Dietary supplementation with heat-killed *Lactobacillus plantarum* L-137 improves growth, immune response, and disease resistance of snakehead (*Channa striata*)

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Abstract. This study aimed to investigate the effects of heat-killed *Lactobacillus plantarum* L-137 (HK L-137) on the growth, survival, intestinal morphology, immune response, and disease resistance of snakehead (*Channa striata*). The fish (7.30±0.07 g) were fed diets supplemented with 0 (control), 2, 4, 10, 20, and 40 mg of HK L-137 kg^{-1} of feed. After feeding (60 days), the fish were injected with *Aeromonas hydrophila* and the cumulative mortality was recorded for 14 days. The results showed that the dietary administration of HK L-137 did not affect the water quality parameters, including water temperature, pH, dissolved oxygen, total ammonia nitrogen, and $N\text{-}NO_2$. HK L-137 supplemented diets significantly improved the growth, protein efficiency ratio, apparent digestibility coefficient, feed conversion ratio, and survival rate of fish. HK L-137 supplemented diets significantly increased the white blood cell count and lysozyme activity, but did not affect the red blood cell count. The height and length of the intestinal villi and the crypt length of the intestine of fish fed HK L-137 were significantly higher than those fed the control diet. The cumulative mortality of fish fed HK L-137 (at 2, 4, 10, 20, and 40 mg kg^{-1} feed) was significantly lower than that of control fish after challenging with *A. hydrophila*. Among the levels of HK L-137, 2 mg HK L-137 kg^{-1} feed showed the lowest cumulative mortality of the fish (6.66%), not significantly different from that of the negative control (3.33%). Altogether, HK L-137 conferred beneficial effects to snakehead.

Key Words: growth performance, heat-killed bacteria, immune response, snakehead.

Introduction. Probiotics are defined as live microorganisms that confer health benefits to the host when they are supplied in adequate amounts (FAO/WHO 2006). In aquatic animals, probiotics have been used as a means of improving growth performance, providing nutritional and enzymatic contributions to the digestion of the host, enhancing immune responses, controlling diseases, and improving water quality or in some cases replacing the use of antimicrobial compounds (Balcázar et al 2006; Tuan et al 2013; Ringø et al 2018; Ringø et al 2020). Several bacterial species belonging to the genera of *Bacillus*, *Lactobacillus*, *Enterococcus*, *Psychrobacter*, *Carnobacterium*, *Pseudomonas*, *Micrococcus*, *Streptomyces*, *Streptococcus*, and *Lactococcus* have been used as probiotics in aquatic animals (Son et al 2009; Tuan et al 2013).

Lactobacillus plantarum is a rod-shaped, Gram-positive, heterofermentative lactic acid bacterium (Dash et al 2015; Dawood et al 2015a). *L. plantarum* has been known to be important in fermenting plant products and is used as a probiotic (Ashenafi & Busse 1991; Son et al 2009). The bacterium is able to adapt to various environmental conditions and is capable of suppressing the growth of many Gram-positive and Gram-negative bacteria by secreting bacteriocin (Dawood et al 2015a). In aquatic animals, the viable form of the bacterium serving as a probiotic has been shown to improve digestive enzyme activities, growth performance, feed utilization efficiency, immunity, disease resistance, survival rate, and it can also inhibit the adhesion and growth of pathogens in aquatic animals (Dash et al 2015). However, because of the instability of viable forms of probiotics, inactivated probiotics appear to be an alternative to live counterparts and can be used with a better safety in open aquatic environments (Dawood et al 2015a; Nguyen et al 2019). In Japanese pufferfish (*Takifugu rubripes*), for example, Biswas et al (2013b) reported that the heat-killed *L. plantarum* 06CC2 isolated from Mongolian dairy products significantly induced the expression of pro-inflammatory cytokines (*Il-1β*, *Il-6*, *Tnf-α*, and *Tnf-N*), cell-mediated immune regulators (*Il-12p40* and *Il-18*), antiviral (*I-ifn-1*) and other regulatory (*Il-2*, *Il-7*, *Il-15*, *Il-21*, *Il-10*, and *Tgf-β1*) cytokines at different time points during the experimental period. Dawood et al (2015b) administered heat-killed bacteria *L. plantarum* L-137 (HK L-137, at 0.025, 0.05, and 0.1% of dry diet) to juvenile red sea bream (*Pagrus major*) for 56 days. Body weight gain, specific growth rate (SGR), feed intake (FI), protein efficiency ratio (PER), serum lysozyme activity (LA), serum alternative complement pathway activity, and mucus secretion were significantly increased by diets supplemented with HK L-137 (Dawood et al 2015b). Previous studies showed that the heat-killed *L. plantarum* strain L-137, provided by House Wellness Foods Corp (Itami, Japan), has been frequently studied to investigate its role in aquatic animals and demonstrated to be able to improve growth performance, immunity, and stress resistance (Yang et al 2016; Dawood et al 2019; Nguyen et al 2019; Duc et al 2020).

Snakehead (*Channa striata*) is widely distributed across southern Asia, southern China, Indochina, and Sunda Islands (Song et al 2013). The fish can live in several habitats, such as rivers, swamps, ponds, canals, lakes, and even rice fields (Song et al 2013). In the Mekong Delta (Vietnam), snakehead is one of the most economically important cultured species, with many different culture models, such as earthen and floating ponds, hapa, and cage systems (Sang et al 2013). Up to date, there is scarce information about the use of HK L-137 as a potential probiotic in snakehead. The aim of this study, therefore, was to assess the effects of HK L-137 on the growth, survival, intestinal morphology, immune response, and disease resistance of snakehead under laboratory conditions. The results of this study provide basic information on the application of probiotics as growth promoters and immune stimulus in the snakehead aquaculture industry.

Material and Method

Experimental fish, HK L-137 preparation, and diets. Healthy juvenile snakehead (7.3±0.07 g) were purchased from a fish hatchery in An Giang Province, Vietnam, in April 2020. Fish were acclimatized in a 4 m^3 composite tank for two weeks at the Wet-Lab, College of Aquaculture and Fisheries, Can Tho University, under laboratory conditions (at temperatures between 27.4-30.6^{o}C, dissolved oxygen between 5.1-5.3, pH between 7.4-7.7, and aeration). Fish were fed a commercial diet (45% crude protein) twice a day (at 08:00 and 16:00) to satiation during the acclimatization period.

Feed LP20 contained 20% HK L-137 and 80% dextrin on a dry-weight basis (House Wellness Foods Corporation, Itami, Japan), with a concentration of approximately 2.0×10^{11} cells g^{-1}. The formulation and chemical composition of the basal diet was prepared following the method from a previous study (Hien et al 2018) (Table 1). The basal diet was prepared to have an iso-nitrogenous (45% crude protein) and iso-caloric (19.69 KJ g^{-1}) content. HK L-137 was thoroughly dispersed in water before being adding to the feed. The basal diet was supplemented with the following levels of HK L-137, to prepare the treatments: 0 (control), 2, 4, 10, 20 and 40 mg kg^{-1}. The diets were steamed at 100°C for 10 min, dried at 45-50°C for 8-10 h to reach 10% moisture, and stored at -20°C until use.

Table 1

The formulation and chemical composition of experimental diets

Ingredients	*Composition (%)*
Kien Giang fishmeal	35.8
Defatted soybean meal	33.4
Cassava	8.26
Rice bran	15.0
Premix mineral and vitamin	2.00
Oil	3.08
Carboxymethyl cellulose	0.40
Lysine	0.40
Methionine	0.28
Fish solution	1.50
Phytase	0.02
Total	*100*
Chemical composition (%)	
Crude protein	45.0
Crude lipid	7.93
Ash	13.6
Carbohydrate	33.5
Energy (kJ g^{-1})	19.6

Note: premix mineral and vitamin (unit kg^{-1}): Vitamin A 2000000 IU; Vitamin D 400000 IU; Vitamin E 6 g; Vitamin B_1 800 mg; Vitamin B_2 800 mg; Vitamin B_{12} 2 mg; Calcium D Pantothenate 2 g; Folic acid 160 mg; Choline Chloride 100 g; Iron (Fe^{2+}) 1 g; Zinc (Zn^{2+}) 3 g; Manganese (Mn^{2+}) 2 g; Copper (Cu^{2+}) 100 mg; Iodine (I^-) 20 mg; Cobalt (Co^{2+}) 10 mg. Fishmeal was purchased from Kien Giang. Cassava and rice bran were local products. CMC, methionine and lysine were products of Evonik.

Feeding trial. The feeding trial was carried out in 500 L composite tanks (filled with 400 L of fresh water) in a recirculating aquaculture system at the Wet-Lab (College of Aquaculture and Fisheries, Can Tho University). The tanks were covered with a nylon net and maintained under a natural light/dark regime. At the beginning of the experiment, the fish were randomly stocked in 24 tanks (four for each diet) at a density of 60 fish $tank^{-1}$. Fish were fed to satiation twice a day at 8:00 and 16:00. The amount of feed consumed in each tank was recorded daily by removing and weighing (dry weight) the excess feed to ascertain intake. During the feeding trial period (60 days), water quality parameters, including water temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), and $N\text{-}NO_2$, were measured. The number of dead fish was recorded. At the end of the feeding, all fish were weighed, and blood samples were collected. The fish were then used for bacterial challenge and digestibility assessment experiments.

Growth performance parameters. At the end of the feeding, the growth performance parameters, namely weight gain (W_g), SGR, FCR, PER, survival rate (SR), and yield (Y) were calculated, with the following formulas:

Weight gain (W_g, %) = $[(W_f - W_i)/W_i] \times 100$

Specific growth rate (SGR, % day^{-1}) = $\{[\text{Ln}(W_f) - \text{Ln}(W_i)]/t\ (60\ \text{days})\} \times 100$

Feed intake (FI) (% $fish^{-1}$ day^{-1}) = $100 \times I/[(Wi + Wf)/2 \times T]$

Feed conversion ratio (FCR) = (amount of consumed feed in dry matter)/(weight gain)

Protein efficiency ratio (PER) = ($W_f - W_i$)/(dry protein intake)

Survival rate (SR, %) = [(Final no. of fish)/(Initial no. of fish)] × 100

Yield (Y, kg m^{-3}) = (Final no. of fish) × (W_f)

Where: W_f - final weight; W_i - initial weight. The W_i was determined before the experiment and W_f was determined after the 60-day experiment.

Digestibility experiment design. At the end of the feeding trial, fish from the same treatments were pooled and randomly introduced to three tanks at a density of 30 fish per tank. The fish were fed a basal diet containing 1% chromium oxide (Cr_2O_3) as the inert marker. After seven days of feeding adaptation, fish feces were collected by the settlement method (Hien et al 2010) for two weeks (or until a sufficient amount of 10 g dry weight was reached). Briefly, fish were fed once at 14:00, the uneaten feed was removed after 2 hours, tanks were cleaned, feces collection bottles were installed, and feces were collected until the next morning. Feces were freeze-dried immediately and kept at -20°C until analysis. The moisture, crude protein, crude lipid, and total ash of both fish feces and diets were analyzed following AOAC (2000). Moisture was determined through drying at 105°C to constant weight. Crude protein was analyzed using the Kjeldahl method. Crude lipid was analyzed using petroleum ether extracted under the Soxhlet method. Ash was determined in a furnace at temperatures of 560-600°C for 6-8 h. The concentrations of Cr_2O_3 in diets and feces were determined according to Furukawa & Tuskahara (1966). Calculations included:

Apparent digestibility coefficient of diet (ADC_{diet}):

$$ADC_{diet} = 1 - (A/B)$$

Apparent digestibility coefficient of nutrient in diet ($ADC_{Nu\text{-}Diet}$)

$$ADC_{Nu\_Diet} = 1 - (A/B) \times (B'/A')$$

Where: A - Cr_2O_3 % in feed; B - Cr_2O_3 % in feces; A' - nutrient % in feed; B' - nutrient % in feces.

Intestinal morphology. Intestinal tract samples fixed in Bouin solution were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to the method described by Krogdahl et al (2003). The paraffin blocks were sectioned (5 mm) in serial sagittal section using a Leica RM 2135 rotary microtome and were stained with hematoxylin and eosin (H&E). The sections were examined using a light microscope to determine villi length and muscle thickness. Photographs were taken with an Olympus digital camera attached to the microscope. 10 random villi from each segment were measured.

Immune response parameters. At the end of the feeding trial, blood samples (n=8 fish per treatment) were used for analyses of hematological parameters and LA. The red blood cell (RBC) count was determined in duplicate for each sample using the Neubauer hemocytometer after dilution with the Natt-Herrick solution (Natt & Herrick 1952). White blood cell (WBC) count was analyzed according to Chinabut et al (1991). The LA was measured from a standard curve generated by the lysis of a Gram-positive bacterium (*Micrococcus lysodeikticus*) according to Ellis (1990). One unit of LA was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} mL^{-1}.

Bacterial challenge test. The bacterial challenge test was immediately conducted at the end of the feeding trial (60 days). The four treatments in the feeding experiment were subdivided. The fish from three tanks (of treatment) were injected intraperitoneally with 0.1 mL of *Aeromonas hydrophila* at the lethal dose (LD_{50}) of 3.1×10^6 CFU mL^{-1}. Fish from three randomly chosen tanks (controls) were also injected with 0.1 mL of sterile physiological saline (0.85%), following the method of Ward et al (2016). During the 14-day post-inoculation period, the fish were fed their respective diets, and the cumulative mortality was noted daily. For moribund fish, the clinical signs were observed and the samples were collected for bacterial isolation. Re-isolation and re-identification of bacteria were carried out according to the method described by Barrow & Feltham (1993) and Panangala et al (2007).

Data analysis. All data were presented as mean value±standard deviation (S.D., n=8). Mean differences of parameters among treatments were tested by one-way ANOVA followed by Duncan's multiple range tests (using MBI SPSS Statistics Version 21). The differences were considered significant at $p<0.05$.

Results and Discussion

Water quality parameters. The effects of HK L-137 on water quality parameters are presented in Table 2. There was no significant difference for all parameters, including water temperature, pH, DO, TAN, and $N\text{-}NO_2$, among the treatments ($p>0.05$), suggesting that the dietary administration of HK L-137 is not correlated to changes in water quality. The probiotics supplemented via feeding were directly consumed by fish and did not consequently affect the culture water (Elsabagh et al 2018). Abareethan et al (2013) have also stated that the levels of ammonia, nitrate, nitrite, and DO in the culture water are significantly reduced by water probiotics rather than feed probiotic application. On the other hand, the decreased ammonia concentration and increased pH, electrical conductivity, total dissolved solids, and salinity were found in the culture system of Nile tilapia (*Oreochromis niloticus*) fed with a mixture of *Bacillus* strains (Elsabagh et al 2018). The differences herein may be explained by differences in the probiotic bacterial species and bacterial forms (live and heat-killed). However, there was no evidence on the presence of HK L-137 in the culture water, which needs to be investigated in further studies to determine whether the dietary administration of HK L-137 is able to control the water quality in the snakehead fish culture system.

Table 2

Water quality parameters during 60 experimental days

Water quality parameters		*HK L-137 (mg kg^{-1}) treatment*					
		0 (Control)	*2*	*4*	*10*	*20*	*40*
Temperature (°C)	AM	27.7±0.65	27.4±0.87	27.6±0.76	27.6±0.75	27.6±0.73	27.6±0.72
	PM	30.5±0.40	30.6±0.43	30.6±0.43	30.6±0.38	30.5±0.36	30.5±0.50
pH	AM	7.4±0.24	7.4±0.26	7.4±0.24	7.4±0.27	7.4±0.29	7.4±0.25
	PM	7.7±0.17	7.7±0.17	7.7±0.18	7.7±0.17	7.7±0.17	7.7±0.17
DO (mg L^{-1})		5.3±0.65	5.2±0.83	5.1±0.74	5.2±0.83	5.0±0.71	5.3±0.80
TAN (mg L^{-1})		0.28±0.15	0.28±0.14	0.33±0.19	0.33±0.23	0.33±0.21	0.31±0.20
$N\text{-}NO_2$ (mg L^{-1})		0.29±0.16	0.28±0.15	0.44±0.60	0.28±0.19	0.27±0.22	0.27±0.14

Note: values represents means of 21 measurements ± SD.

Growth parameters, survival rate and nutrient utilization. Growth performance, survival rate, and feed utilization of snakehead fish were improved by feeding HK L-137-supplemented diets (Table 3). The results revealed that W_f and weight gain of fish fed with HK L-137-supplemented diets (at 2, 4, 10, and 20 mg kg^{-1}) were significantly higher than those in the control ($p<0.05$). The SGR of the fish fed 2 and 4 mg kg^{-1} of HK L-137 was significantly increased compared to that of fish from controls ($p<0.05$). FI was lowest in the fish fed 2 and 10 mg kg^{-1} of HK L-137, significantly different from the control ($p<0.05$), but not from other groups ($p>0.05$). PER was significantly improved in the fish fed 2 mg kg^{-1} of HK L-137, compared to that of fish in the control ($p<0.05$), although there was no significant difference among the fish fed with HK L-137-supplemented diets ($p>0.05$). FCR was significantly decreased in fish fed 2, 10, and 20 mg kg^{-1} of HK L-137 compared to the control ($p<0.05$), but not significantly different from other groups (fed with 4 or 40 mg kg^{-1} of HK L-137). The SR was highest in fish fed 4 mg kg^{-1} of HK L-137, which was significantly different from those of the fish fed the control diet ($p<0.05$). The yield of the fish fed 2, 4, and 10 mg kg^{-1} of HK L-137 was significantly improved compared to the controls ($p<0.05$). The results of this study are similar to the findings for other fish species fed with heat-killed *L. plantarum* (Dawood et al 2015a; Dawood et al 2015b; Dawood et al 2019; Duc et al 2020). For example, a diet supplemented with HK L-137 (at 100 mg kg^{-1} feed) significantly increased performance parameters (final body weight, WG, SGR, FCR) of genetically improved farmed tilapia (GIFT) (*O. niloticus*) compared to fish fed control diet (Dawood et al 2019).

Table 3

Growth parameters, survival rate and nutrient utilization in snakehead (*Channa striata*) fed different diets for 60 days

Growth parameters	*HK L-137 (mg kg^{-1}) treatment*					
	0 (Control)	*2*	*4*	*10*	*20*	*40*
W_i (g)	7.30±0.08^{a}	7.27±0.09^{a}	7.31±0.06^{a}	7.33±0.05^{a}	7.33±0.08^{a}	7.26±0.03^{a}
W_f (g)	64.5±2.62^{c}	69.4±2.94^{a}	69.8±1.77^{a}	68.8±2.47^{ab}	66.6±2.82^{ab}	65.2±2.17^{bc}
Wg (g)	57.2±2.65^{c}	62.2±2.92^{a}	62.5±1.82^{a}	61.5±2.48^{ab}	59.3±2.82^{ab}	57.9±2.19^{bc}
SGR (% day^{-1})	3.63±0.07^{b}	3.76±0.06^{a}	3.76±0.05^{a}	3.73±0.06^{ab}	3.67±0.07^{ab}	3.65±0.06^{ab}
FI (% $fish^{-1}$ day^{-1})	3.88±0.55^{a}	3.14±0.31^{b}	3.35±0.17^{ab}	3.26±0.54^{b}	3.60±0.24^{ab}	3.53±0.17^{ab}
PER	1.71±0.22^{b}	2.13±0.23^{a}	1.99±0.10^{ab}	2.08±0.41^{ab}	1.84±0.13^{ab}	1.86±0.09^{ab}
FCR	1.31±0.19^{a}	1.05±0.11^{b}	1.11±0.07^{ab}	1.09±0.18^{b}	1.21±0.09^{b}	1.19±0.06^{ab}
SR (%)	74.5±4.97^{b}	77.4±2.88^{ab}	80.4±1.59^{a}	79.9±2.35^{ab}	75.5±3.69^{ab}	75.4±3.93^{ab}
Y (kg^{-1} m^3)	5.78±0.48^{c}	6.45±0.16^{ab}	6.73±0.11^{a}	6.60±0.16^{a}	6.02±0.14^{bc}	5.90±0.47^{c}

Note: Wi - initial weight; Wf - final weight; Wg - weight gain; SGR - specific growth rate; FI - feed intake; PER - protein efficiency ratio; FCR - feed conversion ratio; SR - survival rate; Y - yield; the values are means of the three replicate groups ±SD; within a row, different superscripts show significant differences ($p<0.05$).

It has been reported that probiotic bacteria can colonize the host intestine and exert their beneficial effects in improving digestion and absorption of nutrients and promoting the activity of digestive enzymes (Dawood et al 2019). This is associated with the findings of our study, where an increase in digestibility (via ADC) in snakehead fed diets containing HK L-137 was observed (Table 4). In Table 4, the ADC of dry matter, crude lipid, crude ash, and energy in fish fed diets containing HK L-137 were significantly higher than those in the controls ($p<0.05$). On the other hand, the ADC of crude protein was non-significantly increased in the fish fed HK L-137 supplemented diets compared to the controls ($p>0.05$). The increase in ADC in this study is in agreement with the results obtained previously by Rodriguez-Estrada et al (2013), who suggested the significance of heat-killed probiotic-supplemented diets on nutrient digestibility in rainbow trout (*Oncorhynchus mykiss*). Therefore, HK L-137, in this study, was demonstrated to be a growth promoter in snakehead.

Table 4

Apparent digestibility coefficients (ADC %) of diets and nutrients of the diets in snakehead (*Channa striata*) fed the HK L-137-containing diets

Diets		*Dry matter*	*Crude protein*	*Crude lipid*	*Crude ash*	*Energy*
HK L-137 (mg kg^{-1})	0	70.3±0.02^{d}	84.9±0.30^{c}	79.1±2.72^{b}	41.4±2.21^{c}	77.5±0.49^{d}
	2	76.3±0.15^{a}	88.0±0.47^{ab}	84.8±0.61^{a}	52.9±0.38^{a}	82.1±0.14^{a}
	4	75.2±0.34^{b}	87.8±0.84^{ab}	84.7±1.60^{a}	52.1±0.66^{a}	81.2±0.47^{ab}
	10	75.0±0.93^{b}	88.4±0.56^{a}	82.6±0.93^{a}	49.8±5.03^{ab}	81.1±0.27^{ab}
	20	74.4±0.77^{bc}	88.2±0.52^{a}	84.3±0.84^{a}	52.3±1.47^{a}	80.3±0.54^{bc}
	40	73.5±0.14^{c}	87.1±0.32^{b}	82.4±1.60^{a}	47.1±1.94^{b}	79.5±1.44^{c}

Note: values represent means of four replicate groups ±SD; within a column, different superscripts show significant differences ($p<0.05$).

Intestinal morphology. The intestinal morphological changes of snakehead fish fed either control or HK L-137 supplemented diets are presented in Table 5 and Figure 1. The height and length of the intestinal villi were significantly increased in the fish fed with HK L-137 supplemented diets (20 and 40 mg kg^{-1} and 4, 10, and 20 mg kg^{-1}, respectively) compared with those of fish from the controls ($p<0.05$). An enhancement in the crypt length of the intestine of fish fed with 4 and 20 mg kg^{-1} of HK L-137 was observed when compared to the controls ($p<0.05$). This is similar to the results of a previous study of Frouël et al (2008), where both live and heat-inactivated *Lactobacillus* were able to change the morphological structure of the intestine of sea bass (*Dicentrarchus labrax*), which induced important modifications to the gut ultrastructure with the appearance of endocytotic vesicles. It has been stated that changes in the intestinal structure may promote nutrient absorption on the surface area and indirectly improve growth performance (Khojasteh 2012).

Table 5

Intestinal morphometry of snakehead fish fed the HK L-137-containing diets

Diets		*Villus height (μm)*	*Villus width (μm)*	*Crypt (μm)*
HK L-137 (mg kg^{-1})	0	602.7±99.6^{c}	79.9±12.9^{c}	31.3±7.71^{c}
	2	618.4±76.9^{bc}	80.9±9.10^{bc}	32.0±6.91^{c}
	4	611.7±82.1^{bc}	89.1±13.5^{a}	38.8±12.5^{a}
	10	624.8±98.4^{bc}	86.7±13.9^{ab}	32.9±11.2^{bc}
	20	683.7±121.9^{a}	90.6±15.5^{a}	37.3±12.6^{ab}
	40	659.1±118.4^{ab}	84.9±17.6^{abc}	31.8±12.2^{c}

Note: values are means of four replicate groups ±SD; within a column, different superscripts represent significant differences ($p<0.05$).

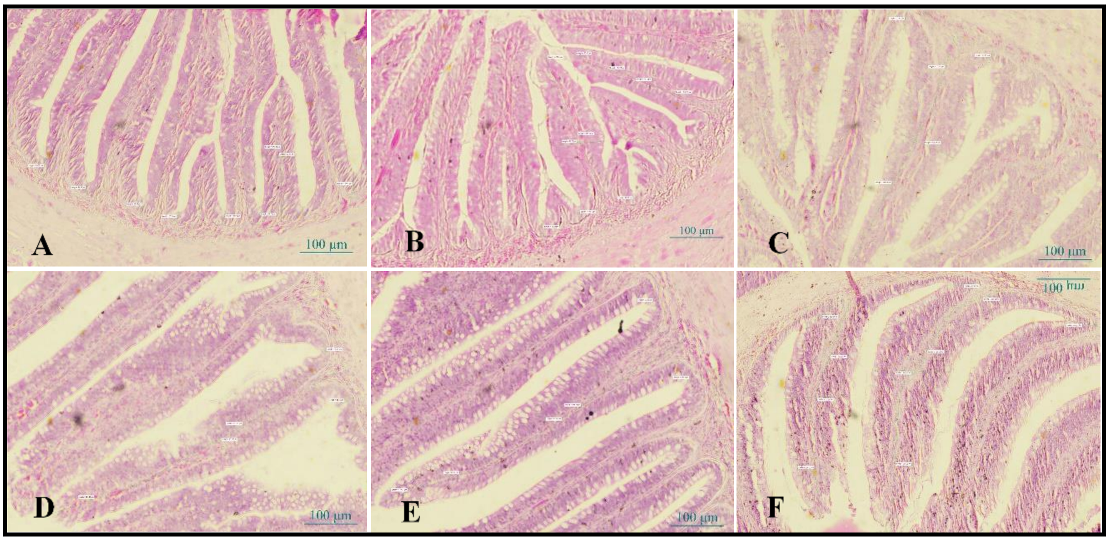

Figure 1. Histomicrograph of intestine of snakehead (*Channa striata*) fed the HK L-137-supplemented diets for 60 days.

Hematological parameters and immune response. RBC and WBC counts of snakehead after 60 days of feeding with HK L-137 supplementation are presented Figure 2A and Figure 2B, respectively. The RBC count in the fish fed 2 mg kg^{-1} of HK L-137 was higher than those fed other diets, but the difference was not significant ($p>0.05$) (Figure 2A). This is similar to the results obtained for *Brycon amazonicus* breeders fed with dietary supplementation of *B. subtilis* (Dias et al 2011), but not in *Clarias gariepinus* juveniles fed with a mixture of *Lactobacillus* and *Bifidobacterium* species (at 1.5 and 2.0 g) (Ayoola et al 2013) or in kutum (*Rutilus frisii kutum*) fry fed with *Bacillus licheniformis* and *Bacillus subtilis* (Azarin et al 2015). The differences herein may be explained by the differences in fish species and developmental stages, as well as by the different probiotic bacteria administered. The WBC count in the fish fed diets supplemented with 2, 4, and 10 mg kg^{-1} of HK L-137 was significantly higher than for those fed control and other diets (supplemented with either 20 or 40 mg kg^{-1} of HK L-137) ($p<0.05$) (Figure 2B). This is consistent with the findings in *O. mykiss* (Faramarzi et al 2011), *Clarius batrachus*

(Dahiya et al 2012), *Clarias gariepinus* (Ayoola et al 2013), *Rutilus frisii kutum* (Azarin et al 2015), and *Acipenser baerii* (Pourgholam et al 2017) fed with probiotic bacteria. The increase in WBC count of snakehead may indicate the main induction of HK L-137 on the innate immunity rather than adaptive immunity, probiotics interacting with the immune cells (i.e., monocytes, macrophages, neutrophils, and natural killer cells) to enhance innate immune responses (Pourgholam et al 2017). The LA of the fish fed diets supplemented with HK L-137 (with the highest in fish fed 10 mg kg^{-1} of HK L-137) was significantly increased compared to the controls ($p<0.05$) (Figure 2C). This indicates the role of HK L-137 in improving the immunity of snakehead, which is similar to previous findings in *O. niloticus* fed HK L-137 (Nguyen et al 2019).

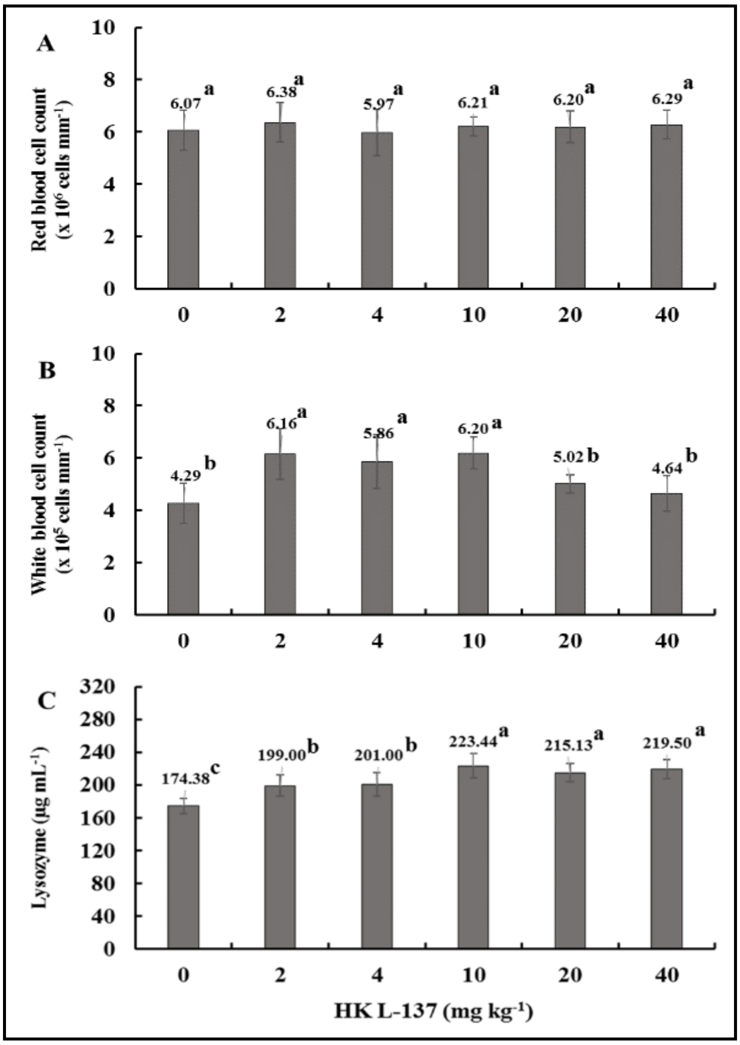

Figure 2. Hematological parameters and immune response in snakehead (*Channa striata*) fed the HK L-137-containing diets for 60 days; A - white blood cell count; B - red blood cell count; C - lysozyme activity. Each bar represents the mean value with SD from 8 fish for each group; values with different superscripts show significant differences ($p<0.05$).

Challenge test. The challenge test study showed that snakehead fed HK L-137 had significantly higher protection against *A. hydrophila* infection (Figure 3). The moribund or dead fish exhibited the clinical signs of hemorrhagic septicemia. As expected, the lowest mortality (3.33%) was recorded in the negative control group, whereas the highest mortality (56.7%) was observed in the positive control. The cumulative mortality of fish fed 4, 10, 20, and 40 mg kg^{-1} of HK L-137 was 26.6%, 33.3%, 30.0%, and 36.7%, respectively, which was significantly lower than those of positive controls ($p<0.05$). Fish fed 2 mg kg^{-1} of HK L-137 displayed a low cumulative mortality (6.66%), which was significantly different from other groups ($p<0.05$), excepting negative control ($p>0.05$).

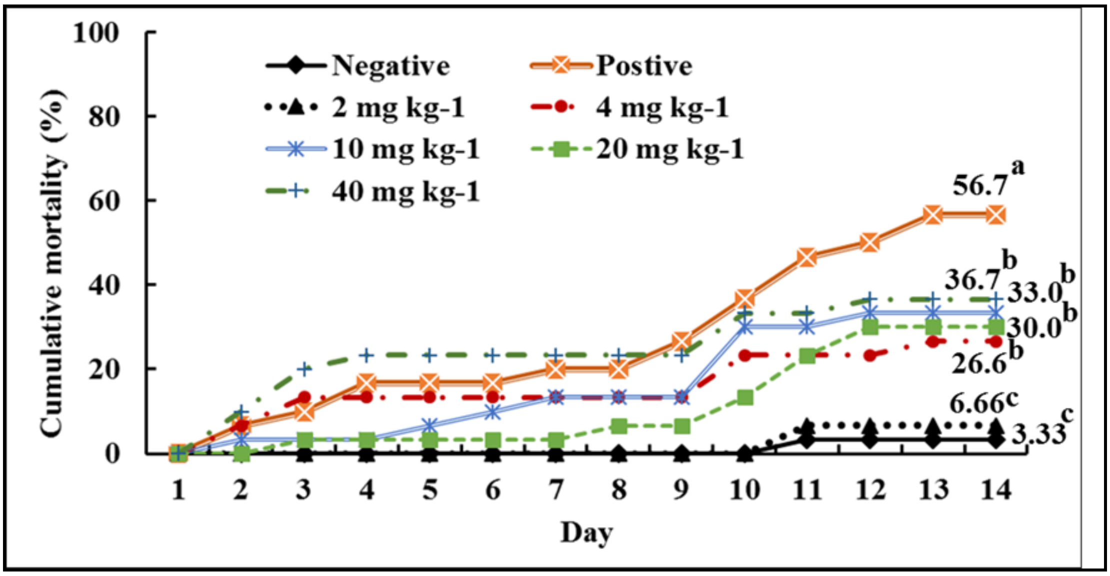

Figure 3. Cumulative mortality of HK L-137-fed snakehead (*Channa striata*) after challenge with *Aeromonas hydrophila* (3.1×10^5 CFU $fish^{-1}$) for 14 days.

The decreased cumulative mortality of HK L-137-fed snakehead challenged with *A. hydrophila* may be related to the stimulation in immunity of the fish. This is in accordance with the findings in Japanese pufferfish administered heat-killed *Lactobacillus paracasei* spp. *paracasei* (06TCa22) (Biswas et al 2013a). Biswas et al (2013a) have reported that the increase of *V. harveyi* removal level, superoxide anion production, and phagocytic activity of the fish fed with a probiotic-supplemented diet are associated with an enhancement in the regulation of cytokines. *Bacillus* probiotics showed action against *Streptococcus iniae* through the enhancement of WBC count and phagocytosis in Nile tilapia (Moustafa et al 2020). Furthermore, *L. plantarum* and *Lactobacillus fructivorans* have been proven to be beneficial for the gut mucosal immunity in gilthead sea bream (*Sparus aurata*) (Picchietti et al 2007). The improvement in the systemic immunity (via alternative complement activity, lysozyme, glutathione peroxidase, respiratory burst, and phagocytic activities) of grouper (*Epinephelus coioide*) by feeding *L. plantarum* has been reported (Son et al 2009). Thus, we suggest that the dietary administration of HK L-137 is important in the increase of immunity and disease resistance of snakehead. However, the mechanisms of action of HK L-137 need to be confirmed in detail in further studies.

Conclusions. The results of this study demonstrated that heat-killed *L. plantarum* (HK L-137) could enhance the growth performance, immune response, survival rate, and disease protection of snakehead, and that it could be used as a potential probiotic in snakehead aquaculture. The levels of HK L-137 at 2, 4, 10, 20, and 40 mg kg^{-1} conferred beneficial effects to snakehead. The level of 2 mg kg^{-1} of HK L-137 produced the highest survival rate of the fish challenged with *A. hydrophila*. However, the mechanisms of action of HK L-137 in snakehead are still unclear and need to be investigated in detail in further studies.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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