new (Panigrahi *et al.*, 2005), but the interest in such safe and high effective functions has been increased rapidly (Gatesoupe, 1999). Various microorganisms have been evaluated as probiotics in aquatic animals (Irianto and Austin, 2002; Vine *et al.*, 2004; Kim and Austin, 2006; Yanbo and Zirong, 2006; Balcázar and Rojas-Luna, 2007; Balcázar *et al.*, 2008; Mirbakhsh *et al.*, 2013; Faeed *et al.*, 2016); however lactic acid bacteria (LABs) are the most used probiotics in aquaculture (Nikoskelainen *et al.*, 2001; Aly *et al.*, 2008; Vendrell *et al.*, 2008; Talpur *et al.*, 2013). Probiotics are microorganisms that beneficially affect the health and survival of the host by selectively stimulating the growth of beneficial gastrointestinal microbial community (Merrifield *et al.*, 2010a,b). The use of probiotics has also been suggested to be an alternative method to prevent and control various aquatic diseases by reducing pathogenic

organisms in the gastrointestinal tract of fish based on its antagonistic activity at the site of colonization on the host's intestine (Askarian *et al.*, 2008; Korkea-aho *et al.*, 2012). Pre-digestion of anti-nutritional factors in the diet, enhancement of non-specific immune responses (Ringø and Gatesoupe, 1998), increasing the fermentation products (Kim and Austin, 2006), improvement of mineral uptake and food absorption have been described as potential beneficial actions of probiotics (Verschuere *et al.*, 2000). Probiotics also may stimulate appetite and digestive enzyme activities (Tovar-Ramirez *et al.*, 2004).

There are several reports available regarding the beneficial influence of bacterial probiotics including live yeast, bifidobacters, *Lactobacillus*, feeding on growth performances and gut microbiota in some aquatic animals such as sea bass (*Dicentrarchus labrax*) (Tovar *et al.*, 2002; Tovar-Ramirez *et al.*, 2004), sea bream (*Sparus aurata*) (Suzer *et al.*, 2008; Sáenz de Rodrigáñez *et al.*, 2009); beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) (Askarian *et al.*, 2008); Tilapia (*Oreochromis niloticus*) (Standen *et al.*, 2013); Rainbow trout (*Oncorhynchus mykiss*) (Korkea-aho *et al.*, 2012; Ramos *et al.*, 2013).

The *Tor gryp*us is one of the most important fish species in southwest Asia (Iran, Iraq, Turkey and Syria) due to its excellent biological characteristics such as fast growth and high resistance against natural stressors, marketable taste, and high economic value.

Recently, this species has been introduced to Cyprinid farms and it has been accounted for among new species in poly-culture systems. Due to lack of information on *Lactobacillus* probiotic application in *Barbus* fish species, this study focused on investigating the effect of two LABs, *Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, isolated from the intestine of *T. grypus* and a standard *Lactobacillus* strain (*Lactobacillus casei* ATTC1608[®]) on growth indices, gut microbiota, and digestive enzymes (α -amylase, lipase, trypsin, chymotrypsin, and alkaline phosphatase) activities. Feeding habit and metabolic flexibility of carbohydrate-protein utilizations was also measured in probiotics fed on *T. grypus*.

Materials and methods

Bacteria

Bacterial isolates were recovered using a previously described method (Irianto and Austin, 2002). Briefly, the entire digestive tracts of *T. grypus* captured from natural water resources of Khuzestan province in Iran were removed and their contents were discarded. One gram of isolated digestive tract was homogenized in tissue grinders (Kontes, Vineland, NJ, USA) on ice and vigorously stirred into 9.0 ml of sterile saline (0.85% w/v). Dilutions at 10^{-2} and 10^{-3} were prepared in fresh saline and 0.1 mL volumes were spread over duplicate plates of de Man, Rogosa and Sharpe (MRS) broth (BD Difco, Sparks, MD,

USA), and incubated at 30°C for up to 3 days. Various numbers of colonies (20-30) were randomly taken and examined for inhibitory effects against the pathogenic *A. hydrophila* (AH04). The in vitro probiotic activity of each colony was determined using an agar diffusion method and its inhibition zone was measured as described by Ruiz *et al.* (1996). Finally, two LAB isolates with the highest inhibitory effects were selected for the following experiments. The *L. plantarum* and *L. bulgaricus* strains used in this study were primarily identified based on colony and cell morphology, Gram staining, biochemical characteristics, and 16S rRNA gene sequencing (GenBank accession number EU520326 and EU520327) (Mohammadian *et al.*, 2016). These strains were grown for 30 h at 37°C in MRS broth (BD Difco, Sparks, MD, USA). *L. casei* ATTC1608[®] was purchased from Pasteur Institute of Iran.

Diet preparation

The control diet was formulated using the ingredients as subsequently described. The proximate analysis of the basal diet according to the AOAC method was: 37.1% for crude protein, 8.8% for crude lipid, 9.6% ash and 390 Kcal per 100 g for gross energy. Probiotic bacterial suspensions were prepared by centrifuging (15min., 4000 rpm) the 72h MRS broth cultured bacteria and resuspending them in PBS at the concentration of Macfarland grade 10 (3×10^9 cfu mL⁻¹). The probiotic-enriched diets were prepared

by gently spraying 16 mL of the prepared bacterial suspension on the control and mixing that part by part in a drum mixer to obtain a final probiotic concentration of 5×10^7 cfug⁻¹. They were packed in sterile propylene containers and stored at 4°C for viability studies for a week. This dose was chosen based on a previously recommended dose (Ghosh *et al.*, 2003; Merrifield *et al.*, 2010). Final concentrations of probiotic bacteria in the diet were confirmed by suspending one gram of food in sterile PBS and culturing the serial diluted food suspension in MRS media. Counted bacteria in the food were almost the same as added probiotic bacteria in all batches of probiotic-enriched diets.

Experimental design

Juveniles of *T. grypup* (45±10 g) (Mean±SD) were transferred from a private cyprinid farm in Khuzestan Province, Iran, to the Lab of Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. The fish were acclimated for 2 weeks in indoor 300 L

fiberglass tanks and were fed with a standard diet (37.1% crude protein, 8.8% crude lipid, 9.6% ash, and 390 Kcal 100g⁻¹ gross energy). Then, after verifying the health status of the fish, they were distributed randomly into 12 aquaria at an initial density of 40 fish per aquarium and divided into four treatment groups, including control (n=40), *L. plantarum* (G1, n=40), *L. bulgaricus* (G2, n=40) and *L. casei* PTTC1608 (G3, n=40 as a positive control). Final concentration of each probiotic was about 5×10^7 cfu g⁻¹ of the diet (Table 1) (Nikoskelainen *et al.*, 2001). The aquaria were supplied with water from external Biofilteres (Athmann, China), at a temperature of 25.9±1.2°C. The fish were fed with probiotic-contained diets for 60 days (twice a day). During the experimental period, the temperature ranged from 24.5 to 28.5°C, salinity was from 0.6±0.11 % and the dissolved oxygen was 5.9±1.3mgL⁻¹. On day 60, feeding with the experimental diet stopped and fish were fed with the control diet for 15 subsequent days. Daily feeding rate was about 2% of biomass.

Table 1: The experimental design and treatment setting up, applied in this study.

Treatment	G1	G2	G3	Control
Probiotics category	<i>L. plantarum</i>	<i>L. bulgaricus</i>	<i>L. casei</i>	Normal saline
Additive quantity (g kg ⁻¹)	5×10^7	5×10^7	5×10^7	0.0

Sampling and analysis of biological parameters

In order to determine growth performance, weight of the all fish in each treatment was measured at the

beginning and 30 and 60 days from that. Growth performance at 30 and 60 days after the feeding was assessed in terms of Body Weight Growth (BWG), Specific Growth Ratio (SGR), Food

Conversion Efficiency (FCE), Food Conversion Ratio (FCR) and Protein Efficiency Ratio (PER). The calculations were performed using the following formulae: $BWG \% = 100 \times (FBW - IBW) / IBW$, $SGR \% = 100 \times (\ln^1 FBW - \ln IBW) / \text{days}$, $FCR = \text{feed consumed} / (FBW - IBW)$, $FCE \% = (FBW - IBW) / \text{feed consumed} \times 100$, $PER = IBW / \text{protein intake}$. IBW is initial body weight, FBW is final body weight and days are days of feeding.

Digestive enzyme activity

To analyze the activity of digestive enzymes, on days 0, 30, and 60 following probiotic feeding and 15 days after treatment cessation, the fish were starved for 24 h and 3 fish were taken randomly. The intestine was dissected out under sterile conditions and at low temperature (around 4°C, near icepack). Then the samples were homogenized in a cold homogenizing buffer containing 50 mM Tris-HCl, pH 8.0 (1:9v/w) followed by centrifugation (13,500 ×g; 30 min at 4°C). The supernatant was collected and kept at -80°C in small portions for later determinations (Rungruangsak-Torrissen *et al.*, 2002; Rungruangsak-Torrissen and Fosseidengen, 2007).

Total protein content of the supernatant was assayed according to a (Bradford, 1976) method using bovine serum albumin as a standard. Trypsin activity was measured using N α -Benzoyl-L-

arginine ethyl ester (BAEE) as the substrate (Erlanger *et al.*, 1961). Banzoyl-L-Tyrosine ethyl ester Ester (BTEE) was used as a substrate to determine enzyme activity of chymotrypsin (Hummel, 1959). The activity of alkaline phosphatase (AP) was measured using p-nitrophenyl phosphate (pNPP) as substrate (Bessey *et al.*, 1946). α -amylase activity was measured according to the modified Bernfeld method as described previously (Areekijserree *et al.*, 2004) using starch solution as substrate. Amylase specific activity was expressed as $\mu\text{mol maltose produced h}^{-1} \text{mg protein}^{-1}$. Lipase activity was determined based on the measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil (Borlongan, 1990). Enzyme activities were measured as the change in absorbance using a spectrophotometer (UV-2802S; Unico, Shanghai, China) and expressed as specific activity (U $\text{mg}^{-1} \text{protein}$) (Sun *et al.*, 2012).

One of the objectives of this study was to select a suitable modified method for improving nutrient utilization. Feeding habit and metabolic flexibility of carbohydrate-protein utilizations to reach this objective was measured in probiotics fed on *T. grypus* according to Hofer and Schiemer (1981) and Thongprajukaew *et al.* (2011). To do this, α -mylase/Trypsin ratio was calculated for each treatment.

¹. Natural logarithm having based 10

Microbiological aspects

Micro floral analyses were done as described by Merrifield *et al.* (2010). Total and *Lactobacillus* counts in fish intestines were determined by plate counting on TSA and MRS agar, respectively. The intestine of the experimental fish (5 fish from each treatment) was sampled just prior to starting on the experimental diets, and 30 and 60 days past the probiotic feeding and to determine the effect of feed cessation on parameters studied the supplemented diets were stopped and normal diets were used for two subsequent weeks (Nikoskelainen *et al.*, 2003). This was done by aseptically dissecting the fish after overdose (1mL/L) of anesthesia (Benzocaine; Sigma-Aldrich Co., St Louis, MO, USA) and removing a portion of the intestine that was finely chopped. All steps were carried out under sterile conditions. One gram of the sample was homogenized with 9 ml of sterilized phosphate buffered saline (PBS, 0.1 M, pH=7.0) and stirred into 1 min in stomacher (Orugan Stomacher, Tokyo, Japan). Subsequently, dilution series were prepared from the homogenate and plated in the MRS and TSA media. The plates were incubated at 30°C for 48 h prior to counting. Confirmation of the isolated bacteria from the gut of *T. grypus* was done in the previous work by using morphological biochemical and molecular tests adopted from Bergey's manual of systematic bacteriology (Holt *et al.*, 1984; Mohammadian *et al.*, 2016).

Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Release 16.0, SPSS, Chicago, IL, USA). Two-way analysis of variance (ANOVA) and general linear model was used to evaluate the effect of time and treatments on each variable. One-way analysis of ANOVA was done to determine the differences between different variables. Differences were considered statistically significant when $p < 0.05$ and the results are expressed as mean \pm SD.

Results

Growth performance

The final weight, weight gain and specific growth rate of *T. grypus* significantly increased when they fed with diets containing *L. bulgaricus* and *L. casei* for 60 days (Table 2). The minimal FCR was observed in fish fed with dietary *L. bulgaricus* while the highest one was observed in the *L. plantarum* fed groups (Table 2). There was a significant higher protein efficiency ratio (PER) in the *L. bulgaricus* fed group (G2) as compared with other treated and control groups ($p < 0.05$). The lowest protein efficiency ratio was found in the fish fed *L. plantarum* containing food (G1).

Table 2: Weight gain (WG), Specific Growth Ratio (SGR), Food Conversion Efficiency (FCE), Food Conversion Ratio (FCR) and Protein Efficiency Ratio (PER) of the *T. grypus* were fed with different experimental diets. Values are presented as mean \pm SD (n = 9). Different small alphabetic letters in the same column show significant differences ($p < 0.05$) and values with a different capital letters in the same row are significantly different ($p < 0.05$).

Indices	Treatment	Day 30	Day 60
WG%	G1	0.23 \pm 0.07 ^{a,A}	0.24 \pm 0.02 ^{a,A}
	G2	0.5 \pm 0.01 ^{b,B}	0.38 \pm 0.01 ^{b,A}
	G3	0.54 \pm 0.04 ^{b,B}	0.35 \pm 0.03 ^{b,A}
	Control	0.26 \pm 0.08 ^{a,A}	0.21 \pm 0.02 ^{a,A}
SGR	G1	0.19 \pm 0.05 ^{a,A}	0.19 \pm 0.01 ^{a,A}
	G2	0.42 \pm 0.009 ^{b,B}	0.30 \pm 0.007 ^{b,A}
	G3	0.42 \pm 0.03 ^{b,B}	0.27 \pm 0.03 ^{c,A}
	Control	0.22 \pm 0.07 ^{a,A}	0.175 \pm 0.02 ^{a,A}
FCR	G1	2.03 \pm 0.71 ^{b,A}	1.8 \pm 0.2 ^{c,A}
	G2	0.52 \pm 0.02 ^{a,A}	0.63 \pm 0.02 ^{a,A}
	G3	0.8 \pm 0.06 ^{a,A}	1.22 \pm 0.13 ^{b,B}
	Control	1.09 \pm 0.39 ^{a,A}	1.39 \pm 0.17 ^{b,A}
FCE	G1	52.8 \pm 16.5 ^{a,A}	55.4 \pm 6.4 ^{c,A}
	G2	191.4 \pm 6.5 ^{c,A}	157.7 \pm 4.9 ^{a,A}
	G3	124.13 \pm 10.4 ^{b,A}	82.29 \pm 8.5 ^{b,B}
	Control	99.62 \pm 33.1 ^{b,B}	72.37 \pm 9 ^{b,B}
PER	G1	1.1 \pm 0.34 ^{a,A}	1.15 \pm 0.13 ^{a,A}
	G2	3.9 \pm 0.13 ^{c,A}	3.27 \pm 0.1 ^{c,B}
	G3	2.58 \pm 0.21 ^{b,A}	1.71 \pm 0.18 ^{b,B}
	Control	2.07 \pm 0.69 ^{b,B}	1.50 \pm 0.18 ^{b,B}

Digestive enzyme activity

The specific activity of trypsin was increased during the 30 days of treatment in G₁ and G₂ groups, while its activity declined at the end of the experiment. The highest specific activity of trypsin was determined in G₁ group on the 30th day of the experiment (2.23 \pm 0.88 U mg protein⁻¹) (Fig. 1). No significant differences were found among the other groups on days 30 or 60 of feeding ($p > 0.05$). We observed no significant changes in trypsin activity 15 days after the probiotic treatment in all tested groups. The specific activity of α -amylase

increased in all experimental groups during the 30 days of probiotic feeding and thereafter slightly decreased until the end of the experiment. Its highest activity (2.3 \pm 0.85 U mg protein⁻¹) was found in G₁ group on day 30 of the experiment (Fig. 2). Treatment cessation had no effect on α -amylase activity in all tested groups.

The specific activity of AP was significantly elevated on the 30th day of the feeding in G₁ and G₂ groups ($p < 0.05$).

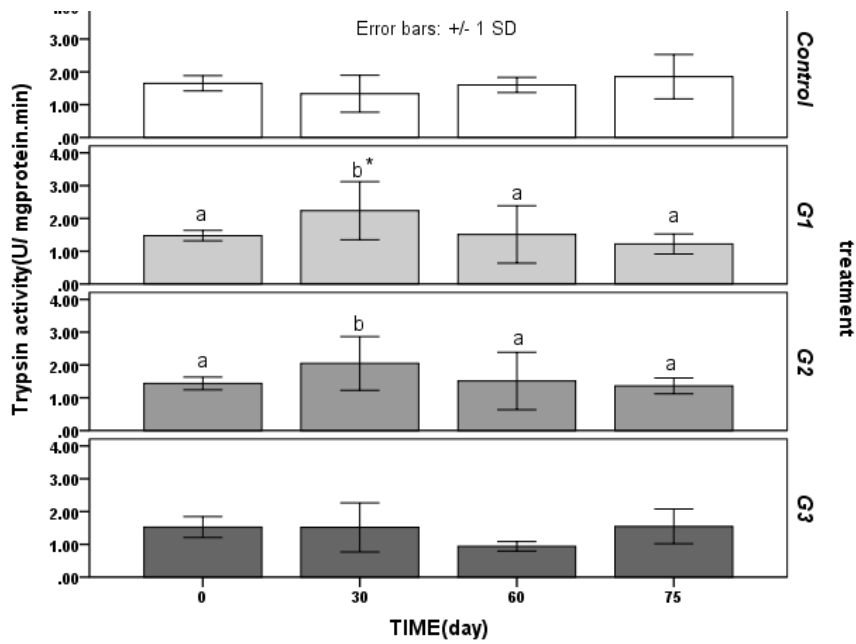


Figure 1: Specific activity of Trypsin of *Tor grypus* treated with G1 (*Lactobacillus plantarum* $\sim 5 \times 10^7$ cfu g^{-1}), G2 (*L. bulgaricus* $\sim 5 \times 10^7$ cfu g^{-1}), G3 (*L. casei* $\sim 5 \times 10^7$ cfu g^{-1}) and without probiotic (control). Data represent as mean \pm SD (n= 9). At each sampling time (column), asterisk shows significant difference ($p < 0.05$) between treatments and control group. In the same row, different letters show significant difference ($p < 0.05$) among different sampling time in each treatment.

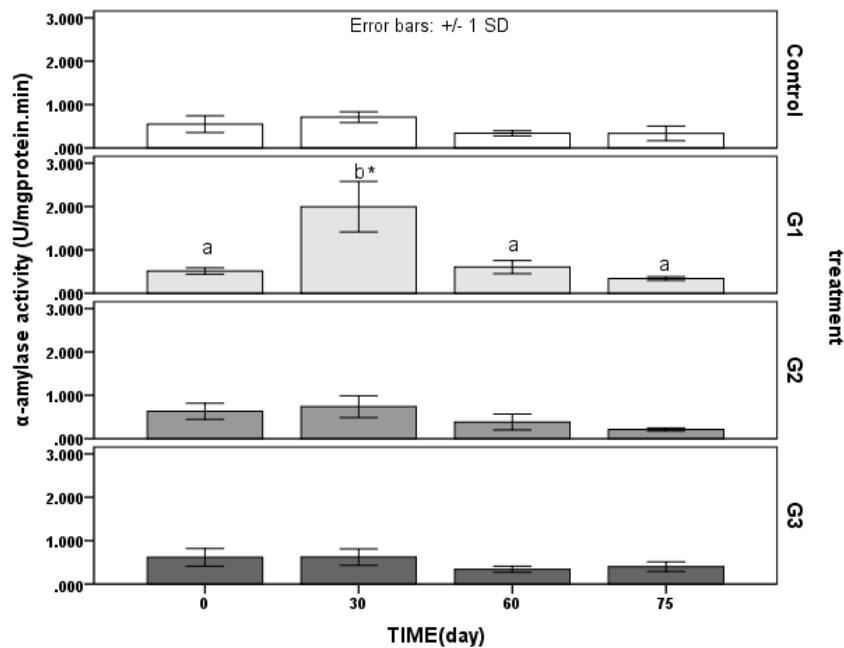


Figure 2: Specific activity of α -amylase of *Tor grypus* treated with G1 (*Lactobacillus plantarum* $\sim 5 \times 10^7$ cfu g^{-1}), G2 (*L. bulgaricus* $\sim 5 \times 10^7$ cfu g^{-1}), G3 (*L. casei* $\sim 5 \times 10^7$ cfu g^{-1}) and without probiotic (control). Values is shown as means \pm standard error (n=9). Legends as mentioned in Fig. 1.

The highest activity of AP (214.9 ± 81.09 U mg protein⁻¹ min⁻¹) was observed on day 30 past the feeding in G1 group (Fig. 3). We found no changes in AP activity of all groups, 60 days after the probiotic feeding. Treatment cessation had no significant effect on activity of AP in all experimental groups.

Chymotrypsin activity was influenced by probiotic administration, so that on the 30th day of the test, G1 groups had the highest chymotrypsin activity and a significant difference with the control group ($p < 0.05$). The results on the 60th day of the feeding showed that except G2 group, all experimental groups had declined chymotrypsin activity. 15 days after the probiotic cessation, chymotrypsin activity decreased in all experimental groups (Fig. 4).

Lipase activity had no significant ($p > 0.05$) differences among the

probiotic-fed groups during the feeding period (Fig. 5).

The highest amylase to trypsin ratio (A/T ratio, $p < 0.05$) was found in fish-fed G1 diet on the 30th day of the treatment. No significant differences ($p > 0.05$) were observed in A/T ratios among the other fish groups at other times of the experiment (Fig. 6).

Microbiological assay

Before the probiotic feeding, the fish had low detectable lactobacilli level in the entire intestines. The viable count of LABs significantly increased in a time-dependent manner in the intestine of fish-fed G2 and G3 probiotics-contained diets. Although fish-fed G1 supplemented diets increased viable counts 30 days after the probiotic feeding, the number of viable LABs in G1 was significantly reduced on the 60th day of the experiment (Table 3).

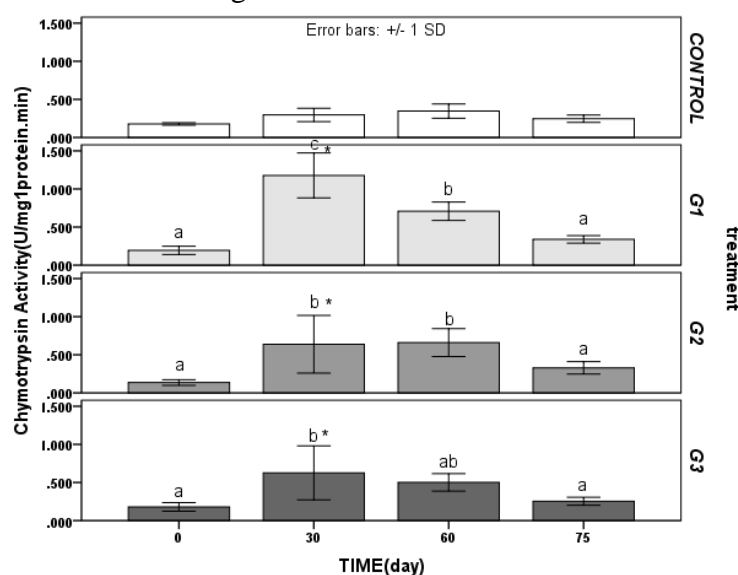


Figure 3: Specific activity of alkaline phosphates (AP) of *Tor gypus* treated with G1 (*Lactobacillus planturum* $\sim 5 \times 10^7$ cfu g⁻¹), G2 (*L. bulgaricus* $\sim 5 \times 10^7$ cfu g⁻¹), G3 (*L. casei* $\sim 5 \times 10^7$ cfu g⁻¹) and without probiotic (control). Values is shown as means \pm standard error (n=9). Legends as mentioned in Fig. 1.

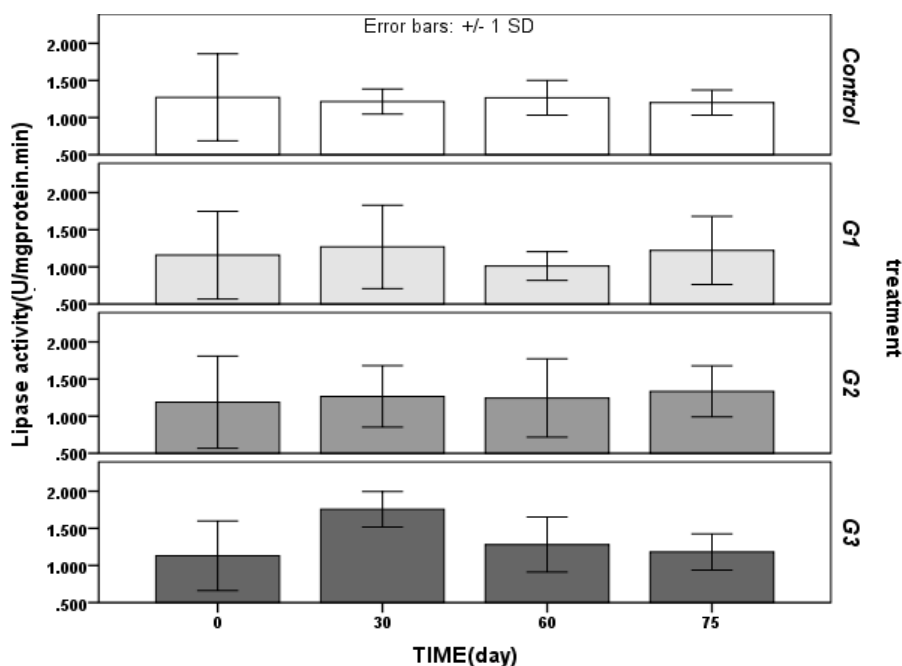


Figure 4: Specific activity of chymotrypsin of *T. grypus* treated with G1 (*Lactobacillus plantarum* $\sim 5 \times 10^7$ cfu g^{-1}), G2 (*L. bulgaricus* $\sim 5 \times 10^7$ cfu g^{-1}), G3 (*L. casei* $\sim 5 \times 10^7$ cfu g^{-1}) and without probiotic (control). Values is shown as means \pm standard error (n=9). Legends as mentioned in Fig. 1.

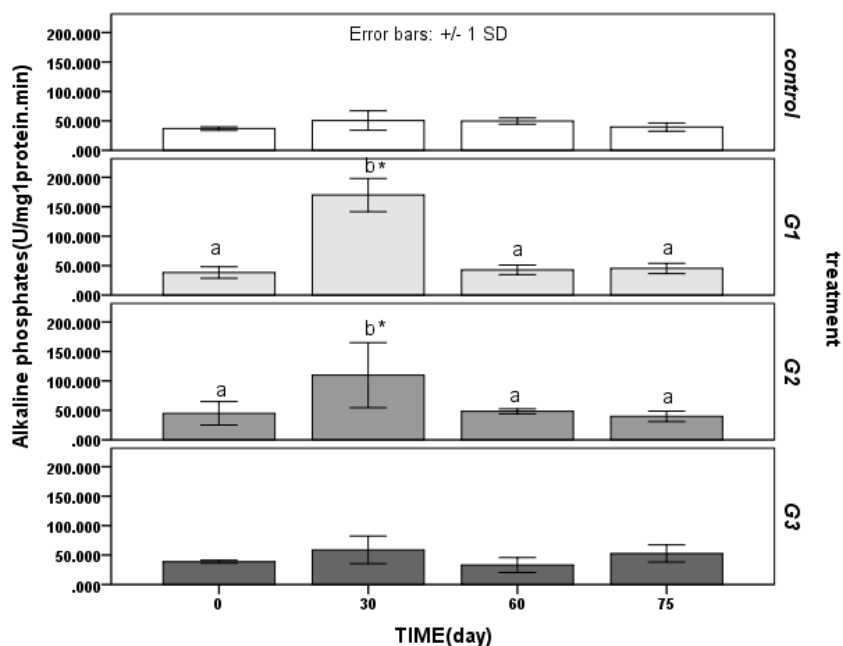


Figure 5: Specific activity of Lipase of *Tor grypus* treated with G1 (*Lactobacillus plantarum* $\sim 5 \times 10^7$ cfu g^{-1}), G2 (*L. bulgaricus* $\sim 5 \times 10^7$ cfu g^{-1}), G3 (*L. casei* $\sim 5 \times 10^7$ cfu g^{-1}) and without probiotic (control). Values is shown as means \pm standard error (n = 9). Legends as mentioned in Fig. 1.

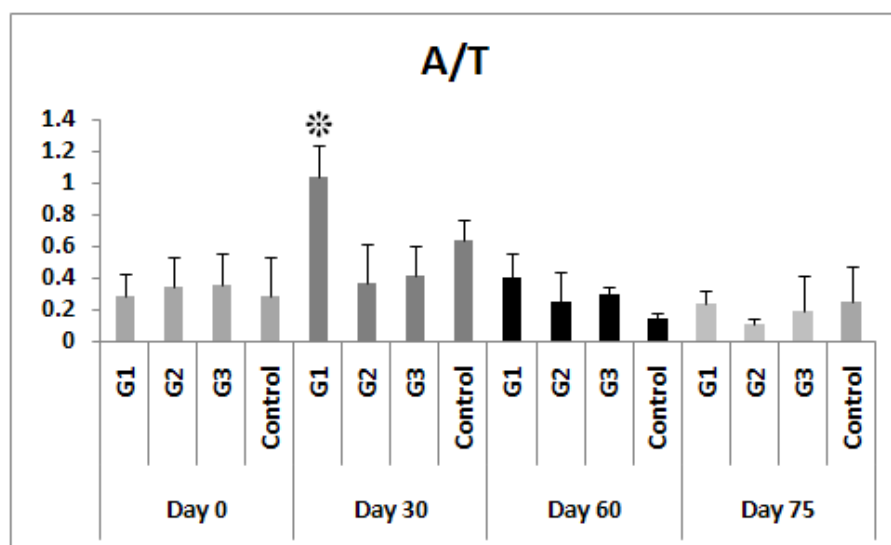


Figure 6: α -amylase/Trypsin ratio in *Tor grypus* treated with G1 (*Lactobacillus planturum* $\sim 5 \times 10^7$ cfu g^{-1}), G2 (*L. bulgaricus* $\sim 5 \times 10^7$ cfu g^{-1}), G3 (*L. casei* $\sim 5 \times 10^7$ cfu g^{-1}) and without probiotic (control). Values is shown as means \pm standard error (n = 9). Legends as mentioned in Fig. 1.

Table 3: Total viable counts, total lactic acid bacteria (LAB), from the digestive tract of *Tor grypus*. MRS: de Man, Rogosa and Sharpe.

	Groups	Time(0)	Time(30)	Time(60)	Time (75/withdraw)
MRS counts (<i>Lactobacillus</i>) 10^2 cfu mL^{-1}	G1	2.3 ^{a,A} ± 16	7.63 ^{b,C} ± 54	6.02 ^{b,B} ± 39.66	5 ^{b,B} ± 42
	G2	3.05 ^{a,A} ± 15	1.15 ^{c,C} ± 67	3.21 ^{c,D} ± 75.66	3 ^{c,B} ± 55.66
	G3	2.51 ^{a,A} ± 16	11.3 ^{bc,B} ± 60	10.4 ^{c,C} ± 74.33	5 ^{c,B} ± 53
	Control	2.51 ^{a,A} ± 14	3.05 ^{a,AB} ± 15	3.51 ^{a,B} ± 17.6	3.05 ^{a,C} ± 74.33
Total counts 10^5 cfu mL^{-1}	G1	48.3 $\pm 8.38^{a,A}$	9.45 ^{b,B} ± 114	15.63 ^{b,C} ± 170.66	20.6 ^{b,B} ± 102
	G2	2 ^{a,A} ± 49	17.6 ^{c,B} ± 150	6.24 ^{c,C} ± 203	14.01 ^{c,B} ± 146
	G3	5.77 ^{a,A} ± 53	19.6 ^{c,D} ± 191	13.52 ^{d,D} ± 217	12.58 ^{c,B} ± 141.33
	Control	5.56 ^{a,A} ± 43	4.58 ^{a,B} ± 57	4.5 ^{a,B} ± 56.33	5.68 ^{a,C} ± 72.33

Discussion

In this study, two LABs (*L. bulgaricus* and *L. planturum*) with probiotic action were isolated from the intestine of *T. grypus* and their effects on growth performance, gut microbial flora, and digestive enzyme activity were determined during a 60-day feeding. We also compared their properties with a standard LAB strain (*L. casei* ATTC1608[®]). Our results showed that *L. bulgaricus* improved the growth

performance of *T. grypus* more effectively when compared with other tested LABs. *T. grypus* fed *L. bulgaricus* supplemented diets showed higher growth performance, SGR, BWG, RGR, PER, FCR and FER in relation to other experimental groups. The promotion of the growth rate, FER and PER in *L. bulgaricus*-fed group occurred concomitantly with increasing protein turnover. This result was in agreement with those observed by

(Gildberg *et al.*, 1995; Byun *et al.*, 1997; Ghosh *et al.*, 2003; Liu *et al.*, 2013). Different action of probiotics on growth performance of treated fish, found in our experiment support the suggestion that each probiotic strain may interact with the host in a different way (Bomba *et al.*, 2002; Sun *et al.*, 2012). It may also be explained by the greater adaptive capacity of *L. bulgaricus* in aquatic environments in comparison to *L. plantarum* and *L. casei*. It was also found that supplementation of food with *L. bulgaricus* could improve the feed utilization of *T. grypus* in higher rates than other probiotics. As a reason based on our supposition, intestinal bacteria shared in the decomposition of nutrients, such as enzymes, minerals and vitamins, and thus, facilitate feed utilization, digestion and absorption. Growth indices such as BWG, PER, FCR and FER increased among *T. grypus* fed on a diet containing *L. bulgaricus* for 60 days. These findings are similar to that obtained by (Khatab *et al.*, 2005; Macey and Coyne, 2005). It has been indicated that probiotics in feeds with a certain concentration display a growth promoting effect and can be beneficial for commercial fish production. In practical terms, this means that probiotic can decrease the amount of feed necessary for animal growth resulting in production cost reduction. The available evidence indicates gastrointestinal bacteria take part in the decomposition of nutrients, provide the microorganisms with physiologically active materials, such as

enzymes, amino acids, and vitamins (Sugita *et al.*, 1998; Waché *et al.*, 2006), and thus, facilitate feed utilization and digestion. This may account for the enhanced FER and PER by dietary *L. bulgaricus* supplementation in the present study (Bairagi *et al.*, 2002; Balcázar and Rojas-Luna, 2007; Bagheri *et al.*, 2008). Another probable reason of this difference can refer to autochthonous characteristic of *L. bulgaricus* which may provide higher digestible nutrients for the host and digests higher dietary protein/amino acids when compared to the allochthonous bacteria like *L. casei*. Considering these findings, we concluded that different LABs especially *L. bulgaricus* isolated from intestine of *T. grypus* can improve the growth performance of cultured juvenile *T. grypus* when administrated as a food additive. Such probiotics are recommended to be used as a commercial growth promoter to facilitate extensive culture of *T. grypus* in future.

Administration of probiotics isolated from the gut of *T. grypus* had effective action on the activities of different digestive enzymes. On day 30 of the probiotic feeding, the specific activities of trypsin, chymotrypsin, α -amylase and AP were higher in all probiotics-fed fishes in relation to the control group. The exception was lipase, activity of which had no difference between probiotic treated and untreated groups during the feeding period. In agreement with results from the previous researches (Francis *et al.*, 2001; Sáenz

de Rodrigáñez *et al.*, 2009; Lara-Flores *et al.*, 2010), we found higher AP activity in *T. grypus* treated with G1 and G2 containing diets for 30 days. The increase in the activity of AP reflects a possible development of brush border membranes of enterocytes that can be stimulated by the probiotics (Cuvier-Péres and Kestemont, 2001). Activities of this brush border enzyme have been reported to be indicators of the intensity of nutrient absorption in the enterocytes of fish (Gawlicka *et al.*, 2000). High AP activity also has been reported to be an indicator of carbohydrate and lipid absorption (Calhau *et al.*, 2000; German *et al.*, 2004). Taken these findings, it has been concluded that higher growth performance rate in *T. grypus* fed probiotics may be due to the improvement of enterocytes function as well as better conversion and utilization of feed in brush border.

The trypsin, α -amylase, and chymotrypsin in the *T. grypus* digestive system may not only be secreted from ingested bacteria but may also be derived from endogenous origin, as in grouper (*Epinephelus coioides*) (Sun *et al.*, 2012) and in beluga (*Huso huso*) (Askarian *et al.*, 2008). Because gram-positive bacteria like members of the genus *Lactobacillus*, secrete a wide range of exoenzymes, the origin of enzymatic activities in fish-fed probiotics could not be distinguished (Moriarty, 1996; Moriarty, 1998; Suzer *et al.*, 2008). It has been confirmed that relatively higher activities of digestive enzymes result in growth performance

improvement. It is believed that probiotics influence digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity; improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization (Mohapatra *et al.*, 2012; Askarian *et al.*, 2008). Our results were in agreement with the study in sea bass (*Dicentrarchus labrax*) larvae (Tovar-Ramírez *et al.*, 2004), common carp (*Cyprinus carpio*) (Yanbo and Zirong, 2006), Indian white shrimp (*Fenneropenaeus indicus*) (Ziaei-Nejad *et al.*, 2006), shrimp (*Litopenaeus vannamei*) (Wang, 2007); Skrodenyte-Arbaciauskiene (2007); gilthead sea bream (*Sparus aurata*, L.) (Suzer *et al.*, 2008); grouper (*E. coioides*) (Sun *et al.*, 2011); rainbow trout (*Oncorhynchus mykiss*) (Andani *et al.*, 2012) demonstrating the improvement of survival rate, growth parameters, and digestive enzyme activities.

Regarding the above results, we encountered contradictory finding because gut enzyme activities were generally lower in *T. grypus* in G2 group than those in fish of G1 group, while G2 group had the highest growth performance. One possibility is that different probiotics in the diet may affect the gut microbiological and biochemical parameters independently (Balcázar and Rojas-Luna, 2007) or it can be speculated that the *L. plantarum* has a wreckful effect on gut morphology and health parameters.

Additional studies are required in order to clarify this hypothesis in detail.

A/T ratio had no association with fish growth because it was at the highest level in G1 group, while the highest fish growth was observed in G2 group. The significantly higher growth performance and A/T ratio in G2 and G1 dietary groups respectively, may indicate higher energy requirement for protein utilization and growth in G1 group than the other groups (Thongprajukaew *et al.*, 2011).

The gut microbiota can play an important role in the health and growth of the aquatic animals (Vine *et al.*, 2004). Our results showed that feeding of *T. grypus* with diet containing *L. bulgaricus* could increase counts of viable LABs. These results were in agreement with previous reports that probiotics have been used as growth promoters in Atlantic salmon and rainbow trout (Robertson *et al.*, 2000), Tilapia (Ferguson *et al.*, 2010), Rainbow trout (Merrifield *et al.*, 2010), Shrimp (Castex *et al.*, 2008) and Siberian sturgeon (Geraylou *et al.*, 2013a; Geraylou *et al.*, 2013b). Two weeks after cessation of feed containing *Lactobacilli*, the number of *gut Lactobacilli* dramatically decreased. This result was in agreement with the observations by other researchers (Vieira *et al.*, 2008; Son *et al.*, 2009; Korkea-aho *et al.*, 2012; Dash *et al.*, 2014) shown in full washing out of probiotic bacteria seven days after the probiotic withdrawal. Findings of this study clearly demonstrate that the probiotic-contained feed must be given

to fish continuously to retain the probiotic-bacteria level in the gut.

In our study when fish were fed on *L. plantarum* containing diets, changes were less apparent in the diversity of the microbiota in 60 days, when compared to those in fish of G2 and G3 groups. The exact mechanism behind this variation was unclear, but probably *L. plantarum* does not harbor in the intestine beyond 4 weeks after administration of the diet. It may be due to structural differences in the cell wall compositions of different LAB strains (Gill, 1998; Geraylou *et al.*, 2013a), or antagonism action of various gut bacteria. In the present study, the highest numbers of LABs concomitant of the highest growth rate were found in the intestine of *T. grypus* fed *L. bulgaricus*. It suggests that the numbers of viable LABs are more efficient than enzyme activity in enhancing the growth parameters of *T. grypus*. To confirm this hypothesis we found that G2 diet could increase counts of the microbiota in the intestine compared to the control and other probiotic groups, but these changes were not translated into increased enzyme activity in the gut. Taking these findings into consideration, we concluded that feeding of *T. grypus* with *L. bulgaricus* may balance intestinal microbial flora resulting in improvement of food absorption and enhancement of growth performance (Irianto and Austin, 2002).

In conclusion, the ability of *L. bulgaricus* and some deal of *L. plantarumas* isolated strains to augment growth performance and enzyme

activity, colonize and modify the intestinal microbiota as a potential probiotic strains were confirmed. Application of this isolated probiotics is recommended as supplementation feed for other cultured fish because the diet with these probiotic bacteria increases digestion, absorption of protein, and other nutrients in the gastrointestinal tract due to the increase of intestinal proteolytic enzyme activity. Finally we recommend a similar study be done on cultured shrimp species, because this industry needs to pay attention to health management.

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