Iranian Journal of Fisheries Sciences
DOI: 10.22092/ijfs.2019.119077

Effects of oral administration of acidifier and probiotic on growth performance, digestive enzymes activities and intestinal histomorphology in *Salmo trutta caspius* (Kessler, 1877)

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Received: September 2018 Accepted: January 2019

Abstract

This study was aimed to evaluate the single and combined effects of acidifier and probiotic on growth performance, digestive enzymes activities and intestinal histomorphology of *Salmo trutta caspius*. The juvenile fish with mean body weight 15±3 g were divided into 8 different treatments (in triplicates), including control, 5, 10 and 15 g sodium diformate kg^{-1} diet, respectively as T1, T2 and T3. Treatments namely T4, T5, T6 and T7 were received diets containing 0.2 g kg^{-1} commercial probiotic Bio-Aqua® in combination with 0, 5, 10 and 15 g sodium diformate kg^{-1} diet for 60 days, respectively. The results showed that T2 and T3 fish growth performance were improved significantly ($p<0.05$), following 30 days after administration, while T1 did not show the same pattern over the 60 days ($p<0.05$). The single probiotic treatment did not induce significant improvements in fish growth rate, digestive enzymes activities and intestinal morphometry though the combined treatments have been showed an intermediate level of improvement. The higher levels of chymotrypsin and trypsin have been observed at day 30 and the higher activities of lipase, protease and amylase could be seen at day 60 in the most acidifier treatments ($p<0.05$). The villi height and the thickness of epithelium have been reduced ($p<0.05$) because of single acidifier while the combined treatments led to either significant increase ($p<0.05$) or no change compared with corresponding single treatment. The addition of 1.0 g sodium diformate kg^{-1} diet can improve the fish growth rate in long-term by changing digestive enzymes activities, and combined treatments of probiotic and acidifier are mostly revealed antagonist effects.

Keywords: Sodium diformate, Dietary organic acids, Growth performance, Enzyme activity, Caspian trout.

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Introduction

Since recent population rising led to a global growing, demand for food and protein supply, the increase in aquaculture productivity is highly desirable; not only owing to several beneficial effects of aquatic animal consumption by human, but also by putting lower pressure on their natural resources (Alexandratos and Bruinsma, 2012; Gormaz *et al*., 2014). Besides, the significant interest in intensive aquaculture development of non-indigenous species, introducing a local species to this sector, can provide efficient and cost-effective alternative source of food that can meet this growing food need without any environmentally or pathogenic threat (Frisch and Murray, 2002; Arthur *et al*., 2010; Saint-Paul, 2018). Regarding this, *Salmo trutta caspius* (Kessler, 1877) as subspecies of brown trout, having been distributed throughout the Caspian Sea and its population was faced with a recent decline due to over exploitation and environmental pollution (Kocabaş and Başçinar, 2013; Sedgwick, 1995; Niksirat and Abdoli, 2009). On the other hand, higher fillet quality and better growth performance (rather than other trout living in this area) nominated this species for large-scale aquaculture in Iran (Kalbassi *et al*., 2006).

The fast-growing development of aquaculture led to increase in new emerging infectious disease to cultured fish. This consequently followed by unrestricted use of antibiotics to control the diseases outbreak and somewhat to ensure the prevention from any infections (Cabello, 2006). The major constraint of this enormous amounts of antibiotics given to the water is the increase in the prevalence of antibiotic-resistance of fish pathogen (other animal and human, as well), justifying the necessity of an alternatives like probiotics in controlling bacterial diseases (Kav and Erganis, 2008; Denev *et al*., 2009). Given that, advance in probiotic applications to prevent and control of pathogenic bacteria in animal farms, particularly in aquaculture are not out-of-mind (Gomez-Gil *et al*., 2000; Robertson *et al*., 2000). These biologically-active compounds, not only boost the quality of water and sediments in the aquaculture ponds, but also can be applied as food additives to enhance aquatic organisms' immunity as well as disease resistance (Merrifield *et al*., 2010 a, b; Mohammadian *et al*., 2019). Therefore, this might be more effective as economic point of view, owing to general believes regarding to the cost-effective prospective of disease prevention in aquaculture industry.

Besides the probiotic, the organic acids and their salts, as acidifiers have been also introduced to livestock nutrition as preservatives (Kim *et al*., 2005) growth premotor by modulating the intestinal micro flora (Canibe *et al*., 2001). They can also improve the digestibility of minerals by reducing the intestinal pH and therefore, realizing some digestive enzymes (Vielma and Lall, 1997).

However, the knowledge of nutritional requirements of acidifier, information on digestibility coefficients of that in combination with other ingredients such as probiotic, and data on the maximum inclusion level of that in fish is still quite rare. In this context, this study has been designed to evaluate the single and combined effects of acidifier and probiotic on growth performance, digestive enzymes activities and intestinal histomorphology of *S. trutta caspius* and therefore, this information might provide the basis for the use of least-cost programming to formulate diets.

Materials and methods

Diet preparation

The control diet was formulated by using the ingredients as subsequently described. The proximate analysis of the basal diet according to the AOAC method incudes 37.1% crude protein, 15% crude lipid, 10% ash, and 390 Kcal $100g^{-1}$ for gross energy. The pH of the diet was measured according to the method described by (Baruah *et al.*, 2005). Briefly, five grams of the feed were macerated in a porcelain mortar and mixed in 50 mL of deionized water for 1 min using a magnetic stirrer. After the diet homogenization, the pH of the solution was measured.

Experimental design

Juveniles *S. trutta caspius* weighing 15±3 g were transferred from fish propagation and cultivation center in Bahonar- Klardasht, Iran, to the aquaculture fish farm. The fish were acclimated for at least 2 weeks in an indoor 400 L cement ponds and were fed with a standard diet. After verifying the health status of the fish, they were distributed randomly into 24 cement ponds at an initial density of 75 fish per tank and divided into 8 treatment groups; including control (had no organic acid salts or probiotic), treatment T1 received a diet containing only 0.5 g sodium diformate kg^{-1} diet, treatment T2 received a diet containing only 1.0 g sodium diformate kg^{-1} diet, treatment T3 received a diet containing only 1.5 g sodium diformate kg^{-1} diet. Treatments T4, T5, T6 and T7 received diets containing 0.2 g kg^{-1} commercial probiotic Bio-Aqua® in combination with 0, 0.5, 1.0 and 1.5 g sodium diformate kg^{-1} diet, respectively.

The tanks were supplied with water from external Biofilteres (Athmann, China), at temperature of 17.1±1.2°C. The fish were fed with sodium diformate and probiotic-contained diets for 60 days (twice a day) at a rate of 2% of biomass. During the experimental period, pH was measured about 7.94±0.11 and the dissolved oxygen was 8.7±1.3 mg L^{-1}.

Sampling and analysis of biological parameters

In order to determine growth performance, weight of all fish in each treatment, was measured at the beginning of experiment, 30 and 60 days after that. All fish were starved for 24h before sampling or biometry and each individual fish then weighed All growth performance and feed utilization parameters, including condition factor (CF, g cm^{-3}), specific growth rate (SGR%), feed conversion ratio (FCR), protein efficiency ratio (PER), daily weight gain (DWG), relative weight gain (RGR) and feed efficiency ratio (FER) were calculated as suggested elsewhere (Mohammadian *et al*., 2017).The survival rate was also evaluated for the whole experimental period.

Digestive enzymes activities

To analyze the activity of digestive enzymes, on days 0, 30, and 60 following probiotic-acidifier feeding, the fish were starved for 24 h and nine fish of each treatment were taken randomly. The intestine has dissected out under sterile conditions 4ºC. Then the samples were homogenized in a cold homogenizing buffer containing 50 mMTris–HCl, pH 8.0 (1:9 v/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 has been assayed according to Bradford (1976) method using bovine serum albumin as a standard. Banzoyl-L-Tyrosine ethyl ester Ester (BTEE) was used as a substrate to determine enzyme activity of chymotrypsin (Hummel, 1959). Trypsin activity was measured using *N* α -*Benzoyl- L -arginine ethyl ester* (BAEE) as the substrate (Erlanger *et al*., 1961). The α-amylase activity has been measured according to the modified Bernfeld method as described previously (Areekijseree *et al*., 2004) using starch solution as substrate. Amylase specific activity has been expressed as μmol maltose produced h^{-1} mg $protein^{-1}$. Lipase activity has been determined based on the measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil (Borlongan, 1990). Protease activity has been measured using casein (Sigma–Aldrich) as the substrate and then the product will react with Folin's reagent (Anson, 1938, with modification). The activity of alkaline phosphatase (ALP) has been measured using p-nitrophenyl phosphate (pNPP) as substrate (Otto *et al*., 1946). Enzyme activities have been measured as the change in absorbance using a spectrophotometer (UV-2802S; Unico, Shanghai, China) and expressed as specific activity, U mg^{1}protein (Sun *et al*., 2012).

Intestinal histomorphology

At the days 30 and 60 from the start of the experiment, the intestine of fish (n=3) were dissected immediately out following euthanizing. The samples were then divided into three different sections, including proximal, middle and posterior parts and separately fixed in 10% neutral phosphate buffered formalin (pH=7.2) and processed using the standard protocol for histopathological examination. After embedding the sample with paraffin wax, three separate cross sections with the thickness of ~5 µm were prepared using a microtome (Microtec CUT4050) and then have been stained with hematoxylin and eosin (H&E) for further histopathological investigations. The villi height, villi width and the thickness of epithelium, lamina propria and muscularis layers were determined under Nikon light microscope (Eclipse E600) by using of AxioVision 8.4 microscope software from Carl Zeiss (Oberkochen, Germany).

Statistical procedure

The normality of data and the homogeneity of variances were analyzed by applying Shapiro-Wilk and Levene's tests, respectively. In order to determine the effects of treatments (acidifier and probiotic) and time on different parameters, Multi-way Analysis of Variance (MANOVA) was applied. The Multiple comparisons (Duncan) were followed if the *p value* on this variable was statistically significant (SPSS, 18). All experimental data were presented as the mean±SD, and the level of significance for all tests was set at $p<0.05$.

Results

In order to evaluate whether different probiotic, acidifier and feeding time may comprise any changes in growth performance, digestive enzymes activities and histomorphometry of intestine, the data were subjected to MANOVA. Obtained results revealed that regardless of whether significant difference originated from those above-mentioned in single treatment or not, the significant interactive effects were observed in the case of growth rate parameters and enzyme activities. Whereas, in the case of intestine morphology, the significant interaction was mostly observed in distal part, though being absolutely in different range to some extent. The exact *p* values for all single and combined effects, which tested prior to other statistical analysis, are provided in Table 1.

Table 1: Multivariate Analysis of Variance (MANOVA) performed for each parameter with its exact *p* value. This table shows the single and interactive effects of probiotic, acidifier and time of different measured parameters.

		Probiotic	Acidifier	Time	Probiotic × Acidifier	Probiotic × Time	Acidifier × Time	Probiotic × Acidifier × Time
CF		0.001	<0.001	0.147	0.002	0.009	<0.001	0.015
SGR		0.118	<0.001	<0.001	<0.001	0.03	<0.001	<0.001
FCR		<0.001	0.063	<0.001	0.066	<0.001	0.021	<0.001
PER		<0.001	<0.001	<0.001	<0.001	0.034	0.001	<0.001
DWG		0.822	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
RGR		0.041	<0.001	<0.001	<0.001	0.005	<0.001	<0.001
FER		0.015	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Chymotrypsin		0.392	0.179	<0.001	<0.001	0.01	0.572	<0.001
Trypsin		0.011	0.083	<0.001	0.03	0.002	0.035	0.016
Amylase		0.706	0.344	<0.001	0.284	<0.001	<0.001	<0.001
Lipase		0.363	0.258	<0.001	<0.001	0.024	0.384	0.001
Protease		0.202	0.03	<0.001	0.105	<0.001	<0.001	<0.001
ALP		<0.001	<0.001	<0.001	0.001	0.012	<0.001	0.068
villi height	Prox.	0.957	0.332	0.923	0.088	<0.001	<0.001	0.079
	Mid.	0.001	0.576	0.002	<0.001	0.725	0.091	0.065
	Dist.	0.606	<0.001	<0.001	0.011	<0.001	<0.001	<0.001
villi width	Prox.	0.218	0.836	0.567	0.859	0.039	0.102	0.740
	Mid.	0.91	<0.001	<0.001	0.569	0.252	<0.001	0.014
	Dist.	0.001	0.013	<0.001	0.011	0.087	0.016	<0.001

Table 1 continued:								
Epithelium	Prox.	0.407	0.527	0.848	0.226	0.045	0.056	0.051
	Mid.	0.059	0.052	0.221	0.119	0.039	<0.001	<0.001
	Dist.	0.050	0.008	0.001	<0.001	0.074	<0.001	<0.001
Lamina propria	Prox.	0.157	0.357	0.465	0.001	<0.001	<0.001	0.001
	Mid.	0.002	0.970	<0.001	0.005	0.157	0.085	0.826
	Dist.	0.661	<0.001	<0.001	0.879	<0.001	0.025	<0.001
Muscularis	Prox.	0.001	0.015	0.622	0.003	0.084	<0.001	0.056
	Mid.	0.025	0.045	0.001	<0.001	0.837	<0.001	<0.001
	Dist.	0.371	0.094	0.419	0.233	0.025	<0.001	0.049

p value with bold-faced type indicated significant differences for the tested parameters.

Growth performance

Over the 60 days feeding trial, there was no mortality observed due to either the probiotic and/or acidifier administrations. The fish fed for 30 days with different levels of acidifier showed no significant changes in CF while the SGR, FCR, PER, DWG, RGR and FER were improved in T2 and T3 groups as compared with control group ($p<0.001$). The T1 group did not show the same changes when compared to control fish. This pattern has been not observed following 60 days of feeding, in which the best growth performance (SGR, PER, DWG, RGR and FER) was for T1. The CF was reduced ($p<0.001$) in all acidifier treatments while FCR did not show any significant different when compared with control. The probiotic-fed group (T4) has been showed better SGR, FCR and FER rather than control following 30 days while other parameters did not differ from control in this group. Significant increase ($p<0.001$) in CF, PER and FER were observed in T4 as compared with control on day 60 (Table 2).

Table 2: The effects of different diets on growth performance and feed utilization of *Salmo trutta*.

parameters	Time	Control	T1	T2	T3	T4	T5	T6	T7	*p* value
CF (g cm^{-3})	30	1.29±0.10^{bc}	1.30±0.21^{bc}	1.33±0.19^{bc}	1.45±0.13^{b}	1.10±0.03^{bc}	1.10±0.16^{bc}	1.23±0.02^{bc}	1.85±0.08^{a}	<0.001
	60	1.34±0.09^{b}	1.06±0.02^{d}	1.22±0.05^{c}	1.24±0.02^{c}	1.45±0.04^{a}	1.35±0.05^{b}	1.37±0.03^{ab}	1.45±0.05^{a}	<0.001
	p value	<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
SGR (%)	30	0.305±0.004^{d}	0.251±0.017^{e}	0.446±0.032^{b}	0.608±0.032^{a}	0.314±0.006^{d}	0.406±0.004^{c}	0.423±0.002^{bc}	0.404±0.006^{c}	<0.001
	60	0.125±0.019^{c}	0.266±0.076^{a}	0.122±0.036^{c}	0.161±0.033^{bc}	0.198±0.034^{bc}	0.237±0.053^{ab}	0.236±0.040^{ab}	0.190±0.028^{bc}	0.006
	p value	<0.001	0.753	<0.001	<0.001	0.005	0.005	0.001	<0.001	
FCR	30	2.25±0.03^{c}	3.07±0.20^{a}	1.67±0.13^{e}	1.21±0.07^{f}	2.52±0.05^{b}	1.76±0.01^{e}	1.81±0.01^{de}	1.94±0.03^{d}	<0.001
	60	5.17±0.86^{ab}	3.11±1.15^{a}	5.56±1.86^{a}	3.75±0.77^{bc}	3.01±0.61^{a}	2.63±0.65^{a}	2.33±0.44^{a}	3.05±0.46^{a}	0.006
	p value	0.004	0.959	0.023	0.005	0.243	0.084	0.112	0.053	
PER	30	1.19±0.02^{e}	0.88±0.05^{g}	1.62±0.12^{c}	2.22±0.13^{b}	1.06±0.02^{f}	2.56±0.02^{a}	1.48±0.00^{d}	1.38±0.02^{d}	<0.001
	60	0.53±0.08^{c}	0.93±0.29^{ab}	0.52±0.16^{c}	0.74±0.16^{bc}	0.91±0.17^{ab}	1.07±0.26^{ab}	1.18±0.22^{a}	0.89±0.14^{ab}	0.007
	p value	<0.001	0.751	0.001	<0.001	0.218	0.001	0.077	0.005	
DWG	30	0.126±0.002^{e}	0.089±0.005^{f}	0.197±0.014^{b}	0.290±0.018^{a}	0.117±0.002^{e}	0.173±0.001^{c}	0.172±0.001^{c}	0.149±0.002^{d}	<0.001
	60	0.060±0.009^{b}	0.114±0.035^{a}	0.065±0.020^{b}	0.099±0.022^{ab}	0.088±0.016^{ab}	0.126±0.030^{a}	0.120±0.022^{a}	0.086±0.013^{ab}	0.019
	p value	<0.001	0.350	0.001	<0.001	0.043	0.059	0.017	0.002	
RGR	30	19.00±0.27^{d}	15.94±1.00^{e}	26.54±1.68^{b}	34.30±1.50^{a}	19.52±0.36^{d}	24.47±0.23^{c}	25.34±0.12^{bc}	24.36±0.33^{c}	<0.001
	60	8.26±1.22^{c}	16.75±4.45^{a}	8.08±2.29^{c}	10.51±2.04^{bc}	12.78±2.09^{bc}	15.10±3.11^{ab}	15.02±2.36^{ab}	12.30±1.73^{bc}	0.005
	p value	<0.001	0.775	<0.001	<0.001	0.005	0.007	0.002	<0.001	
FER	30	44.36±0.76^{e}	32.58±2.10^{g}	60.01±4.49^{b}	82.43±5.11^{a}	39.58±0.94^{d}	56.58±0.62^{c}	55.02±0.34^{bc}	51.37±0.86^{c}	<0.001
	60	19.69±3.22^{c}	34.73±10.77^{ab}	19.27±5.97^{c}	27.43±6.21^{bc}	34.03±6.51^{ab}	39.55±9.62^{ab}	43.77±8.17^{a}	33.26±5.39^{ab}	0.008
	p value	<0.001	0.751	0.001	<0.001	0.217	0.038	0.076	0.076	

CF: Condition factor, SGR: Specific growth rate, FCR: Feed conversion ratio, PER: Protein efficacy rate, DWG: Daily weight gain, RGR: Relative growth rate and FER: Feed efficiency ratio. All data appears as mean and SD. Control: 0 probiotic+0 acidifier, T1: 0 probiotic+0.5 acidifier, T2: 0 probiotic+1.0 acidifier, T3: 0 probiotic+1.5 acidifier, T4: 0.2 probiotic+0 acidifier, T5: 0.2 probiotic+0.5 acidifier, T6: 0.2 probiotic+1.0 acidifier, T7: 0.2 probiotic+1.5 acidifier g kg^{-1} diet. Significance between treatments at each specific sampling time is indicated by different letters in each row. Significant difference between sampling time (i.e., 30 and 60) in each treatment was shown by their specific *p* value.

The combined treatment of acidifier and probiotic have been compared with their corresponding single treatment and the results indicated that only T7 had a higher ($p<0.05$) CF as compared with either T4 (single probiotic-fed group) or T3 (single acidifier) during both 30 and 60 days of experiment. All combined treatments showed significant increases ($p<0.05$) in SGR as compared with T4 at day 30 and the only T5 showed higher SGR as compared with T1 at the same time. The T6 was enhanced the SGR of fish, treated for 60 days as compared with T2 while no significant changes were observed in comparison with single probiotic treatment. Better FCR was has been observed following all combined treatments when compared with their specific simple acidifier- or probiotic-fed groups at day 30. This was not continued for 60 days and even some treatment like T7 showed an increase in the FCR as compared with T3. The PER has been reduced in combined treatments (T6 and T7) compared with simple acidifier treatments while showed a significant increase compared with T4 on day 30. The most combined treatment on day 60 did not show any significant changes in comparison with single treatment. All combined treatments indicated significant increases in DWG, RGR and FER compared with T4 on day 30 while this pattern was not continued till the end of experiment, i.e., day 60 (Table 2).

Digestive enzymes activities

The intestine enzymes activities at the beginning of the experiment (day 0) did not show any significant ($p<0.05$) changes between different treatments. Over the 30 days, the single acidifier (T1 and T2) and probiotic (T4) led to the increase ($p<0.05$) in the level of this enzyme compared with control. The only combined treatment (T5) was significantly ($p<0.05$) lessened the level of chymotrypsin as compared with the single acidifier (T1) or probiotic treatments. Although there were no significant changes following either single treatment of acidifier or probiotic over the 60 days of administration, the combined treatment (T6) showed a significant rise in the level of this enzyme as compared with T2 (Fig. 1).

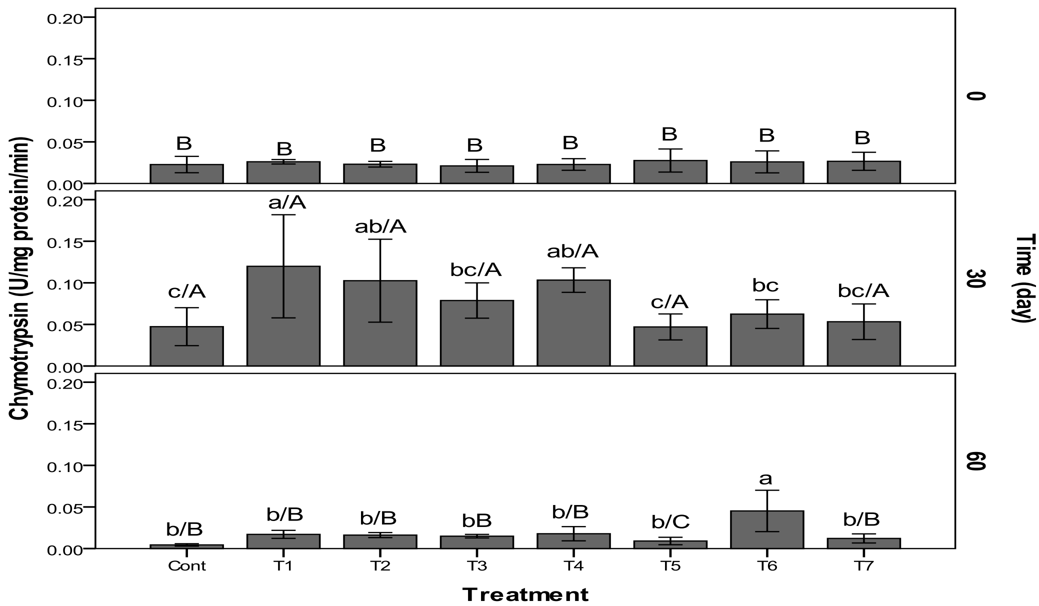

Figure 1: The effects of different probiotic diets on chymotrypsin of *Salmo trutta caspius* intestine over the 30 and 60 days, single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letter express significant difference among different sampling time (p<0.05).

Trypsin enzyme activity was significantly higher (p< 0.05) in fish fed with single diet of acidifier compared to the control at day 30, while probiotic group did not show any significant changes at the same time. The combined feeding trial (T5 and T6) induced significant decrease in the level of trypsin at day 30 when compared to only the single acidifier treatments (Fig. 2).

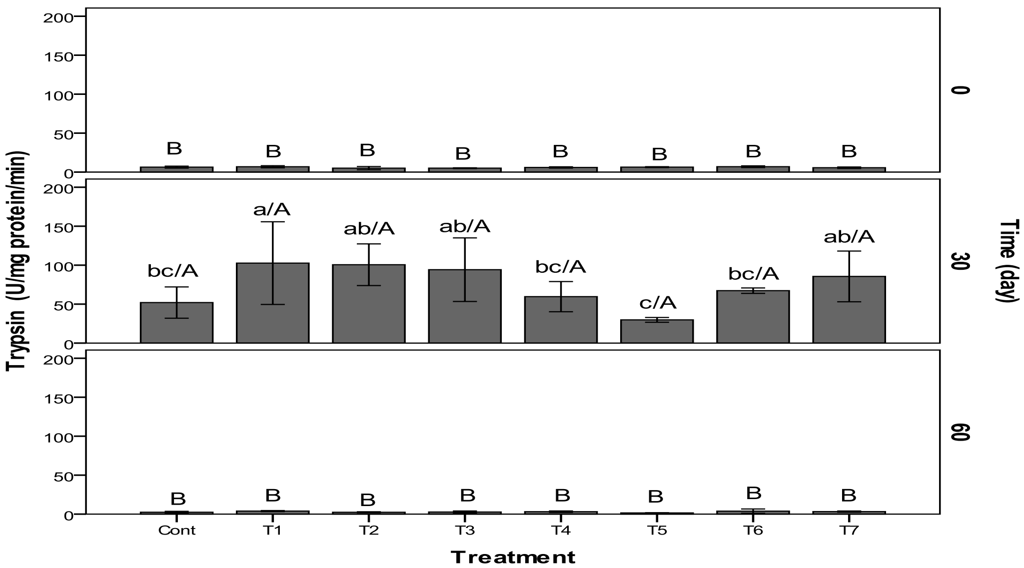

Figure 2: The effects of different probiotic diets on trypsin of *Salmo trutta* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letter express significant difference among different sampling time (p<0.05).

The α-Amylase enzyme activity has been significantly lower ($p<0.05$) in fish fed with single acidifier- or probiotic- supplemented diet when compared to the control group at day 30. Although the combined treatment led to significant rises in the level of this enzyme when compared with their specific single treatment at day30, there were no significant changes in comparison with the single probiotic-fed group (Fig. 3). Over the 60 days supplemented feeding period, the α-Amylase enzyme was elevated significantly as compared to the control and the combined treatment led to significant decrease in the level of this enzyme as compared with single acidifier group (Fig. 3).

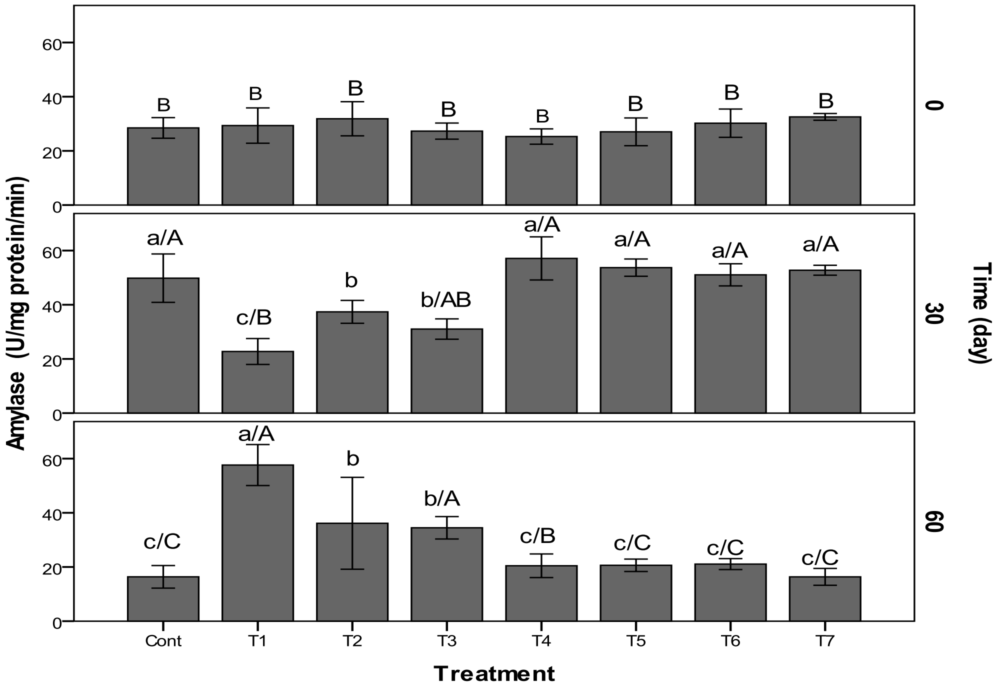

Figure 3: The effects of different probiotic diets on amylase of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.
All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letter express significant difference among different sampling time ($p<0.05$).

A significant increase in the level of gut lipase has been also observed between single acidifier treatment (T1) and control over the 60 days of feeding experiment whereas this significant change could not be observed when fish have been fed with only probiotic. The only combined treatment that showed a significant change in the level of lipase was T5 that showed lower activity when compared to either single acidifier or probiotic-fed group (Fig. 4). Protease enzyme activity was significantly lower ($p<0.05$) in fish fed with single acidifier groups compared to the control group following 30 days post feeding. The combined treatments cause significant increase in the level of protease as compared its single acidifier corresponding group but not with probiotic-fed group. The higher level of this enzyme could be observed following 60 days feeding with single acidifier group while single probiotic –fed group did not act the same. The combined treatment at the same time showed a significant decrease in the level of this enzyme when compared to their corresponding single acidifier group (Fig. 5).

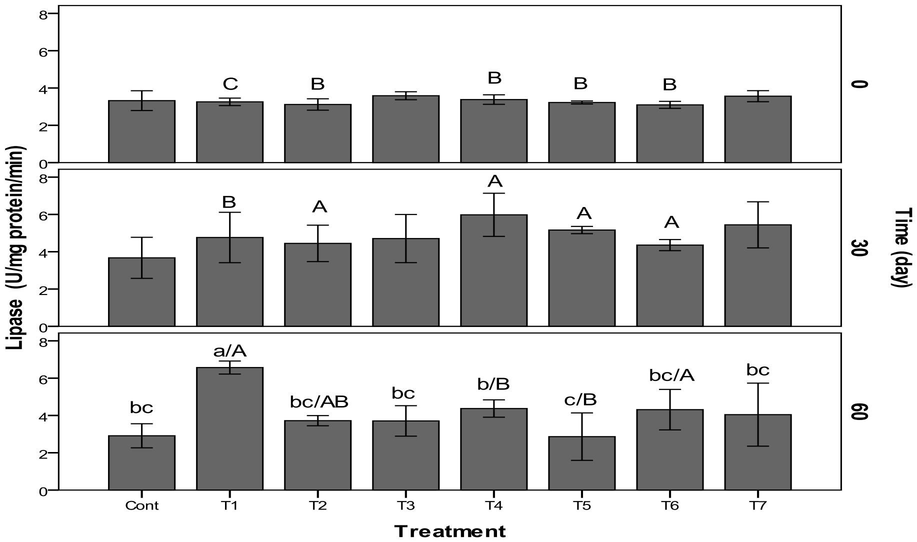

Figure 4: The effects of different probiotic diets on protease of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letter express significant difference among different sampling time (p<0.05).

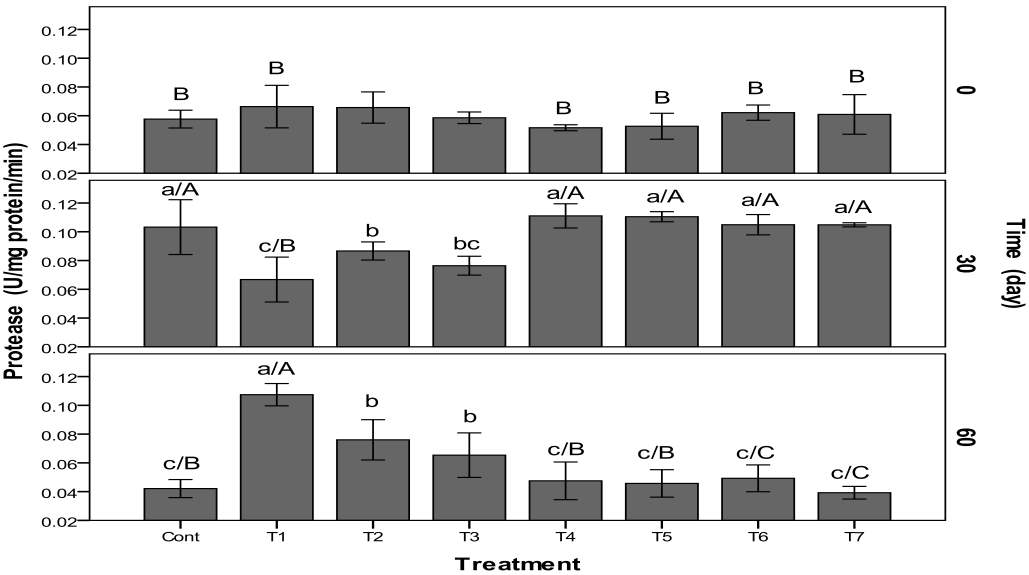

Figure 5: The effects of different probiotic diets on lipase of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letter express significant difference among different sampling time (p<0.05).

Gut ALP activity was significantly lower (p<0.05) in only T3 acidifier fed groups compared to the fish fed with the control diet at day 30. The ALP activity was significantly higher (p<0.05) in treatments T5 and T7 as compared to their corresponding single acidifier groups at the same time. In addition, ALP was reduced significantly following all single acidifier group following 60 days of feeding. All combined treatment led to significant decrease in the level of this enzyme when compared to single probiotic-fed group (Fig. 6). All measured enzymes have been showed their highest activity at day 30 as compared to other sampling time as well.

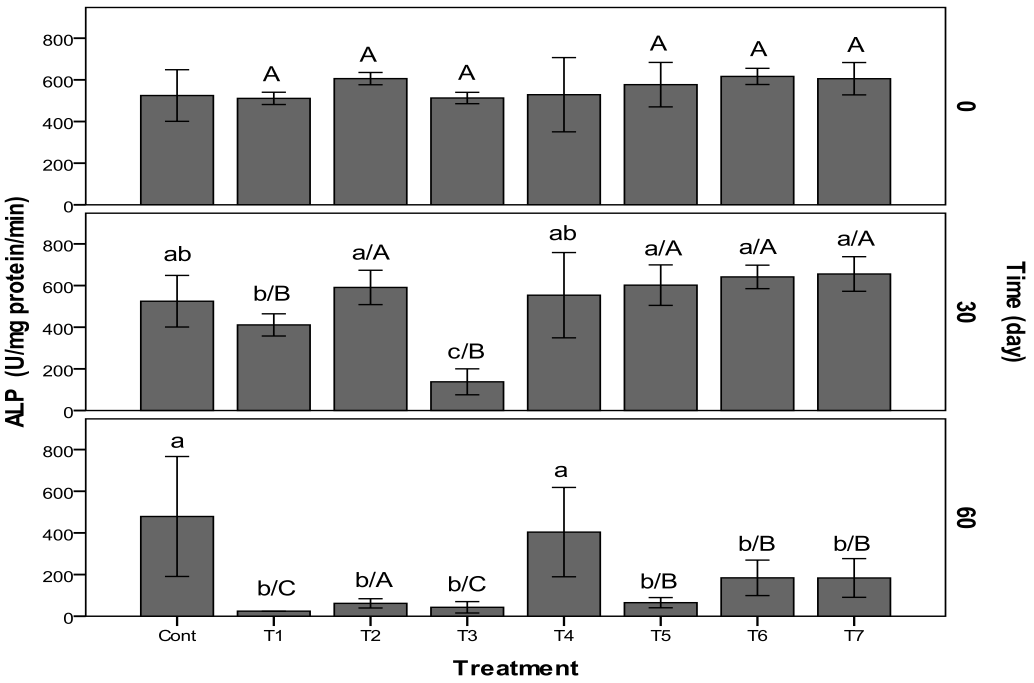

Figure 6: The effects of different probiotic diets on alkaline phosphatase of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.
All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letter express significant difference among different sampling time (p<0.05).

Intestinal histomorphology

Lower villi height in proximal area has been observed in T1 and T2 as compared with control at day 30 while all single acidifier treatments led to significant increase in this parameter at day 60. The villi height was only elevated in the proximal and distal parts of intestine following probiotic treatment at day 30 while in other areas the similar pattern has not been observed. The combined treatments mostly showed significant increase or no significant change in the height of villi when compared to single corresponding treatment (Fig. 7).

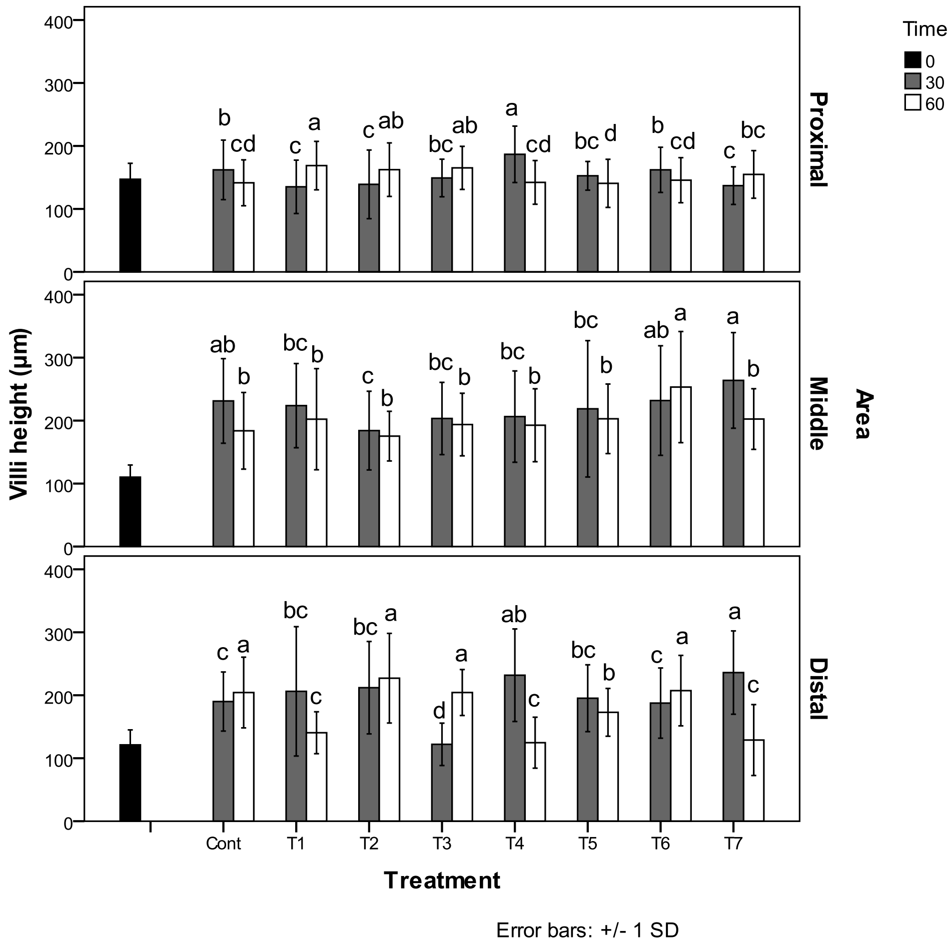

Figure 7: The effects of different probiotic diets on villi height of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments ($p<0.05$). Comparison was made only among the treatment at each specific sampling time.

The T1 and T3 led to significant changes in the villi width in different parts of intestines when compared with the control. The probiotic did not affect the villi width in different parts of intestines when compared with the control. The combined treatment did not show any significant changes in this parameter in the proximal and middle part of intestine while some significant reduction could be observed in distal area when compared to single treatment (Fig. 8).

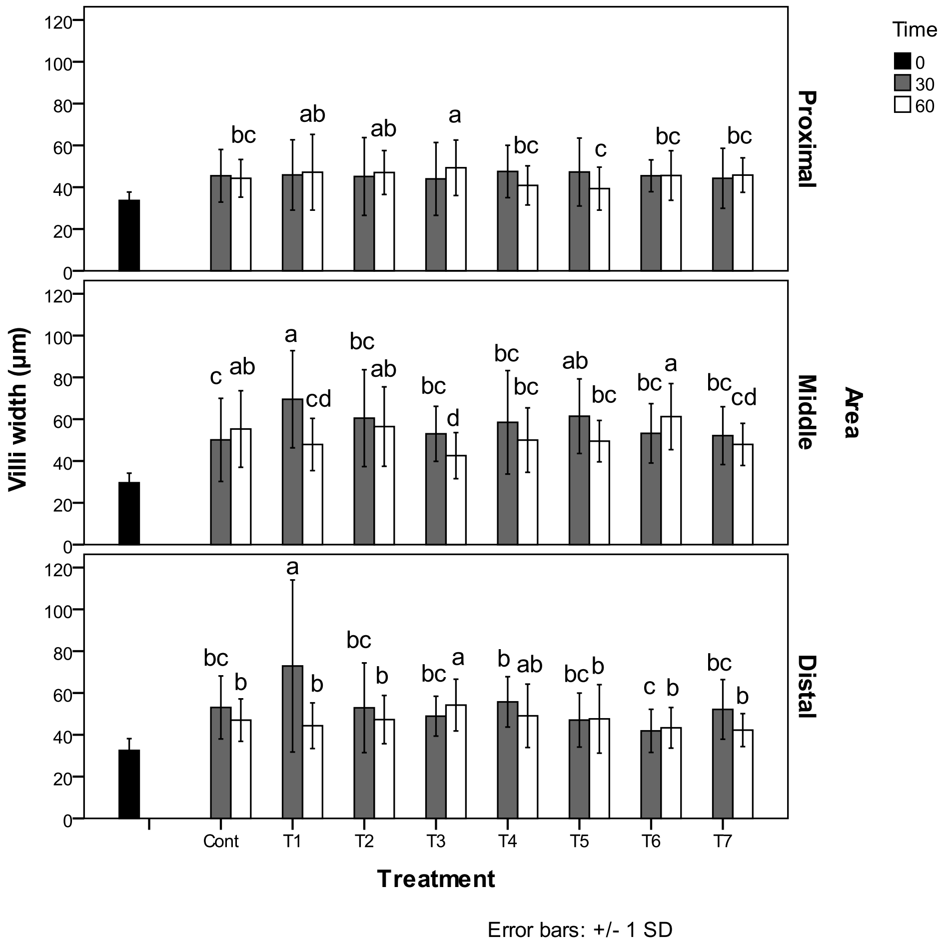

Figure 8: The effects of different probiotic diets on villi width of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments ($p<0.05$). Comparison was made only among the treatment at each specific sampling time.

The most changes in the case of epithelium thickness in single acidifier treatment have been observed in the proximal and middle parts of intestine following 60 days treatment with T1 and T3. Although the epithelium layer of intestine was thicker rather than control group in the distal part, there was a significant decrease and even no significant change in other parts of intestines could be observed. The probiotic feeding resulted in either significant or insignificant decrease in the epithelium thickness in the proximal and middle parts of intestine while significant increase has been observed in the distal part following the same treatment. In the proximal part of intestine, there was no significant change in epithelium thickness of this species following combined treatments as compared with corresponding single acidifier treatments. The thickness of epithelium in combined treatments was elevated in the proximal and middle parts of intestines following 60 days administration as compared with single probiotic treatment while in other parts we did not find the same pattern (Fig. 9).

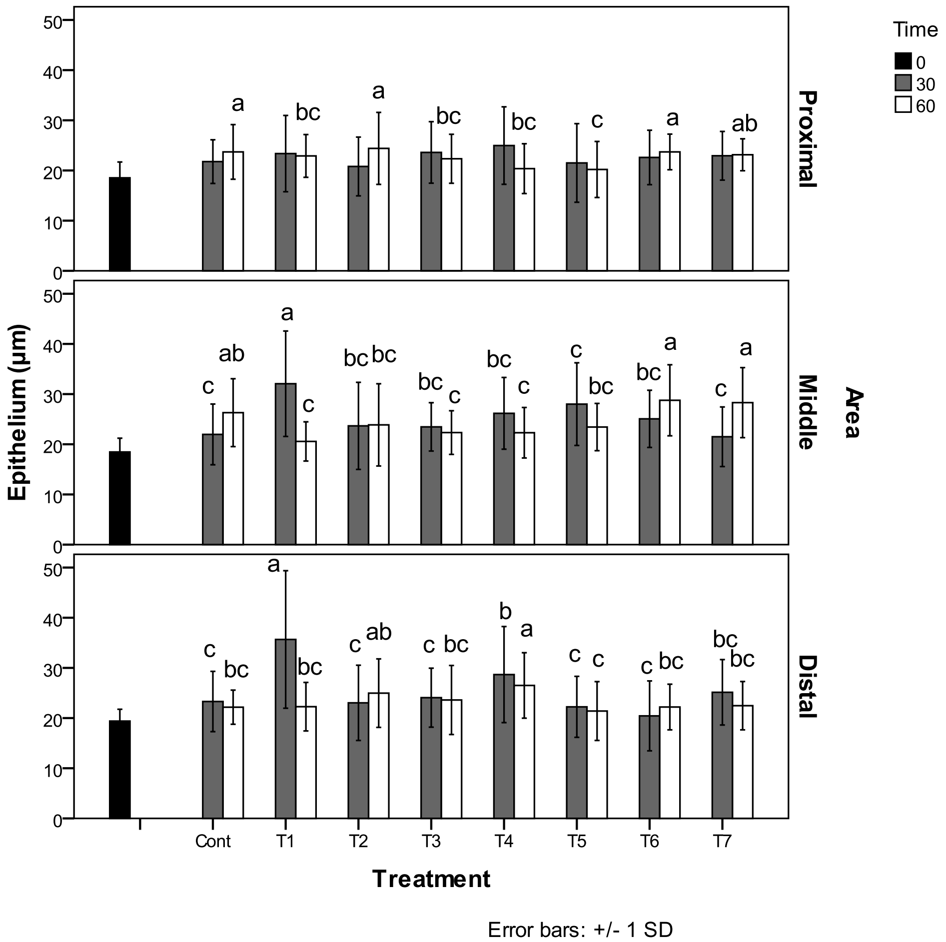

Figure 9: The effects of different probiotic diets on epithelium thickness of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments (p<0.05). Comparison was made only among the treatment at each specific sampling time.

Different levels of acidifier administration had not been affected the thickness of lamina propria in the proximal and middle parts of intestine following 30 days of exposure while these treatments led to increase in the thickness of muscularis in the middle and distal areas of intestine. The thickness of lamina propria and muscularis layers in different parts of intestine was mostly not affected by probiotic or acidifier feeding. The thickness of lamina propria and muscularis layers following combined exposure had not shown any significant changes in the middle part of intestine when compared to single acidifier or probiotic (Figs. 10 and 11).

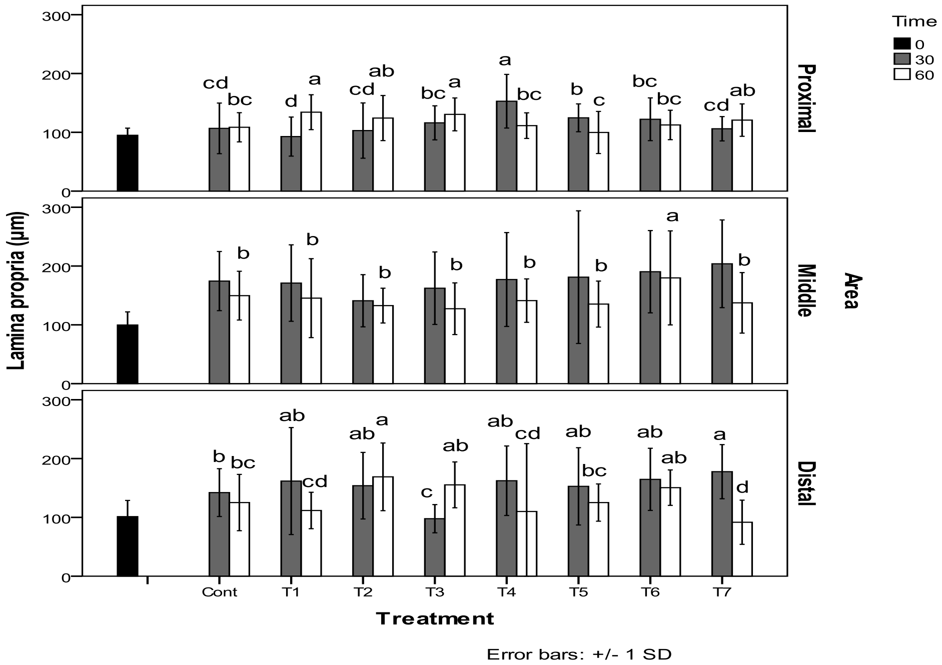

Figure 10: The effects of different probiotic diets on lamina propria thickness of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments (p<0.05). Comparison was made only among the treatment at each specific sampling time.

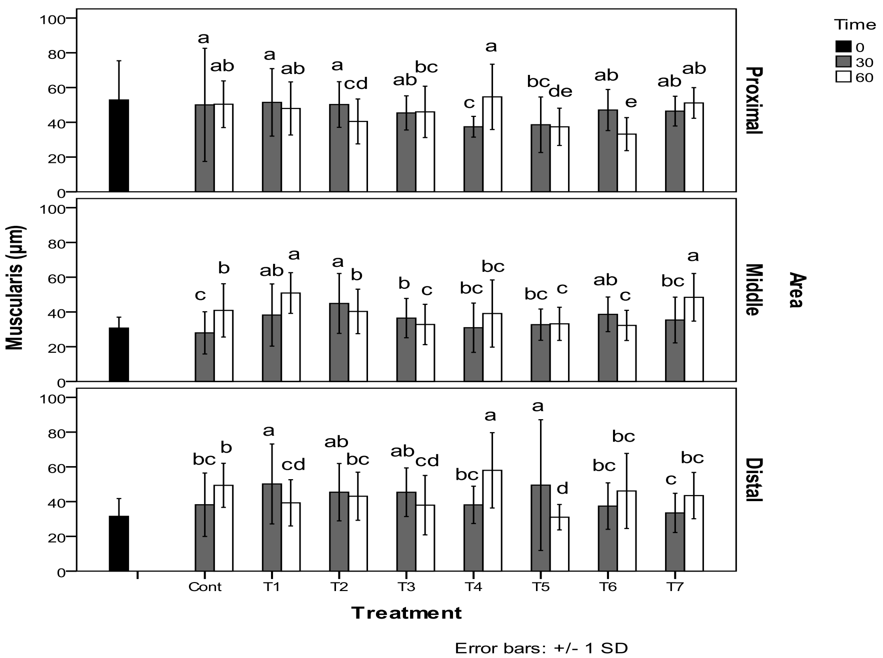

Figure 11: The effects of different probiotic diets on muscularis layer thickness of *Salmo trutta caspius* intestine over the 30 and 60 days single and combined diets of acidifier and probiotic.

All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Treatment codes as mentioned in Table 2. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments (p<0.05). Comparison was made only among the treatment at each specific sampling time.

Discussion

Effects of acidifier

In the present study, the T2 and T3 indicated significant improvements in fish growth performance following 30 days of administration. This has not been continued over the 60 days of feeding trial, in which the T1 showed better growth performance. Previous studies demonstrated that different dietary acidifiers could enhance the growth performance and the feed utilization in various aquatic species. Regarding this, Wassef *et al*. (2017) reported sodium diformate (3%) as growth promoter in *Dicentrarchus labrax* following 13 weeks and Elala and Ragaa (2015) reported that *Oreochromis niloticus* growth performance was elevated following 60 days commercial acidifier (i.e., Aquaform containing potassium diformate). They revealed that lower doses of acidifier, 0.1% did not act the same as higher doses (0.2 and 0.3%). Furthermore, *Rutilus kutum* growth performance was enhanced because of 0.25% dietary sodium propionate for 7 weeks (Hoseinifar *et al*., 2016). Whereas, other studies on red hybrid tilapia for a shorter period of time (2 weeks) and *Pagrus major* for 75 days did not show similar improvement in growth rate (Hossain *et al*., 2007, Ng *et al*., 2009). It is possible that longer feeding with higher inclusion of acidifier lessens the beneficial role of that to trigger fish growth rate, owing to internal interaction with normal physiological function of gut microbiota. Therefore, it was resulted in lower dose of acidifier manifest the signs of better function (by comparing the data obtained at day 60 *vs*. day 30). However, the mechanism underlying the growth promoting of acidifier did not investigated here but it previously demonstrated that these compounds can clearly reduce the intestine pH of host (Ng *et al*., 2009) and consequently stimulate the pepsin activity and therefore, improving the protein digestibility (Thaela *et al*., 1998). This apparently observed in higher PER, obtained following sodium diformate administration in the present study.

It is interesting to note that, inter-specific difference among species, type of organic acids and their administrated level, and different cultural system potentially affect the growth-promoting effects of dietary acidifiers (Thaela *et al*., 1998). However, these criteria should be addressed in a closer look to pursue the exact of role of each in the future studies.

The inclusion of organic acids in the diets of red drum, *Sciaenops ocellatus*, resulted in higher activity of several digestive enzymes (Castillo *et al*., 2014). This was in agreement to our findings, in which the higher levels of chymotrypsin and trypsin have been observed at day 30 and the higher activity of lipase, protease and amylase could be seen at day 60 in most acidifier treatments. In this respect, increase in digestibility of proteins, lipids and amino acids in *O. mykiss* fed with acidifier was also reported (Morken *et al*., 2011). The increase in the level of these digestive enzymes activities might be due to releasing of secretin, which is, in turn, dependent on the intestinal pH (Castillo *et al*., 2014). In contrast to other findings who reported either improvement of phosphorus absorption and/or higher activity of ALP, because of organic acid feeding (Vielma and Lall, 1997; Hossain *et al*., 2007; Castillo *et al*., 2014), we did not find the same increase in the level of intestinal ALP during 30 and 60 days of acidifier administration. This however, might be the results of organic acid types and dose, which was not unique among the different studies.

Another possible reason for the better growth performance of this species as a result of acidifier was related to the fact that the uptake (via passive diffusion) of dietary acidifiers could perhaps provide the required energy for renewing the intestinal epithelia (Vielma and Lall, 1997; Wassef *et al*., 2017) as well as higher surface area for more absorption of nutrients (Awad *et al*., 2008). This phenomenon, however, did not totally observe in the present study, in which the villi height was only elevated following 60 days of acidifier administration in the proximal area of fish intestine. In addition, the microvillus were wider in acidifier treatments though histological layer thickness has been reduced. These, together, could possibly increase the absorption of nutrient within the fish intestine and thereby improve the growth performance. In other domestic animals, like pigs and present broiler chickens, the positive effect of acidifier and organic acids were observed in the case of higher villi height (Jia *et al*., 2010; Kum *et al*., 2010).

Effects of probiotic

The probiotic-fed group (T4) mostly led to insignificant change in growth performance. Nevertheless, improvements of growth performance were observed in *O. mykiss* fed with different probiotics, including *E. faecium* , *L. plantarum* and *L. casei* containing diets (Merrifield *et al*., 2010a; Merrifield *et al*., 2010b; Andani *et al*., 2012), implying the probiotic-fed fish utilized dietary nutrients more efficiently. The improvement of feed utilization or conversion in probionts supplemented groups could likely be owing to the increase in digestive enzymes activities, induced by probiotics (Yanbo and Zirong, 2006; Suzer *et al*., 2008). The increase in digestive enzymes activities and therefore, improved feed utilization through the use of probiotics has also been reported in *O. mykiss* as results of other bacterial strains, like *L. casei* and *L. plantarum* or even in other fish species, like *Sparus aurata*, fed with *Lactobacillus* sp. (Suzer *et al*., 2008; Andani *et al*., 2012). Obtained results suggested that higher chymotrypsin, and trypsin activities only following 30 days of administration. The ALP, protease, lipase and amylase did not show any significant changes, in which the formers might be responsible for better-feed utilization and therefore, growth performance. The higher intestine ALP activity as compared to single acidifier treatments indicates the intensity of nutrient absorption in the enterocytes of fish (Gawlicka *et al*., 2000), which can be responsible for more carbohydrates and lipids uptake

(Calhau *et al*., 2000). Previous studies explained that how probiotics (especially *L. bulgaricus*) are able to stimulate these enzymes activities within the brush border of fish enterocytes (Cuvier-Péres and Kestemont, 2001; Mohammadian *et al*., 2017).

Whatever the underlying cause(s), the physiologically-active compounds (enzymes, amino acids, vitamins and etc.) of these bacterial strains could likely facilitate feed utilization and digestion due to their specific metabolic and trophic functions (Waché *et al*., 2006; Denev *et al*., 2009; Mohammadian *et al*., 2017). On the other hand, exoenzyme secretion, being originated from probiotics, could produce proteolytic, amylolytic, cellulolytic, lipolytic and chitinolytic influences to induce the better fish growth performance (Moriarty, 1998; Gutowska *et al*., 2004). However, it seems that the improvement of these enzymes following this probiotic treatment have not been strong enough too able to induce fish growth rate.

The histomorphological observations revealed that the villi height were only elevated following probiotic treatment in the proximal and distal parts following 30 days feeding trial and likewise the others have not been shown any significant different compared to control. No changes in the villi height was also reported in *S. aurata* fed with *B. subtilis* for 4 weeks (Cerezuela *et al*., 2012). Other studies demonstrated the beneficial impact of probiotic, like *L. rhamnosus* on villus height of *O. niloticus* (Pirarat *et al*., 2011). Furthermore, the probiotic treatment did not mostly change other histomorphological parameters, measured in the fish intestine here, similar to what found by Cerezuela *et al*. (2012), in which no significant changes in lamina propria thickness has been observed in *S. aurata* fed with *B. subtilis* for 4 weeks. However, our findings clearly suggest that some improvement of fish growth performance (i.e., SGR, FCR and FER) in this treatment did not dependent on the remodeling of intestine to increase the absorption surface area.

Combined treatment

To the best of our knowledge, lack of information regarding to the joint effects of acidifier and probiotic on fish health limits further discussion. However, absence of a similar pattern for all combined treatment might be related to the competition of both agents (i.e., acidifier and probiotic), occurred at the same time. Previously, it has been reported that 40 days administration with combined treatment of prebiotic and acidifier not only able to induce any improvement in the growth performance of *O. mykiss* but also this treatment have a significant negative effects on SGR and FCR (Tabrizi *et al*., 2012). Our findings indicate that parameters, measured as growth performance criteria did not change or even slightly declined as compared with their corresponding single treatment of acidifier. This suppressive effects of joint treatment might be related to the reduction occurred because of probiotic administration. For instance, the PER has been reduced in most combined treatment as compared with their single acidifier treatment whereas there was a significant increase in PER as compared with single probiotic treatment, suggesting some antagonist activity of acidifier and probiotic. However, the mechanism supposed to be the result of this change was the lowering of the gut pH following dietary supplementation with sodium diformate. This kind of additive might have antagonist effect on the allochthonous or even autochthonous beneficial lactic acid bacteria of intestine (Liu et al., 2013). In other treated animals, it has been shown that even single prebiotic or acidifier can induce the intestinal absorption surface area but the combined treatment did not act the same (Das *et al*., 2012). In addition, the antagonist effect of acidifier with other dietary compounds like phytase was previously confirmed when the growth performance of *Pangasianodon Hypophthalmus* did not change significantly at higher doses (Le Thanh *et al*., 2017).

In conclusion, the results obtaining for the present study indicated that the single acidifier did not show a similar trend at different feeding duration. However, present study may elucidate that how acidifier can improve the *S. trutta caspius* growth performance to some extent. The addition of 1.0 g sodium diformate kg^{-1} diet in long-term can improve the fish growth rate by changing the feed utilization rate and digestive enzyme activities. The applied probiotic here could increase the growth performance of this species, likely owing to increased digestive enzyme activity. The higher growth performance observed at this treatment, cannot be accounted as changes ~~as~~ which has been observed in the intestine morphology. However, this conclusion cannot be generalized for other probiotics or even for higher or lower doses of that. Since no researches on the effect of acidifier on *S. trutta caspius* is available yet, our findings support the beneficial effects of this compound on this species for the first time but the effects of other organic acids should be addressed as a comparison to the present findings. Further studies should be designed to evaluate the effects of acidifier and its combination with probiotic on fish health status and its preventive effects against pathogenic bacteria. This, however, can reveal the effectiveness of this kind of diets on health management of aquatic species practice.

Acknowledgement

This work was funded by a Grant from Tehran University and Shahid Chamran University of Ahvaz Research Council (Grant No: 636410, 1392.4.6).

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