Iranian Journal of Fisheries Sciences 20(3) 879-900 2021
DOI: 10.22092/ijfs.2021.350956.0

Research Article

Effects of different levels of Sodium diformate and Formic acid salt on growth performance, digestive enzymes, and innate immunological parameters of Beluga (*Huso huso*) juveniles

Jedi Mostafaloo A.[1]; Hedayatifard M.[1*]; Keshavarz M.[1]; Mohammadian T. [2,3]

Received: March 2020 Accepted: August 2020

Abstract

The purpose of this study is to evaluate the effects of Sodium diformate (NDF) and Formic acid salt on the growth performance, nutrition indices, innate immune system, and digestive enzymes activity of 420 *Huso huso* (30.55±1.72 g) were randomly divided into seven experimental treatments. All groups were fed with the experimental diets for 60 days. The experimental diets contained 0.05%, 0.1% and 0.15% NDF and 0.05%, 0.1% and 0.15% Formic acid. Diet control was without any Acidifier. Results show that the best FCR and SGR were observed in the group fed with 0.1% supplemented food by NDF and formic acid (p<0.05) on the 30_{th} and 60_{th} day , respectively. The results show that the immunological parameters including total immunoglobulin (by 0.1% NDF), serum bactericidal activity (by 0.1 %NDF and 0.1 %Formic acid), and lysozyme activity (by 0.05 and 0.1% NDF) were affected significantly by Acidifier (p<0.05). The highest activity of trypsin and chymotrypsin was significant in groups 0.1 % supplemented food by NDF (p<0.05) in the first 30 days. It can be concluded that the addition of 0.1% of NDF to beluga diets improves growth parameters decreases FCR, increases immunity levels and digestive enzymes activity.

Keywords: Acidifier, *Huso huso,* Growth performance, Immune parameters, Digestive enzymes activity

1-Department of Fisheries, Qaemshahr Branch, Islamic Azad University, Qaemshahr, Iran.
2-Department of Clinical Sciences, College of Veterinary Medicine Shahid Chamran University of Ahvaz, Ahvaz, Iran.
3-Excellence Center of Warm Water Fish Health, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
*Corresponding author's Email: hedayati.m@qaemiau.ac.ir

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[DOR: 20.1001.1.15622916.2021.20.3.15.8]

Introduction

Fish consumption and global fish demand have increased because of population growth (FAO, 2012). The aquaculture expansion will help to overcome the growth of fish demand and declining capture fisheries (Tacon and Metian, 2013). Besides the significant interest in intensive aquaculture development of non-endemic species, introducing a local species to this sector can provide an 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). Beneficial aquaculture products depend on nutritious feeding that can affect their health, weight, reproduction, and quality of meat in cultured fish (Tacon and Metian, 2013). The sturgeon is one of the most valuable fish, like Beluga *Huso huso* as species of Acipenseridae have been distributed throughout the Caspian Sea and its population faced with a recent decline due to over-exploitation, environmental pollution and destruction of spawning places. Emerging problems in natural habitats of these fish species have propelled researchers to pay much attention to their artificial reproduction, rehabilitation and restocking of sturgeon population and nutrition requirements. Beluga is one of the most important sturgeon species for aquaculture because of its adaptability with rearing conditions, rapid growth, and valuable caviar. Despite good growth indices of Beluga under natural conditions, improvement of the growth and nutrition indices under artificial conditions is an important objective. At present, the major issue in commercial aquaculture is the improvement of nutrition and feeding protocols to enhance fish growth and health conditions. The growth rate and disease resistance are two very important factors in aquaculture. On the other hand, microbial diseases are a serious threat in intensive fish farming. They have led to significant economic losses. Therefore, it is necessary to apply the required measures in these fields to improve the immunity and survival rate of fish. Some efforts have been directed toward preparing a nutritionally balanced diet for this species during the last decade (Storebakken *et al.*, 2000; Mohseni *et al.*, 2006) whilst the nutritional requirements and application of food supplementation totally remained uninvestigated for this species. Regarding the side effects of antibiotics, limited use of them, and low vaccine efficiency in fish farms, the development of other methods for disease control and the increased growth rate has been given special attention. Artificial feeds play a main role in the successful intensive aquaculture and represent the major production cost of the system. Today, one of the food additives used in aquatic animals for this purpose are organic acids. The use of dietary acidifier is an approach to promote the health conditions of fish farms. Acidifiers and their salts (mainly Na, K, or Ca) have the potential to stimulate

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growth parameters, improve welfare, and increase disease resistance and feed quality when added in sufficient amounts in diets. Dietary inclusion of citric acid/formic acid escalated growth performances and enhances the bioavailability of minerals, including phosphorus, magnesium, calcium, and iron in rainbow trout (*Oncorhynchus mykiss*), Asian Seabass (*Lates calcarifer*), Caspian Sea brown trout (*Salmo trutta caspius*) and sea bream (*Pagrus major*) (Vielma and Lall, 1997; Jun-sheng *et al.*, 2006; Kalantarian *et al*., 2020; Mohammadian *et al.*, 2020; Reshahri *et al*, 2020). Previous studies demonstrated the beneficial effects of salts of formic acid on growth performance (Ng and Koh, 2016; Hoseinifar *et al*., 2017), feed utilization, gut microbiome, innate immune responses (Abu Elala and Ragaa 2015; Wassef *et al*., 2017) and disease resistance (Ramli *et al*., 2005; Ng *et al*., 2009) (in different farmed fish species. Organic acids, like acetic, butyric, citric, formic, lactic, malic, propionic and sorbic acid have been shown to improve health and growth performance in terrestrial and marine organisms. Despite some efforts directed toward the preparation of a nutritionally balanced diet for this sturgeon species during the last decades, its nutritional requirements and the application of acidifiers and information on digestibility coefficients and data on the maximum inclusion level of that in fish are still remained uninvestigated. This work aimed to assess the efficacy of two acidifiers, sodium diformate and formic acid in different concentrations on growth performance, digestive enzyme activity and some immunological responses of juvenile *Huso huso.*

Materials and methods

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 includes 54.1% crude protein, 15% crude lipid, 0/3% fiber, and 390 Kcal/ 100g 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 *Huso huso* weighing 30.55±1.72 g was transferred from fish propagation and cultivation center in Shahid Rajaee Sari, Iran. The fish were acclimated for at least 2 weeks in an indoor 2000 L pond and were fed with a standard diet. After verifying the health status of the fish, they were distributed randomly into 21 ponds at an initial density of 20 fish per tank and divided into 7 treatment groups; including an experimental diet containing 0.05, 0.1 and 0.15 % sodium diformate (NDF) and 0.05, 0.1 and 0.15 % Formic acid (Formi). Diet control was without any Acidifier. The tanks were supplied with

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water from external biofilteres (Athmann, China), at a temperature of 20.1±1.2°C. The fish were fed with sodium diformate and Formi-contained diets for 60 days (twice a day) at a rate of 2% of the biomass. During the experimental period, temperature, alkalinity, pH, and the dissolved oxygen were measured 17.04±1.31, 250±27, 7.94±0.11 and 7.7±1.3 mg/L, respectively.

Fish growth and sample collection

To determine growth performance, the weight of all fish in each treatment was measured at the beginning of the experiment, 30 and 60 days afterward. Fish were starved for 24 h and their weight (BW_f) and length were individually measured at accuracy levels of 0.1 g and 1 mm, respectively. For evaluating serum humoral immunity, six fish from each tank (n = 18 fish per diet treatment) were anesthetized with 2-phenoxyethanol (2-phenoxyethanol at 0.3 mL l^{-1}; Merck, Schuchardt, Germany) for bleeding from the caudal vein with un-heparinized syringes. Blood specimens were transferred into vials and allowed to clot at room temperature of 25°C, then, samples were centrifuged (room temperature, 1600 *g*, 5 min), and sera were extracted and stored at −80°C until further analysis. For the evaluating digestive enzymes activities, three fish per tank (n = 9 fish per diet treatment) were randomly sampled, euthanized with overdose the same anesthetic, and immediately eviscerated on the ice surface. The alimentary tract was dissected, adherent adipose and connective tissues carefully removed and stored at −80°C until further analysis.

Growth performance and survival rate

To determine growth parameters, all fish were starved for 24h before sampling or biometry and each fish was then weighed. All growth performance and feed utilization parameters, including Body Weight Growth (BWG), Weight gain, 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× (FBW–IBW)/ IBW, WG= FBW–IBW, SGR%=100× (lnFBW–lnIBW)/ days, FCR=feed consumed/ (FBW–IBW), FCE%=(FBW–IBW)/feed consumed×100, PER=IBW/protein intake. IBW is initial body weight, FBW is final body weight and days are days of feeding (Al-Dohail *et al*., 2009; Mohammadian *et al*., 2017). The survival rate was also evaluated for the completely experimental period.

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 turbid metric assay. Briefly, sodium

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Citrate buffer (0.02 M, pH=5.8, Sigma–Aldrich) was used. The Citrate 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*., 2019b). To measure total immunoglobulin in fish serum, the total protein (TP) content of serum was determined according to the Biuret method. The basis of this method is a formation of a Cu^{2+}-protein complex in alkaline reagent and then measuring optical density at 540 nm by a spectrophotometer. Serum albumin (Alb) was also measured at 540 nm using the bromcresol green complex (Aldrich *et al*., 1998). Finally, total globulin was calculated by subtracting of Alb from TP. Agarose plates containing rabbit red blood cells were applied for detecting the activity of 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 *A. hydrophyla* 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.

Digestive enzyme activity

To analyze the activity of digestive enzymes, on days 0, 30, and 60 following acidifier feeding, the fish were starved for 24h, and nine fish of each treatment were taken randomly. The intestine was dissected out under sterile condition at 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). The total protein content of the supernatant was 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 the 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 was measured according to the modified Bernfeld method as described previously (Areekijseree *et al*., 2004) using starch solution as a substrate. Amylase specific activity was expressed as µmol maltose produced h^{-1} mg $protein^{-1}$. Lipase activity was determined based on the measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in the stabilized emulsion of olive oil (Borlongan, 1990). Protease activity

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was 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) was measured using p-nitrophenyl phosphate (pNPP) as a substrate (Otto *et al.,* 1946). Enzyme activities were 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).

Statistical procedure

Data was analyzed using SPSS ver.16.0 (Chicago, Illinois, USA). All data are presented as mean±standard error of the mean calculated from three biological replicates. Arcsine transformations were conducted on data expressed as a percentage. After confirmation of normality and homogeneity of the variance, one way ANOVA was performed at a significance level of 0.05, and Duncan's procedure was used for multiple comparisons.

Results

Growth performance

Over the 60 days feeding trial, there was no mortality observed due to the acidifier administrations. All parameters of growth performance except FCR significantly decreased with time of experiment in all treatments (even control). The fish fed for 30 days with different levels of acidifier showed significant changes in CF, SGR, FCR, PER, DWG, RGR, and FER were improved in 0.1% sodium diformate (NDF) and 0.05% sodium diformate groups as compared with the control group (p<0.05) (Table 1). The other group did not show the same changes when compared to the control fish. This pattern was not observed following 60 days of feeding, in which the best growth performance (SGR, PER, DWG, RGR, and FER) was for 0.1% Formi acidifier. The FCR did show significant differences when compared with the control group except for the 0.05% sodium diformate group (Table 2).

Digestive enzyme activities

The chymotrypsin enzyme activity at the beginning of the experiment (day 0) did not show any significant (p<0.05) changes between different treatments. Over the 30 days, all acidifier groups except the control group has increased (p<0.05) the activity in the level of this enzyme compared to the beginning of the experiment.

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Table 1: Growth performance, feed utilization and survival rate of *Huso huso* fed different levels of dietary SDF and Formi for 30 days. Values are presented as the mean ± SE. n=3.

Treatment	WI	WF	WG	CF	SGR	FCR	PER	DWG	RGR	FER
0.05 SD	31.8±0.04	54.48±0.4^{ab}	122.6±0.26^{b}	1.39±0.03^{a}	5.27±0.01^{b}	0.51±0.00^{d}	3.61±0.007^{b}	4.09±0.008^{b}	79.41±0.06^{b}	94.90±0.42^{b}
0.1SD	34.1±1.1	67.56±0.8^{a}	133.42±0.1^{a}	1.47±0.03^{a}	5.30±0.005^{a}	0.50±0.00^{e}	3.70±0.003^{a}	4.45±0.003^{a}	79.63±0.03^{a}	99.66±0.16^{a}
0.15SD	32.2±1.03	44.43±0.75^{b}	112.2±0.19^{c}	1.28±0.03^{b}	5.00±0.01^{e}	0.58±0.001^{b}	3.21±0.005^{d}	3.74±0.006^{c}	77.69±0.07^{e}	173.09±0.3^{d}
0.05FA	31.1±0.8	41.05±0.3^{b}	109.99±0.06^{e}	1.26±0.01^{cb}	5.04±0.008^{d}	0.57±0.00^{c}	3.23±0.001^{c}	3.67±0.002^{e}	77.98±0.05^{d}	74.25±0.09^{c}
0.1FA	31.8±0.94	39.44±0.5^{b}	107.64±0.06^{f}	1.18±0.02^{c}	4.93±0.001^{f}	0.59±0.00^{a}	3.13±0.001^{e}	3.59±0.002^{f}	77.20±0.01^{f}	169.12±0.1^{e}
0.15FA	31.3±0.84	43.34±0.2^{b}	112.07±0.06^{d}	1.22±0.03^{cb}	5.07±0.01^{d}	0.57±0.001^{c}	3.23±0.006^{c}	3.74±0.007	78.18±0.12^{d}	174.45±0.3^{c}
CONTROL	30.6±1.31	41.82±0.2^{b}	111.12±0.26^{d}	1.29±0.02^{b}	5.12±0.01^{c}	0.57±0.001^{c}	3.23±0.00^{c}	3.71±0.006^{d}	78.45±0.09^{c}	74.16±0.29^{c}

*WI: Initial weight, WF: Final weight WG: Body weight gain, 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. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. Different letters refer to difference between treatments (p<0.05).

Table 2: Growth performance, feed utilization, and survival rate of *Huso huso* fed different levels of dietary SDF and Formi for 60 days. Values are presented as the mean ± SE. n=3.

Treatment	WI	WF	WG	CF	SGR	FCR	PER	DWG	RGR	FER
0.05SD	154.4±0.4	315.17±*2.3*^{c}	160.69±0.28^{g}	0.85±0.009^{d}	2.38±0.005^{g}	0.95±0.001^{a}	2.02±0.003^{f}	5.36±0.009^{f}	104.02±0.33^{g}	105.26±0.18^{f}
0.1SD	167.5±0.8	353.60±*1.5*^{a}	186.04±0.51^{f}	0.91±0.008^{b}	2.49±0.006^{f}	0.85±0.002^{d}	2.26±0.006^{c}	6.20±0.01^{a}	111.03±0.42^{f}	117.45±0.32^{c}
0.15SD	144.4±0.7	322.51±*1.6*^{b}	178.08±0.3^{b}	0.92±0.01^{b}	2.68±0.002^{b}	0.84±0.001^{e}	2.28±0.003^{b}	5.94±0.01^{c}	123.30±0.13^{b}	118.51±0.20^{b}
0.05FA	141.05±0.5	312.68±*2.2*^{e}	171.64±0.29^{c}	0.92±0.001^{b}	2.65±0.002^{c}	0.88±0.001^{c}	2.19±0.003^{d}	5.72±0.009^{d}	121.69±0.18^{c}	113.77±0.19^{d}
0.1FA	139.4±05	320.48±*3.1*^{b}	181.04±0.39^{a}	0.92±0.01^{b}	2.77±0.003^{a}	0.81±0.001^{f}	2.38±0.005^{a}	6.03±0.01^{b}	129.84±0.22^{a}	124.±0.27^{a}
0.15FA	143.3±0.2	307.8±*2.1*^{c}	164.46±0.82^{e}	0.89±0.006^{c}	2.55±0.009^{e}	0.90±0.004^{b}	2.14±0.001^{e}	5.48±0.02^{e}	114.74±0.60^{e}	111.32±0.56^{e}
CONTROL	141.8±0.2	306.63±*2.6*^{c}	164.81±0.46^{d}	0.95±0.004^{a}	2.57±0.005^{d}	0.90±0.002^{b}	2.14±0.006^{e}	5.49±0.01^{e}	116.21±0.33^{d}	111.26±0.31^{e}

* WI: Initial weight, WF: Final weight WG: Body weight gain, 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. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. Different letters refer to difference between treatments (p<0.05).

The 0.1% sodium diformate, 0.05% sodium diformate, and 0.1% Formi acidifier groups were significantly (p<0.05) increased the level of chymotrypsin as compared with the control group. Although there was a significant decrease, following in all acidifiers group except formi-groups over the 60 days of administration (Fig.1). Trypsin enzyme activity was significantly higher (p< 0.05) in fish fed with diets containing acidifier as compared to the control at day 30, while 0.1% and 0.15% sodium diformate group higher levels between other groups at this time. But the highest value was observed in 0.1% formic acid and the lowest in control Feeding trial at day 60 (Fig.2). The α-Amylase and lipase enzyme activity were significantly lower at day 30 (p<0.05) in fish fed with acidifier-sodium diformate supplemented diet when compared to the beginning of the experiment (day 0).

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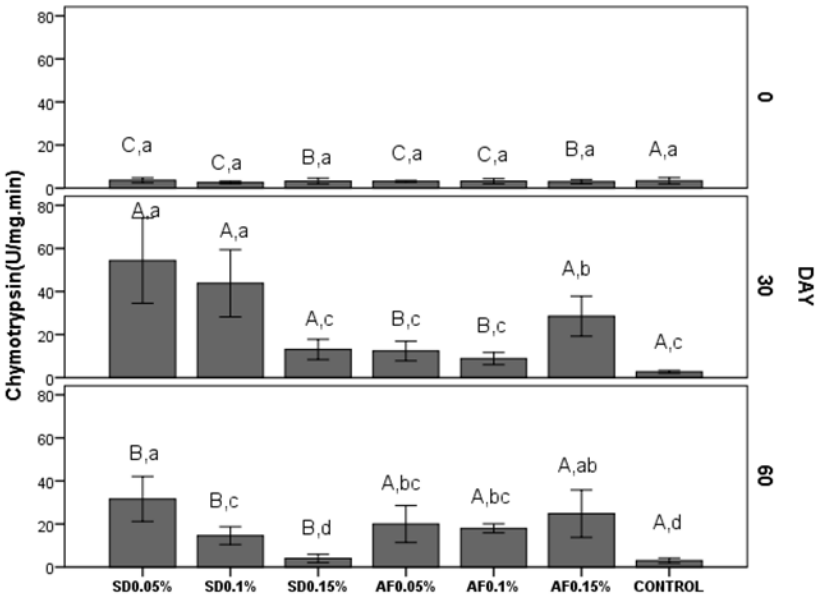


Figure 1: Chymotrypsin activity of *H huso* fed different levels (0 [control], 0.05, 0.1 and 0.15 %) of dietary Sodium diformate and Formi for 60 days. The capital letters letter shows the significant difference among sampling time and Means with the different letter is lower case letters significantly different between treatments. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. (Using one-way ANOVA and Duncan test, P value: 0.05).

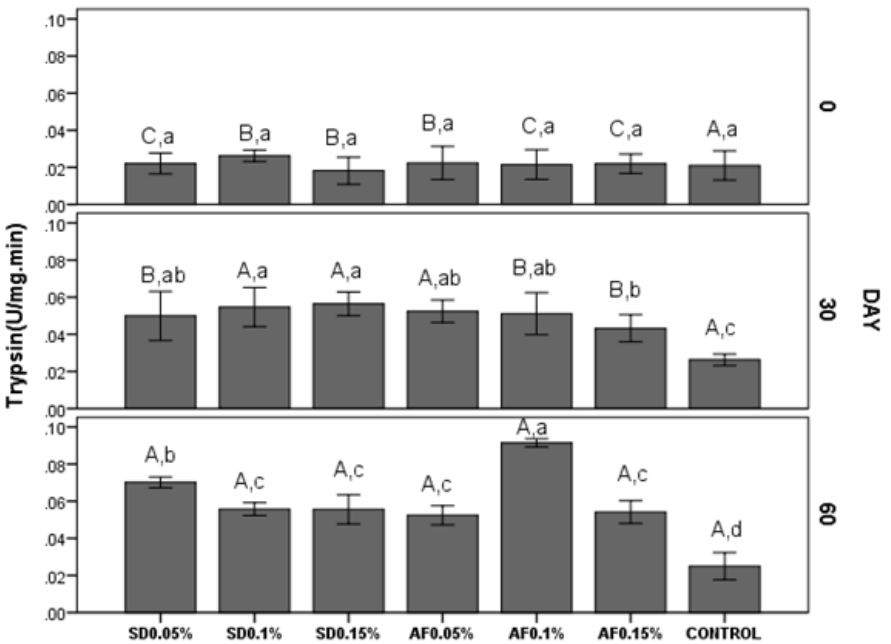


Figure 2: Trypsin activity of *H. huso* fed different levels (0 [control], 0.05, 0.1 and 0.15 %) of dietary Sodium diformate and Formi for 60 days. The capital letters letter shows the significant difference among sampling time and Means with the different letter is lower case letters significantly different between treatments. Treatment A: 0.05% Sodium diformate, B: 0/1% Sodium diformate, C: 0/15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. (Using one-way ANOVA and Duncan test, P value: 0.05).

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Although the 0.05%, 0.1% and, 0.15% formic acid led to significant rises in the level of this enzyme when compared with other treatments and control groups at day60 (Fig. 3). Protease enzyme activity significantly increased in all treatments after 30 days (p<0.05), but except 0.05% and 0.1% sodium diformate, Protease enzyme activity in other treatments was significantly lower in day 60 (p<0.05) compared to the 30 days post-feeding (Fig. 4). The higher level of this enzyme was observed following 30 days feeding with 0.15% sodium diformate group while after 60 days post-feeding group did not act the same (Fig. 5). The gut ALP activity was significantly higher (p<0.05) in all acidifier fed groups compared to fish fed with the control diet at day 30. The ALP activity was significantly higher (p<0.05) in the treatment 0.05% formic acid and 0.1% formic acid as compared to their other acidifier groups at the same time. In addition, ALP reduced significantly following all acidifier group following 60 days of feeding (Fig. 6).

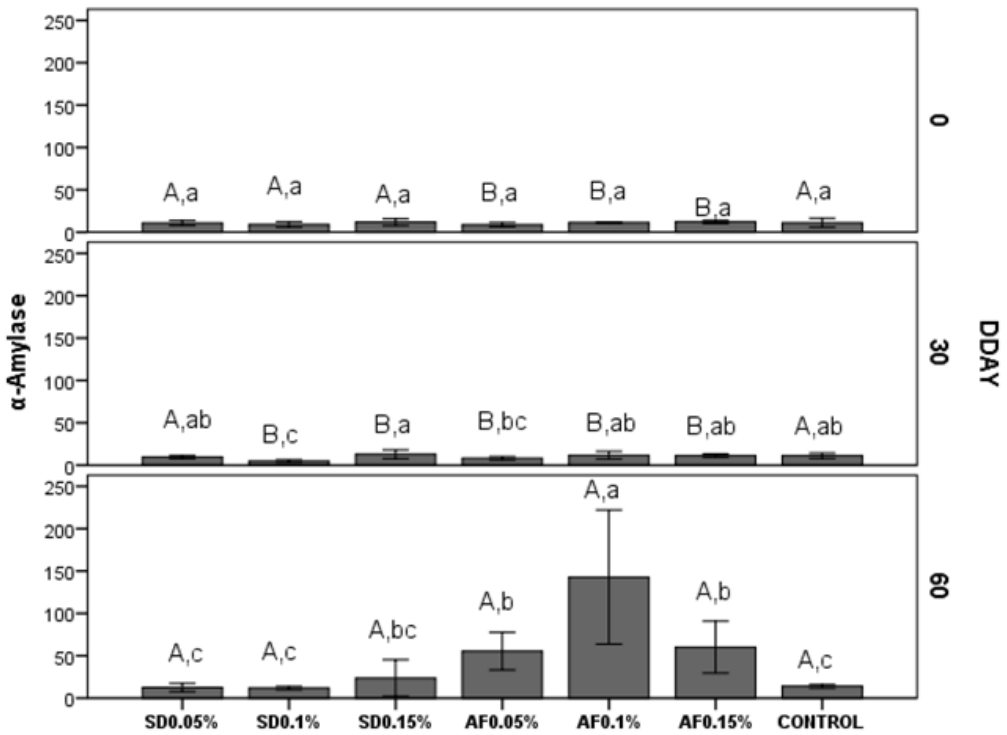


Figure 3: α-Amylase activity of *H. huso* fed different levels (0 [control], 0.05, 0.1 and 0.15 %) of dietary Sodium diformate and Formi for 60 days. The capital letters letter shows the significant difference among sampling time and Means with the different letter is lower case letters significantly different between treatments. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. (Using one-way ANOVA and Duncan test, P value: 0.05).

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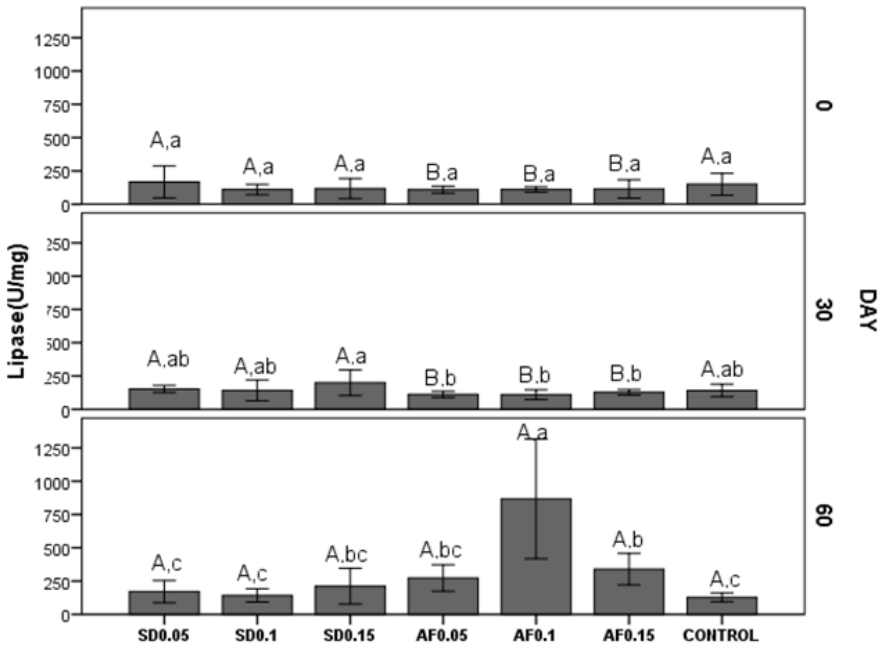


Figure 4: Lipase activity of *H. huso* fed different levels (0 [control], 0.05, 0.1 and 0.15 %) of dietary Sodium diformate and Formi for 60 days. The capital letters letter shows the significant difference among sampling time and Means with the different letter is lower case letters significantly different between treatments. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. (Using one-way ANOVA and Duncan test, P value: 0.05).

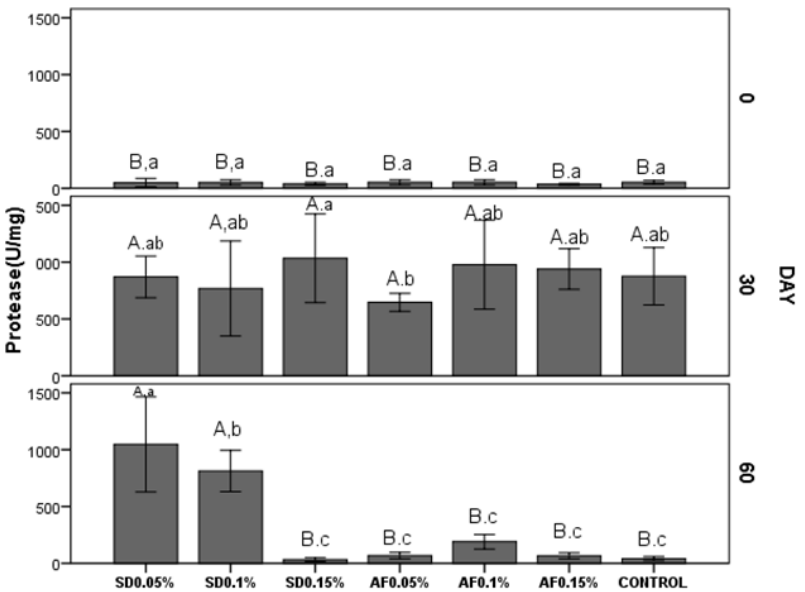


Figure 5: Protease activity of *H. huso* fed different levels (0 [control], 0.05, 0.1 and 0.15 %) of dietary Sodium diformate and Formi for 60 days. The capital letters letter shows the significant difference among sampling time and Means with the different letter is lower case letters significantly different between treatments. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. (Using one-way ANOVA and Duncan test, P value: 0.05).

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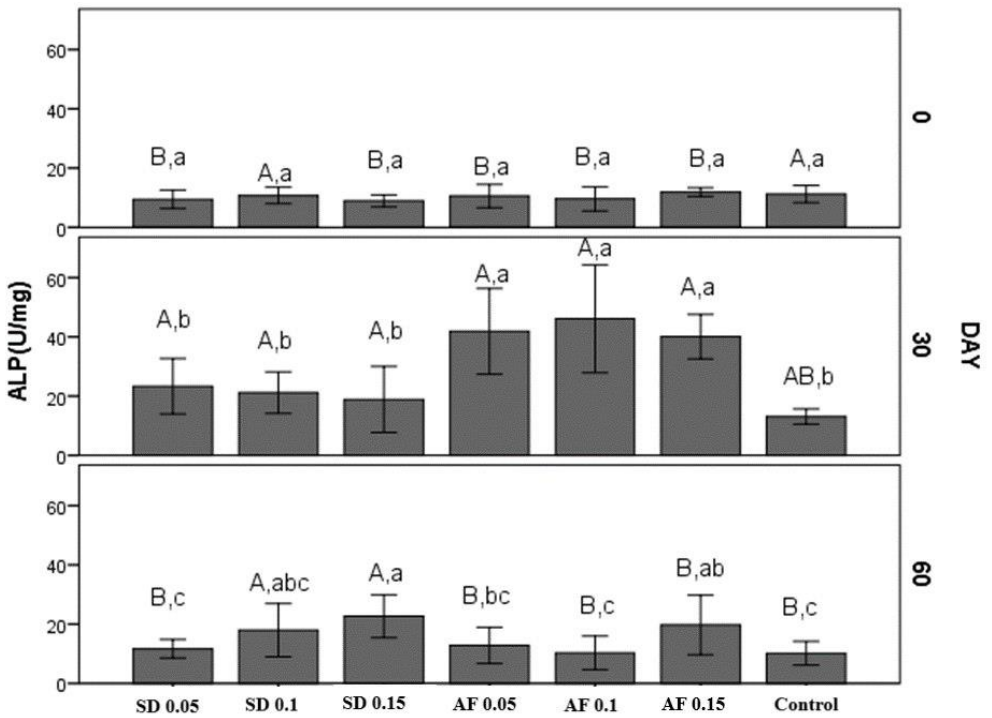


Figure 6: ALP activity of *H. huso* fed different levels (0 (control), 0.05, 0.1 and 0.15 %) of dietary Sodium diformate and Formi for 60 days. The capital letters letter shows the significant difference among sampling time and Means with the different letter is lower case letters significantly different between treatments. Treatment A: 0.05% Sodium diformate, B: 0.1% Sodium diformate, C: 0.15% Sodium diformate, D: 0.05% Formi, E: 0.1% Formi, F: 0.15% Formi. (Using one-way ANOVA and Duncan test, P value: 0.05).

Non-specific immune responses

The results of one-way ANOVA of non-specific immune response parameters of fish fed with a diet containing different concentrations of sodium diformate and Formic acid salt (Formi) have been presented in "Table 3". Serum lysozyme activity increased at 30 days in all acidifier treated groups except 0.15% sodium diformate and 0.1% Formi group and a significant increase was observed in 0.1% sodium formate and 0.05% Formi compared to the start of the experiment (p<0.05). Meanwhile, after 60 days the highest and lowest in serum lysozyme activity were observed in 0.1% sodium and 0.1% Formi groups respectively (p<0.05). In comparison to the controls, complement activity was not significantly different in all acidifier supplemented groups in sampling at day 30 (p>0.05). After 60 days, the complement activity showed the increase values in all acidifier groups (p>0.05), However, no significant differences were observed between the control and the other treated groups (p>0.05). Total immunoglobulin at day 30, all treatments had a significant difference with the control group (p<0.05). The highest value of this parameter was observed in 0.1% sodium deformate and the lowest value in the control group.

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Table 3: Immune parameters of *Huso huso* fed different levels of dietary SDF and Formi for 30 and 60 days. Values are presented as the mean±SE. n=9.

Factors	Time	0.05 SD	0.1SD	0.15SD	0.05FA	0.1FA	0.15FA	CONTROL
Lysozyme	0	172.2± 12.82^{A,a}	151.85±61.6^{B,a}	173.33±82.2^{A,a}	162.96±36.51^{B,a}	170.92±58.79^{A,a}	151.1±57.52^{AB,a}	174.9±61.6^{A,a}
	30	241.81±18.1^{A,ab}	233.33±69.9^{A,a}	151.85±55.1^{A,c}	214.81±50.0^{A,ab}	166.66±33.7^{A,c}	207.4±60.72^{A,ab}	168.5±57.5^{A,c}
	60	182.22±18.59^{A,bc}	266.66±61.26^{A,a}	207.4±62.32^{A,ab}	144.44±41.57^{B,bc}	196.29±51.4^{A,bc}	133.33±44.4^{B,c}	159.2±73.5^{A,bc}
Complement	0	3.8±0.57^{A,a}	3.9±0.44^{B,a}	3.8±0.83^{A,a}	4±1^{A,a}	4±0.81^{A,a}	4.2±0.54^{A,a}	3.83±0.04^{B,a}
	30	3.83±0.98^{A,a}	3.83±0.4^{AB,a}	4.16±1.16^{A,a}	4±0.63^{A,a}	3.66±0.51^{A,a}	4±0.89^{A,a}	3.16±0.4^{B,a}
	60	4.1±1.32^{A,a}	4.1±0.98^{A,a}	4.33±0.51^{A,a}	3.83±0.75^{A,a}	4.5±1.04^{A,a}	4.66±1.21^{A,a}	4.5±0.54^{A,a}
Globulin	0	3.08±0.31^{B,a}	3.15±0.21^{B,a}	2.973±0.52^{B,a}	2.97±0.51^{B,a}	2.95±0.34^{B,a}	2. 8±0.32^{B,a}	3.13±0.19^{A,a}
	30	4.8±0.64^{A,b}	6.3±0.57^{A,a}	5.72±0.4^{A,ab}	5.48±0.46^{A,ab}	6.13±1.27^{A,a}	5.12±0.98^{A,b}	3.08±0.25^{A,c}
	60	3.04±0.8^{B,a}	2.71±0.41^{B,ab}	2.23±0.26^{B,b}	2.76±0.15^{B,ab}	3±0.4^{B,a}	2.5±0.59^{B,ab}	2.42±0.32^{B,ab}
Bactricidal	0	351.8± 45^{a,A}	364.6±34^{a,A}	367.3±36^{a,A}	344.3±46^{a,A}	360.6±58^{a,A}	374.3±59^{a,A}	344.3±46^{a,A}
	30	346.8± 61^{a,A}	298.5± 22^{b,B}	304.3±46^{b,AB}	297.3±33^{b,B}	321.3±54^{a,A}	262.6±40^{b,B}	346.3± 73^{a,A}
	60	356.5± 44^{a,A}	274.6±46^{b,B}	368.5±73^{a,A}	346.4±35^{a,A}	255.8± 46^{b,B}	341.3± 73^{a,A}	566.3±59^{a,A}

*The capital case letter shows significant difference between feeding times within the same Acidifiers level and Means with the different letter is lower case letters significantly different between Acidifiers levels within the same feeding time (p<0.05, using one-way ANOVA).

On day 60, no significant differences were observed between the control and the acidifier groups (p>0.05) (Fig. 6). Serum bactericidal activity increased by 0.1% and 0.15% sodium formate and 0.05% formic acid treated groups compared to the control group in 30 days (p<0.05). Only on day 60 significant differences were observed between the control and 0.1% sodium formate (p<0.05). It should be noted that whatever more bacterial colonies in the serum were lower; the greater bactericidal capacities of the serum were showed.

Discussion

Commercial feed producers tried to find additive with positive efficacy due to the little information about preparation of diets for brackish water species like *H. Huso*. The use of short-chain organic acids and their salts is increasing due to its beneficial effects as a dietary supplement in animals (Abu Elala and Ragaa, 2014; Mohamadi Saei *et al*., 2016). The effect of using organic acids on fish performance varies widely and depends on many factors such as fish species, age, types, and levels of organic acids used (Tran-Ngoc *et al.*, 2018). The growth factor in fish fed on organic acids is believed to increase and enhance mineral and nutrient digestibility (Omosowone *et al*., 2018). There have been some studies of ineffectively and even reports of the negative effects of these supplements. Fish feeds should be adequately supplemented with natural feed additives such as Sodium di format (Liebert *et al*., 2010) or Formi acidifier, which have generated increasing interest in the industry. This is the first study to investigate the effect of different levels of sodium formate and formic acid on growth performance, innate immune response, and digestive

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enzyme activity in *Huso huso*. Fish fed diet containing 0.1% sodium diformate had the highest final bodyweight that was associated with the higher FI as well as improved health status in this group. Body weight gain and specific growth rate in fish fed 0.1% sodium formate at day 30 and 0.1% Formi at second 30 days after start experiment were higher than other groups. The growth-promoting effects of NDF in this study can be explained by the pH reduction of gut that may stimulate pepsinogen activity and/or synthesis (Castillo *et al*., 2014), enhance proliferation of beneficial acid-tolerant bacteria like LAB (Luckstadt, 2008) and increase digestibility of dietary nutrients and minerals that may consequently improve nutrients absorption and growth rate (Ng *et al*., 2015; Hoseinifar *et al*., 2016). In this study, food conversion ratio (FCR) in-group 2 also showed a significant decrease compared to the other groups and the control group. Previous research has shown that the use of citric acid as an attractant in the diet of *Huso huso* has increased and improved growth and nutrition parameters (*Sudagar et al., 2010*). Hassaan *et al*. (2014) express that organic acids reduced FCR in *O. niloticus* and improved growth indices, which is in parallel with our results. The results of our work up to 60 days are in accordance with the studies of DeWet *et al.* (2005) on rainbow trout (*Oncorhynchus mykiss*) when fed an organic acid blend supplement mainly consisting of formate and sorbate, and Wassef *et al*. (2017) reported sodium diformate (3%) as growth promoter in *Dicentrarchus labrax* following 13 weeks. Abu Elala *et al.* (2015) stated that adding 0.2% and 0.3% potassium di-formate to fish feed (*Oreochromis niloticus*) improved feed intake, weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio and improve protein digestibility. Khaled (2015) stated that the addition of sodium formate (NDF) to the diet of tilapia fish has potential beneficial effects on the growth and feed intake of this fish. Also, some other studies revealed that formic acid salts did not influence growth performance in various farmed fish species such as red hybrid tilapia (Ng *et al*., 2009; Ebrahimi *et al*., 2017) and African sharp tooth catfish (*Clarias gariepinus*) (Asriqah *et al*., 2018; Omosowone *et al.*, 2018). Acidifier affected most of the growth parameters in the present study in the second 30 days of the experiment, but the specific growth rate, weight gain, protein efficiency ratio, and condition factor increased in the group that feeds diet containing 0.1% formic acid. There was a significant difference between the other groups and the control group in the second 30 days. Sugiura *et al*. (1998) state that the addition of formic acid increased the absorption of magnesium and calcium in *O.mykiss*. Some researchers have also stated (Pandey and Satoh, 2008; Ng *et al.*, 2009) that organic acids have not significant effect on aquatic growth factors, which is inconsistent with the results of this

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study, which may be due to inorganic acids or species. According to the findings of this study, the use of organic acids mixture (0.1% sodium dichromate) can be used as a growth stimulator and reduce FCR in diet and consequently causes reduce the cost of production.

All growth indices in all groups decreased during the second 30 days of the experiment compared to the first 30 days. Long-term dietary supplementation with 0.1% sodium diformate in the diet may reduce the role of acidifier and decrease the growth performance of fish due to internal interactions with the physiological normal functions of the fish's gastrointestinal microbiota. However, in the long-term, 0.1% Formi treatment was able to improve the growth process in fish which fed this acidifier supplement for 60 days. The results showed that the amount and type of acidifier could affect the function of the gastrointestinal tract of various fish. In this study, by comparing the data obtained on day 60 *vs*. day 30, suitable dose and appropriate acidifier manifests the signs of better function.

Evaluation of digestive enzyme activity level can be used as a suitable indicator to compare fish growth coefficient, food intake, and digestive capacity. In the present study, digestive enzymes trypsin and chymotrypsin were significantly increased on day 30_{th} in groups of treated with 0.1% sodium diformate but by day 60_{th} with decreased activity. Proteolytic enzymes were found in the sodium diformate groups, however, dietary treatments supplemented with Formic acid, especially 0.1% group, increased digestive enzymes trypsin and chymotrypsin, which it was in line with the results of fish growth performance. In agreement with our findings, 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). Trypsin and chymotrypsin are proteolytic enzymes that can increase activity by Ca^{2+} and Mg^{2+}. Many lactic acid bacteria produce a wide variety of exogenous digestive enzymes that are responsible for the uptake of minerals (Zhou *et al.,* 2009). Probably the reason for the increase in trypsin and chymotrypsin can be attributed to the increase in the number of lactic acid bacteria in the intestine and the enzymes secreted by these bacteria. However, the activity of alkaline phosphatase and protease activity was shown significantly different from other enzymes. Alkaline phosphatase activity was decreased in sodium diformate treatments on day 30_{th} of the experiment, but 0.1% formic treatment showed a significant increase in this time. However, the total protease activity increase was only observed on day 60 in 0.05% and 0.1% sodium diformate treatments, which was probably related to the positive effect of these treatments overall growth process of *Huso huso* but we did not observe this trend in Formi treatments.

The immune parameters were another aspect of the beneficial effects

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of dietary administration of SDF on fish physiology. Acidifiers used in the present study, especially 0.1% sodium diformate and 0.1% Formi treatments on day 30_{th} and 0.1% sodium diformate on day 60, were provoked serum lysozyme activity as compared to control. Similar to our results, previous studies revealed that Sodium propionate could boost lysozyme activity in other fish species such as Caspian white fish and *Oreochromis niloticus* (Hoseinifar *et al.,* 2016; Reda *et al*., 2016). However, in contrast to our findings, supplementing diets with NaDF, humic or citric acids did not have any influence on lysozyme serum in rainbow trout (Yilmaz *et al.,* 2018), common carp (Krome *et al.,* 2018) and turbot (Dai *et al.,* 2018). Feeding of the juvenile grouper *Epinephelus fuscoguttatus* with 1.0 or 2.0 g/kg sodium alginate-containing diet showed increased immune response activities (Chiu *et al.,* 2008). They suggested that different levels of this acidifier in the diet, as well as the duration of the feeding trial, might not be enough to induce serum lysozyme activity. The results of the present study revealed that fish fed diets supplemented with 0.1% sodium diformate had the highest total globulin contents and the other treatments showed intermediate values on day 30 but we faced a significant decrease in all treatments except control treatment at day 60 compared to day 30. In this regard, it has been shown that dietary supplementation of various dietary such as malic acid (Hassan *et al.,* 2015) and NaDF (Wassef *et al.,* 2017) increased serum total protein in Nile tilapia and European sea bass, respectively. This process in our study may be due to an increase in the number of lactic acid bacteria that in the short term lead to an increase in serum immunoglobulin. In parallel with our result, Khattab *et al.* (2005) showed a reduction in plasma total proteins in fish fed with diet containing probiotics. The data for the serum total protein, which is a reflection of innate immunity (Wiegertjes *et al.,* 1996). Sun *et al.* (2010) and Mohammadian *et al.* (2016) observed that probiotic-supplemented diets stimulated serum Ig level in *E. coioides* and *Tor grypus* ,respectively, until day 30, after which the Ig levels decreased in probiotic supplemented groups. These reports suggest that the augmentation of immunoglobulin levels is a short-term phenomenon attributable to probiotics. We observed a similar trend in the bactericidal parameter. This might be related to higher bactericidal activity following acidifier treatment, which is, in turn, is responsible for the production of reactive oxygen species. The cause could be due to organic acids can influence the indigenous intestinal flora, which is necessary for the development of the gut immune system. In this study, the Acidifier-fed groups had no significant difference in complement activities rather than the control group. There is limited information available regarding the effects of acidifiers on fish immune responses (Ng *et al.,* 2017). There are very contradictory results in some non-

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specific immune system of the *Huso huso*.

In conclusion, the results obtaining for the present study indicated that the acidifier did not show a similar trend at different feeding duration (between day 30 and day 60) on growth performance and immune responses. Generally, it can be stated that adding 0.1% sodium diformate for 30 days has the best efficiency, but if used for 60 days, it reduces the usefulness for beluga but 0.1% formic acid in the second 30 days showed better performance than the first 30 days, this treatment is indicating a long term oral administration better than the short term. Compared to acidifier treatments it can be concluded that sodium diformate in concentration , especially the 0.1 % at day 60 in the feed significantly increased and improve the immune system, growth performance and reduce the feed conversion ratio (FCR).

Acknowledgments

This work was funded by a grant from Islamic Azad University of Qaemshahr and Excellence Center of Warm Water Fish Health of Shahid Chamran University of Ahvaz.

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