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Research Article

The Effects of Different Levels of Potassium Diformate and Calcium Diformate on Growth, Digestion Enzyme Activity, Antioxidant Capacity, Intestinal Flora, Stress Markers, and Some Serum Biochemical Analytes in Juvenile Beluga *Huso huso*

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Potassium diformate (KDF) and calcium diformate (CaDF) are organic acids that modulate growth performance, stress, and biochemical status. So, the present study aimed to investigate the effects of different levels of potassium diformate and calcium diformate on growth performance, stress markers, oxidant/antioxidant status, microbial flora, and some serum biochemical analytes in juvenile Beluga (mean weight: 34 ± 4.5 g). Juvenile Beluga fed control food or diet supplemented with different levels of KDF (1, 1.5, and 2 g/kg) and CaDF (1, 1.5, and 2 g/kg) for 60 days. The results showed that fish fed the 0.15% (1.5 g/kg) KDF showed the favorable growth value at 30 days and relatively less, 0.15% CaDF improved significantly ($P<0.05$) fish growth performance following 60 days of application ($P<0.05$). The results showed that dietary administration of KDF and CaDF significantly ($P<0.05$) increased digestive enzymes. Moreover, elevated biochemical parameters were observed in *H. huso* fed KDF and CaDF supplemented the diet. Serum oxidant/antioxidant status was significantly ($P<0.05$) improved in the KDF and CaDF treatments than the control group. Moreover, dietary administration of KDF and CaDF significantly ($P<0.05$) decreased stress markers of *H. huso* after 60 days. The liver enzymes activities considerably altered in the KDF and CaDF groups compared with the control group after 60 days. Furthermore, dietary administration of KDF and CaDF significantly ($P<0.05$) increased intestinal lactic acid bacteria (LAB) of *H. huso* after 60 days. Based on the results of this study, it appears that incorporating KDF and CaDF into the diet of *H. huso* can have positive effects on their growth performance and physiological response. The study found that a short-term use of 0.15% (1.5) g/kg of KDF for 30 days was the most effective in promoting growth. However, the potential use of 0.2% (2 g/kg) of KDF and 0.15% (1.5 g/kg) of CaDF for a longer period of 60 days may also be beneficial.

1. Introduction

Aquaculture is an important contributor in many countries. However, in large-scale production facilities and due to the fast growth of aquaculture, where aquatic animals are exposed to stressful conditions, problems related to disease, and deterioration of environmental conditions often occur and result in serious economic losses. This has led to the indiscriminate use of antibiotics to slow the spread of disease and, to some extent, stop infectious agents from appearing [1]. Concerted research efforts have therefore been focused on optimizing production through environmentally friendly alternatives to the use of chemotherapeutic agents [2, 3]. Researchers are thus interested in finding alternatives to antibiotics that have comparatively less negative effects. Probiotics, plant extracts, organic acids, etc., are some examples of these compounds. Organic acids are viewed as feed additives or dietary supplements providing scope for more robust nutrition

without the need for antibiotic-type growth promoters, which have been banned from use within the EU since January 2006. By balancing the intestinal microbiota in animal feed, these substances and their salts referred to as "acidifiers" have been introduced as a maintenance and growth booster. By bringing down the pH of the stomach, acidifiers help with mineral digestion and also trigger the release of certain enzymes [4].

Studies on the use of acidifiers in the fish diet for promoting growth and improving hematological and biochemical parameters, stress markers, oxidant/antioxidant status, and microbial flora have been conducted [5–15]. It has been shown that acidifiers such as formic, lactic, butyric, propionic, and malic acids, as well as potassium diformate (KDF), calcium diformate (CaDF), sodium diformate (SDF), and calcium diformate (CaDF), increase the health and development of a variety of aquatic creatures [16]. The beluga (*H. huso*) is a highly prized fish species known for its quick adoption of tailored meals, quick development in comparison to other acipensers, excellent quality meat, great stress tolerance, and relatively high market value [17–19]. On the southern shores of the Caspian Sea, in Eastern Europe and in Japan, this type of sturgeon is one of the most prominent farmed fish [20, 21]. To ensure the sustainable production of beluga sturgeon, it is crucial to optimize its nutritional requirements, select appropriate diets, and provide effective dietary supplements. Given the significance of beluga sturgeon in Iran and globally, it is noteworthy that this species lacks teeth but remains a carnivore, which underscores the presence of a robust digestive system suitable for high-protein diets. Therefore, the utilization of acidifiers as a dietary supplement holds considerable importance. According to the research that has been done on sturgeon and other fish in the past [22, 23], considering that potassium diformate and calcium diformate acidifiers (used in this study) have stronger acid properties and probably due to the unique structure of the digestive system of these fishes (the stomach acidity of sturgeon fish is higher than that of fishes that have been studied before such as salmon and tilapia), the concentration and type of organic acid used In this study, it will be required to a lesser extent. All of this makes it essential to further our knowledge of how calcium and potassium diformate affect aquatic organisms. The goal of this study was to evaluate the effects of various potassium and calcium diformate concentrations on the development efficiency, stress indicators, oxidant/antioxidant status, microbial flora, and a few serum biochemical analytes in juvenile Beluga *H. huso*.

2. Materials and Methods

2.1. Fish. The Animal Ethics Committee of Shahid Chamran University in Ahvaz gave its approval to all studies, which were carried out in compliance with generally accepted ethical standards (approval number: EE/98.11.2.51020/scu.ac.ir). Eight hundred forty juvenile Beluga (mean weight: 34 ± 4.5 g) were delivered to the aquatic animal health research lab of the school of veterinary medicine at Shahid Chamran University of Ahvaz from one of the fish farms in Ahvaz. Fish were given commercial meals twice daily for the 2 weeks that they were acclimated to the lab's 12 hr light/12 hr dark photoperiod. Following the acclimatization phase, fish were randomly dispersed among 21 tanks (each tank containing 1,500 L sand-filtered water and was connected to a flow-through system (2 L/min) and had 40 fish) with comparable water volume, quantitative, and qualitative conditions using seven treatments. The physicochemical characteristics of the water throughout the experiment were as follows: temperature (°C): 22.5 ± 0.7, dissolved oxygen (mg/L): 7 ± 0.3, salinity (ppt): 1.2, pH: 7.8 ± 0.3, and total hardness (ppm): 250 ± 27.

2.2. Diet Preparation and Rearing Period. The base diets (FFS2 (diet contained 44% crude protein, 14% crude fat, and 2% crude fiber) and GFS1 (diet contained 42% crude protein, 14% crude fat, and 2% crude fiber), Alltech Coppens Co., Germany) were created for the experimental diets, and they were supplemented with potassium and calcium diformate (Bioproducts bahman Co., Tehran, Iran) using the techniques described in other research [5, 6]. In a nutshell, acidifiers were measured out at the required quantities (1, 1.5, and 2 g/kg), dissolved in normal saline, and then added to meals with gelatin. Using a meat grinder, the resulting doughy mixture was formed into pellets. The pellets were then bagged and kept in a refrigerator at 4°C until they were needed, when they were air dried at ambient temperature for 1 hr. Every 10 days, the weight of the fish was recorded (digital scale, Mahak Company, Iran) to calculate the ratio of the diet. Fish were fed 3% of their body weight each day, three times a day at 08:00, 14:00, and 20:00 throughout the course of the research. The technique suggested by Baruah et al. [24] was used to test the pH of the feed. In a nutshell, 50 mL of deionized water and 5 g of feed were combined for 1 min with a magnetic stirrer in a China plant. The pH of the solution was tested after feed homogenization. Experimental diets were included control (without supplementing KDF and CaDF), KDF 0.1% (1 g/kg potassium diformate), KDF 0.15% (1.5 g/kg potassium diformate), KDF 0.2% (2 g/kg potassium diformate), CaDF 0.1% (1 g/kg calcium diformate), CaDF 0.15% (1.5 g/kg calcium diformate), and CaDF 0.2% (2 g/kg calcium diformate).

2.3. Sampling. For blood samples and digestive enzyme assays, three fish were chosen at random from each tank, anesthetized with 0.5 mL/L of 2-phenoxyethanol, and brought out of the tanks. A 2.5 mL sterile syringe was used to take blood from the caudal vein and utilize the blood to create serum (three fish per replication, a total of nine fish per treatment). The blood samples were then allowed to coagulate at room temperature before being centrifuged for the purpose of separating the serum (3,000 g for 10 min at 4°C) [25]. After that, each sample was kept at −80°C until analysis.

2.4. Growth Measurement. At the beginning (0 days), middle (30 days), and end of the trial (60 days), total fish biomass in every tank was anesthetized (0.5 mL/L of 2-phenoxyethanol) and their weight (g) and length (cm) were measured

individually. The data obtained from each group were used to calculate the feed utilization and growth parameters. Daily weight gain (DWG), weight gain (WG), specific growth ratio (SGR), condition factor (CF), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated for each group as follows:

$$\mathrm{DWG} = \frac{(\mathrm{WF} - \mathrm{WI})}{\mathrm{days}}, \quad (1)$$

$$\mathrm{SGR\,(body\ weight\,(\%)/days)} = ((\mathrm{Ln}\,W_F - \mathrm{Ln}\,W_I)/\mathrm{t}] \times 100, \quad (2)$$

$$\mathrm{CF} = \frac{(\mathrm{FW} \times 100)}{\mathrm{Standard\ length}^3\,(\mathrm{cm})}, \quad (3)$$

$$\mathrm{FCR} = \frac{\mathrm{Feed\ intake\,(g)}}{\mathrm{Weight\ gain\,(g)}}, \quad (4)$$

$$\mathrm{PER} = \frac{\mathrm{Protein\ intake\,(g)}}{\mathrm{Weight\ gain\,(g)}}, \quad (5)$$

where W_I is initial body weights, W_F is final body weights (g), and t is the trial duration in days.

2.5. Digestive Enzyme Activity. After 0, 30, and 60 days of feeding with various doses of potassium and calcium diformate, the activity of chymotrypsin, trypsin, α-amylase, lipase, protease, and ALP (alkaline phosphatase) was measured in triplicates for each tank (using pooled samples from each tank). On an ice bath, intestinal samples were thoroughly homogenized with PBS buffer (1 : 5 w/v). After centrifuging the mixture at 12,000 rpm for 20 min at 4°C, the supernatant was removed and utilized to measure the activity of digestive enzymes. When calculating the total proteins in the crude enzyme extracts using the Bradford technique, bovine serum albumin was utilized as the reference [26]. Using *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and *N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates, respectively, the levels of chymotrypsin and trypsin activity were kinetically determined [27, 28]. Using soluble starch as the substrate hydrolyzable to maltose when it was treated with 3,5-dinitrosalicylic acid solution, the amount of α-amylase activity was evaluated [29]. Using a spectrophotometer, the hydrolysis of p-nitrophenolemyristate was used to assess the lipase enzyme activity in crude enzyme extract. In this process, p-nitrophenylemyristate is broken down into myristate and p-nitrophenol by the lipase enzyme in the presence of sodium cholate, creating a yellow color [30]. Two percent azocasein substrate solution in 50 mM tris/HCl buffer at pH = 7.5 and a spectrophotometer were used to evaluate the activity of the protease enzyme [31]. Using p-nitrophenyl phosphate (PNPP) as a substrate and a commercially available colorimetric kit, the activity of intestinal ALP was measured (Pars Azmoon Co., Tehran, Iran).

2.6. Serum Biochemical Assessment

2.6.1. Serum Biochemical Assessment. Serum activity of ALP (alkaline phosphatase) by IFCC method, AST (aspartate aminotransferase) by diazo reaction method, ALT (alanine transaminase) by IFCC method, LDH (lactate dehydrogenase) by DGKC method, CPK (creatine phosphokinase) by IFCC method, and concentration of total protein by biuret method, albumin by bromocresol green method, total cholesterol by CHOD-PAP method, glucose by glucose oxidase method, calcium by cresol phethalein complex method, and phosphorus by ammonium molybdate UV method was determined via spectrophotomey utilizing commercial kits (Pars Azmoon, Karaj, Iran) and a biochemical autoanalyzer (BT-1500, Italy). Serum sodium and potassium levels were assessed by flame photometry (Sherwood Scientific, UK). Serum cortisol levels were assayed using a commercial ELISA kit (Monobind, USA).

2.7. Oxidative Stress Indicators. The serum SOD (superoxide dismutase) activity was determined using a commercial kit (RANSOD kit, Randox Com, UK) on microplate-reader (Synergy HT, BioTek, USA) according to the manufacturer's protocol, which measured the conversion of superoxide anion to hydrogen peroxide.

The activity of CAT (catalase) was assayed according to the method described by Koroluk et al. [32]. Serum CAT was determined based on the decomposition rate of hydrogen peroxide. Briefly, 5 μL of the serum sample, 100 μL of H_2O_2 (10 mM), 50 μL of tris HCl buffer (50 mM, pH = 7.8) were poured into microplate wells. After 10 min at room temperature, 100 μL of 4% ammonium molybdate was added to wells. Then, the optical absorption rate of the samples against the control by a microplate analyzer was read at 410 nm and expressed as IU/mg protein.

The serum GSH (glutathione) activity was assayed using the method of Ellman [33]. Briefly, the reaction solution consisted of 15 μL of the serum sample, 260 μL of sodium phosphate buffer (Na_2HPO_4, 0.1 M) containing EDTA (1 mM, pH = 8) and DTNB reagent (0.01 M). Five microliters of Elman's reagent was added to this solution. Then, this solution was incubated at room temperature for 15 min and then read at 412 nm against the blank sample using the microplate reader. The GSH activity was stated as μmol/L serum.

The serum MDA (malondialdehyde) level was measured according to the method of Uchiyama and Mihara [34]. Briefly, 25 μL of each sample was mixed with the working solution (250 mL of 20% trichloroacetic acid (TCA) and 100 mL of 0.6% TBA) and kept in a hot water bath for 30 min. The solution after cooling was centrifuged at 5,000 g for 5 min. After that, samples optical absorption was detected spectrophotometrically at 535 nm. Finally, the results were expressed as nmol/mg protein.

2.8. Total Count and Lactic Acid Bacteria. In this study, posterior intestine samples were aseptically removed and homogenized with sterile PBS (1 : 10 w/v). The samples were diluted with 10^2–10^5 serial dilutions and cultured superficially in de Man, Rogosa, and Sharpe (MRS, BD, Sparks, MD, USA) agar

and Trypticase Soy Agar (TSA, Sigma–Aldrich) medium (aerobic and anaerobic bacteria) for lactobacillus count and total count, respectively. A total of 48 hr of incubation at 29°C was required to count the colonies, and the amount of counting was expressed as a denary logarithm per milliliter of homogenized suspension (CFU) [35].

2.9. Statistical Analysis. SPSS-20 software was used to carry out the statistical analysis for this investigation (SPSS Inc., USA). Using the Kolmogorov–Smirnov and Levine tests, the data's normality and variance homogeneity were examined. One-way analysis of variance (ANOVA) was used to assess the data, and Tukey's test was then performed. The significant threshold was set at $P < 0.05$. Each experimental group's mean and standard error (SE) are used to represent all data.

3. Results

3.1. Growth Performance. The results of growth parameters in different groups are presented in Table 1. On the 30th day of the experiment, there was no significant difference in the WI and CF of *H. huso* ($P > 0.05$). WF was significantly elevated in fish fed with a diet containing 0.15 KDF compared to the control group ($P < 0.05$). In our study, fish fed the 0.15 g/kg KDF showed the favorable FCR value, while the worst value of ($P < 0.05$) FCR was recorded in the CaDF 0.1 group. SGR was significantly elevated in fish fed with a diet containing 0.1 and 0.15 KDF compared to the control while the value of this factor decreased in the other groups ($P < 0.05$). In addition, PER was significantly elevated in fish fed with a diet containing 0.1 and 0.15 KDF compared to the control ($P < 0.05$). Moreover, DWG was significantly elevated in fish fed with a diet containing 0.1, 0.15, and 0.2 KDF, and 0.2 CaDF compared to the control while the value of this factor decreased in the CaDF 0.15 groups ($P < 0.05$). At the end of the feeding experiment, there was no significant difference in the CF value of *H. huso* ($P > 0.05$). Fish fed the 0.15 g/kg KDF showed the higher WI and WF values compared to the control group ($P < 0.05$). In our study, fish fed the 0.15 g/kg CaDF showed the favorable FCR value, while the worst value of ($P < 0.05$) FCR was recorded in the 0.1 g/kg KDF group. SGR was significantly elevated in fish fed with a diet containing 0.1 and 0.15 CaDF and 0.2 KDF compared to the control while the value of this factor decreased in the 0.1 and 0.15 KDF and 0.2 CaDF groups after 60 days ($P < 0.05$). Also, DWG and PER were significantly increased in fish fed with diet containing 0.15, 0.2 KDF and 0.1, 0.15 CaDF groups while the value of these factors decreased in the 0.1 KDF group compared to the control after 60 days ($P < 0.05$).

3.2. Digestive Enzymes. Figure 1 represents the effects of supplemented KDF and CaDF foods on the activity of the digestive enzyme of *H. huso*. On the 30th day of the experiment, there was a significant increase in the trypsin, protease, and ALP levels of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P < 0.05$). There was a significant increase in the chymotrypsin level of the CaDF 0.1%, 0.15%, and 0.2% and KDF 0.15% groups compared to the control group ($P < 0.05$). Also, the statistical analysis of results revealed that 0.15% and 0.2% of KDF and CaDF significantly increased the α-amylase levels compared to the control group ($P < 0.05$). Moreover, a remarkable increase of lipase level was observed in the KDF 0.1%, 0.15%, and 0.2% groups compared to the control group ($P < 0.05$). At the end of the feeding experiment, there was a significant increase in the trypsin level of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P < 0.05$). There was a significant increase in the chymotrypsin level of the CaDF 0.15%, and KDF 0.15% groups compared to the control group ($P < 0.05$). Also, the statistical analysis of results revealed that 0.1%, 0.15%, and 0.2% of CaDF significantly increased the lipase levels compared to the control group ($P < 0.05$). Moreover, a remarkable increase of α-amylase level was observed in the CaDF 0.1%, 0.15%, and 0.2% and KDF 0.2 groups compared to the control group ($P < 0.05$). Furthermore, a remarkable increase of protease level was observed in the CaDF 0.1%, 0.15%, and 0.2% and KDF 0.15% groups compared to the control group ($P < 0.05$). There was a significant increase in the ALP level of *H. huso* in the CaDF 0.1%, 0.15%, and 0.2% and KDF 0.15% and 0.2% groups compared to the control group ($P < 0.05$).

3.3. Serum Biochemical Parameters. Table 2 represents the effects of supplemented KDF and CaDF foods on biochemical parameters of *H. huso*. On the 30th day of the experiment, there was no significant difference in the serum potassium, creatine phosphokinase, and phosphorus levels of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P > 0.05$). Also, there was a significant increase in the serum calcium and cholesterol levels of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P < 0.05$). Moreover, a remarkable increase was observed in the total protein levels in the KDF 0.15 and 0.2 and CaDF 0.1, 0.15, and 0.2 groups compared to the control group ($P < 0.05$). Furthermore, the statistical analysis of results revealed that 0.1 and 0.15 KDF and 0.1 and 0.2 CaDF significantly increased the albumin levels compared to the control group ($P < 0.05$). A remarkable decrease was observed in the sodium level in the KDF 0.2 group compared to the control group ($P < 0.05$). Cortisol level was significantly decreased in the KDF 0.15 and CaDF 0.2 and 0.15 groups compared to the control ($P < 0.05$). Also, glucose level was significantly decreased in fish fed with a diet containing 0.1, 0.15, and 0.2 KDF and 0.15 CaDF compared to the control ($P < 0.05$). At the end of the feeding experiment, there was no significant difference in the blood total protein, potassium, creatine phosphokinase, and phosphorus levels of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P > 0.05$). There was a significant increase ($P < 0.05$) in the serum total cholesterol of the KDF 0.15 groups compared to the control group ($P < 0.05$). Also, the statistical analysis of results revealed that KDF and CaDF 0.2 significantly increased the albumin levels compared to the control group ($P < 0.05$). Moreover, a remarkable increase was observed in the sodium level in supplemented except CaDF 0.2 group compared to the control group ($P < 0.05$). Calcium level was significantly elevated in fish fed with a diet containing 0.15 CaDF compared to the control while the level of this factor decreased

Table 1: Growth performance of *Huso huso* fed feed supplemented with different levels of potassium diformate and calcium diformate for 60 days.

Parameters	Groups	Day 30	Day 60
Initial weight (IW)	Control	31.3 ± 7.8^{a}	144.4 ± 3.28^{b}
	Potassium diformate 0.1	33.1 ± 9.2^{a}	156.0 ± 9.2^{ab}
	Potassium diformate 0.15	35.5 ± 9.7^{a}	170.0 ± 9.17^{a}
	Potassium diformate 0.2	33.2 ± 9.4^{a}	146.2 ± 9.34^{b}
	Calcium diformate 0.1	33.3 ± 5.4^{a}	143.1 ± 5.14^{b}
	Calcium diformate 0.15	33.3 ± 0.1^{a}	141.2 ± 5.2^{b}
	Calcium diformate 0.2	32.1 ± 5.2^{a}	145.2 ± 8.12^{b}
Final weight(FW)	Control	144.4 ± 3.28^{b}	310.6 ± 33.24^{b}
	Potassium diformate 0.1	156.0 ± 9.2^{ab}	318.2 ± 87.2^{ab}
	Potassium diformate 0.15	170.0 ± 9.17^{a}	357.3 ± 3.57^{a}
	Potassium diformate 0.2	146.2 ± 9.34^{b}	326.5 ± 21.84^{ab}
	Calcium diformate 0.1	143.1 ± 5.14^{b}	316.6 ± 38.54^{ab}
	Calcium diformate 0.15	141.2 ± 5.2^{b}	324.4 ± 18.82^{ab}
	Calcium diformate 0.2	145.2 ± 8.12^{b}	311.7 ± 5.92^{b}
Condition factor (CF)	Control	0.44 ± 0.04^{a}	0.43 ± 0.4^{a}
	Potassium diformate 0.1	0.41 ± 0.04^{a}	0.47 ± 0.04^{a}
	Potassium diformate 0.15	0.43 ± 0.17^{a}	0.46 ± 0.17^{a}
	Potassium diformate 0.2	0.41 ± 0.1^{a}	0.47 ± 0.1^{a}
	Calcium diformate 0.1	0.41 ± 0.05^{a}	0.47 ± 0.05^{a}
	Calcium diformate 0.15	0.40 ± 0.09^{a}	0.44 ± 0.09^{a}
	Calcium diformate 0.2	0.41 ± 0.1^{a}	0.45 ± 0.1^{a}
Specific growth rate (SGR)	Control	5.05 ± 0.51^{c}	2.55 ± 0.09^{d}
	Potassium diformate 0.1	5.2 ± 0.31^{a}	2.36 ± 0.14^{g}
	Potassium diformate 0.15	5.24 ± 0.25^{a}	2.47 ± 0.05^{f}
	Potassium diformate 0.2	4.94 ± 0.11^{e}	2.66 ± 0.17^{b}
	Calcium diformate 0.1	4.98 ± 0.18^{d}	2.63 ± 0.08^{c}
	Calcium diformate 0.15	4.87 ± 0.91^{f}	2.75 ± 0.15^{a}
	Calcium diformate 0.2	5.01 ± 0.38^{d}	2.53 ± 0.12^{e}
Feed conversion ratio (FCR)	Control	1.32 ± 0.01^{b}	1.89 ± 0.24^{b}
	Potassium diformate 0.1	1.23 ± 0.02^{c}	1.94 ± 0.01^{a}
	Potassium diformate 0.15	1.18 ± 0.03^{d}	1.85 ± 0.05^{d}
	Potassium diformate 0.2	1.32 ± 0.01^{b}	1.84 ± 0.03^{e}
	Calcium diformate 0.1	1.36 ± 0.04^{a}	1.87 ± 0.02^{c}
	Calcium diformate 0.15	1.34 ± 0.04^{a}	1.8 ± 0.05^{f}
	Calcium diformate 0.2	1.3 ± 0.07^{b}	1.89 ± 0.04^{b}
Protein efficiency ratio (PER)	Control	1.73 ± 0.41^{c}	1.44 ± 0.16^{d}
	Potassium diformate 0.1	1.85 ± 0.18^{b}	1.38 ± 0.01^{e}
	Potassium diformate 0.15	1.93 ± 0.03^{a}	1.57 ± 0.13^{b}
	Potassium diformate 0.2	1.72 ± 0.03^{c}	1.54 ± 0.17^{b}
	Calcium diformate 0.1	1.68 ± 0.02^{c}	1.49 ± 0.18^{c}
	Calcium diformate 0.15	1.7 ± 0.3^{c}	1.59 ± 0.17^{a}
	Calcium diformate 0.2	1.74 ± 0.13^{c}	1.44 ± 0.12^{d}
Daily weight gain (DWG)	Control	3.71 ± 0.22^{d}	5.55 ± 0.39^{d}
	Potassium diformate 0.1	4.13 ± 0.18^{b}	5.4 ± 0.24^{e}
	Potassium diformate 0.15	4.49 ± 0.13^{a}	6.24 ± 1.32^{a}
	Potassium diformate 0.2	3.78 ± 0.21^{c}	5.98 ± 1.07^{c}
	Calcium diformate 0.1	3.71 ± 0.22^{d}	5.76 ± 0.18^{c}
	Calcium diformate 0.15	3.63 ± 0.16^{e}	6.07 ± 0.23^{b}
	Calcium diformate 0.2	3.78 ± 0.21^{c}	5.53 ± 0.72^{d}

Different lowercase letters in each column indicate significant differences between the values ($P < 0.05$). Data were expressed as means ± SEM.

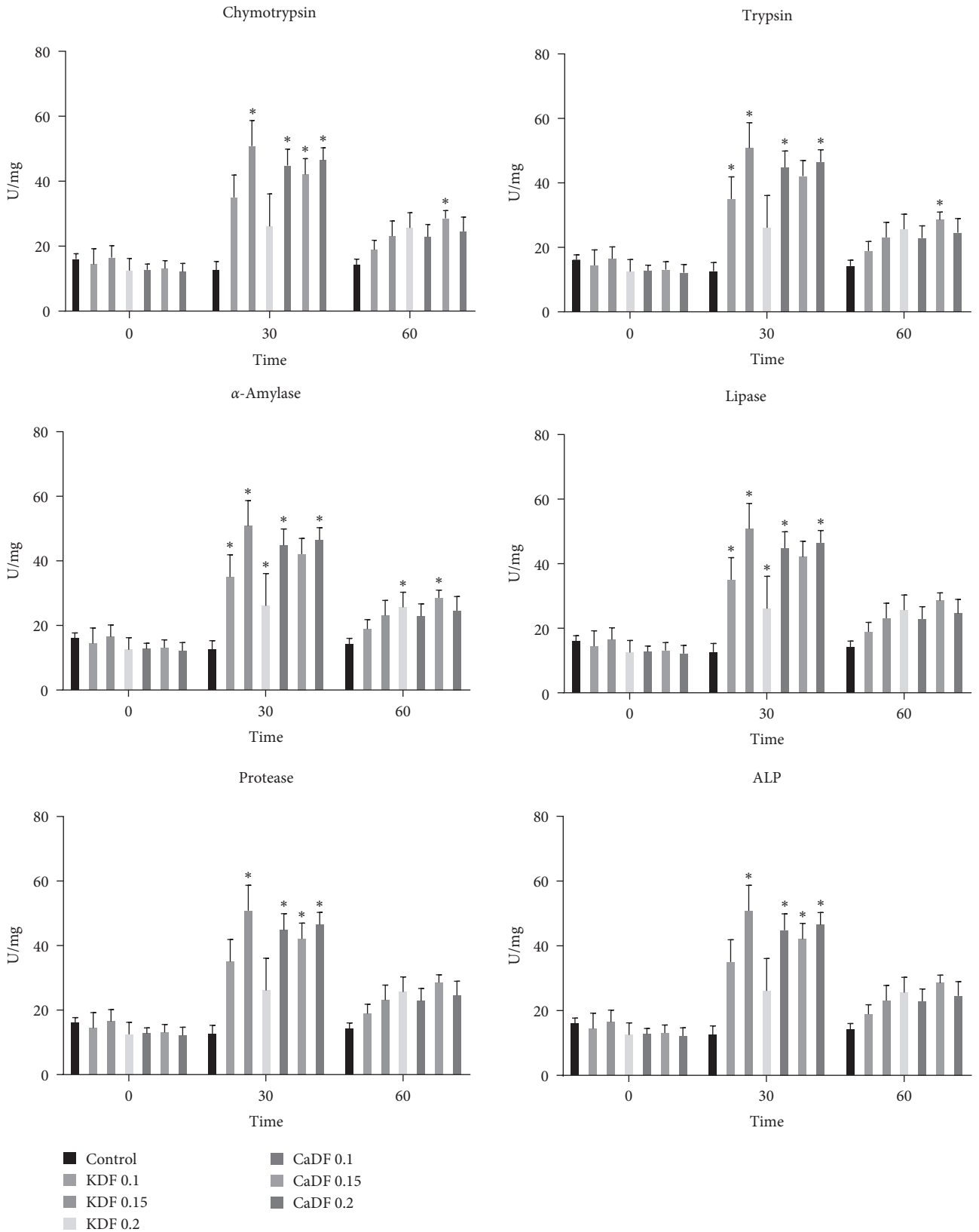

FIGURE 1: The activities of digestive enzymes in *H. huso* fed feed supplemented with different levels of potassium diformate and calcium diformate for 60 days. *Indicates statistically significant differences between each of the experimental groups at various sampling time points ($P<0.05$). Data were expressed as means ± SEM ($n=9$).

Table 2: Biochemical parameters in *H. huso* fed feed supplemented with different levels of potassium diformate and calcium diformate for 60 days.

Parameters	Groups	Day 0	Day 30	Day 60
Total protein (mg/dL)	Control	3.89 ± 0.1^{Aa}	4.99 ± 0.06^{Ba}	3.92 ± 0.05^{ABa}
	Potassium diformate 0.1	4.06 ± 0.19^{Aa}	4.91 ± 0.17^{Bb}	4.10 ± 0.25^{Aa}
	Potassium diformate 0.15	4.08 ± 0.05^{Aab}	5.61 ± 0.11^{Aa}	3.55 ± 0.15^{Bb}
	Potassium diformate 0.2	4.0 ± 0.13^{Aa}	5.26 ± 0.07^{Aa}	3.88 ± 0.15^{ABb}
	Calcium diformate 0.1	3.99 ± 0.04^{Aab}	5.36 ± 0.35^{Aa}	3.82 ± 0.29^{ABb}
	Calcium diformate 0.15	3.89 ± 0.09^{Ab}	5.21 ± 0.15^{Ab}	3.87 ± 0.17^{ABb}
	Calcium diformate 0.2	3.98 ± 0.18^{Ab}	5.39 ± 0.26^{Aa}	3.92 ± 0.12^{ABb}
Albumin (mg/dL)	Control	0.85 ± 0.05^{Aa}	0.81 ± 0.08^{Ba}	0.88 ± 0.04^{Aa}
	Potassium diformate 0.1	$0.88 \pm 0.05^{A,b}$	$1.17 \pm 0.1^{A,a}$	$0.99 \pm 0.11^{A,b}$
	Potassium diformate 0.15	$0.85 \pm 0.12^{A,b}$	$1.11 \pm 0.1^{A,a}$	$0.81 \pm 0.11^{A,b}$
	Potassium diformate 0.2	$0.82 \pm 0.06^{A,b}$	$0.95 \pm 0.08^{AB,a}$	$0.760 \pm 0.025^{B,b}$
	Calcium diformate 0.1	$0.85 \pm 0.13^{A,b}$	$1.63 \pm 0.08^{A,a}$	$0.81 \pm 0.11^{A,b}$
	Calcium diformate 0.15	$0.88 \pm 0.03^{A,b}$	$0.94 \pm 0.06^{AB,a}$	0.85 ± 0.12
	Calcium diformate 0.2	$0.87 \pm 0.03^{A,b}$	$1.29 \pm 0.06^{A,a}$	$0.72 \pm 0.04^{B,b}$
Total cholesterol (mg/dL)	Control	73.61 ± 7.1^{Aa}	70.62 ± 2.6^{Ba}	67.28 ± 3.18^{Ba}
	Potassium diformate 0.1	$77.72 \pm 7.19^{A,a}$	72.2 ± 6.3^{Ba}	$82.66 \pm 1.71^{A,a}$
	Potassium diformate 0.15	$78.1 \pm 10.2^{A,b}$	99.0 ± 4.12^{Aa}	$72.76 \pm 4.54^{B,b}$
	Potassium diformate 0.2	$74.05 \pm 8.8^{A,a}$	81.62 ± 1.41^{Aba}	$68.16 \pm 2.5^{B,a}$
	Calcium diformate 0.1	$71.13 \pm 8/14^{A,a}$	86.66 ± 4.35^{Aba}	$71.6 \pm 3.49^{B,a}$
	Calcium diformate 0.15	$72.45 \pm 7.9^{A,b}$	72.66 ± 5.33^{Ba}	$87.4 \pm 3.15^{A,a}$
	Calcium diformate 0.2	$74.26 \pm 8.98^{A,b}$	73.0 ± 3.4^{Ba}	$38.65 \pm 1.4^{B,b}$
Calcium (mg/dL)	Control	8.6 ± 1.1^{Aa}	7.4 ± 1.3^{Ba}	8.84 ± 1.1^{BCa}
	Potassium diformate 0.1	$8.6 \pm 2.19^{A,b}$	$10.5 \pm 2.3^{A,a}$	$9.53 \pm 1/31^{B,ab}$
	Potassium diformate 0.15	$8.1 \pm 3.05^{A,b}$	$12 \pm 1.32^{A,a}$	$9.7 \pm 2.14^{B,b}$
	Potassium diformate 0.2	$7.5 \pm 1.13^{A,b}$	$12.7 \pm 4.41^{A,a}$	$7.2 \pm 14.15^{C,b}$
	Calcium diformate 0.1	$8.38 \pm 1.14^{A,b}$	$11.4 \pm 4.5^{A,a}$	$9.47 \pm 2.49^{B,b}$
	Calcium diformate 0.15	$7.67 \pm 2.9^{A,b}$	$12.6 \pm 3.3^{A,a}$	$12.19 \pm 1.95^{A,a}$
	Calcium diformate 0.2	$8.74 \pm 4.8^{A,b}$	$11.3 \pm 2.9^{A,a}$	$7.18 \pm 1.4^{C,b}$
Potassium (mg/dL)	Control	3.68 ± 1.07^{Ab}	2.85 ± 0.75^{Ab}	2.7 ± 0.51^{Ab}
	Potassium diformate 0.1	3.4 ± 1.47^{Ab}	4.36 ± 0.94^{Ab}	3.23 ± 0.57^{Ab}
	Potassium diformate 0.15	3.36 ± 1.42^{Ab}	2.32 ± 0.6^{Ab}	2.9 ± 0.7^{Ab}
	Potassium diformate 0.2	3.45 ± 1.44^{Ab}	1.56 ± 0.68^{Ab}	2.46 ± 0.35^{Ab}
	Calcium diformate 0.1	3.53 ± 0.47^{Ab}	1.7 ± 0.44^{Ab}	3.22 ± 0.65^{Ab}
	Calcium diformate 0.15	3.38 ± 1.1^{Ab}	2.14 ± 0.49^{Ab}	2.48 ± 0.88^{Ab}
	Calcium diformate 0.2	3.7 ± 1.98^{Ab}	2.96 ± 0.94^{Ab}	5.72 ± 0.65^{Ab}
Creatine phosphokinase (mg/dL)	Control	214.57 ± 3.08^{Ab}	365.5 ± 2.47^{Aa}	365.17 ± 2.56^{Aa}
	Potassium diformate 0.1	214.57 ± 14.68^{Ab}	358.6 ± 1.7^{Aa}	365.8 ± 2.47^{Aa}
	Potassium diformate 0.15	$216.36 \pm 2.54^{A,b}$	$356.25 \pm 5.37^{A,a}$	$363.2 \pm 5.49^{A,a}$
	Potassium diformate 0.2	$217.88 \pm 3.90^{A,b}$	$353.66 \pm 2.88^{A,a}$	$362.5 \pm 5.92^{A,a}$
	Calcium diformate 0.1	$218.07 \pm 2.09^{A,b}$	$369.5 \pm 4.03^{A,a}$	$360 \pm 2.2^{A,a}$
	Calcium diformate 0.15	$214.41 \pm 2.56^{A,b}$	$358.25 \pm 1.31^{A,a}$	$364.25 \pm 6.65^{A,a}$
	Calcium diformate 0.2	$215.72 \pm 3.83^{A,b}$	$364.33 \pm 2.2^{A,a}$	$376.6 \pm 4.44^{A,a}$
Sodium (mg/dL)	Control	146.3 ± 18.12^{Aa}	172.0 ± 44.9^{Aa}	161.3 ± 46.54^{Ba}
	Potassium diformate 0.1	137.4 ± 15.45^{Aa}	120.0 ± 12.52^{Aa}	127.0 ± 14.73^{Ba}
	Potassium diformate 0.15	158.3 ± 49.11^{Aa}	139.4 ± 1.84^{Aa}	135.8 ± 26.71^{Ba}
	Potassium diformate 0.2	144.1 ± 27.69^{Aa}	96.4 ± 15.8^{Bb}	123.2 ± 20.93^{Ba}
	Calcium diformate 0.1	134.1 ± 20.51^{Aa}	120.6 ± 9.5^{Aa}	130.2 ± 15.02^{Ba}
	Calcium diformate 0.15	154.5 ± 47.14^{Aa}	130.8 ± 8.5^{Aa}	126.4 ± 10.92^{Ba}
	Calcium diformate 0.2	146.5 ± 30.82^{Ab}	148.0 ± 27^{Ab}	232.6 ± 21.64^{Aa}
Phosphorus (mg/dL)	Control	4.32 ± 0.48^{Aa}	4.68 ± 1.31^{Aa}	3.77 ± 0.3^{Aa}
	Potassium diformate 0.1	5.58 ± 0.29^{Aa}	5.06 ± 1.0^{Aa}	5.37 ± 0.54^{Aa}
	Potassium diformate 0.15	4.79 ± 1.74^{Aa}	5.06 ± 0.13^{Aa}	4.41 ± 0.0^{Aa}

Table 2: Continued.

Parameters	Groups	Day 0	Day 30	Day 60
	Potassium diformate 0.2	4.91 ± 1.16^{Aa}	5.24 ± 0.38^{Aa}	4.76 ± 0.11^{Aa}
	Calcium diformate 0.1	4.91 ± 0.61^{Aa}	5.3 ± 0.46^{Aa}	4.94 ± 0.39^{Aa}
	Calcium diformate 0.15	4.25 ± 0.35^{Aa}	5.77 ± 0.43^{Aa}	5.13 ± 1.04^{Aa}
	Calcium diformate 0.2	4.64 ± 1.02^{Aa}	5.28 ± 0.65^{Aa}	4.74 ± 0.24^{Aa}
Cortisol (mg/dL)	Control	16.5 ± 1.6^{Aa}	17.6 ± 6.1^{Aab}	17.8 ± 0.37^{Aa}
	Potassium diformate 0.1	15.24 ± 2.9^{Aa}	14.3 ± 2.43^{Ab}	14.9 ± 3.5^{Aa}
	Potassium diformate 0.15	14.55 ± 1.8^{Aa}	8.11 ± 2.27^{Bbc}	9.09 ± 2.73^{Bab}
	Potassium diformate 0.2	15.04 ± 2.97^{Aa}	12.97 ± 3.77^{Ab}	6.1 ± 1.45^{Bb}
	Calcium diformate 0.1	16.63 ± 1.7^{ABa}	21.68 ± 7.16^{Aa}	11.69 ± 2.5^{Bab}
	Calcium diformate 0.15	15.21 ± 3.0^{Aa}	3.98 ± 0.49^{Bc}	5.83 ± 1.25^{Bb}
	Calcium diformate 0.2	15.16 ± 2.2^{Aa}	3.19 ± 0.24^{Bc}	13.49 ± 7.1^{Aa}
Glucose (mg/dL)	Control	76.6 ± 3.11^{Aa}	80.33 ± 11.7^{Aa}	78.0 ± 0.18^{Aa}
	Potassium diformate 0.1	79.9 ± 7.19^{Aa}	67.6 ± 8.13^{Ba}	68.0 ± 5.1^{Aa}
	Potassium diformate 0.15	73.5 ± 3.5^{Aa}	61.0 ± 8.82^{Aa}	69.83 ± 4.34^{Aa}
	Potassium diformate 0.2	71.33 ± 4.8^{Aa}	54.0 ± 5.41^{Cb}	72.21 ± 5.21^{Aa}
	Calcium diformate 0.1	79.16 ± 6.14^{Aa}	84.0 ± 4.35^{Aa}	69.8 ± 7.49^{Aa}
	Calcium diformate 0.15	76.2 ± 1.93^{Aa}	59.0 ± 4.33^{BCb}	59.4 ± 4.15^{ABb}
	Calcium diformate 0.2	71.0 ± 4.98^{Aa}	79.0 ± 3.24^{Aa}	51.8 ± 0.4^{Bb}

Different lowercase letters indicate statistically significant differences between each of the experimental groups at various sampling time points (row) ($P<0.05$). Different capital letters denote significant differences between the experimental groups at a specified time point (column) ($P<0.05$). Data were expressed as means $\pm$ SEM ($n=9$).

in the KDF 0.2 ($P<0.05$). Also, cortisol level was significantly decreased in 0.15 and 0.2 KDF and 0.15 CaDF groups compared to the control ($P<0.05$). Furthermore, glucose level was significantly decreased in fish fed with a diet containing 0.2 CaDF compared to the control ($P<0.05$).

3.4. Liver Enzymes. Table 3 represents the effects of supplemented KDF and CaDF foods on the liver enzymes of *H. huso*. On the 30th day of the experiment, there was no significant difference in the serum ALP levels of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P>0.05$). AST level was significantly decreased in fish fed with a diet containing KDF 0.15 compared to the control ($P<0.05$). Moreover, ALT level was significantly increased in CaDF 0.15 and KDF 0.1 groups compared to the control ($P<0.05$). LDH level was significantly decreased in fish fed with a diet containing 0.15 and 0.2 of KDF and 0.15 and 0.2 of CaDF compared to the control ($P<0.05$). At the end of the experiment, there was no significant difference in the serum ALT levels of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P>0.05$). AST level was significantly decreased in fish fed with a diet containing 0.1 and 0.15 CaDF and 0.2 KDF compared to the control ($P<0.05$). Moreover, the ALP level was significantly increased in 0.1 CaDF and 0.2 KDF groups compared to the control ($P<0.05$). LDH level was significantly decreased in fish fed with a diet containing 0.15 and 0.2 KDF and 0.1 and 0.2 CaDF compared to the control ($P<0.05$).

3.5. Oxidative Stress Indicators. The effects of KDF and CaDF on the oxidative stress indicators of *H. huso* were presented in Table 4. On the 30th day of the experiment, there was a significant increase in the serum CAT activity of *H. huso* in the KDF 0.1 and 0.15 groups compared to the control group ($P<0.05$). There was a significant increase in the serum SOD and GSH activities of the CaDF 0.15 and KDF 0.15 groups compared to the control group ($P<0.05$). Moreover, a remarkable decrease of MDA was observed in the CaDF 0.1 and KDF 0.15 groups compared to the control group ($P<0.05$). At the end of the feeding experiment, there was a significant increase in the serum CAT activity of *H. huso* in all supplemented KDF and CaDF groups compared to the control group ($P<0.05$). There was a significant increase ($P<0.05$) in the serum SOD and GSH activities of the CaDF 0.15 and KDF 0.15 groups compared to the control group ($P<0.05$). Also, a remarkable decrease of MDA was observed in all supplemented KDF and CaDF groups except KDF 0.1 group compared to the control group ($P<0.05$).

3.6. Total Count and Lactic Acid Bacteria. The effects of KDF and CaDF on the total count and lactic acid bacteria in the intestine of *H. huso* were presented in Table 5. On the 30th day of the experiment, the total count of bacteria was significantly increased in the KDF 0.1 group compared to the control group ($P<0.05$). In addition, lactic acid cultivable bacterial counts were found at significantly higher numbers in all groups fed with diets containing KDF and CaDF than in the control group ($P<0.05$). After 60 days of feeding with diets containing KDF and CaDF, no significant alteration in the total count of bacteria was observed between the groups ($P>0.05$). Moreover, lactic acid cultivable bacterial counts were found at significantly higher numbers in the groups fed with diets containing KDF and CaDF than in the control group ($P<0.05$).

Table 3: Liver enzymes in *H. huso* fed feed supplemented with different levels of potassium diformate and calcium diformate for 60 days.

Parameters	Groups	Day 0	Day 30	Day 60
Alkaline phosphatase (ALP)	Control	453.7 ± 66.6^{Aa}	438.2 ± 133.2^{Aa}	415.5 ± 8.7^{Aa}
	Potassium diformate 0.1	420.8 ± 26.8^{Aa}	432.8 ± 20.2^{Aa}	429.8 ± 20.8^{Aa}
	Potassium diformate 0.15	466.8 ± 80.2^{Ab}	517.6 ± 54.4^{Aa}	466.6 ± 57^{Ab}
	Potassium diformate 0.2	414.0 ± 37.8^{Aab}	347.0 ± 90.1^{Bb}	491.6 ± 240.3^{Aa}
	Calcium diformate 0.1	433.3 ± 17^{Aa}	383.7 ± 84.9^{Bb}	500.6 ± 127.2^{Aa}
	Calcium diformate 0.15	458.0 ± 51.1^{Aa}	397.0 ± 57.2^{ABb}	445.1 ± 60.8^{Aa}
	Calcium diformate 0.2	469.8 ± 41.6^{Aa}	482.3 ± 122.1^{Aa}	449.6 ± 106.6^{Aa}
Aspartate aminotransferase (AST)	Control	4.3 ± 1.07^{Aa}	4.4 ± 0.77^{Aa}	4.0 ± 1.58^{Aa}
	Potassium diformate 0.1	4.23 ± 1.05^{Aa}	3.93 ± 1.68^{Ab}	3.77 ± 1.06^{ABb}
	Potassium diformate 0.15	4.26 ± 1.75^{Aa}	2.62 ± 8.66^{Bb}	3.4 ± 0.5^{Bb}
	Potassium diformate 0.2	4.07 ± 1.56^{Aa}	5.0 ± 1.91^{Aa}	2.4 ± 0.5^{Bb}
	Calcium diformate 0.1	4.41 ± 1.98^{Aa}	4.25 ± 1.87^{Aa}	2.8 ± 0.48^{Bb}
	Calcium diformate 0.15	4.5 ± 1.07^{Aa}	3.0 ± 0.6^{Bb}	2.83 ± 0.94^{Bb}
	Calcium diformate 0.2	4.91 ± 1.62^{Aa}	5.75 ± 1.75^{Aa}	4.0 ± 1.58^{Ab}
Alanine transaminase (ALT)	Control	0.91 ± 0.14^{Aa}	0.82 ± 0.44^{Aa}	0.86 ± 0.33^{Aa}
	Potassium diformate 0.1	0.73 ± 0.14^{Aa}	1.55 ± 0.27^{Aa}	0.88 ± 0.19^{Ba}
	Potassium diformate 0.15	0.77 ± 0.19^{Aa}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	Potassium diformate 0.2	0.81 ± 0.2^{Aa}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	Calcium diformate 0.1	0.83 ± 0.22^{Aa}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	Calcium diformate 0.15	0.73 ± 0.08^{Aa}	1.33 ± 0.08^{Aa}	0.68 ± 0.19^{Ba}
	Calcium diformate 0.2	0.85 ± 0.2^{Aa}	0.75 ± 0.07^{Aa}	0.96 ± 0.21^{Ba}
Lactate dehydrogenase (LDH)	Control	9.12 ± 0.36^{Aa}	9.1 ± 0.92^{Aa}	9.66 ± 0.57^{Aa}
	Potassium diformate 0.1	10.4 ± 1.37^{Aa}	10.51 ± 1.7^{Aa}	10.18 ± 1.5^{Aa}
	Potassium diformate 0.15	9.86 ± 1.24^{Aa}	4.51 ± 0.58^{Bb}	6.74 ± 1.39^{ABb}
	Potassium diformate 0.2	9.3 ± 1.43^{Aa}	5.85 ± 1.26^{Bb}	5.96 ± 0.76^{Bb}
	Calcium diformate 0.1	9.41 ± 1.32^{Aa}	7.49 ± 0.34^{ABab}	6.23 ± 0.45^{ABb}
	Calcium diformate 0.15	10.98 ± 1.85^{Aa}	5.62 ± 0.6^{Bb}	7.66 ± 1.4^{ABab}
	Calcium diformate 0.2	10.04 ± 1.8^{Aa}	5.41 ± 0.63^{Bb}	5.85 ± 0.99^{Bb}

Different lowercase letters indicate statistically significant differences between each of the experimental groups at various sampling time points (row) ($P < 0.05$). Different capital letters denote significant differences between the experimental groups at a specified time point (column) ($P < 0.05$). Data were expressed as means ± SEM ($n = 9$).

4. Discussion

To our knowledge, no study had previously investigated the impacts of CaDF and KDF on growth parameters in this species. In our study, feeding of fingerling *H. huso* with diets containing 2 g/kg KDF and 1.5 g/kg CaDF for 60 days improved the growth parameters. Our results are in accordance with those of Hassaan et al. [15] who observed the highest growth performance in Nile tilapia (*Oreochromis niloticus*) fed with 10 g/kg KDF. Various studies show that the use of organic acids due to improving the metabolism and digestibility of proteins and minerals in the intestine improves growth and nutrition beside enhanced appetite and changed the composition, diversity, and/or activity of the population of beneficial bacteria in the gut microbiota while inhibiting pathogenic bacteria in aquatic species [5, 6, 12, 13, 36, 37].

In this study, after 30 days of treatment with 1.5 g/kg and 1 g/kg KDF, there was a notable improvement in SGR, FCR, PER, and FER compared to the control group. However, other parameters showed no significant differences. The enhanced growth performance in the initial 30 days is attributed to the positive impact of the prescribed concentration of KDF in the feed. Contrastingly, in the following 60 days, a decline in growth factors was observed in all groups, particularly in the 1.5 g/kg KDF. Prolonged feeding with 1.5 g/kg of KDF may reduce its positive effects, potentially interfering with the microbiota of the fish's digestive tract and normal physiological functions. In a related study by Kalantarian et al. [38], treatment with 1.5 g/kg of CaDF on the 60th day showed improved digestive enzyme activity and intestinal morphology, resulting in enhanced fish growth. The role of acidifiers in the long-term digestive health of different fish depends on their type and concentration in a dose-dependent manner. It is important to note that organic acids, which are weak acids, have different acid strengths at specific pH levels based on their degree of ionization and deionization. The pKa values indicate acidity, with lower pKa values correlating with greater acidity. For example, propionic acid has a pKa of 4.88, butyric acid 4.82, acetic acid 4.76, and formic acid 3.75. This illustrates the different acid strengths of the diformate acidifiers. The different acid strengths of the diformate acidifiers are highlighted by the different pKa values. In addition, simpler molecular structures with shorter chains increase acid activity and facilitate passage through cell walls. As an

Table 4: Antioxidant responses in *H. huso* fed feed supplemented with different levels of potassium diformate and calcium diformate for 60 days.

Parameters	Groups	Day 0	Day 30	Day 60
Catalase (U/mg)	Control	1.91 ± 0.77^{Aa}	1.12 ± 0.39^{Ab}	1.85 ± 0.51^{Ac}
	Potassium diformate 0.1	$1.43 \pm 0.25^{C,a}$	4.1 ± 1.36^{Aa}	2.11 ± 0.32^{Bb}
	Potassium diformate 0.15	$1.04 \pm 0.3^{B,a}$	3.7 ± 0.25^{Aa}	2.87 ± 0.07^{ABb}
	Potassium diformate 0.2	$0.04 \pm 1.4^{B,a}$	0.95 ± 0.34^{Bb}	2.53 ± 0.21^{Ab}
	Calcium diformate 0.1	$1.60 \pm 0.14^{B,a}$	1.31 ± 0.51^{Bb}	3.45 ± 0.36^{Aa}
	Calcium diformate 0.15	$1.33 \pm 0.26^{B,a}$	0.98 ± 0.27^{Bb}	4.28 ± 0.67^{Aa}
	Calcium diformate 0.2	$1.92 \pm 0.32^{B,a}$	1.0 ± 0.26^{Bb}	4.49 ± 0.86^{Aa}
Superoxide dismutase (U/mg)	Control	4.61 ± 0.31^{Aa}	4.17 ± 0.83^{Ab}	4.87 ± 0.4^{Ab}
	Potassium diformate 0.1	4.6 ± 0.8^{Aa}	5.86 ± 0.36^{Aab}	5.13 ± 1.29^{Ab}
	Potassium diformate 0.15	4.79 ± 1.33^{a}	7.7 ± 0.23^{Aa}	4.78 ± 1.05^{Ba}
	Potassium diformate 0.2	4.67 ± 0.18^{Aa}	5.47 ± 1.85^{Aab}	4.9 ± 0.75^{Ab}
	Calcium diformate 0.1	4.19 ± 0.84^{Aa}	4.01 ± 0.6^{Ab}	4.78 ± 0.78^{Ab}
	Calcium diformate 0.15	4.86 ± 0.63^{Ba}	7.58 ± 2.32^{Aa}	7.8 ± 1.1^{Aa}
	Calcium diformate 0.2	4.52 ± 1.11^{Aa}	3.44 ± 1.13^{Ab}	5.21 ± 0.64^{Ab}
Glutathione (U/mg)	Control	0.15 ± 0.05^{Aa}	0.11 ± 0.03^{Ac}	0.12 ± 0.05^{Ab}
	Potassium diformate 0.1	0.16 ± 0.06^{Aa}	0.18 ± 0.08^{Ac}	0.17 ± 0.07^{Ab}
	Potassium diformate 0.15	0.15 ± 0.05^{Aa}	0.17 ± 0.07^{Ac}	0.14 ± 0.04^{Ab}
	Potassium diformate 0.2	0.12 ± 0.02^{Ba}	0.13 ± 0.03^{Bc}	0.42 ± 0.12^{Aa}
	Calcium diformate 0.1	0.12 ± 0.02^{Ba}	0.21 ± 0.02^{Abc}	0.15 ± 0.07^{Bb}
	Calcium diformate 0.15	0.18 ± 0.06^{Ba}	0.24 ± 0.03^{Ab}	0.16 ± 0.06^{Bb}
	Calcium diformate 0.2	0.16 ± 0.06^{Ba}	0.52 ± 0.02^{Aa}	0.14 ± 0.04^{Bb}
Malondialdehyde (U/mg)	Control	16.89 ± 4.17^{Aa}	13.44 ± 2.7^{Aa}	15.11 ± 1.6^{Aa}
	Potassium diformate 0.1	13.77 ± 3.56^{Aa}	14.99 ± 4.06^{Aa}	17.13 ± 5.75^{Aa}
	Potassium diformate 0.15	13.8 ± 2.02^{Aa}	6.37 ± 1.23^{Bb}	7.46 ± 3.51^{Bb}
	Potassium diformate 0.2	15.32 ± 5.28^{Aa}	14.3 ± 4.64^{Aa}	5.29 ± 1.01^{Bc}
	Calcium diformate 0.1	13.08 ± 4.76^{Aa}	8.09 ± 2.14^{Bb}	5.88 ± 1.44^{Bc}
	Calcium diformate 0.15	12.76 ± 4.48^{Aa}	13.64 ± 3.9^{Aa}	8.15 ± 2.38^{Bb}
	Calcium diformate 0.2	14.66 ± 4.55^{Aa}	12.57 ± 2.9^{Aa}	8.83 ± 0.8^{Bb}

Different lowercase letters indicate statistically significant differences between each of the experimental groups at various sampling time points (row) ($P<0.05$). Different capital letters denote significant differences between the experimental groups at a specified time point (column) ($P<0.05$). Data were expressed as means ± SEM ($n=9$).

acidifier, formic acid is more effective than butyric acid (88.12) and sorbic acid (114.12) because it has fewer mol/kg of pure substance (46.3). The functional divergence of KDF and CaDF in this study is due to their unique properties. KDF has a lower pKa and fewer mol/kg compared to other acids used in aquaculture. This makes it more effective in cellular processes. However, dietary supplementation of KDF at 12 and 15 g/kg did not improve growth performance in hybrid tilapia [39]. Kakavand et al. [40] observed similar effects of improved KDF in the freshwater sturgeon, sterlet (*Acipenser ruthenus*), but the concentration used in their study was 9 g/kg feed, which is much higher than the concentration used in our study. Differences in nutrition and digestive system structure between *A. ruthenus* and *H. huso* may account for the observed difference in concentration. Due to their typically low stomach pH, it is recommended that a lower amount of acidifier be added to fish feed to optimize palatability. A high concentration of acidifier may not have a beneficial effect and could negatively impact the palatability of the food.

The results indicate that fish fed with both KDF and CaDF for 60 days had significantly higher activities of intestinal trypsin, α-amylase, lipase, and ALP. Therefore, increased activity of digestive enzymes was the primary cause of the ability of KDF and CaDF to stimulate growth performance. When aquatic animals are young or when their diet is high in protein, the concentration of hydrochloric acid in their stomach decreases, leading to an increase in pH. This increase can adversely affect the activity of the enzyme pepsin and the secretion of pancreatic enzymes, resulting in digestive disorders. Adding acidifiers to the diet can solve this problem and help digest food [41]. Pepsinogen is rapidly converted to pepsin at a pH of 2, but slowly at a pH between 5 and 6. In addition, pepsin works best in an acidic environment with a pH of 2–3.5, and its activity is significantly faster than at higher pH levels [42, 43]. Similar results regarding digestive enzymes were seen in hybrid tilapia fed potassium diformate (KDF) [39], *Oncorhynchus mykiss* fed Dietary Prima-Lac® and potassium diformate (KDF) [10], *Lateolabrax japonicus* fed citric, lactic, and phosphoric acids [44], and *Litopenaeus vanami* [45]. Trypsin, a proteolysis enzyme, contributes to fish development and feed intake, but chymotrypsin is active when food availability or supply is constrained [46]. ALP and protease enzyme activity increased in the potassium and calcium

Table 5: Total viable and lactic acid bacterial counts from the intestine of *H. huso* fed feed supplemented with different levels of potassium diformate and calcium diformate for 60 days.

Parameters	Groups	Day 0	Day 30	Day 60
Total count bacteria ($\times 10^5$ CFU/g)	Control	93 ± 14.2^{Ba}	203.0 ± 24.5^{Aa}	241.3 ± 47.5^{Aa}
	Potassium diformate 0.1	87.3 ± 1.3^{Ca}	176.0 ± 19.5^{Bb}	238.2 ± 35.3^{Aa}
	Potassium diformate 0.15	91 ± 12.0^{Ba}	240.0 ± 27.16^{Aa}	276.0 ± 66.1^{Aa}
	Potassium diformate 0.2	96.0 ± 15.2^{Ba}	249.0 ± 19.6^{Aa}	279.0 ± 33.5^{Aa}
	Calcium diformate 0.1	91.0 ± 9.7^{Ba}	219.0 ± 22.64^{Aa}	274.0 ± 25.5^{Aa}
	Calcium diformate 0.15	95.0 ± 15.2^{a}	246.0 ± 19.6^{Aa}	286.0 ± 33.5^{Aa}
	Calcium diformate 0.2	91.0 ± 9.7^{Ba}	220.0 ± 22.64^{Aa}	275.0 ± 25.5^{Aa}
Lactic acid bacteria ($\times 10^2$ CFU/g)	Control	34.0 ± 2.2^{Aa}	31.0 ± 3.05^{Ac}	37.6 ± 3.51^{Ac}
	Potassium diformate 0.1	44.0 ± 2.3^{Ba}	134.0 ± 7.63^{Ab}	141.45 ± 26.2^{Ab}
	Potassium diformate 0.15	45.0 ± 6.5^{Ba}	163.0 ± 1.15^{Aa}	166.66 ± 3.2^{Aab}
	Potassium diformate 0.2	46.0 ± 4.5^{Ca}	112.0 ± 14.3^{Bb}	172.33 ± 10.4^{Aab}
	Calcium diformate 0.1	47.0 ± 5.2^{a}	117.0 ± 10.3^{Bb}	166.33 ± 10.4^{Aab}
	Calcium diformate 0.15	51.0 ± 4.5^{Ca}	121.0 ± 14.3^{Bb}	181.33 ± 10.4^{Aa}
	Calcium diformate 0.2	47.0 ± 5.2^{Ca}	128.0 ± 10.3^{Bb}	158.33 ± 10.4^{Aab}

Different lowercase letters indicate statistically significant differences between each of the experimental groups at various sampling time points (row) ($P < 0.05$). Different capital letters denote significant differences between the experimental groups at a specified time point (column) ($P < 0.05$). Data were expressed as means ± SEM ($n = 9$).

diformate treatments on day 30 of the experiment. However, on day 60 of the experiment, all acid treatments showed a significant decrease in activity compared to day 30. Additionally, increased enterocyte nutritional absorption in fish is indicated by increased intestinal ALP activity, which is critical for both carbohydrate and lipid absorption [47, 48]. On day 60 of the 0.15% CaDF treatment, protease enzyme activity was higher compared to the other treatments, which may be attributed to the positive effect of these treatments on the overall growth process of the *H. huso*. The process of activity of lipase and α-amylase enzymes was similar, so the 0.15% KDF treatment on day 30 showed a significant increase in activity compared to other treatments, but the 0.15% CaDF treatment showed a significant increase on day 60. During the first 30 days of the experiment, a concentration of 0.15% KDF may increase the secretion or release of cholecystokinin by lowering the pH in the digestive tract, which in turn may significantly increase pancreatic secretions such as digestive enzymes to stimulate digestion.

Our findings showed that the 0.2 of KDF and CaDF have beneficial effects on the serum albumin level of *H. huso*. Several studies demonstrated that different organic acids simultaneously increased serum albumin values in different fish species [7, 8, 15, 49]. As an organism's response to internal and external circumstances, blood serum protein is a highly responsive biochemical system. An increase in serum albumin concentration in fish indicates an improved level of innate immunity that may also be linked to improved function of organs involved in protein production, including the liver [50]. Hence, in the current study, the increased serum albumin values might be attributed to the elevated values of protein synthesis in liver tissue of fish fed with KDF and CaDF-incorporated diets. In this study, cholesterol levels were highest in the 0.15% KDF acidifier treatment. All acidifier treatments showed a significant increase compared to the control group on day 30. However, on day 60, the acidifier treatments, particularly 0.1% KDF and 0.15% NaDF, showed a significant increase compared with the other treatments and the control group. The possible relationship between the increase or decrease in blood cholesterol in acidifier-treated fish on day 30 and the duration of use and appropriate concentration of acidifier on the synthesis, absorption, and metabolism of cholesterol is unclear. Further research should be conducted in this area to determine the indirect effects of acidifiers, such as an increase in lactic acid-producing bacteria, on the activity of the enzyme HMG CoA reductase, which plays a crucial role in cholesterol biosynthesis.

Cortisol and glucose are indicators of stress that are found in the blood [51, 52]. In response to stressful situations, cortisol is released into the blood from internal tissues. Glucose is produced in the liver through glycogenolysis (the breakdown of glycogen into glucose) or gluconeogenesis (the breakdown of protein into glucose) to meet the energy needs of cells [53–55]. Therefore, the lower levels of these in *H. huso* fed KDF and CaDF probably indicate a higher resistance to common stresses under laboratory conditions. In contrast to our findings, Yılmaz and Ergün [56] reported no significant difference in serum cortisol and glucose levels in *O. mykiss* fed diets containing trans-cinnamic acid. This difference may be attributed to variations in the type of organic acid, species, and dosage used in different studies. Research has shown that acidic anions can facilitate the absorption of mineral cations, including calcium [57]. In our study, the addition of CaDF and KDF significantly affected serum calcium and sodium levels, possibly due to improved mineral absorption and utilization by enteric epithelial cells [58]. Similar to Hassaan et al.'s [15] findings, our study also observed an increase in serum calcium levels in Nile tilapia (*O. niloticus*) fed a diet containing either 5 g or 10 g/kg KDF. Our results demonstrate that the use of KDF and CaDF improves the haematopoietic system of *H. huso* and that these supplements are safe to use as feed.

This is based on the observed significant reductions in AST and LDH levels in *H. huso* liver cells. This study suggests that acidified diets, such as the supplemented KDF and CaDF diets, may have potential benefits in improving fish health, as these diets may improve gut balance and digestibility, reduce toxins, and improve immune status, as reported in previous studies [10, 59]. These findings are in line with previous studies conducted on Nile tilapia [60–62] and rainbow trout [10], as well as other fish species, where acidified diets were found to improve fish health and performance. However, it is important to note that the effects of acidified diets on fish health may vary depending on the specific fish species, the composition and dose of organic acids used, and other environmental factors. These results demonstrate the potential benefits of acidified diets in aquaculture, particularly in improving fish health and reducing the risk of disease outbreaks.

As biomarkers of oxidative stress, compounds and substances such as CAT, SOD, GSH, and MDA (As an indicator of lipid peroxidation) serve as indicators of damage [63]. Acidifiers improve the state of oxidative stress in organisms and reduce the risk of infection in organisms by increasing antioxidative and antimicrobial activity [25, 64, 65]. With the increased probability of the presence of lactic acid bacteria in the digestive tract following the use of acidifiers, the stress indicators in the blood, decrease. This may be due to the antistress effects of the beneficial bacteria present in the digestive tract of fish fed with the diet. The food contains acidifier, which improves the antioxidant defense mechanism and reduces stress indicators in the blood [66]. The present results showed that supplemented KDF and CaDF diets stimulated serum SOD and especially CAT activities which are two pioneer antioxidant enzymes. Eventually, these cumulatively suppress lipid peroxidation and increase the health index. Similar to our results, Hassaan et al. [15] observed that the activities of SOD and CAT were highest in Nile tilapia (*O. niloticus*) fed diet containing either 5 g or 10 g/kg KDF. Also, Nascimento et al. [67] showed that citric acid minimizes oxidative stress in Amazonian fish (*Colossoma macropomum*) when fed plant protein-based diets. Moreover, Huang et al. [44] showed that an acidifier blend (citric, lactic, and phosphoric acids) reduces MDA levels in juvenile Japanese sea bass (*L. japonicus*). Hence, the results of this study revealed upregulation of SOD, CAT, and MDA in fish fed with KDF and CaDF, showing that KDF and CaDF reduced cell damage. Moreover, these results indicated that treatment with the acidifiers (KDF and CaDF) leads to a reduction in lipid peroxidation, which leads to the induction of the secretion of antioxidant enzymes and the elimination of free radicals and eventually improved the oxidative stability responses in *H. huso*. However, these studies show that organic acids and their salts can protect the fish body from cellular oxidative damage. Finding out the various details of the effect of organic acids on antioxidant defense responses will be the subject of future studies.

Analysis of the gut microbiota in this study showed that the total number of bacteria in the 0.1% KDF treatment was significantly lower than in the other treatments on day 30 of the experiment. However, examination of the lactic acid bacteria in this study showed that the total number of lactic acid bacteria in the 0.15% KDF treatment was significantly higher than in the other treatments on day 30 of the experiment. In the control group, only a very limited number of colonies were isolated, which was not statistically significant (less than 10 colonies in 0.01 dilution). At the end of the 60-day experiment, the gut microbiota of fish fed 0.15% CaDF showed the highest number of lactic acid bacteria. Although there was a significant difference between the acidifier treatments in terms of the number of lactic acid bacteria, the fish fed calcium diformate had a higher number of lactic acid bacteria. The study found a strong correlation between the establishment of bacteria in the intestines and the stimulation of growth responses in *H. huso*. The reason for this finding is that the acidifier, in the desired concentration, affects the intestinal conditions for the penetration of lactic acid bacteria into the intestinal mucosa within the first 30 days. Organisms capable of deep mucosal penetration are able to colonize the intestinal mucosa, but the long-term effects of this acidifier are reduced. CaDF, which has different structural characteristics to KDF, was found to have a positive effect on the longevity of lactic acid bacteria in *H. huso* gut. It is interesting to note the potential benefits of organic acids in altering the intestinal environment to prevent the growth of harmful bacteria. The findings of Castillo et al. [68] suggest that KDF and CaDF may induce low pH in the intestine, which can inhibit the proliferation of pathogenic microorganisms. Similarly, Reyshari et al. [11] observed increased lactic acid bacterial counts in Asian sea bass fed diets supplemented with NaDF, further supporting the idea that organic acids can have a positive impact on gut microbiota. However, it is also important to consider the findings of Dai et al. [9], indicating that citric acid supplementation did not affect the gut microbiota of *Scophthalmus maximus* L. This suggests that the effects of organic acids on gut microbiota may vary depending on the specific type of acid and the species of fish being studied. Further research is necessary to fully understand the mechanisms underlying their effects and to explore their applications in aquaculture.

5. Conclusion

The study provides evidence that incorporating KDF and CaDF into the diet of *H. huso* has positive effects on their growth performance, feed utilization, stress markers, oxidant/antioxidant status, microbial flora, and some biochemical parameters. Moreover, these results highlighted the potential use of KDF (1.5 g/kg) for use at 30 days and KDF (2 g/kg), CaDF (1.5 g/kg) for use at 60 days as an additive in *H. huso* diets. However, further research is needed to confirm these findings.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure

Preprint version of this paper has been published previously [69].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Takavar Mohammadian conceptualized the study. Takavar Mohammadian, Mehrzad Mesbah, Seyedeh Misagh Jalali, and Mohammad Reza Tabandeh designed and supervised the study. Abdollah Beit Sayah wrote and Takavar Mohammadian and Mehrzad Mesbah revised the manuscript draft. Abdollah Beit Sayah and Takavar Mohammadian performed in vitro experiments related to acidifier. Takavar Mohammadian, Mehrzad Mesbah, and Seyedeh Misagh Jalali conducted in vitro evaluations of acidifiers and reviewed the manuscript. Takavar Mohammadian, Mehrzad Mesbah, and Mohammad Reza Tabandeh conducted data analysis. All authors have read and agreed to the published version of the manuscript.

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