Dietary supplementation with heat-killed *Lactobacillus plantarum* L-137 improves growth, survival rate, and immune response of snakehead (*Channa striata*) in practical Hapa culture

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Abstract. This study investigated the effects of heat-killed *Lactobacillus plantarum* L-137 (HK L-137) on the growth, survival, and immune response of snakehead (*Channa striata*) cultured in practical field conditions with Hapa nets. The fish (13.7±4.73 g fish^{-1}) were fed diets supplemented with 0 (control), 2, and 4 mg of HK L-137 kg^{-1} of feed. After feeding for 215 days, all fish were harvested. The results showed that HK L-137 supplemented diets improved the growth, protein efficiency ratio, feed conversion ratio, and survival rate of fish. HK L-137 supplemented diets also significantly increased the white blood cell count and lysozyme activity, but did not affect the red blood cell count. Hence, overall, dietary supplementation with HK L-137 enhance survival, growth and immune response of snakehead cultured under typical farming conditions.
Key Words: *Channa striata*, growth performance, heat-killed bacteria, pond condition.

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, improving water quality, and 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 for aquatic animals (Son et al 2009; Tuan et al 2013).

Lactobacillus plantarum is a rod-shaped, lactic acid bacteria, Gram-positive and heterofermentative (Dash et al 2015; Dawood et al 2015a), widely used in fermenting plant products and as a probiotic (Ashenafi & Busse 1991; Son et al 2009). The bacterium can 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). As a probiotic in aquatic animals, the viable living forms of the bacterium have been shown to improve the activity of digestive enzymes, growth performance, feed utilization efficiency, immunity, disease resistance, and 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 living forms of probiotics, inactivated probiotics appear to be an alternative to their live counterparts, and can be used more safely in open aquatic environments (Dawood et al 2015a; Nguyen et al 2019). In Japanese pufferfish (*Takifugu rubripes*), for example, Biswas et al (2013) reported that the heat-killed *Lactobacillus plantarum* 06CC2 isolated from Mongolian dairy products significantly induced the expression of pro-inflammatory cytokines (Il-1β, Il-6, Tnf-ɑ, 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 red sea bream (*Pagrus major*) for 56 days. It was determined that HK L-137 can improve specific growth rate (SGR), body weight gain, protein efficiency ratio (PER), feed intake (FI), serum lysozyme activity (LA), serum alternative complement pathway activity, and mucus secretion in juvenile red sea bream (Dawood et al 2015b). Other studies have shown that HK L-137 provided by House Wellness Foods Corp (Itami, Japan) improves growth performance, immunity, and stress resistance in fish when used as a probiotic (Yang et al 2016; Dawood et al 2019; Nguyen et al 2019; Duc et al 2020; Hien et al 2021b; Liem et al 2021; Duc et al 2022; Hien et al 2022).

Snakehead (*Channa striata*) is widely distributed across southern Asia, southern China, Indochina, and the Sunda Islands (Song et al 2013). The fish can live in a wide range of habitats, such as rivers, swamps, ponds, canals, lakes, and even rice fields (Song et al 2013). In the Mekong Delta, the fish 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). The use of formulated feed is common in snakehead culture (Hien et al 2018). Dietary supplementation with HK L-137 at the rate of 2 mg kg^{-1} has been shown to enhance the growth performance, immune response, survival rate, and disease protection of snakehead under laboratory conditions (Hien et al 2021a). However, there is very little information on the use of HK L-137 as a potential probiotic in snakehead aquaculture. The aim of this study, therefore, was to assess the effects of HK L-137 on the growth, survival rate, and immune parameters of snakehead under practical farming conditions.

Material and Method

Experimental fish, HK L-137 preparation, and diets. Healthy juvenile snakehead (13.7.30±4.73g $fish^{-1}$) were purchased from a fish hatchery in Dong Thap Province, Vietnam in April 2021. Fish were acclimatized for two weeks in a 27-m^2 Hapa inside a pond of 3000 m^2 at the Research Station of Viet Thang Feed Joint Stock Company, Dong Thap province, under prevailing water conditions (temperatures of 30.8-33.2^{o}C, dissolved oxygen concentrations of 5.1-5.3 mg L^{-1}, and a pH range of 7.16-7.55). Fish were fed a commercial diet with 45% crude protein (Viet Thang Feed Joint Stock Company, Dong Thap province) twice a day (at 08:00 and 16:00) to satiation during the acclimatization period.

For the feeding trial, fish were fed one of three experimental diets, a basal diet (control with no added HK-137), a diet containing 2 mg kg^{-1} HK L-137, and a diet containing 4 mg kg^{-1} HK L-137. The source of HK L-137 was LP20 feed, which contained 20% HK L-137 and 80% dextrin on a dry-weight basis, with a concentration of approximately 2×10^{11} cells g^{-1} (House Wellness Foods Corporation, Itami, Japan). The basal diet was formulated by the Viet Thang Feed Joint Stock Company, Vietnam, and had the chemical composition shown in Table 1. The basal diet was prepared to have an iso-nitrogenous (45% crude protein) and iso-caloric (19.69 KJ g^{-1}) content.

The feeding trial was conducted in one large pond (3000 m^2), with three replicate hapas for each dietary treatment. Each hapa of 81 m^3 (3x9x3 m in LxWxH) was randomly stocked at a density of 181 fish m^{-2} and the fish were cultured for a period of 215 days, until market size was attained. During the trial, fish were fed to satiation twice a day at 8:00 and 16:00. Feed consumption was recorded as the difference between the dry weight of feed given and the dry weight of uneaten feed. Water temperature and pH were monitored weekly with a YSI 556 meter (Yellow Springs Instruments, Yellow Springs, Ohio, USA); NH_4^+-N and NO_2^--N were monitored weekly by the indophenol and diazonium methods, respectively (APHA 2012). Dead fish were recorded and removed daily. At the end of the experiment, all fish were weighed (live weight), and blood samples were collected.

Table 1

The chemical composition of basal diets*

Chemical composition	Content
Crude protein (%)	40
Crude lipid (%)	6
Ash (%)	5
Ethoxyquin (mg kg^{-1})	<150
Total phosphorus (%)	0.8
Total lysine (%)	1.5
Energy (kJ g^{-1})	19

Note: * - Viet Thang Feed Joint Stock Company, Vietnam (GLOBAL G.A.P.).

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

Weight gain (W_g, g $fish^{-1}$) = (Wf - Wi)

Specific growth rate (SGR, % day^{-1}) = [Ln (W_f) - Ln (W_i)/t (215 days)] × 100

Feed intake FI (% $fish^{-1}$ day^{-1}) = 100×I/[(Wi + Wf)/2×215 days]

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^{-2}) = Total yield/27 m^2 Hapa

Where: initial weight (W_i, g) was determined before the experiment, final weight final (W_f, g) was determined after the 215-day experiment, and I was the total feed $fish^{-1}$.

Immune response parameters. At the end of the feeding trial, blood samples (n=9 fish per treatment) were used for analyses of hematological parameters and serum lysozyme activity (LA). The red blood cell (RBC) count was determined in duplicate for each sample using a Neubauer hemocytometer after dilution with Natt-Herrick solution (Natt & Herrick 1952). White blood cell (WBC) count was analyzed according to Chinabut et al (1991). Serum lysozyme activity was read from a standard curve generated by the lysis of a Gram-positive bacterium (*Micrococcus lysodeikticus*) according to the method of 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}.

Data analysis. All data were presented as mean value±standard deviation (S.D., n=9). 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. All hapas were located in the same 3000 m^2 pond, so all fish were cultured in the same practical water quality conditions (Table 2), irrespective of their diet. Water quality was suitable for the normal growth of fish. The effects of probiotics on water quality appear to vary with the bacterial composition of the probiotic, whether or not the bacteria are living or have been heat-killed, and whether they are supplied in the feed or added directly to the water. In general, probiotics provided in the feed are consumed directly by fish and do not affect water quality significantly (Elsabagh et al 2018), although the same authors reported decreased ammonia concentration and increased pH, electrical conductivity, total dissolved solids, and salinity in the culture system when Nile tilapia (*Oreochromis niloticus*) were fed with a mixture of *Bacillus* strains (Elsabagh et al 2018). Probiotics consisting of organisms added directly to the pond water have been reported to reduce the levels of ammonia, nitrite, and dissolved oxygen in the pond (Abareethan et al 2013). In the case of HK L-137, further work is needed to determine whether dietary administration of HK L-137 is able to control water quality in the snakehead fish culture system.

Table 2
Average water quality parameters in the pond during the 215 experimental days

Water quality parameters		*Value*
Temperature (°C)	AM	30.15±0.85
	PM	31.41±1.29
pH	AM	7.85±8.01
	PM	8.01±0.84
NH_4^+-N (mg L^{-1})		0.072±0.063
NO_2^--N (mg L^{-1})		0.016±0.001

Note: values represent means of 30 measurements ± SD.

Growth parameters, survival rate and nutrient utilization. W_g, SGR, FCR, and PER were all improved by the addition of HK L-137 to the diet at a rate of 4 mg kg^{-1} (Table 3). However, for some unknown reason, fish fed 2 mg kg^{-1} HK L-137 in their diet had a lower weight gain and specific growth rate than fish fed the control diet without HK L-137 (Table 3). The FI and FCR of fish fed 2 and 4 mg kg^{-1} of HK L-137 were marginally lower than for fish in the control, whereas PER was marginally higher in fish fed 2 and 4 mg kg^{-1} of HK L-137, compared to that of fish in the control. The survival rate (SR) was highest in fish fed 2 mg kg^{-1} of HK L-137. However, despite these relatively modest differences in performance between diets, there was no significant difference in yield (Table 3). Broadly, the results of this study are similar to those 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; Hien et al 2021a; Hien et al 2021b; Liem et al 2021; Duc et al 2022; Hien et al 2022). 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 215 days

Growth parameters	*HK L-137 (mg kg^{-1}) treatment*		
	0 (Control)	*2*	*4*
W_i (g)	13.7±4.73	13.7±4.73	13.7±4.73
W_f (g)	917.4±9.87	853.3±63.7	927.4±32.3
W_g (g)	903.7±9.87	839.6±63.8	913.6±32.3
SGR (% day^{-1})	1.95±0.01	1.92±0.04	1.96±0.02
FI (% $fish^{-1}$ day^{-1})	1.48±0.03	1.47±0.03	1.43±0.02
PER	1.63±0.03	1.65±0.06	1.67±0.04
FCR	1.53±0.02	1.52±0.05	1.49±0.03
SR (%)	54.1±1.81	57.0±4.52	52.7±3.52
Y (kg m^{-2})	90.0±2.02	87.9±1.44	88.5±3.48

Note: W_i - initial weight; W_f - final weight; W_g – Weight gain; SGR – Specific growth rate; FI – Fed intake; PER – Protein efficiency ratio; FCR – Feed conversion ratio; SR – Survival rate; Y – Yield; The values are means of the three replicate groups ±SD.

Hematological parameters and lysozyme activity. RBC, WBC counts of snakehead, and the lysozyme activity after 215 days of feeding with HK L-137 supplementation are presented Figure 1A, Figure 1B, and Figure 1C, respectively.

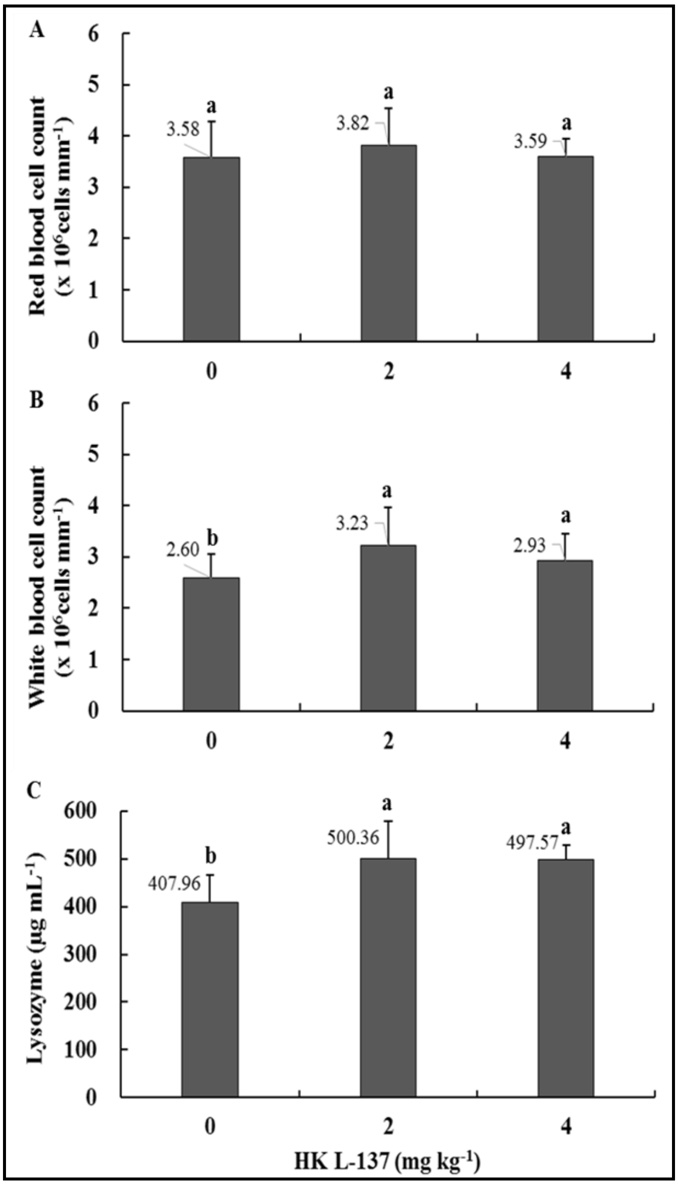

Figure 1. Hematological parameters and immune response in snakehead (*Channa striata*) fed HK L-137-containing diets for 215 days; A - white blood cell count; B - red blood cell count; C - lysozyme activity. Each bar represents the mean value with SD from 9 fish for each group; values with different superscripts show significant differences ($p<0.05$).

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 1A). This is similar to the results obtained for *Brycon amazonicus* breeders fed with dietary supplementation of *B. subtilis* (Dias et al 2011), but not for *Clarias gariepinus* juveniles fed with a mixture of *Lactobacillus* and *Bifidobacterium* species (at 1.5 and 2 g) (Ayoola et al 2013) or in kutum (*Rutilus frisii kutum*) fry fed with *Bacillus licheniformis* and *B. 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 and lysozyme concentrations in the fish fed diets supplemented with 2, and 4 mg kg^{-1} of HK L-137 were significantly higher than for those fed control ($p<0.05$) (Figures 1B and 1C). This is consistent with the findings in *O. mykiss* (Faramarzi et al

2011), *Clarius batrachus* (Dahiya et al 2012), *C. gariepinus* (Ayoola et al 2013), *R. frisii kutum* (Azarin et al 2015), and *Acipenser baerii* (Pourgholam et al 2017) fed with probiotic bacteria, and it would appear that the addition of probiotics to the diet helps to improve the immune response of many fish species. The increase in WBC count of snakehead in response to HK L-137 may indicate an improvement in innate immunity rather than in adaptive immunity, where probiotics interact 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 2 mg kg^{-1} of HK L-137) was significantly increased compared to the controls ($p<0.05$) (Figure 1C). HK L-137 improves the immunity of snakehead, as also observed in *O. niloticus* fed with HK L-137 (Nguyen et al 2019). However, in our trial, there was no consistent benefit of an improvement in immune responses on growth or yield.

Conclusions. The results of this study demonstrated that heat-killed *L. plantarum* (HK L-137) could enhance the growth performance, immune response, and survival rate under practical farm culture conditions, and that it could be used as a potential probiotic in snakehead aquaculture. The level of 2 mg kg^{-1} of HK L-137 produced the highest survival rate and a lower FCR.

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

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