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Research Article

Immunological, oxidative stress, and biochemical responses of *Salmo trutta caspius* orally subjected to *Bacillus* probiotics (*Bacillus subtilis* and *B. licheniformis)* and sodium diformate

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Abstract

This study was aimed to evaluate the single and combined effects of sodium diformate as an acidifier and bacilli probiotics (*Bacillus subtilis* and *B. licheniformis*) on some immunological, oxidative stress and biochemical responses in juvenile *Salmo trutta caspius* for 60 days. Hundred juvenile fish weighing 12.6±1.4 g was divided into 4 different treatments, including control, 0.4 g *B. subtilis*+*B. licheniformis* /kg diet, 0.5 g sodium diformate/kg diet and their combination (0.4 g *B. subtilis* + *B. licheniformis*+0.5 g sodium diformate/kg diet). Blood samples (9 fish/treatment) were withdrawn at two different time intervals (days 30 and 60). Results showed that bacilli probiotics treatment led to significant increases in the serum lysozyme and complement activities (ACH_{50}) at days 30 and 60, and sodium diformate only increased this parameter at day 30 as compared with the control one. The ACH_{50} indicated a significant decrease following sodium diformate treatment at the same time. Serum bactericidal activity was declined following either acidifier or probiotic treatment. Serum catalase activity was elevated in all treatments, either significant or insignificant as compared with control. Serum malondialdehyde (MDA) was not changed significantly during 30 days of the experiment. On the contrary, bacilli probiotics and sodium diformate led to increase and decrease in MDA, respectively. Our data suggest that sodium diformate applied here was much more successful in improving the fish health status rather than bacilli probiotics. Combined treatment revealed a mid-level of responses except for ACH_{50}, in which a higher level was observed.

Keywords: Acidifier, Probiotic, Antioxidant defense, Immunity system, Caspian trout

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Introduction

The important role of nutrients for fish health status and therefore, a higher aquaculture production is not obscure today though the costs of fish diet preparation at a commercial scale are still very high (Tacon, 1996). Especially, the role of dietary supplements in achieving better growth performance (Castillo *et al.,* 2014), and improving of health condition is a crucially new important issue associated with aquaculture sector where the emerging infectious and non-infectious diseases are the major obstacles to sustainable aquaculture development (Cabello, 2006). The application of some dietary supplements such as probiotics has raised hope (Soltani *et al.,* 2018; Soltani *et al.,* 2019). A recent review of the application of *Bacillus* probiotics, including *B. subtilis* and *B. licheniformis* in aquaculture has demonstrated several beneficial effects (Soltani *et al.,* 2019). However, still more data are required to assess the efficacy and potency of bacilli probiotics in many commercial fish species.

Moreover, organic acids with different salt compounds such as sodium, potassium and calcium, accounted as acidifiers, have been also introduced to aquaculture nutrition as the non-nutritive ingredients (Xie *et al.,* 2003). These acidifiers can improve fish health status via providing undesirable bacteria flora in the gastrointestinal tract of fish (Canibe *et al.,* 2001; Morken *et al.,* 2011; Soltani *et al.,* 2019). They can also stimulate the foregut mineral uptake and pepsin activity by lowering the intestinal pH (Jun-sheng *et al.,* 2006; Lückstädt, 2008). However, despite some data on terrestrial animals (Hollister *et al.,* 1990; Cakir *et al.,* 2008; Dizaji *et al.,* 2012), minimum data is available regarding the co-efficacy of probiotics such as bacilli bacteria and the acidifier on the fish immune-physiological variables. For instance, supplementation of diet with potassium diformate and *Bacillus* spp. illustrated a higher growth rate in juvenile turbot, *Scophthalmus maximus* (Fuchs *et al.,* 2015).

Salmo trutta caspius (Kessler, 1877) is an endemic salmonid species of Caspian Sea, and is a valuable commercial alternative species as part of a sustainable aquaculture development program in the Caspian Sea regions where the wild fish population is in danger. Some countries such as Iran are currently performing the restoking program for endangered fish species, including Caspian trout (Sedgwick, 1995; Niksirat and Abdoli, 2009; Karami *et al.,* 2018; Kalantarian *et al.,* 2019). Also, despite some efforts directed toward the preparation of a nutritionally balanced diet for this salmonid species during the last decades (Ramezani, 2009), its nutritional requirements and the application of dietary supplementation still remain uninvestigated.

This work aimed to assess the efficacy of two *Bacillus* probiotics (*B. subtilis* and *B. licheniformis*) and sodium diformate in either single or combined forms on some immunological,

oxidative stress, and biochemical responses of juvenile *S. trutta caspius*.

Materials and methods

Experimental animals

Apparently healthy juvenile *S. trutta caspius* weighing 12.6±1.4 g were obtained from Bahonar Salmon Fish Center, Klardasht, Iran. The fish were acclimated for at least 2 weeks in an indoor 400 L cement pond and were fed twice daily with a control diet (37.1% crude protein, 15% crude lipid, 10% ash, and 390 Kcal/100g for gross energy) prior to the experiment. Water quality parameters, including dissolved oxygen (8.7±1.3 mg/L), temperature (17.1±1.2°C) pH (7.94±0.11) and total ammonia nitrogen (< 0.01 mg/L) were measured, and the natural photoperiod were maintained constant during the experiment.

Experimental design

Four different groups containing 25 juveniles *S. trutta caspius* were assigned to different treatments, including control (without any supplements), 0.4 g Bacilli probiotics®/kg diet, 0.5 g sodium diformate/kg diet and their combination (0.4 g Bacilli probiotics+0.5 g sodium diformate/kg diet). The Bacilli probiotics contained *Bacillus subtilis* (1.6×10^{12} CFU/kg) and *Bacillus licheniformis* (1.6×10^{12} CFU/kg). The experiment lasted for 60 continuous days and all fish were fed at a rate of *ad libitum* (twice a day). The pH of the diet was measured each week by mixing 5 g of the food in a porcelain mortar. To complete macerating, 50 mL of deionized water was added. The homogenized food was used for pH determination (Baruah *et al.*, 2005; Mohammadian *et al.*, 2017).

Sampling

In the present study, the blood samples were taken at two different time intervals, *i.e.*, days 30 and 60 from the beginning of the experiment. At each time, 9 fish were randomly withdrawn from each treatment. To do this, all fish were starved for 24 h before sampling. Fish were anesthetized with 100 mg/L Clove oil (Coyle *et al.*, 2004). Approximately 1 mL of whole blood was collected from the caudal peduncle region and, serum was separated from blood cells by using centrifugation technique (3,500 ×g for 5 min).

Serum Immunity parameters

Separated serum was used for determining immunity parameters, including lysozyme activity, total globulin, alternative complement activity (ACH_{50}) and bactericidal activity. Lyophilized *Micrococcus lysodeikticus* was used to determine serum lysozyme activity according to the turbidometric assay. Briefly, sodium phosphate buffer (0.02 M, pH=5.8, Sigma–Aldrich) was used. The phosphate buffer-free serum sample was applied as a negative control. The absorbance was recorded at 450 nm and expressed in the unit of lysozyme per mL serum when causing a reduction of 0.001 per min at 22°C (Sharifuzzaman and Austin 2009; Mohammadian *et al.*, 2019_b). In order to measure total

immunoglobulin in fish serum, total protein (TP) content of serum was determined according to the Biuret method. The basis of this method is the formation of a Cu^{2+}-protein complex in an alkaline reagent and then measuring optical density at 540 nm by a spectrophotometer. Serum albumin (Alb) was also measured at 540 nm using bromcresol green complex (Aldrich *et al.*, 1998). Finally, total globulin was calculated by subtracting Alb from TP. Agarose plates containing rabbit red blood cells were applied to detect the activity of the alternative complement pathway (ACH_{50}). Several holes (diameter=3 mm) were punched on a plate and then filled with 15 µL of serum. After 24 h of incubation at room temperature, the zone of lysis was measured and expressed as an arbitrary unit per ml of serum (Barta, 1993). Serum bactericidal activity was determined by incubating (90 min at 25 °C) the mixture of the diluted sera and *L. garvieae* as previously described by Kajita *et al.* (1990). The bactericidal activity of serum was expressed as a percentage of the ratio of CFU in the experimental group to those in the control group.

Oxidative stress

Blood sera were prepared for either antioxidant enzymes or lipid peroxidation, *i.e.*, myeloperoxidase (MPO), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) in treated and control fish.

Serum MPO activity was measured by adding 15 µL of serum in the combination of 135µLphosphate buffer. Then, 50 µL of TMB (3,3′,5,5′-tetramethylbenzidine, 2 mM, Sigma) and H_2O_2 (5 mM) were added. The color reaction was stopped by adding 25 µL of H_2SO_4 (1 N) following 2 min (Quade and Roth, 1997). The absorbance was recorded at 450 nm in a plate reader. Serum CAT activity was determined by measuring the absorbance when initial decrease was observed at 240 nm. The decrease in absorbance was the result of H_2O_2 addition during 1 min (Beers and Sizer, 1952). The MDA activity in fish serum was determined according to the complex formation of malondialdehyde and thiobarbituric acid. The absorbance at 532 nm indicates the formation of this complex and can be calculated the activity of MDA (Buege and Aust, 1978). The SOD activity was also determined by the amount of reduction of nitrobluetetrazolium (NBT) by superoxide radicals which have been produced by adding xanthine/xanthine oxidase (Beauchamp and Fridovich, 1971). Samples without serum were used as blanks. All assays were performed in duplicates. The activity was expressed as a µmol per gram protein of serum.

Serum biochemical parameters

Serum cholesterol level was measured based on cholesterol hydrolysis and production of H_2O_2 in the samples. The generation of color following this reaction was read at 576 nm (Allain *et al.*, 1974). Triglyceride was determined

according to the enzymatic reaction of GPO/Trinder. The color generation was measured at 510 nm (Fossati and Prencipe, 1982). Serum glucose was determined according to the method namely GOD-PAP by measuring formed H_2O_2 by adding phenol and 4-aminoantipyrine in the presence of peroxidase at 546 (Burtis *et al.*, 2012). Lactate Dehydrogenase contents of serum were the rate of pyruvate reduction to lactate. This reaction was performed in the presence of NADH (Wróblewski and Ladue, 1955).

Statistical procedure

If normality of data and homogeneity of variance were achieved, analyze of variance (Multi-way ANOVA) was applied used hereto elucidate the effects of treatments (acidifier and probiotic) and time on different measured parameters. Duncan's complimentary test was also applied at *P*-value lower than 0.05. Statistical analyses were carried out by using SPSS 18 program and data are presented as mean±SD (standard deviation) for all cases.

Results

Combined effect

The multivariate analysis of variance was applied to examine the combined effect of Bacilli probiotics, sodium diformate and time on immunity, oxidative stress and biochemical responses in serum of *S. trutta caspius*. Our results showed that acidifier and time, acidifier and Bacilli probiotics, and acidifier, Bacilli probiotics and time have significant (p<0.05) interactive effects on serum lysozyme activity. Amongst TP, Alb, and Glb, treatment involved in the Bacilli probiotics and time resulted in interactive effects. The ACH_{50} and bactericidal activities showed significant (p<0.05) interactive effect. The MPO was only affected by the combined treatment of Bacilli probiotics and sodium diformate. All interactive analyses were significant (p<0.05) in the case of MDA. While CAT showed a significant (p<0.05) interactive effect except for sodium diformate in the combination of time, the SOD activity was only significant (p<0.05) in the combined treatment of Bacilli probiotics and time. Cholesterol and LDH did not show any combined significant (p<0.05) effects whilst serum glucose was significant (p<0.05) following all combined treatments (Table 1).

Effect of time

At two different sampling times (days 30 and 60), the fish sera were analyzed for immunity, oxidative stress, and biochemical responses and obtained data for each treatment were compared. Serum lysozyme activities in single Bacilli probiotics or sodium diformate treatment showed significant (p<0.05) increase at day 60 as compared to day 30 (Fig. 1). TP and Glb showed significant (p<0.05) decreases following 60 days of Bacilli probiotics administration in comparison to day 30 (Figs. 2 and 3), while serum Alb (day 60 *vs.* day 30) was only elevated in combined treatment of Bacilli probiotics+sodium diformate

(Fig. 4). Time had only significant (p<0.05) effect on ACH_{50} in Bacilli probiotics treatment with significant (p<0.05) decrease in day 60 as compared with day 30 (Fig. 5). The combined treatment of Bacilli probiotics and sodium diformate showed a significant (p<0.05) decrease in the serum bactericidal activity following 60 days (Fig. 6).

Table 1: Antioxidant responses, Liver enzymes, Innate immune system and Biochemical factor in *S. trutta caspius* fed feed supplemented with different levels of Sodium diformate and *Bacillus* probiotics for 60 days.

	Bacilli probiotics	Sodium diformate	Time	Bacilli probiotics× Time	Sodium diformate× Time	Bacilli probiotics× Sodium diformate	Bacilli probiotics× Sodium diformate× Time
Lysozyme	**<0.001**	0.202	**<0.001**	0.053	**0.006**	**0.001**	**<0.001**
TP	0.697	0.388	0.298	**<0.001**	**0.006**	0.276	0.528
Alb	**<0.001**	0.406	0.973	0.703	0.974	0.663	0.082
Glb	**0.005**	0.271	0.329	**0.015**	0.318	0.670	0.821
Complement	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Bactericidal	**<0.001**	**<0.001**	**<0.001**	**0.002**	**0.023**	**0.002**	0.773
MPO	0.628	**0.002**	**<0.001**	0.449	0.534	**0.019**	0.246
MDA	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	0.196
CAT	**0.011**	0.068	0.407	**0.002**	0.790	**<0.001**	**0.003**
SOD	0.586	0.778	0.866	**0.001**	0.798	0.448	0.617
Cholesterol	**0.001**	0.815	0.096	0.194	0.309	0.379	0.094
Tg	**0.002**	0.262	0.118	0.286	0.813	**0.031**	0.519
Glucose	0.076	0.062	0.445	**0.049**	**<0.001**	**0.039**	**0.001**
LDH	0.569	0.915	0.094	0.569	0.450	0.934	0.703

P-value with bold-faced type indicated significant (p<0.05) differences for the tested parameter.

While MPO did not show any significant (p<0.05) changes among two different times in all treatments, MDA revealed either significant (p<0.05) rise or decline in all treated fish (Figs. 7 and 8). The CAT not only showed a significant (p<0.05) decrease in the control group following day 60 (*vs.* day30) but also indicated a similar reduction in both Bacilli probiotics and Bacilli probiotics+sodium diformate treatments (Fig. 9). The SOD activity was also significantly (p<0.05) declined in the control group at day 60. In contrast, treatment namely Bacilli probiotics+sodium diformate led to a significant increase in the serum SOD when compared day 60 *vs.* day 30 (Fig. 10).

Serum cholesterol and triglyceride were significantly (p<0.05) reduced in the control fish as day 60 compared with day 30. Cholesterol was also lowered in Bacilli probiotics +sodium diformate treatment at day 60 (Figs. 11 and 12). Although serum glucose was significantly (p<0.05) elevated in control fish, sodium diformate led to a significant decrease in that as compared to day 60 *vs.* day 30 (Fig. 11). Figure 12 indicates that serum LDH did not change

significantly (p<0.05) among two different sampling times.

Effect of treatment

Serum lysozyme activity was not changed in sodium diformate or Bacilli probiotics + sodium diformate treatments compared with control while, Bacilli probiotics treatment led to higher lysozyme activity at day 30 as compared to other treated groups. This parameter showed a similar trend to some degrees during day 60; *i.e.*, a significant increase in Bacilli probiotics or sodium diformate treatment while Bacilli probiotics+sodium diformate treatment did not show any significant change as compared to control (Fig. 1). TP, Alb, and Glb did not show any significant changes in treated fish as compared to control in both time intervals (Figs. 2-4). At day 30, the ACH_{50} was elevated in Bacilli probiotics and Bacilli probiotics +sodium diformate treatment as compared to the control group. Single sodium diformate treatment led to a significant decrease in serum ACH_{50} as compared to other groups. Sodium diformate resulted in a significant decrease in ACH_{50} while Bacilli probiotics did elevate as compared with control. A higher level of ACH_{50} activities was observed in Bacilli probiotics +sodium diformate treatment when compared with control at day 60 (Fig. 5). Bactericidal activity of serum was lessened either significantly or insignificant when compared to control at both sampling times (Fig. 6).

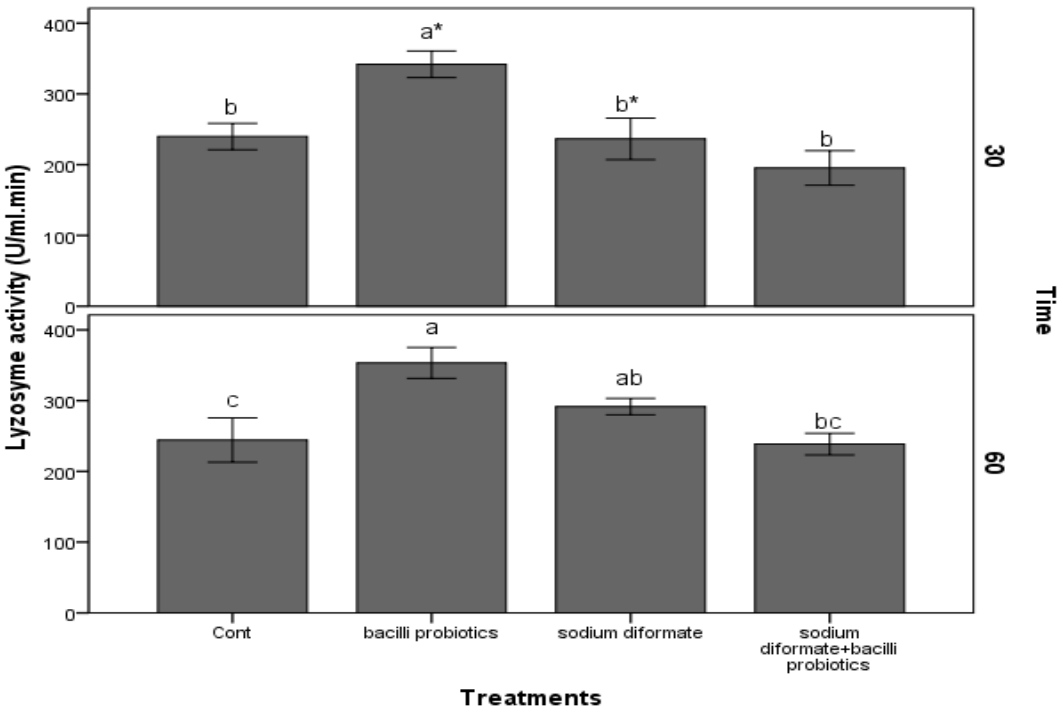

Fig. 1: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum lysozyme activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different alphabetic letters on each bar indicate significant differences amongst each specific time interval (p<0.05). Asterisk indicates a significant difference between two sampling times (30 vs. 60 days).

The MPO was not changed in Bacilli probiotics or Bacilli probiotics+sodium diformate treatments at day 30 while higher MPO activity was observed in sodium diformate treatment at that time. No significant changes were also observed at day 60 (Fig. 7). Although MDA was not changed significantly (p<0.05) at day 30, higher (Bacilli probiotics) and lower (sodium diformate and Bacilli probiotics +sodium diformate) activity of MDA were observed following 60 days of the experiment (Fig. 8). Higher CAT activity in the serum of fish, treated with either Bacilli probiotics or its combination was observed at day 30 when compared to control or sodium diformate. At day 60, a significant (p<0.05) increase in CAT was observed in Bacilli probiotics and sodium diformate (Fig. 9). The SOD activity in serum was not affected by our treatments in both time intervals (Fig. 10).

Serum cholesterol and LDH were not changed significantly (p<0.05) as results of either Bacilli probiotics and/or sodium diformate (Fig. 11-14). Serum triglyceride was only reduced following sodium diformate treatment at day 60 as compared to control (Fig. 12). Following 30 days of the experiment, serum glucose was elevated only in sodium diformate treatment as compared to other treatments. Sodium diformate and Bacilli probiotics +sodium diformate treatments led to a significant (p<0.05) decrease in serum glucose at day 60 (Fig. 13).

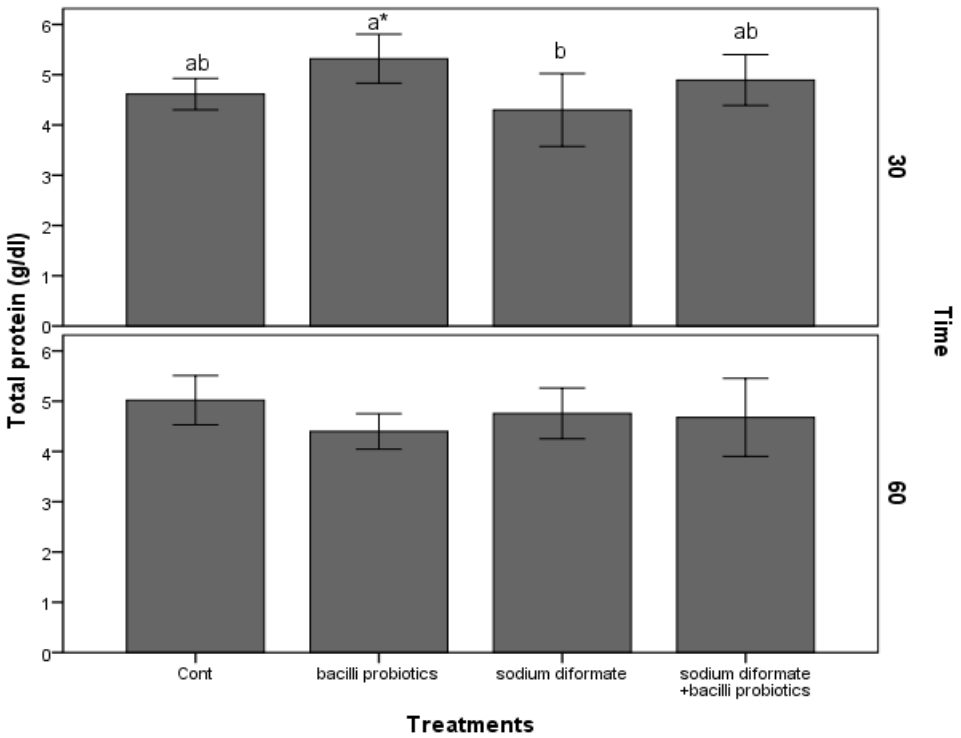

Fig. 2: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum total protein activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

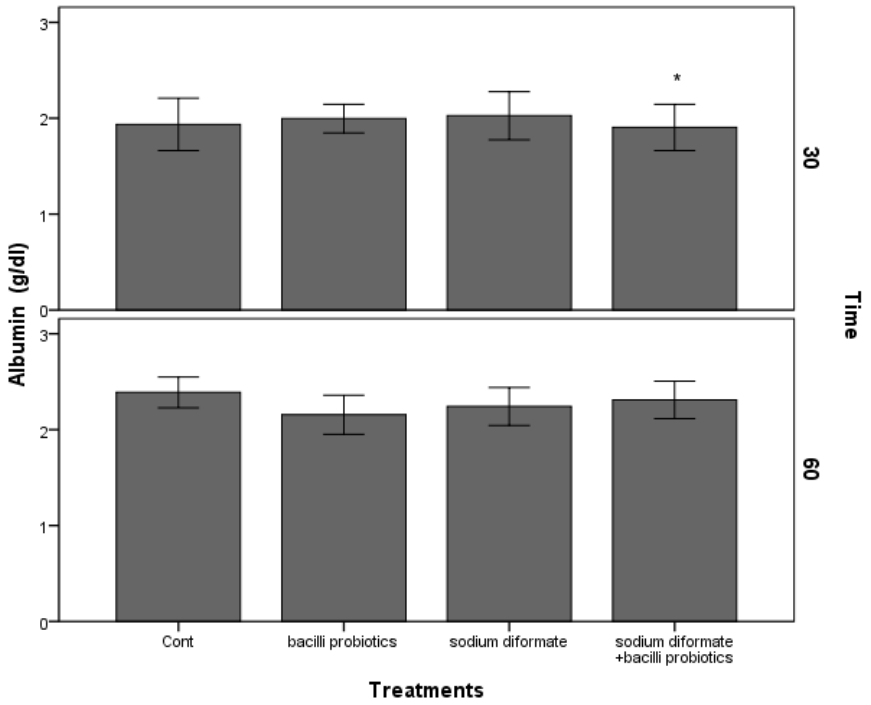

Fig. 3: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum albumin activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

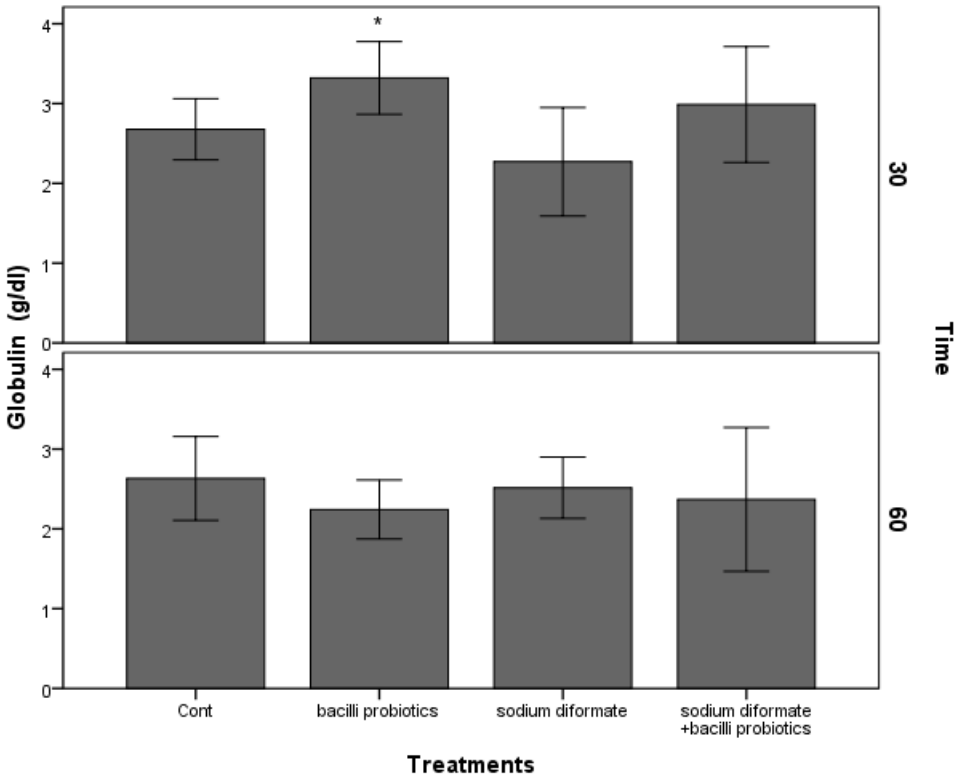

Fig. 4: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum globulin activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

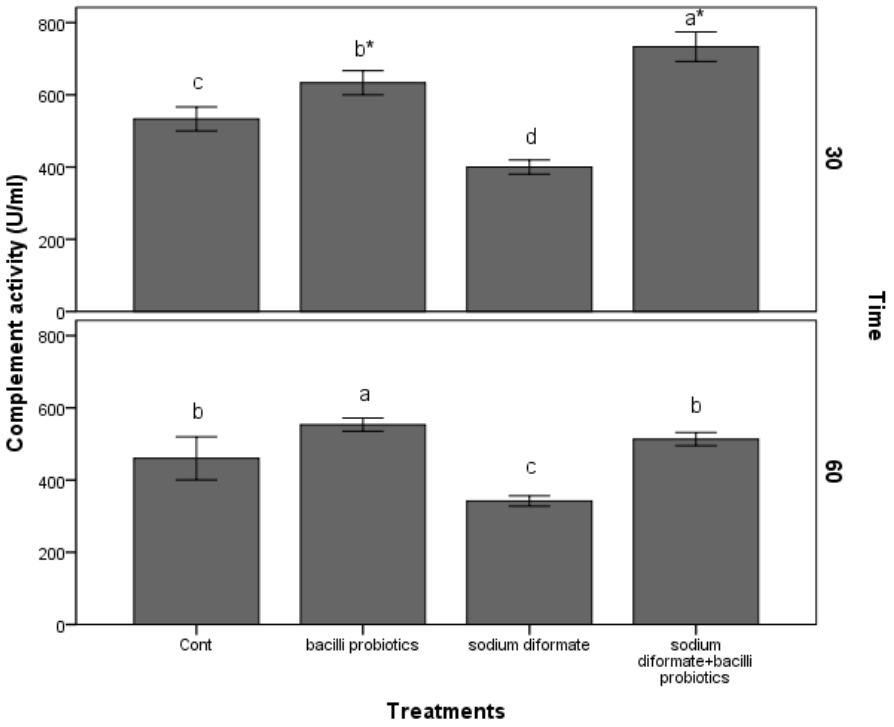

Fig. 5: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum ACH_{50} activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

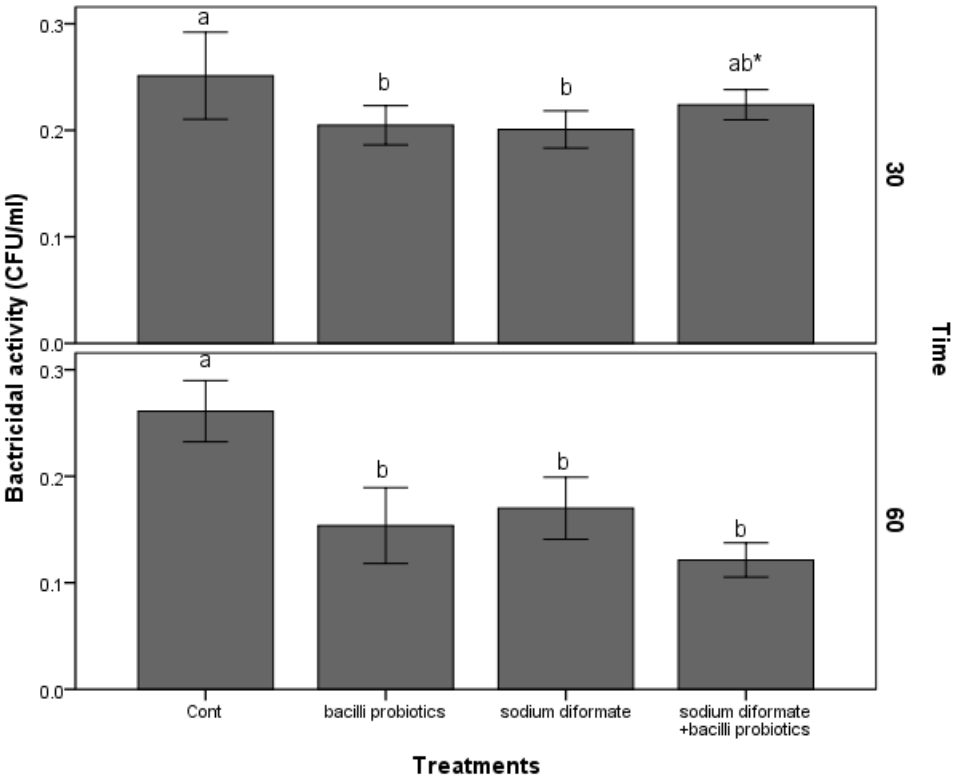

Fig. 6: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum bactericidal activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

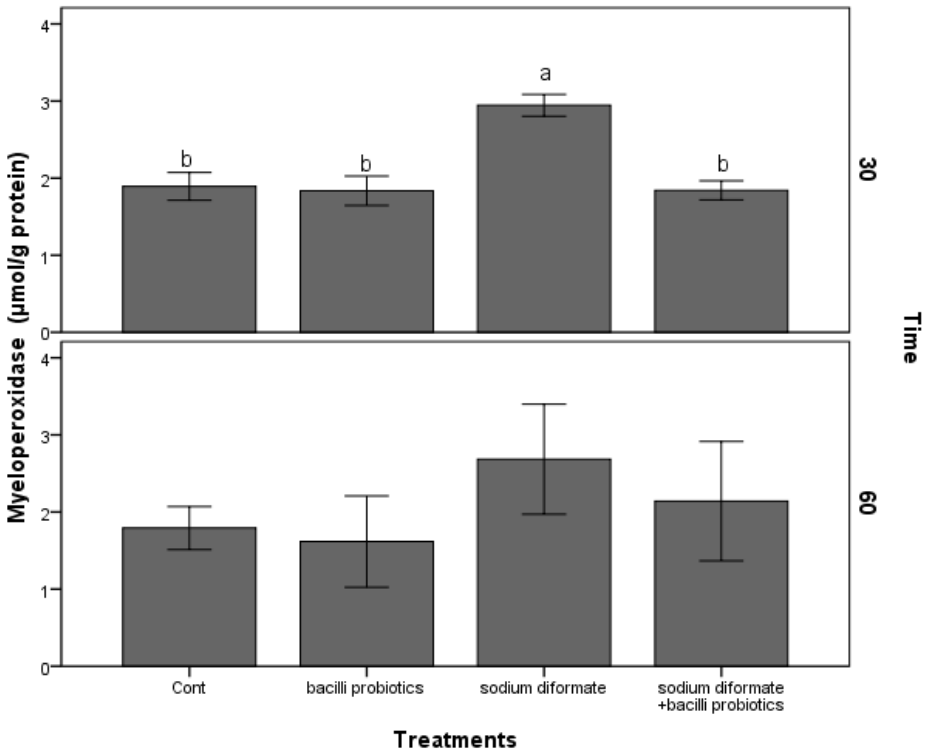

Fig. 7: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum MPO activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

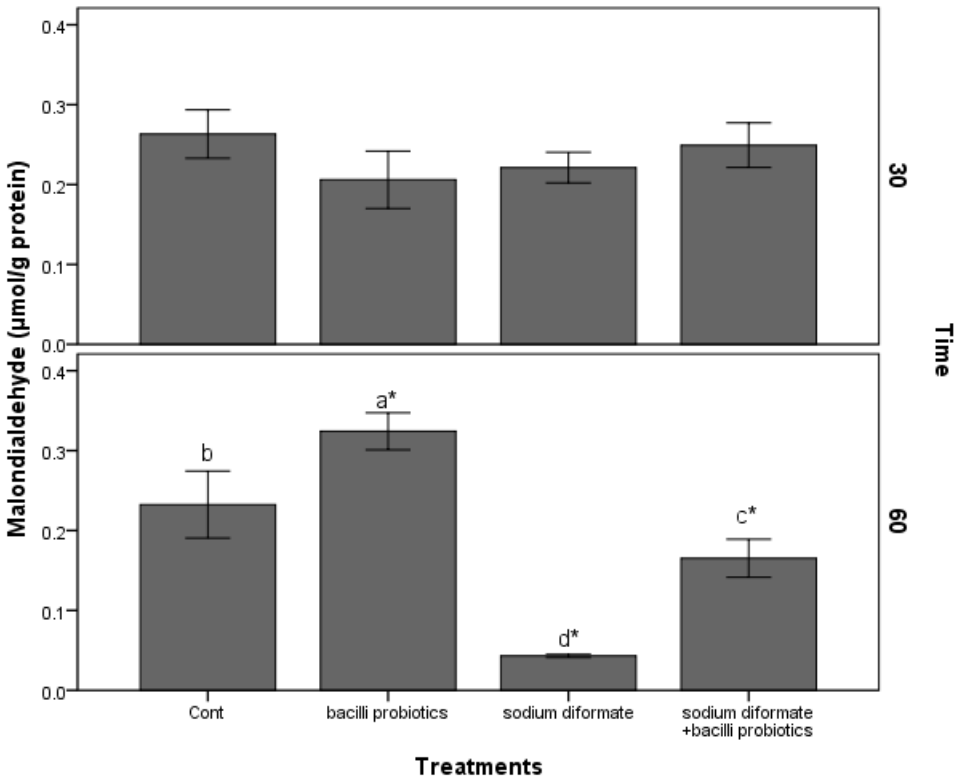

Fig. 8: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum MDA activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

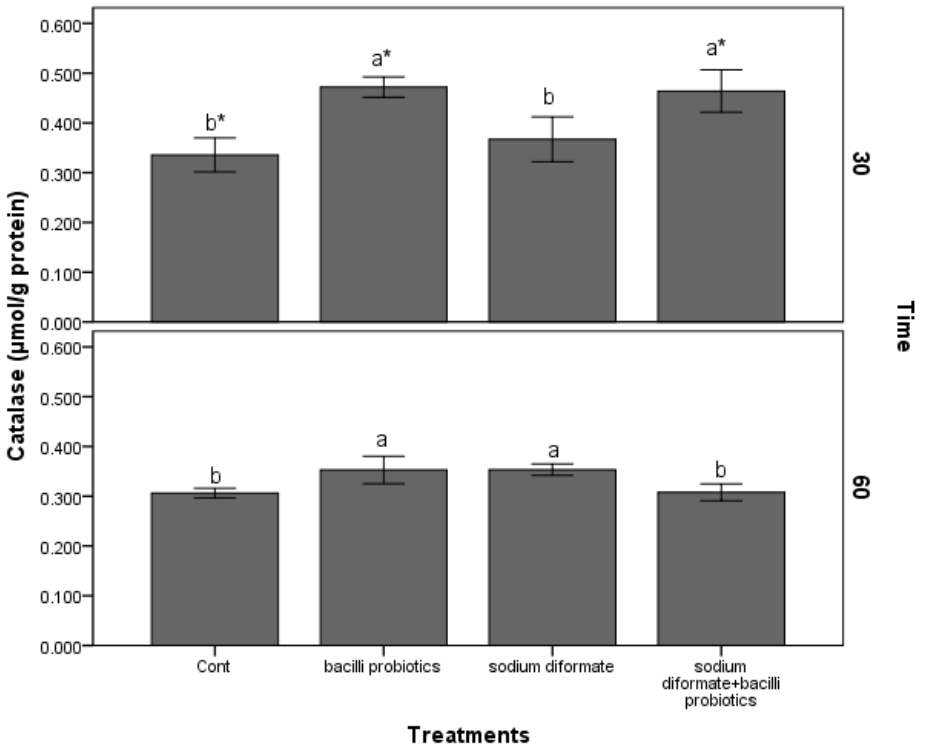

Fig. 9: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum CAT activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

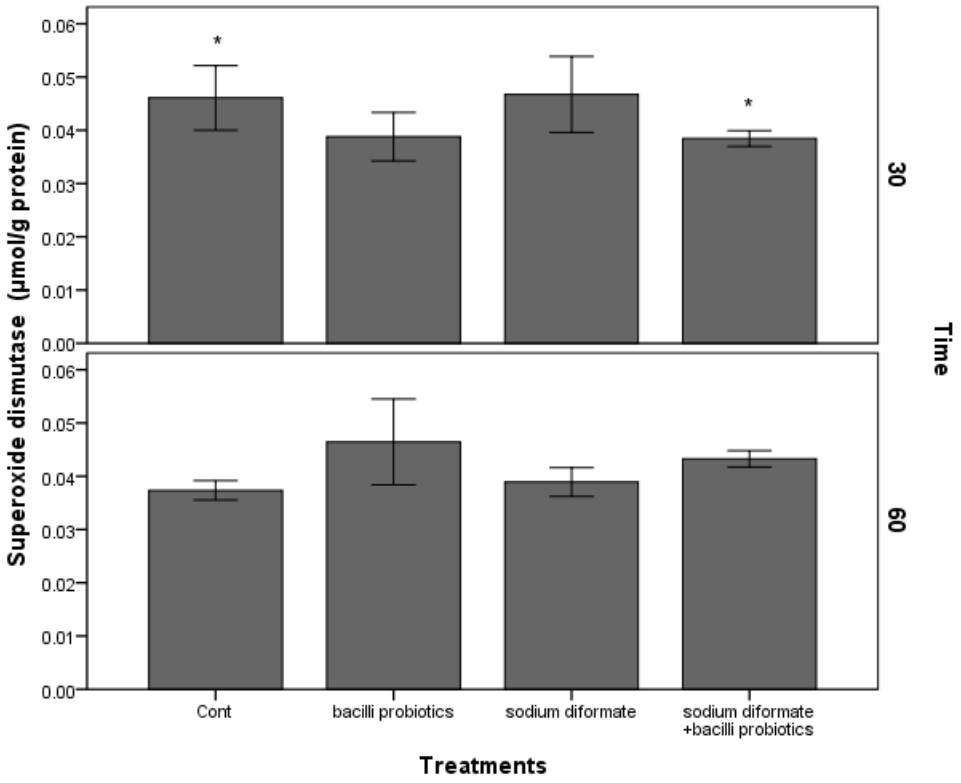

Fig. 10: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum SOD activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

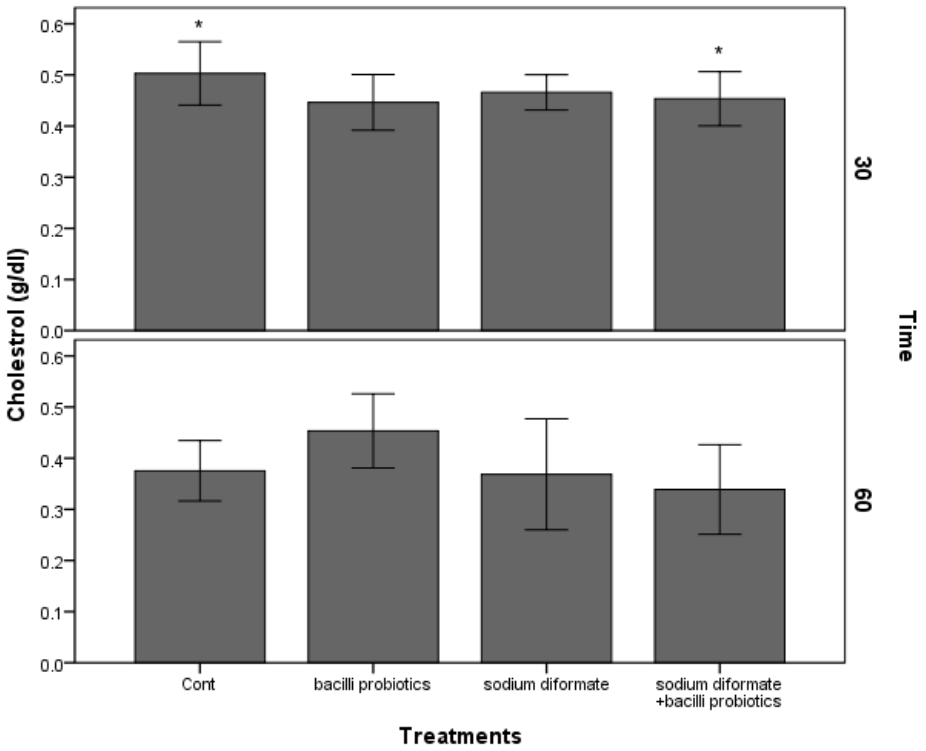

Fig. 11: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum cholesterol following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

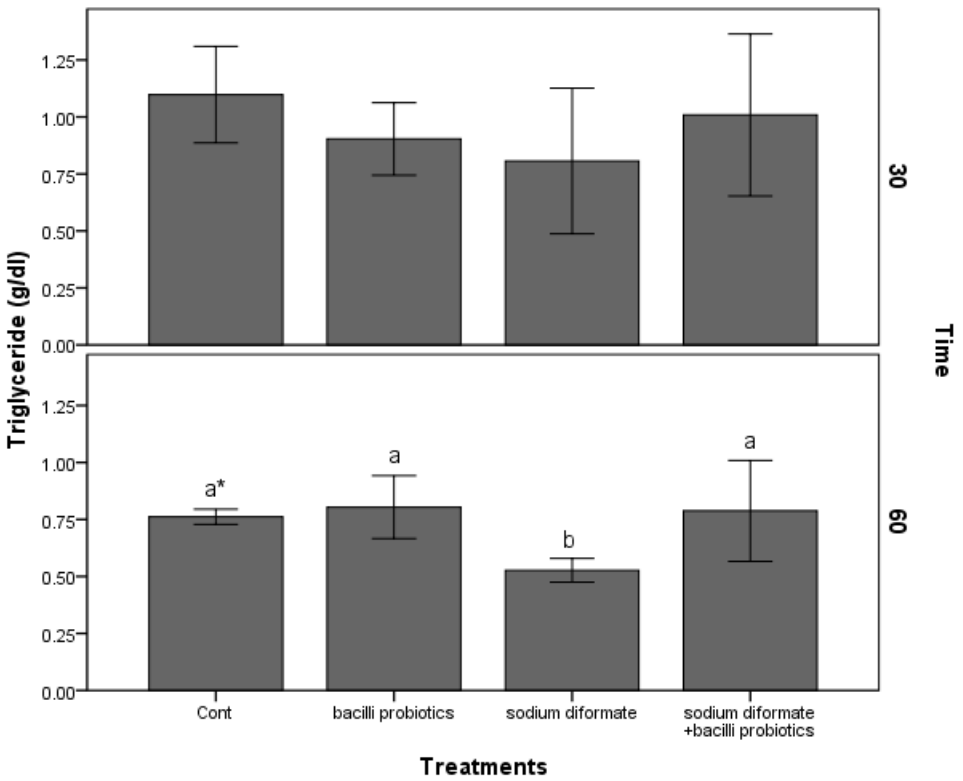

Fig. 12: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum triglyceride following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

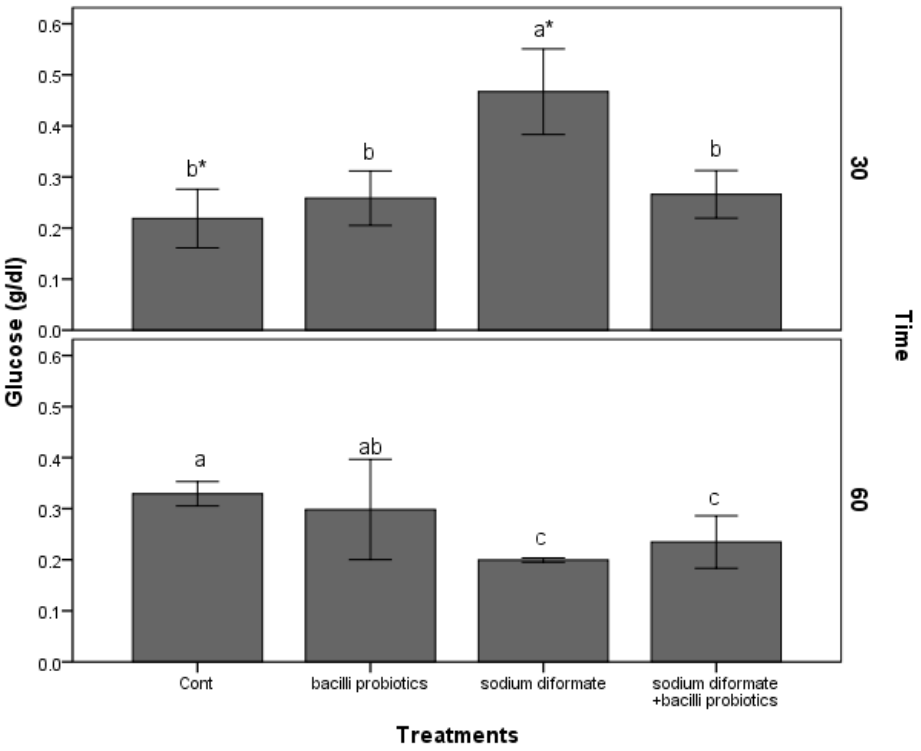

Fig. 13: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum glucose following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

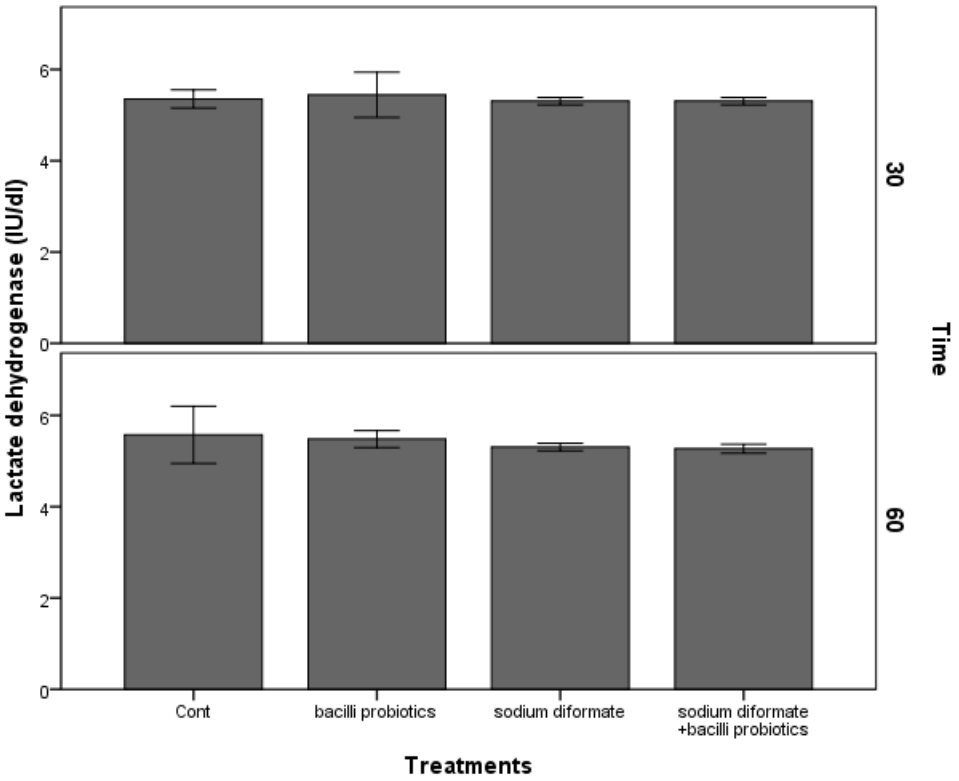

Fig. 14: The effects dietary of Bacilli probiotics, sodium diformate, and its combination on serum LDH activity following 30 and 60 days. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD.

Discussion

In the present study, we examined the single and combined effects of probiotics (Bacilli probiotics) and acidifier (sodium diformate) on some immunological, antioxidant defense, and biochemical parameters of *S. trutta caspius* during 30 and 60 days of dietary supplementation. Our results showed that Bacilli probiotics can change the immunity response of this species even following 30 days of experiments (60 days as well). The probiotics used in the present dose, can provoke serum lysozyme activity as compared to fish fed without Bacilli probiotics. Previous studies revealed that *Bacillus sp*. can boost lysozyme activity in other fish species such as *Oreochromis niloticus* and *Epinephelus coioides* (Aly *et al.*, 2008; Sun *et al.*, 2012). Contrary to our findings, Geng *et al.* (2011) reported that different levels of *B. subtilis* did not affect serum lysozyme activity in *Rachycentron canadum* for 8 weeks. They suggested that different levels of this bacterium in the diet, as well as the duration of the feeding trial, might not be enough to induce serum lysozyme activity. However, another study explained that a longer period of feeding (4 *vs*. 8 weeks) with *Bacillus amyloliquifaciens* did not affect serum lysozyme activity in *Catla catla* (Das *et al.*, 2013). Moreover, different lysozyme activity in the probiont fed fish could likely be owing to the type of fish and bacteria origin, which are different among experiments mentioned above (Geng *et al.*, 2011).

TP, Alb, and Glb levels of serum did not change significantly following Bacilli probiotics feeding in *S. trutta caspius*, which is not consistent with other studies (Zhang *et al.*, 2013). However, it might explain that this level of probiotic was not able to trigger the synthesis of protein which can be responsible for the production of immunity defense protein (Magnadottir, 2010). The ACH_{50} was elevated following 30 days of Bacilli probiotics administration. Similar to our findings, Geng *et al.* (2011) and Panigrahi *et al.* (2004) reported a higher level of complement in the serum of *R. canadum* and *Oncorhynchus mykiss* fed with *Bacillus sp*. diet. However, a similar increase but with a lesser extent was observed following 60 days of experiment in which a lower level of ACH_{50} was found at day 60 vs. day 30. This might, however, the time course for the induction of immune response by probiotics differs with respect to the type of immune parameter (Ai *et al.*, 2011). Because lysozyme has bactericidal activity, any increase in the serum lysozyme manifests the higher bactericidal activity (Alexandratos and Bruinsma, 2012), which has been observed in the present study. Most studies indicated a higher level of bactericidal activity in the serum of the fish fed with probiont, some studies showed a significant decline; for instance, Mohammadian *et al.* (2019) reported lower bactericidal activity as a result of lactic acid bacteria administration in *O. mykiss*.

Among oxidative stress responses, MDA and CAT were higher following

Bacilli probiotics treatment at day 60 rather than the control (untreated) group. This might be due to the role of *Bacillus sp*. as an antigen, which can only extend lipid peroxidation in the fish body and was able (at the tested dose) to induce antioxidant enzyme secretion system, which can effectively remove excess free radicals produced by high metabolism and adverse environmental stress, regulate the body's free radical balance and repair the damage of tissues and organs (Weifen *et al.*, 2012). On the contrary, other related parameters like SOD and MPO were not changed in the present study. Weifen *et al.* (2012) reported that higher serum SOD and no change in MPO following 4 weeks *Bacillus sp*. administration of *Ctenopharyngodon idellus*. In agreement with our findings, Sun *et al.* (2012) reported no significant change in serum SOD of *Epinephelus coioides* as a result of *Bacillus sp*. feeding. This might be related to lower bactericidal activity following Bacilli probiotics treatment, which is, in turn, responsible for the production of reactive oxygen species (Di Giulio *et al.*, 1993; Sun, 2010). Therefore, the higher phagocytosis process produces an elevated level of CAT or even not changed SOD in fish blood. However, the higher CAT or unchanged SOD activities will increase the level of lipid peroxidation in the fish body; consequently higher activity of MDA will be expected (Ayala *et al.*, 2014).

Blood biochemical analyses often provide vital information for the health assessment and management of cultured fish. The nutritional status, environmental stressors, diseases, and even immunological responses could be pursued by measuring the serum biochemical parameters (Abdel-Tawwab *et al.*, 2010). Nevertheless, the applied probiotics within the present study did not affect any serum biochemical parameters, demonstrating no adverse effect of Bacilli probiotics, tested dose on *S. trutta caspius* health status.

In the present study, 30 days of sodium diformate treatment did not lead to a significant change in serum lysozyme activity. Similar to what we found, Wassef *et al.* (2017) reported an increase in serum lysozyme activity of *Dicentrarchus labrax* following 13 weeks. Potassium diformate had also a significant effect on serum lysozyme activity in *O. niloticus* (Elala and Ragaa, 2015). Previously, some researchers reported significant increases in serum lysozyme activity of *Rutilus frisii kutum* as a result of 7-weeks of acidifier administration (Hoseinifar *et al.*, 2016). Reda *et al.* (2016) indicated that even higher doses of acidifier could enhance fish lysozyme activity, lower doses of that at the end of the feeding trial did not show any significant changes, which is in agreement with our findings that there was no significant change in the serum lysozyme activity was observed following 30 days sodium diformate feeding. It seems our acidifier dose (0.5 g sodium diformate/kg) was not strong enough to evoke this protein synthesis at a limited time.

Similar to probiotic treatment, TP, Alb, and Glb levels of serum did not change significantly following sodium diformate. Similarly, Bio Acid Ultra had no effect on the total serum protein of *O. mykiss* and *Huso huso*, manifesting that this level of acidification did not cause any metabolic stress (Khajepour and Hosseini, 2012; Safari *et al.,* 2016). Contrary to our finding, sodium diformate led to a significant increase in serum TP, Alb, and Glb of *D. labrax* (Wassef *et al.,* 2017). However, our findings showed that the inclusion of sodium diformate did not non-specific immune response in this species.

Serum ACH_{50} was reduced following 30 or even 60 days of sodium diformate administration. Contrary to our finding, Hoseinifar *et al.* (2016) reported a higher level of ACH_{50} in the serum of *Rutilus frisii kutum*. However, the present study possibly revealed that longer feeding with higher inclusion of sodium diformate lessens the beneficial role of that to trigger the fish immune system.

Furthermore, serum bactericidal activity was reduced as a result of sodium diformate treatment. On the contrary, sodium diformate led to higher serum bactericidal activity in *O. niloticus* (Reda *et al.,* 2016). It, therefore, resulted in an improvement in fish immunity. This discrepancy, however, might be the results of organic acid types and dose, which were not unique among the different studies.

Serum MPO and MDA were only elevated at 30 and 60 days of sodium diformate feeding trial, respectively while serum SOD and CAT were not changed at both feeding times. Higher serum activity of SOD following sodium alginate treatment was reported by Chiu *et al.* (2008). It is interesting to note that, the inter-specific difference among species, types of organic acids and their administrated level, and different cultural systems potentially affect the antioxidant defense system. LDH and cholesterol were not changed as a result of sodium diformate while triglyceride was reduced at day 60. Serum glucose level was elevated at day 30 and this was not continued over the 60 days, *i.e*., a significant reduction was observed. Dietary acidic Calcium Sulfate led to the lower hemolymph glucose level in *Litopenaeus vannamei* (Anuta *et al.,* 2011). They anyway concluded that a lower level of glucose possibly reflects the stressful condition of acidifier feeding. However, this conclusion could not be generalized for other feeding trials as can be seen in other reports (Khajepour and Hosseini, 2011). The lack of information regarding the effect of acidifiers on oxidative stress response in fish limits further discussion. However, the relevance of each measured parameter should be addressed in a closer look to find any underlying mechanism of these changes.

Although the application of both probiotics and acidifiers in aquaculture management is of great attention either combined and/or even a single investigation of the acidifier with other dietary agents is completely rare. It, therefore, limits a complete discussion and should be addressed in future studies. However, the absence of a

similar pattern for all combined treatments might be related to the competition of both agents (*i.e*., acidifier and probiotic), which occurred at the same time. The only limited works on the combined effect of probiotics and acidifiers on other animals were already available (Hollister *et al.,* 1990; Cakir *et al.,* 2008; Dizaji *et al.,* 2012). In addition, the effects of different acidifiers in combination with prebiotics were investigated by Tabrizi *et al.* (2012) in which they found better growth performance of *O. mykiss*. \the effect of *S. maximus* diet with potassium diformate and *Bacillus* spp. was also examined on the growth performance of fish by Fuchs *et al*. (2015).

However, it seems that the improvement of the immune response, antioxidant defense system, and serum biochemical response following this probiotic treatment (Bacilli probiotics at the present dose) was not as strong as it can able to induce juvenile *S. trutta caspius* health status. Our experiment provides evidence that when this species was administrated by sodium diformate at the tested doses did not act the same as the probiotics. A similar trend at different feeding durations (30 and 60 days) could not be observed in the case of immune, oxidative, and biochemical responses. On the other hand, combined treatment of Bacilli probiotics and acidifier revealed a mid-level response in most cases. However, this conclusion could not support other probiotics or acidifiers, or even higher or lower doses of those. Further studies should be designed to evaluate the effect of the acidifier and its combination with the probiotics on fish health status and its preventive effects against pathogenic bacteria. This, however, can reveal the effectiveness of this kind of diet on the health management of aquatic species practice.

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