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Effect of feed supplementation with effective microorganisms (em) bokashi on hepatopancreas and gut histology, growth performance, and survival rate of freshwater crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823)

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Abstract: A sixty-day feeding trial was conducted to investigate the effect of dietary effective microorganisms (EM) Bokashi on organ histology, growth performance, and survival rate of freshwater crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Bokashi serum and powder consisted of two major microorganisms as *Bacillus amyloliquefaciens* spp. *plantarum* and *Lactobacillus plantarum*. Four treatment groups were established in control (without bokashi), bokashi powder 1% and 5% supplemented to diets. Bokashi serum was added at a ratio of 0.01% (v/v) of the total volume of the tank water once a week into which there was a constant flow of fresh water at a flow rate of 1.2 L min^{-1}. The crayfish were fed ad libitum with treatment diets for 60 days. The results showed that there was no significant difference in growth performance among the groups, but survival rates were significantly different between groups ($p < 0.05$). As a result of the microscopic examination of the intestine, pathological changes such as inflammatory cell infiltrations in the interstitial tissue, and lack of B, F, and R epithelial cells were found in the groups that were supplemented with bokashi while the control group exhibited normal tissue histology. As a result of this study, the addition of nonindustrial bokashi to crayfish diets had no effect on growth. In addition, nonindustrial bokashi products supplementation to crayfish diets caused severe pathological findings in hepatopancreas and guts and a low survival rate.

Key words: Crayfish, *Pontastacus leptodactylus*, bokashi, histology, growth performance

1. Introduction

Crayfish are an important component of aquaculture, however, bacterial and viral diseases are an increasingly serious threat, causing considerable economic losses [1]. Therefore, antibiotics and chemical control agents are widely applied to eliminate these pathogens effectively and to stop and treat diseases in aquaculture systems [1]. However, using antibiotics in aquaculture production systems leads to residual antibiotic accumulation in wild fish, plankton, and sediments [2]. Furthermore, the compounds commonly used for water treatment and disease control in aquaculture can reduce the innate immunity and disease resistance of crayfish [1]. All crustaceans, including crayfish, lack a highly specific adaptive immune system, instead, they oppose foreign pathogens primarily via innate immunity [3]. Therefore, the use of probiotics and medicinal plants for protecting health is even more important in crustacean aquaculture.

Probiotics are microorganisms with beneficial effects for hosts such as enhanced immunity, improved digestion, and protection from pathogens [4]. Probiotics do not produce residues or drug resistance in animals [5]. Effective Microorganisms (EM) Bokashi probiotics are microbial strains that have not been processed technologically [6].

The roots of bokashi are entwined with the traditional natural farming philosophy prevalent in Korea and other parts of Asia. The EM active serum is a synergistic consortium of beneficial microorganisms in anaerobic conditions, a group of more than one type part of microbes that work together so that each organism benefits, rather than harms, the other organisms [7]. It is primarily composed of three groups of microorganisms: lactic acid bacteria, photosynthetic bacteria, and yeast [8]. The anaerobic fermentation process involves the production of an organic acid such as, copious quantities of lactic acid, a smaller amount of butyric, propionic, and benzoic acid

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among the others. The acids lower the pH and start the fermentation process. The low pH environment has the ability to inhibit or destroy pathogens [9]. Bokashi powder (bran) is a carbon substrate that is an energy source for microorganisms that is inoculated by a specific set of beneficial microorganisms [7]. The beneficial effects of EM on animals are known as increased weight gain, improved feed digestibility, reduced mortality, and improved health. Szewczuk et al. [10] found that supplemented diets with Bokashi significantly improved the growth performance of Holstein calves. Laskowska et al. [11] indicated that supplementation of pig feed with EM Bokashi activated enhancing immune processes protecting the body against infection. Rybarczyk et al. [12] reported that carcasses of the pigs fed with bokashi probiotic had a higher meat percentage and lower fat content than the carcasses of pigs from the control group.

There are very few studies on the use of bokashi products in aquaculture. Muhasdika and Johan [13] indicated that the highest *Moina sp.* population was found on the treatment with a dosage of 1 g L^{-1} bokashi fertilizer. Rodriguez et al. [14] reported that EM Bokashi promoted population growth, in reared of *Tetraselmis suecica* alga. The dietary inclusion of bokashi leachate (5%) enhanced the feed intake and growth performance of the red tilapia fingerlings [15].

The crayfish do not have a true adaptive immune response. For this reason, the losses of crayfish are high in intensive culture. This study is aimed to increase the survival rate and growth of crayfish with the treatment of bokashi probiotic, in liquid or solid form.

2. Materials and methods

2.1. Animal ethics

Formal ethics approval is not necessary for the laboratory trial with invertebrates.

2.2. Preparation of effective microorganisms (EM) bokashi

2.2.1. Preparation of EM Bokashi serum

Rice was rinsed with warm water by swirling gently. Milky-colored rice wash, which is a rich source of carbohydrates, was obtained. The rice-washed water was stored in a loosely closed plastic jar for a week under dark and room temperature (21 °C) conditions. The serum turned into three distinct layers: top, middle (Lactic Acid Bacteria culture), and bottom. The lactic acid bacteria culture, which is the mixture of lactic acid and other bacteria, was separated from the rest. Then, it was mixed with milk (1:10 v/v). After about 1-week (fermentation under dark and room temperature (21 °C) conditions), the mixture was turned into two layers: solid (curd) and serum layer (whey). The whey separated was mixed with molasses (1:1) [16]. The resultant mixture is called bokashi serum.

2.2.2. Bokashi powder

Bokashi powder was prepared as follows: 100 mL of molasses or beet molasses was mixed in 5 L of chlorine-free water. 100 mL of bokashi serum was added. In 5.5 kg of wheat bran, 64 g of clay and 32 g of rock salt were added. Water was gradually added to this bran. The mixture should be lumpy like a dough, stick to the hand, and water should not drip when squeezed. This dough was placed into vacuumed bags in a dark room for 2 weeks.

Identification of the microorganisms in the bokashi serum was performed by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry, Bruker Daltonics GmbH, Bremen, Germany). Microorganism Identification device located in Hatay Mustafa Kemal University Plant Health Clinic Application and Research Center (The two major microorganisms were identified: *Bacillus amyloliquefaciens* spp. *plantarum* and *Lactobacillus plantarum*.)

2.3. Chemical analysis of EM Bokashi serum

The chemical analysis of the serum was performed and presented in Table 1. pH, organic matter, total organic carbon, and total nitrogen were measured according to APHA standard methods [17]. Water-soluble potassium oxide (K_2O) of serum was determined based on BS EN 15477.

2.4. Crayfish rearing conditions and experiment design

Pontastacus leptodactylus mean weighing 16 g and with a total length of 8 cm were collected by using fyke nets of (16 mm) mesh size from Eğirdir Lake in March 2020, Turkey [18]. A total of 84 crayfish were transported to the Faculty of Eğirdir Fisheries at Isparta University of Applied Sciences and randomly stocked 5 individual/m^2 in tanks (120 × 120 cm) with an open water flow system (1.2 L min^{-1}). The crayfish were fed ad libitum with broodstock commercial trout feed (45% protein, 20% lipid) twice a day at 9 am and 19 pm for 15 days for adaptation to pellet feed and the environment. Then, four treatment groups were established as follows: 1) Control (without bokashi application); 2) Bokashi powder 1% supplemented to the diet 3) Bokashi powder 5% supplemented to the diet, and finally 4) Only bokashi serum was added to their water at a

Table 1. Chemical analysis of the bokashi serum.

Parameters	Values
pH	3.7
Volatile solid (%)	2.5
Total organic carbon (%)	1.7
Total nitrogen (%)	0.18
Water soluble potassium oxide (K_2O) (%)	0.19

rate of 0.01% (v/v) once a week to the tank. During addition of Bokashi serum, the water flow into the tank was stopped for 5 h. The crayfish were fed ad libitum with treatment diets for 60 days. Three isonitrogenous (350 g/kg^{-1}) and isoenergetic (4354 kcal/kg^{-1}) diets were formulated by adding 1% and % 5 bokashi powder and without bokashi powder (control). Bokashi powder and other ingredients were thoroughly mixed homogeneously in a mixer. Diets were passed through a mincing machine with a 1.5 mm sieve, and the spaghetti-like strands were dried. Moisture, crude protein, crude fibre, and ash contents of the diets were determined according to standard AOAC methods [19]. The total lipids were determined by the chloroform-methanol extraction method [20]. The formulation and proximate composition of experimental diets are given in Table 2.

The bokashi serum group (Group 4) was fed with the control diet. Each treatment was replicated thrice. Pipes of 8 cm diameter and 14 cm length were placed as shelters for crayfish in each tank to prevent cannibalism. Leftover feed and feces were siphoned out every other day before the commencement of the next feed. The mean culture water temperature of crayfish was 18 ± 1 °C. The level of dissolved oxygen concentration ranged from 6.15 to 6.35 mg L^{-1}.

2.5. Histopathological methods

For histopathological analysis, hepatopancreas tissue samples of 6 individuals from each treatment were removed from the proximal region of the intestine under the carapax, and gut tissue samples were taken from the tail. Then, tissue samples were fixed in 10% buffered formalin solution during the necropsy. Hepatopancreas and gut samples were routinely processed by an automatic tissue processor equipment (Leica ASP300S, Wetzlar, Germany), embedded in paraffin, and sectioned to 5-µm thickness by a Leica RM2155 rotary microtome (Wetzlar, Germany). Sections were stained with hematoxylin-eosin (HE) and examined under an Olympus CX41 light microscope (Olympus Corporation, Tokyo, Japan). Histopathological changes were graded in a blinded manner. Morphometric evaluation and microphotographs were made using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

2.6. Statistical analysis

A one-way analysis of variance (ANOVA) was used to compare growth performance among treatments. All data were analyzed using the SPSS computer program (SPSS 2000). Duncan test was used to determine the significant difference between treatment means ($p = 0.05$).

Table 2. Formulation and proximate composition of experimental diets (%).

Ingredients (%)	**Control**	**Bokashi Powder 1%**	**Bokashi Powder 5%**
Fish meal	25	25	25
Soybean	26	26	26
Corn gluten meal	10	10	10
Wheat	29.45	28.15	23
Bokashi Powder	0	1	5
Fish oil	6.55	6.85	8
Vit-min	2	2	2
Pellet binder	1	1	1
Chemical analyses			
Protein (%)	35.33	35.19	35.18
Lipid (%)	9.61	9.89	11.12
Ash (%)	8.01	8.06	8.28
Energy (kcal/kg)	4354.25	4354.77	4354.27

Vitamin premix; per kg, 4000,000 IU vitamin A, 480,000 IU vitamin D3, 40,000 mg vitamin E, 2400 mg vitamin K3, 4000 mg vitamin B1, 6000 mg, vitamin B2, 40,000 mg niacin, 10,000 mg calcium D-pantothenate, 4000 mg vitamin B6, 10 mg vitamin B12, 100 mg D-biotin, 1200 mg folic acid, 40,000 mg vitamin C and 60,000 mg inositol; Mineral premix; per kg 23,750 mg Mn, 75,000 mg Zn, 5000 mg Zn, 2000 mg Co, 2750 mg I, 100 mg, Se, 200,000 mg Mg.
NFE: Nitrogen Free Extract = 100 - (% Moisture + % Crude protein + % Crude lipid + % ash + % Crude fiber)
GE: Gross energy (kcal/kg) = [(% crude protein × 5.72 + % crude lipids × 9.50 + % cellulose × 4.79 + % NFE × 4.03) × 1000]/100 [30]

3. Results

The growth performance values of crayfish in all 4 groups are given in Table 3. No statistical differences were found in terms of final weight, weight gain, and SGR ($p > 0.05$). However, survival rates were significantly different among treatments ($p < 0.05$).

At the histopathological examination of the hepatopancreas, normal tissue architecture was observed in crayfish in the control group. In this group, in the normal hepatopancreas tissue, large vacuoles were observed at the cytoplasms of B-cells, small vacuoles were identified in the cytoplasms of R-cells and basophilic nonvacuolated appearance was observed at the F-cells of the tubules (Figure 1A). However, the treated group's histopathological examination of the hepatopancreas revealed pathological findings such as sloughing of tubular cells of the hepatopancreas. Enlarged nuclei of the epithelial cells and disruption of the regular cell layer were also common findings. Inflammatory cell infiltrations in the interstitial tissue and lack of B, F, and R epithelial cells were noticed in hepatopancreases of some crayfish belonging to treated groups. The level of the pathological changes was increased related to the dose of the probiotic addition (Figures 1B, C, and D).

Microscopical examination of the intestine revealed normal tissue histology in the control group (Figure 2A). The most common findings of bokashi supplemented groups were loss of the epithelial cells, and the severity of the pathological findings increased with the increase in the concentration of the bokashi (Figures 2B, C, and D).

4. Discussion

The bokashi supplementation to diets of crayfish caused severe pathological findings in the hepatopancreas and guts. The necrotic cells and loss of the epithelial cells were observed in the gut of crayfish fed with diet supplementation bokashi products. In addition, the necrotic cells, loss of tubular epithelial cells, slight and moderate inflammatory cell infiltrations were seen in the hepatopancreas. Contrary to the current study, Foysal et al. [21] indicated that black soldier fly based diets supplemented with probiotic bacteria *Lactobacillus plantarum* significantly improved gut health and survival rate of *Cherax cainii*. Zheng et al. [22] reported that supplementation of cell-free extract of *L. plantarum* in diets increased the enterocytes height of *Litopenaeus vannamei*. Reda and Selim [23] indicated that the heights of the intestinal villi increased with the increase of the *Bacillus amyloliquefaciens* level in the diet of *Oreochromis niloticus*. In the present study, while nonindustrial bokashi powder and serum contained two main microorganisms *Bacillus amyloliquefaciens spp. plantarum* and *Lactobacillus plantarum*, they may also contain different microorganisms that were not detected. We know that *Bacillus amyloliquefaciens sp. plantarum* and *Lactobacillus plantarum* are used as probiotics. However, any undefined bacteria, contaminating the serum might have adversely affected the survival rate and pathology of the crayfish.

In the present study, no improvement was observed in the growth performance of *Pontastacus leptodactylus* fed with diets supplemented with Bokashi. No statistical differences were found in terms of final weight and weight gain, however, survival rates were significantly different among the groups.

Contrary to the current study, positive results have been observed in growth in previous studies; Lim et al. [15] pointed out that dietary inclusion of Bokashi leachate (5%) enhanced the feed intake and growth performance of the red tilapia fingerlings and reduced the crude fiber content in the diets. Muhasdika and Johan [13] indicated that the highest Moina sp. population was found in the treatment

Table 3. The growth performance of crayfish fed nonindustrial bokashi products.

	Control	Bokashi Powder 1%	Bokashi Powder 5%	Bokashi Serum	Df	F	P
Initial weight (g)	16.84 ± 0.58	16.86 ± 0.61	16.67 ± 0.61	16.77 ± 0.82	3	0.02	0.99
Final weight (g)	21.33 ± 0.74	21.14 ± 0.96	20.04 ± 0.41	20.95 ± 0.67	3	0.63	0.61
Initial length (cm)	8.98 ± 0.15	8.67 ± 0.05	9.00 ± 0.06	8.98 ± 0.13	3	2.10	0.14
Final length (cm)	9.68 ± 0.25	9.25 ± 0.17	9.40 ± 0.06	9.38±0.3	3	1.37	0.28
Survival rate (%)	100.00 ± 00^{b}	90.47 ± 4.76^{b}	38.10 ± 4.76^{a}	57.14 ± 0.00^{a}	3	73.29	0.00
Weight gain (g)	4.49 ± 0.17	4.28 ± 0.40	3.50 ± 0.34	4.19 ± 0.25	3	1.99	0.19
Specific growth rate (SGR) % day^{-1}	0.39 ± 0.01	0.37 ± 0.02	0.32 ± 0.04	0.37 ± 0.03	3	1.24	0.36

Mean values in the same row with different superscripts differ significantly ($p < 0.05$). The growth parameters were calculated with the following formulas at the end of 60 days. Data expressed as Mean ± SE. Weight gain = (final body weight – initial body weight). Specific growth rate (SGR) (% day^{-1}) = [(ln final body weight – ln initial body weight) / days] × 100. Survival rate = [(Final number of fish)/(Initial number of fish)] × 100.

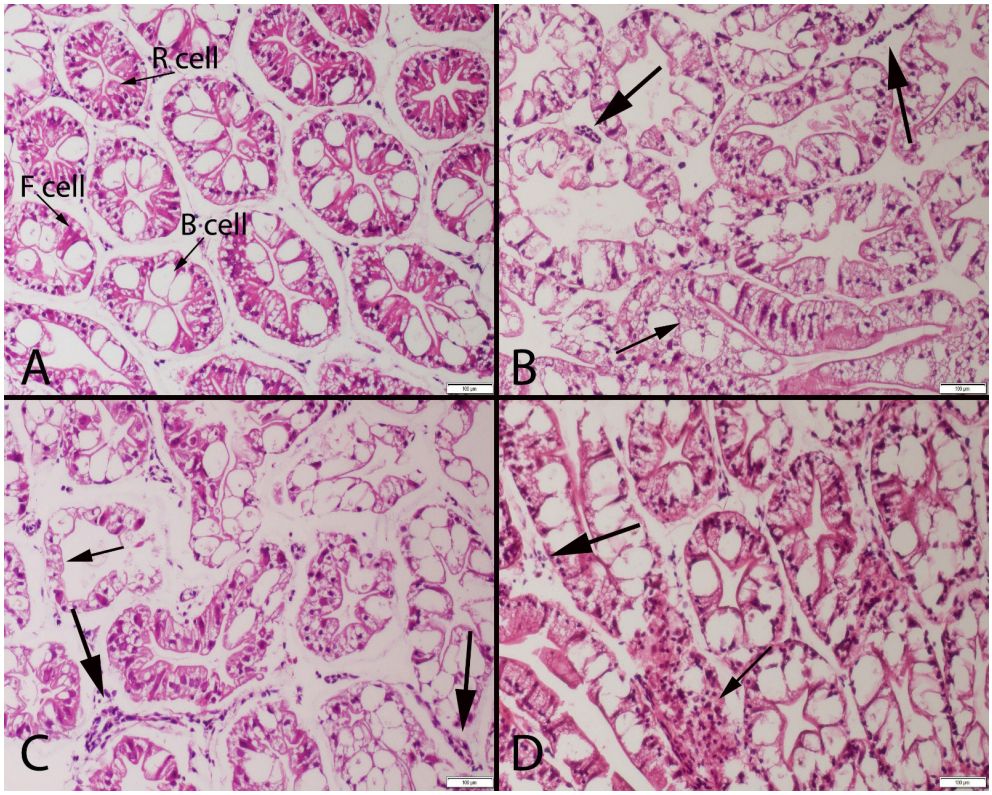

Figure 1. Histopathological examination of the hepatopancreases. (A) Normal structure of hepatopancreas in the control group. (B) Decreased big vacuolated B cells sloughing of tubules cells (thin arrow) and slight inflammatory cell infiltrations (thick arrows) in serum bokashi added group. (C) loss of tubular epithelial cells (thin arrow) and moderate inflammatory cell infiltrations (thick arrows) in 1% bokashi powder added group. (D) Severely degenerated and necrotic cells (thin arrow) moderate inflammatory cell infiltrations (thick arrow) and severely distorted tissue architecture in 5% bokashi powder added group. HE. Bars = 100 µm.

with a dosage of 1 g L^{-1} bokashi obtained with cow dung, rice bran, and household wastewater. Rodriguez et al. [14] reported that EM-Bokashi promoted population growth, improved environmental quality, and presented less production costs in the production of *Tetraselmis suecica* algae. The difference between these studies [13,14,15], showing beneficial effects, and the current study may stem from the difference in the microbial population of bokashi used in such studies.

Similar to the current study, Llario et al. [24] indicated that the application of *B. amyloliquefaciens* did not produce significant differences in water quality or in the growth performance of *L. vannamei*. Contrary to the current study, Lai et al. [25] reported that *B. amyloliquefaciens* supplement to diets of crayfish *Procambarus clarkii* significantly increased the survival rate of crayfish against the white spot syndrome virus. In addition, *B. amyloliquefaciens* supplement also influenced immune parameters. Xu et al. [5] showed that *Bacillus amyloliquefaciens* supplementation could effectively enhance white spot syndrome virus (WSSV) resistance of *P. clarkii*. While the reported studies used only one species, the current study used two species namely, *Bacillus amyloliquefaciens* spp. *plantarum* and *Lactobacillus plantarum*.

Similar to the current study, Nedaei et al. [26] indicated that *L. plantarum* supplementation diets of *A. leptodactylus* (synonymy *Pontastacus leptodactylus*) did not show significant differences in the growth performance parameters and survival rate compared to the control. Valipour et al. [27] recorded no significant difference in growth parameters of narrow clawed crayfish fed *L. plantarum*. On the contrary, *L. plantarum* inclusion in Pacific white shrimp diets significantly improved growth performance and feed utilization [28]. Mohammadi et al. [29] reported that *Oreochromis niloticus* fed *L. plantarum* containing diet presented significantly superior weight gain and average daily growth over other diets. The difference between the above studies showing beneficial effects and the present study is that *Bacillus amyloliquefaciens spp. plantarum* and *Lactobacillus plantarum* bacteria were not used alone in the current study. The nonindustrial bokashi products used in the present study are mixed products dominated by these two bacteria. The mixed products may have had an antagonistic effect.

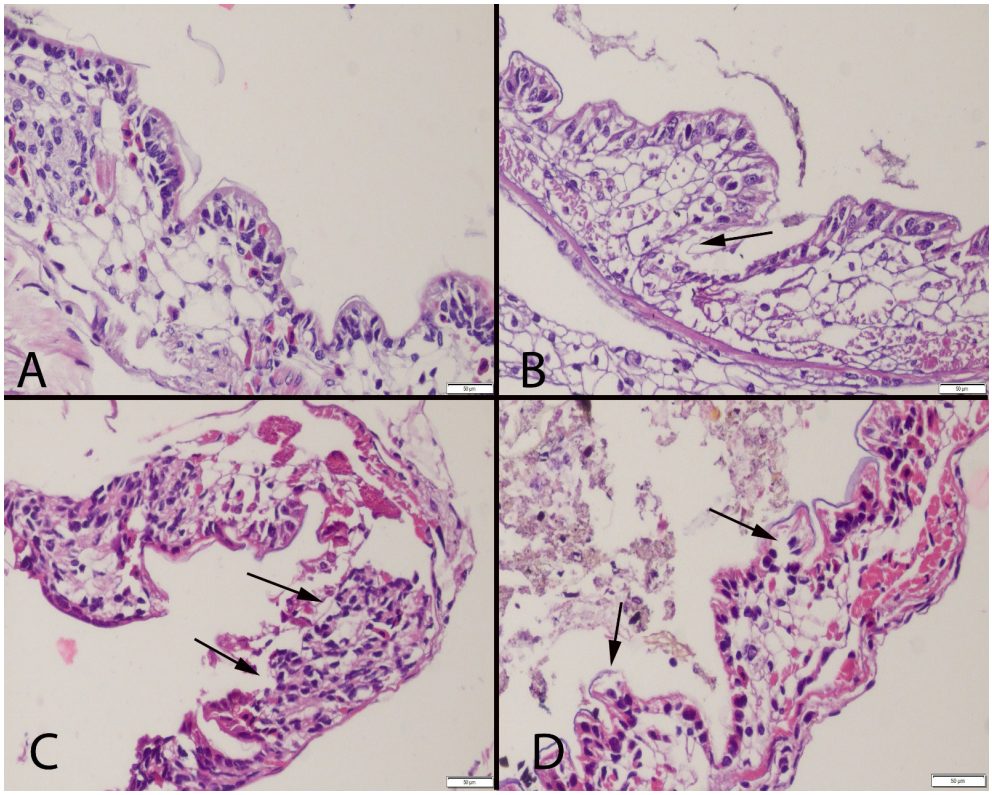

Figure 2. Microscopical appearance of the large intestine. (A) Normal gut architecture in control group. (B) loss of the epithelial cells (arrow) in serum bokashi added group. (C) marked epithelial loss (arrows) in 1% bokashi powder added group. (D) Severely degenerated and necrotic cells (arrows) in 5% bokashi powder added group. HE. Bars = 50 µm.

As a result of this study, the addition of nonindustrial bokashi (5%, 1% powder, and serum) to crayfish diets had no effect on growth. In addition, nonindustrial bokashi products supplementation to crayfish diets caused severe pathological findings in hepatopancreas and guts and low survival rate.

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