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Effects of Heat-Killed *Lactobacillus plantarum* L-137 on Growth Performance, Feed Utilization, Immune Response, and Survival Rate in Red Tilapia (*Oreochromis* sp.)

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ABSTRACT

This study aimed to evaluate the effects of heat-killed *Lactobacillus plantarum* L-137 (HK L-137) on growth, feed utilization, immune response, and survival rate of cage-cultured red tilapia (*Oreochromis* sp.). The cages were located in Dong Thap (DT), Vinh Long (VL), and An Giang (AG) provinces (Vietnam). The formulated diets contained iso-nitrogenous (30.5% crude protein) and iso-lipid (5.2% lipid) compounds and were supplemented with HK L-137 at either 0 (control) or 4 mg/kg. Results indicated HK L-137 had positive effects on the weight gain, final weight, protein efficiency ratio, feed conversion ratio, survival rates, and yield of the fish as compared to the control. The red blood cell count was significantly higher in fish fed with HK L-137 than those in the control group in DT (HK L-137: 2.67 cells/mm^3 *vs.* control: 2.28 cells/mm^3) and AG (HK L-137: 2.83 cells/mm^3 *vs.* control: 2.47 cells/mm^3) at 4 months (P<0.05). The white blood cell count was significantly increased in fish fed with HK L-137 cultured in three locations at 2 and 4 months as compared to the control (P<0.05). The lysozyme activity of the fish fed HK L-137 was significantly higher than those fed a control diet in three locations at 2 and 4 months of feeding (P<0.05). Taken together, the results of this study demonstrated the role of HK L-137 in improving growth performance, feed utilization, and immune response of red tilapia.

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Keywords: Growth performance; Heat-killed *Lactobacillus plantarum* L-137; Immune response; Red tilapia; Survival

1. Introduction

Red tilapia (*Oreochromis* sp.) contributes to 8% of the total world fish production (in 2016) and ranks fourth among global fishery commodities [1]. In the Mekong Delta of Vietnam, red tilapia is one of the most important species, being cultured in different systems such as garden and farm ponds, rice fields, and cages; of these, cage culturing is considered the most productive system due to the high culture density (80-100 fish/m^3/crop) [2]. The extension of cage culture has, however, led to the spread of disease in the cultured red tilapia [3-4]. Swelling of the head and eyes, along with body hemorrhaging caused by bacterial pathogens have been previously reported to be the most common in cage-cultured red tilapia in the Mekong Delta [5]. The application of antibiotics, such as a mixture of sulfonamide with trimethoprim, amoxicillin, doxycycline, and florfenicol for controlling disease in red tilapia is popular [5]. Nevertheless, the improper use of antibiotics can influence the microbial communities harbored in the guts of fish, increase the spread of antibiotic-resistant bacteria, and leak residual runoff to the surrounding environment [6-7]. These may cause problems that indirectly affect human health; thus, the development of antibiotic alternatives that are bio-friendly and stimulate the growth and health of aquatic animals has become crucially important [8]. The application of probiotics or prebiotics is considered by many to be the best choice in sustainable aquaculture [9-12].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [13]. The major mechanisms of action of probiotics in the host are known to enhance epithelial barrier function, improve adhesion of probiotic cells to intestinal cells, and inhibit pathogen colonization by occupying adhesion sites, production of antibacterial substances, and regulation of the immune function [14]. Previous studies have shown that probiotics confer multiple health benefits, including improved growth and immune response promotion, nutritional and enzymatic contribution, and disease controlling, in aquatic animals [8, 15]. To some extent however, live probiotics may negatively influence the aquaculture environment through interactions with the ecosystem [16]. Previously, it was found that both live and dead probiotics (such as *Clostridium butyricum* CB2) exhibit an increase in immune response and protect Chinese drum (*Miichthys miiuy*) against bacterial infection [17]. Moreover, dietary supplementation with inactivated bacteria has improved the growth, immunity, and disease resistance in fish species [7, 18, 19]. Several studies have demonstrated the beneficial properties of heat-killed *Lactobacillus plantarum* L-137 (HK L-137) as a feed additive (including promotion of growth, immune response, and tolerant ability) in aquatic animals, such as Nile tilapia (*Oreochromis niloticus*) [16, 18], striped catfish (*Pangasianodon hypophthalmus*) [20], and sea cucumber (*Apostichopus japonicas*) [21]. To our knowledge, the effects of HK L-137 on red tilapia have not yet been investigated. Thus, the present study aimed to determine the effects of dietary supplementation with HK L-137 on the growth performances, feed utilization, immune response, and survival rate of red tilapia. The results of this study provide basic information on the application of HK L-137 as a growth promoter and immune stimulant in the red tilapia aquaculture industry.

2. Materials and Methods

2.1 HK L-137, diet, experimental fish, and feeding trial

HK L-137, containing 20% HK L-134 and 80% dextrin on a dried-weight basis, was

made by House Wellness Foods Corporation (Itami, Japan). The concentration of HK L-134 in dry weight is 2×10^{11} cfu/g. The chemical composition of the diets, containing iso-nitrogenous (30.5% crude protein) and iso-lipid (5.2% lipid) compounds are shown in Table 1. The feed produced by Viet Thang Feed Joint Stock Company (Vietnam) were supplemented with HK L-137 at 0 (control group) and 4 mg/kg (HK L-137-supplemented group).

Table 1. The formulation and chemical composition of commercial diet for red tilapia culture (dry matter basis).

Items (Ingredients, g.kg^{-1} dry diet)	Control diet	Diet with 4 mg/kg of HK L-137
Rice bran	195.90	195.88
Wheat	50.00	50.00
Cassava	115.00	115.00
Soybean meal	537.00	537.00
Fish meal	30.00	30.00
Meat and Bone meal	20.00	20.00
Limestone	10.50	10.50
DCP	5.00	5.00
NaCl	5.00	5.00
Fish oil	25.00	25.00
Choline (60)	1.00	1.00
Lysine	0.50	0.50
Methionine	2.50	2.50
Feed LP20 (20% HK L-137)	0.00	**0.02**
Phytase	0.10	0.10
Premix mineral and vitamin	2.50	2.50
Total (g)	**1,000**	**1,000**
Chemical composition (%)		
Crude Protein	30.5	30.5
Crude fat	5.2	5.2
Ash	9.2	9.2
NFE	10.57	10.57
Crude fiber	3.1	3.1
Gross energy (KJ/g^{-1})	10.81	10.81

Source: Viet Thang Feed Joint Stock Company, Vietnam

The floating cages used for culturing red tilapia were distributed along the Hau River, located in the Dong Thap (DT), Vinh Long (VL), and An Giang (AG) provinces (Mekong Delta, Vietnam) (Table 2). The cages in these provinces were selected for the purpose of the study to confirm the consistent role of HK L-137 in red tilapia when cultured in different locations along the Mekong River. There were two cages (used for control and experiment) at each province. The cage volumes were 360 m^3/cage (6×12×5 m for both control and HK L-137-supplemented group in DT), 252 m^3/cage (6×12×3.5 m for both groups in VL), 398 m^3/cage (6×13×5.1 m for the control in AG), and 250 m^3/cage (5×10×5 m for the HK L-137-supplemented group in AG). The healthy red tilapia had an average body weight of 37 g (in DT), 40 g (in VL), and 83 g (in AG) (Table 2) and were purchased from a local fish hatchery.

Table 2. The basic information of the feeding trial.

Treatment	Volume of cage (m^3)	Stocking density (fish/m^3)	Period culture (day)
Dong Thap province			
Control	360	59	195
HK L-137	360	59	195
Vinh Long province			
Control	252	71	138
HK L-137	252	71	138
An Giang province			
Control	398	163	142
HK L-137	250	160	142

Before the feeding, the fish were stocked in cages with 59 (in DT), 71 (in VL), and 163 and 160 fish/m^3 (for control and HK L-137-supplemented cage, respectively, in AG). Fish were fed to satiation twice per day at 8:00 and 16:00. The amount of feed consumed by the fish was carefully recorded daily. The dead fish were recorded and removed. Fish were fed for 195 (DT), 138 (VL), and 142 days (AG). The survival rate (SR, %), specific growth rate (SGR, %/day), feed conversion ratio (FCR), protein efficiency ratio (PER), and weight gain of the fish was determined at the end of the feeding. During the feeding trial, the water temperature was measured using a thermometer, the pH value (at 7:00 and 15:00) was measured weekly using a pH meter (HANA, USA), and N-NO_2 and TAN (NH_3/NH_4) were tested weekly using Test Sera (Germany). The water temperature and pH were 28.4-32.3°C and 7.6-8.1 (in DT), 27.8-31.5 and 7.5-8.0 (in

VL), and 28.1-31.6 and 7.5-8.0 (in AG), respectively.

2.2 Growth and feed utilization parameters

At the beginning and end of the feeding trial, a total of 30 fish were randomly collected and measured for the initial weight (Wi, g), final weight (Wg, g), and yield (kg/m3). The growth performance and feed utilization parameters were calculated as follows:

SR = [(number of fish at the end of experiment)/(number of initial fish)] × 100

SGR = (ln(Wf)– ln(Wi))×100/t

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

PER = (Wf – Wi)/(protein intake)

2.3 Sample collection and immune parameter analyses

Blood samples (n=8 per group) used for hematological parameter and lysozyme activity analyses were collected from the fish fed with HK L-137 at the end of the feeding trial. The red blood cell (RBC) count was measured in duplicate for each sample using the Neubauer hemocytometer after dilution with the Natt-Herrick solution [22]. White blood cell (WBC) count was analyzed according to the method previously described by Chinabut *et al.* [23]. The lysozyme activity was measured from a standard curve generated by the lysis of a Gram-positive bacterium (*Micrococcus lysodeikticus*) according to Ellis [24]. One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} mL^{-1}.

2.4 Data analysis

Immune parameters (RBC, WBC, and lysozyme activity) are presented as mean±standard deviation (S.D., n=8). Mean differences of analyzed parameters between HK L-137-supplemented and control groups were tested by *t*-test using SPSS Version 21.0 software. The differences were considered significant at $P<0.05$.

3. Results and Discussion

Red tilapia culturing, using the floating cage culture system in the Mekong Delta of Vietnam, commonly employs antibiotics to protect the fish from pathogens [5]. For safe aquaculture production, strategies using probiotics, prebiotics, and symbiotics have been developing and emerging, which are able to promote the growth performance and immune response of cultured animals [6]. In environmentally friendly sustainable aquaculture, the application of prebiotics and probiotics has also become an alternative method of antibiotic treatment. The use of probiotics in aquaculture has been previously reported to be beneficial in increasing growth and immune response, controlling disease, and aiding digestion by the host [8, 15]. Among them, heat-killed *L. plantarum* has exhibited probiotic properties that improve the growth and health status of fish and shrimp aquaculture [16, 18, 20, 21, 25-32]. This is, to our knowledge, the first comprehensive study on the effects of HK L-137 on the growth, feed utilization, and immune response of red tilapia.

3.1 Growth performance of red tilapia

Growth performance, survival rate, and feed utilization of red tilapia (cultured in DT, VL, and AG) fed different diets are shown in Table 3 and Table 4, respectively. The results showed that weight gain, SGR, PER, and yield of fish in the HK L-137-supplemented group were higher than those in the control. Meanwhile, the FCR was observed to be significantly lower in the fish fed a HK L-137-supplemented diet (4 mg/kg) compared to those fed the control diet.

Table 3. Growth parameters of cage-cultured red tilapia.

Treatment	Wi (g)	Wf (g)	Wg (g)	Y (Kg/m^3)
Dong Thap province				
Control	37	831.1	794.1	16.7

HK L-137	37	876.2	839.2	18.9
Vinh Long province				
Control	40	622.4	582.4	16.3
HK L-137	40	643.8	603.8	17.1
An Giang province				
Control	83	442.4	359.4	25.4
HK L-137	83	467.1	384.1	39.2

Note: W_i: initial mean weight; W_f: final mean weight; Wg: mean weight gain; Y: yield

Table 4. Survival rate and nutrient utilization of cage-cultured red tilapia.

Treatment	SGR (%/day)	PER	FCR
Dong Thap province			
Control	1.60	1.66	1.73
HK L-137	1.62	1.73	1.65
Vinh Long province			
Control	1.99	1.45	1.98
HK L-137	2.01	1.55	1.84
An Giang province			
Control	1.18	1.42	2.01
HK L-137	1.22	1.55	1.84

Note : SR: survival rates; SGR: specific growth rate; PER: protein efficiency ratio; FCR: feed conversion ratio.

The increased growth performance (*via* weight gain and SGR) and feed utilization (*via* PER and FCR parameters) of red tilapia fed a dietary supplementation of HK L-137 observed in this study were very similar to results obtained from previous investigations on fish species. In Nile tilapia (*O. niloticus*), diets supplemented with HK L-137 (at 20-50 ppm) improved the growth rate and enhanced phagocytic and lysozyme activity of the fish [18]. Similarly, Duc *et al.* [20] revealed that final weight, weight gain, and SGR of juvenile striped catfish (*P. hypophthalmus*) fed diets supplemented with HK L-137 (at 20 and 50 ppm) were enhanced compared to fish fed basal diets. Furthermore, Dawood *et al.* [25] found that, in red sea bream (*Pagrus major*), diets with doses (10, 100, 1000, and 2000 mg/kg diet) of heat-killed *L. plantarum* (HK-LP) promoted the growth of fish. Thus, in the present study, it can be concluded that HK L-137 may play an important role in improving growth performance in red tilapia. Interestingly, it has been reported that an increase in the growth of fish fed a dietary supplementation of HK-LP was associated with an enhancement in health status, gastric development and/or enzymatic secretion, and digestibility [25]. The higher growth of HK L-137-fed fish, in this study, may be attributable to the increase in digestive enzyme production and apparent digestibility coefficient, which is similar to that in rainbow trout (*Oncorhynchus mykiss*) [19] and genetically improved farmed tilapia (GIFT, *O. niloticus*) [16] after feeding with heat-killed probiotic-supplemented diets. Moreover, it could be suggested that the increased growth of red tilapia herein is related to the modulation of HK L-137 on the expression of growth-related genes. This is in accordance with the observation of Dawood *et al.* [16] that HK L-137 upregulated the growth-related gene (IGF-I) and the glucose regulation gene (G6PD) and downregulated the fatty acid synthase (FAS) gene in GIFT (*O. niloticus*). However, the specific mechanisms of action of HK L-137 on the growth performance and feed utilization of red tilapia are still unclear and will require further research to be understood.

3.2 Immune parameters

The results of immune parameter analyses in red tilapia (both control and HK L-137-supplemented group) are illustrated in Table 5. The lysozyme activity of fish in the HK L-137-supplemented group (in three locations) significantly increased compared to those in the control group, after two and four months of the culture period ($P<0.05$) (Table 5). After a 2- and 4-month culture period, the average WBC count of the fish in the HK L-137-supplemented group was significantly higher than that of the control group, at three locations ($P<0.05$), excluding the fish cultured in VL, where no significant difference was seen between the two groups after a 2-month feeding (Table 5). The average RBC count of fish in the HK L-137-supplemented group was not significantly different from the control at two months of the culture period in three locations ($P>0.05$); meanwhile, it was significantly higher in fish fed HK L-137 cultured in DT and AG at four months of the culture period ($P<0.05$) (Table 5).

Table 5. Lysozyme activity, red blood cell (RBC) count and white blood cell (WBC) count on the paired comparison of cage-cultured red tilapia after 2 and 4 months of the culture period.

Treatment	2 months			4 months			
	RBC ($\times 10^6$ cells/mm^3)	WBC ($\times 10^5$ cells/mm^3)	Lysozyme (µg/mL)	RBC ($\times 10^6$ cells/mm^3)	WBC ($\times 10^5$ cells/mm^3)	Lysozyme (µg/mL)	Survival rate (%)
Dong Thap province							
Control	1.89±0.09^a	1.19±0.15^b	155.8±9.91^b	2.28±0.24^b	1.49±0.28^b	171.5±8.22^b	34.0
HK L-137	2.10±0.15^a	1.55±0.20^a	186.3±8.61^a	2.67±0.18^a	1.99±0.29^a	224.5±7.52^a	36.5
Vinh Long province							
Control	1.98±0.18^a	1.21±0.06^a	127.0±27.4^b	2.04±0.16^a	1.25±0.24^b	136.8±27.1^b	36.5
HK L-137	2.06±0.21^a	1.48±0.44^a	181.1±31.3^a	2.30±0.14^a	1.67±0.18^a	205.4±10.1^a	37.1
An Giang province							
Control	1.70±0.14^a	0.82±0.32^b	115.0±19.8^b	2.47±0.24^b	1.33±0.25^b	119.1±16.4^b	35.2
HK L-137	1.92±0.12^a	1.45±0.34^a	164.3±14.3^a	2.83±0.11^a	1.80±0.24^a	195.9±35.5^a	52.5

Note: The mean value from 8 fishes for each group with standard deviation (SD), value with the same letters are not significantly different ($P>0.05$).

In this feeding trial, the higher survival rate was recorded in the HK L-137-supplemented group (at 4 mg/kg diet) rather than the control group. This is in line with observations previously made in white leg shrimp (*Litopenaeus vannamei*) fed a diet containing 100 ppm of HK L-137 [27]. The increase in survival rate of red tilapia herein may relate to the role of HK L-137 in stimulating immunomodulatory activity in the intestine of the host. It has been reported that heat-killed lactic acid bacteria (such as *Lactobacillus paracasei* spp. *paracasei*, *L. plantarum* (strain 06CC2), *Bacillus amyloliquefaciens* FPTB16, *B. subtilis* FPTB13, *Pseudomonas aeruginosa* VSG2, and *Enterococcus faecalis*) stimulate cytokine responses (associated with gene expression of pro-inflammatory cytokines, cell-mediated immune regulators, and antiviral and regulatory cytokines) and the respiratory burst and peroxidase content in fish species [31-35]. Lysozyme activity plays an important role in the innate immunity of fish [36]. Lysozyme attacks and causes damage to the outer cell wall of bacteria, which allows additional lysozymes to reach and injure internal cell structures [36-37]; the higher the lysozyme activity, the higher the capacity in killing pathogenic bacteria. In this study, the results showed that the lysozyme activity of red tilapia fed an HK L-137-supplemented diet (with 4 mg/kg) was significantly higher than those fed a control diet. Similarly, an increase in lysozyme activity of Nile tilapia (*O. niloticus*) was found in the HK L-137-supplemented groups (with 20 and 50 ppm) [18]. This is also in agreement with the findings of published works on Chinese drum (*M. miiuy*) fed *C. butyricum* CB2 [17], red sea bream (*P. major*) fed heat-killed *L. plantarum* (LP20) [25], olive flounder (*Paralichthys olivaceus*) fed heat-killed *Bacillus* sp. SJ-10 [7], and Indian major carp (*Labeo rohita*) fed heat-killed *P. aeruginosa* strain VSG2 [38]. In this study, higher lysozyme activity was found in the fish of the HK L-137-supplemented group, in addition to this groups higher RBC and WBC counts. It has been known that RBC and WBC play important roles in both the innate and adaptive immune responses, and an increase in the abundance of these cells implies a strong immune system [39-40]. Herein, the increase in the number of RBC and WBC in fish fed HK L-137 was found, which suggests the important role of HK L-137 in the enhancement of the immune system of red tilapia. The results of this study are similar to those seen in rainbow trout (*O. mykiss*) [41-42], mrigal carp (*Cirrhinus mrigala*) [43], and Siberian sturgeon (*Acipenser baerii*) [44] fed diets supplemented with probiotic bacteria. However, the RBC count in the fish fed HK L-137 cultured in VL was not found to be significantly

higher than the control at 4 months of the culture period ($P>0.05$). This may be explained by differences in water quality and flow conditions among culture locations, which will require further investigation in future studies. Thus, the increase in non-specific immune responses may protect the host against pathogens and stresses [25, 29, 32], which possibly enhanced the survival of red tilapia during the feeding trial.

4. Conclusion

The results of this study demonstrate the role of dietary supplementation of HK L-137 (at 4 mg/kg) in improving the growth performance, immune response, and survival rate of cage-cultured red tilapia. HK L-137 can be introduced into diets to improve the growth and health of red tilapia in cage cultures. However, the mechanisms of action HK L-137 has on the growth and immune stimulation in red tilapia, and the factors that affect the size of the impact of HK L-137 supplementation on red tilapia in cage cultures need to be investigated in more detail in future studies. Moreover, the findings of this study are required to be confirmed through disease challenge investigations.

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