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RESEARCH ARTICLE

Effect of a Probiotic *Lactobacillus plantarum* CR1T5 Dietary Supplements on Non-specific Immunity in Black Eared Catfish (*Pangasius larnaudii*)

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Abstract

The effects of a probiotic *Lactobacillus plantarum* CR1T5, at various concentrations(0, 10^6, 10^7, 10^8 and 10^9cfu g^{-1}) in dietary supplements for Black Eared Catfish (*Pangasius larnaudii*) were investigated. Fish were randomly allocated into 5 treatments with 3 replications and reared for 60 days. The growth performance, non-specific immunity and disease resistance were determined at 15, 30, 45 and 60 days. The results revealed that at day 15 the fish received probiotic dietary supplements at 10^7, 10^8 and 10^9 cfu g^{-1} had significantly higher Specific growth rate (SGR) and lower feed conversion ratio (FCR) than the fish received control diet. On the other hand, fish fed 10^6cfu g^{-1} dietary supplement had significantly higher SGR and lower FCR at day 45. For immune parameters, the respiratory burst activity was significantly increased after 30 days. Probiotic diets at 10^8 and 10^9cfu g^{-1} significantly improved complement activity after 15 days and significantly enhanced lysozyme activity after 45 days of feeding. All fish received probiotic dietary supplement displayed a significantly increased survival rate post-challenge with *A. hydrophila.* This study found that the optimum probiotic dietary supplement at 10^8 cfu g^{-1} had significantly improved growth performance, immune stimulation and disease resistance in *P. larnaudii*.

Keywords: Black Eared Catfish, *Lactobacillus plantarum*, *Pangasius larnaudii*, non-specific immunity, probiotic.

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INTRODUCTION

Black Eared Catfish (*Pangasius larnaudii*) is a large freshwater fish species in genus *Pangasius* which has been found in large rivers of the Chao Phraya and Mekong basins (Rainboth, 1996)[1]. *P. larnaudii* has an important value in the markets as ornamental fish. The propagation of this fish can now be successful, however, there are many problems which lead to low survival rate. One of the major problems is infectious disease. The farmers usually use antibiotics and chemicals *for disease prevention and control.* Using antimicrobial drugs and chemical compounds not only increase production cost, but the long-term uses of antimicrobial drugs also has cause the evolution of resistant strains of bacteria. It is important to develop the alternative method, like probiotic bacteria, to solve this problem. Probiotic is the bacteria associated with beneficial effects to humans and animals. It is used as biological control agents of disease in aquaculture to replace conventional drugs and reduce the use of antibiotics, Probiotics are microorganisms which generally recognized as safe (GRAS) (Brien *et al.,* 1999)[2] to human and animal including aquatic animal.

Lactobacillus is one of the most *important* lactic acid bacteria*.* It is characterized as gram positive, rod sharp, non-motile and non-spore forming that produce lactic acid as a major product of fermentative metabolism (Ringo and Gatesoupe, 1998)[3]. *Lactobacillus* has been used as probiotic in aquaculture. It improves production and enhances immune system especially the non-specific or innate immune response in fish. The primary line of defense in fish is the skin and mucus membranes. However, when pathogenic microorganisms enter the fish, cellular and humoral innate defense mechanisms are activated. At present, there are few reports on the effects of *Lactobacillus*, particularly *Lactobacillus plantarum* on lysozyme activity, respiratory burst activity and complement activity which have been frequently used as indicators of nonspecific immune functions. The increases in these activities improve health performance of fish by controlling pathogens (Verschuere *et al.,* 2000)[4]

The aims of this study were to determine the effects of *Lactobacillus plantarum* CR1T5 dietary supplements on non-specific immunity and to investigate the optimal concentration of *L. plantarum* CR1T5 on growth performance, non-specific immunity and survival rate in *P. larnaudii*.

MATERIALS AND METHODS

Preparation of Experimental Diets and Probiotic Supplementation

In this study, commercial pelleted feed containing at least 30% protein was used as a basal diet. Probiotic culture of *L. plantarum* CR1T5 prepared following Meidong *et al.* (2017)[5] was obtained from Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand. Cell suspension of *L. plantarum* CR1T5 was mixed with basal diet at 0, 10^6, 10^7, 10^8 and 10^9cfu ml^{-1}, air dried and kept in sealed containers at 4°C for immediate uses. These feed mixtures were prepared daily.

Preparation of Experimental Fish (Black Eared Catfish, *Pangasius larnaudii*)

Two weeks prior to the experiments, black eared catfishes were maintained in 1000 litre tanks and acquired to feed on a basal diet twice a day. Fishes with weight ranging between 82.84±3.35 g were allocated in a completely randomized design (CRD). Fifteen aquaria with 150 L capacity were used as experimental unit. Each aquarium contained 10 fish. Five levels of probiotic mixture i.e. 0, 10^6, 10^7, 10^8 and 10^9cfu g^{-1} with 3 replications were assigned. During the experiment, water quality was maintained (temperature, pH and DO were 26.8-27.8°C, 6.6-7.5 and 6.0-7.0 mg l^{-1}, respectively) within the acceptable range for aquaculture (Stickney, 2009)[6].

Sample Collection

Data were collected at 15, 30, 45 and 60 day intervals. One day prior to the data collection, experimented fish were abstained from feeding. Fish were anaesthetized with MS-222. Two individuals from each replicate were randomly taken for measuring growth performance and feed efficiency. For Blood sampling, a 26G-syringe and ordinary 1-ml plastic syringe were administered to draw blood through caudal vein.

For lysozyme and complement activities, another 0.5 ml blood sample was drawn with uncoated syringe, then transferred to microcentrifugal tube and sit for 1 hr at room temperature and at 4°C for solidification. Serum was extracted using centrifuge at 3000 rpm for 5

mins and kept at -20°C. For nitrobluetetrazolium reduction activities, 1 ml of blood sample was drawn with hypodermic needle coated with heparin and transferred to a conical tube already containing 3 ml of Roswell Park Memorial Institute Medium (RPMI 1640). Samples were kept ice cold for lymphotic leucocyte extraction by centrifugal technique.

Non-specific Immunoassay

Lysozyme activity assay

Lysozyme activity assay explained by Obach *et al.*(1993).[7] was followed. Twenty-five ml of serum from Black Eared Catfish was placed in a well-plate, 3 wells per sample followed by addition of 0.02% *Micrococcusly sodeikticus*in 175 ml of 0.05M PBS, pH 6.2. Kinetic mode was measured every 0.5 min-interval for 5 mins at 530 nm. Lysozyme concentration obtained was later compared against standard concentration of hen egg white lysozyme (HEWL) and reported as milligram per milliliter (mg ml^{-1}).

Respiratory burst activity

An assay of respiratory burst activity explained by Secombes (1990)[8] was followed with a slight modification. Prepared 175 ml leucocyte samples of $6x10^6$ cell ml^{-1} were placed on 96-well plate, topped with 25 ml NBT each well and incubated at room temperature for 2 hrs. Clear supernatant was discarded, remaining solution was then carefully washed with 125 ml absolute methanol twice and air-dried. 125 ml of 2N KOH followed by 150 ml of DMSO were later added. The respiratory burst activity was expressed as A655 nm. Spontaneous O_2^- Production = (Absorbance NBT reduction of sample – Absorbance of blank)

Alternative complement

Method of complement activity study by Yano (1992)[9] was modified. Three ml of rabbit red blood cell (RaRBC) was prepared in a 15ml conical tube and centrifuged at 2500 rpm for 5 mins. Clear supernatant was discarded. Remaining RaRBC was washed twice with PBS and spun at 2500 rpm for 5 mins and finally washed three times with EGTA-mg GVB at 2500 rpm for 5 mins at room temperature. Final RaRBC concentration was made up to $2x10^8$ cell ml^{-1} with EGTA-mg GVB.

Fish serum was diluted 20 folds from 50 ml serum in 0.01 M EGTA-mg GVB 450 ml to given ratios of 1:20, 1:40, 1:80, 1:160 and 1:320. Conical tube containing only 0.01 M EGTA-mg GVB 20 ml and another containing 100% cell lysis in 3.4 ml deionized distilled water were used as control. Every conical tube was added with 100 ml RaRBC, incubated at 20°C for 90 mins, shaken every 15-20 mins, followed by addition of 3.15 ml of 85% NaCl and lastly centrifuged at 2500 rpm, 4°C for 5 mins. OD was measured at 414 nm. Complement activities was expressed as ACH50 unit'ml^{-1}

ACH50 value(unit ml^{-1}) = 1 / k x (reciprocal of the serum dilution) x 0.5

Where K is the amount of serum giving 50 % hemolysis, 0.5 is the correction factor since this assay was performed on half scale of the original method.

Challenge test

After 60 days of probiotic diet supplementation, ten individuals Black Eared Catfish was randomly selected and injected with 0.1 ml of *Aeromonas hydrophila* FW52, (~10^7cfu ml^{-1}) into abdominal cavity (Tongpim *et al*., 2009)[10]. Mortality and Relative Percent Survival were observed over 14 days. Noted that *A. hydrophila* suspension was a courtesy of Department of Microbiology, KhonKhaen University. RPS = (1-mortality in treatment group/ mortality in control group) x 100

Analytical and Statistical Calculation

The data were expressed as mean ± standard deviation. Through SPSS commercial statistical package, data were analyzed using analysis of variance (ANOVA) for determining the significant differences over control values. The significance level was set at $p< 0.05$.

RESULTS

Growth Performance

The effects of probiotic strain *L. plantarum* CR1T5 supplement on growth performance and feed efficiency in *P. larnaudii* were show in Table 1 and 2. Fish received probiotic supplements at 10^7, 10^8 and 10^9cfu g^{-1} had significantly higher Specific growth rate (SGR) and lower feed conversion ratio(FCR) than fish that received the control diet at day 15. While, fish fed 10^6cfu g^{-1} *L. plantarum* CR1T5 had significantly higher SGR and lower FCR at day 45.

Immune Response

Lysozyme Activity

The effects of *L. plantarum* CR1T5 dietary supplements in *P. larnaudii* are presented in Fig.

Table 1. Effect of *L. plantarum* CR1T5 supplement on growth performance of *P. larnaudii.*

Diets	SGR(days)			
	15	30	45	60
Control	0.73±0.02[a]	0.77±0.04[a]	0.76±0.02[a]	0.80±0.02[a]
L. plantarum10^6cfu g^{-1}	0.77±0.04[ab]	0.82±0.02[ab]	0.85±0.02[b]	0.87±0.02[b]
L. plantarum10^7cfu g-1	0.81±0.06[b]	0.87±0.0 2[bc]	0.87±0.0 1[b]	0.88±0.0 1[b]
L. plantarum10^8cfu g^{-1}	0.83±0.02[b]	0.88±0.03[bc]	0.91±0.03[c]	0.89±0.02[bc]
L. plantarum10^9cfu g^{-1}	0.83±0.02[b]	0.92±0.05[c]	0.95±0.02[d]	0.93±0.12[c]

Data represent the mean (±S.E.); The mean values in same column with different letters are significantly different ($p < 0.05$)

Table 2. Effect of *L. plantarum* CR1T5 supplement on feed efficiency of *P. larnaudii.*

Diets	FCR (days)			
	15	30	45	60
Control	2.59±0.06[a]	2.45±0.13[a]	2.50±0.08[a]	2.35±0.08[a]
L. plantarum 10^6cfu g^{-1}	2.47±0.15[ab]	2.28±0.05[ab]	2.18±0.07[b]	2.12±0.07[b]
L. plantarum 10^7cfu g^{-1}	2.34±0.19[b]	2.15±0.05[bc]	2.17±0.04[bc]	2.13±0.04[b]
L. plantarum 10^8cfu g^{-1}	2.27±0.07[b]	2.13±0.09[bc]	2.05±0.08[cd]	2.08±0.06[bc]
L. plantarum 10^9cfu g^{-1}	2.26±0.06[b]	2.02±0.13[c]	1.94±0.04[d]	2.00±0.04[c]

Data represent the mean (±S.E.); The mean values in same column with different letters are significantly different ($p < 0.05$)

1a. The serum activities in fish fed 10^8cfu g^{-1} and 10^9cfu g^{-1} probiotic supplement had significantly ($p<0.05$) increased when compared to the control diet both at day 45 and day 60.

Respiratory Burst Activity

The results of dietary supplement to respiratory burst activity are shown in Fig. 1b. All supplement significantly improved respiratory burst activity ($p<0.05$) at 30, 45 and 60 days.

Alternative Complement Pathway (ACH50 Activity)

The serum ACH50 activity in probiotics dietary supplement at 10^8cfu g^{-1} and 10^9cfu g^{-1} had significantly ($p<0.05$) increased at 30, 45 and 60 days. whereas the ACH50 activity in fish received probiotic diets at 10^7cfu g^{-1} and 10^6 cfu g^{-1} were significantly different from the control group at 30 and 45 days. (Fig. 1c)

Challenge Test

Relative percent survival (RPS) of *P. larnaudii* is shown in Fig. 2. All fish received probiotic dietary supplement displayed a significant different RPS post-challenge with *A. hydrophila.*

DISCUSSION

Lactobacillus plantarum is a lactic acid bacterium which has been used as probiotic in aquaculture to improve growth performance, increase survival rate, resistance against pathogen and enhance immune system in aquatic animals. The results in this study indicated that *L. plantarum* CR1T5 dietary supplements fed on *P. larnaudii* at 10^7, 10^8 and 10^9 cfu g^{-1} caused lower feed conversion rate and higher growth performance. *L. plantarum* CR1T5 diet supplements may promote the growth performance and feed efficiency. The improvement of nutrient utilization in fish has resulted from the ability to compete with pathogens for essential nutrients, adhesion for binding to the wall of the digestive tract of fish and the ability of cells to produce metabolites and enzymes (Pandiyan *et. al.,* 2013)[11]. Pathogens that cannot adhere and colonize in digestive tract are released by excretion. As a result, microorganisms maintain balance in digestive tract which lead to better performances of growth and feed efficiency of fish. In a previous study, Nile tilapia (*Oreochromis niloticus*) fed with 1% probiotic mixture of

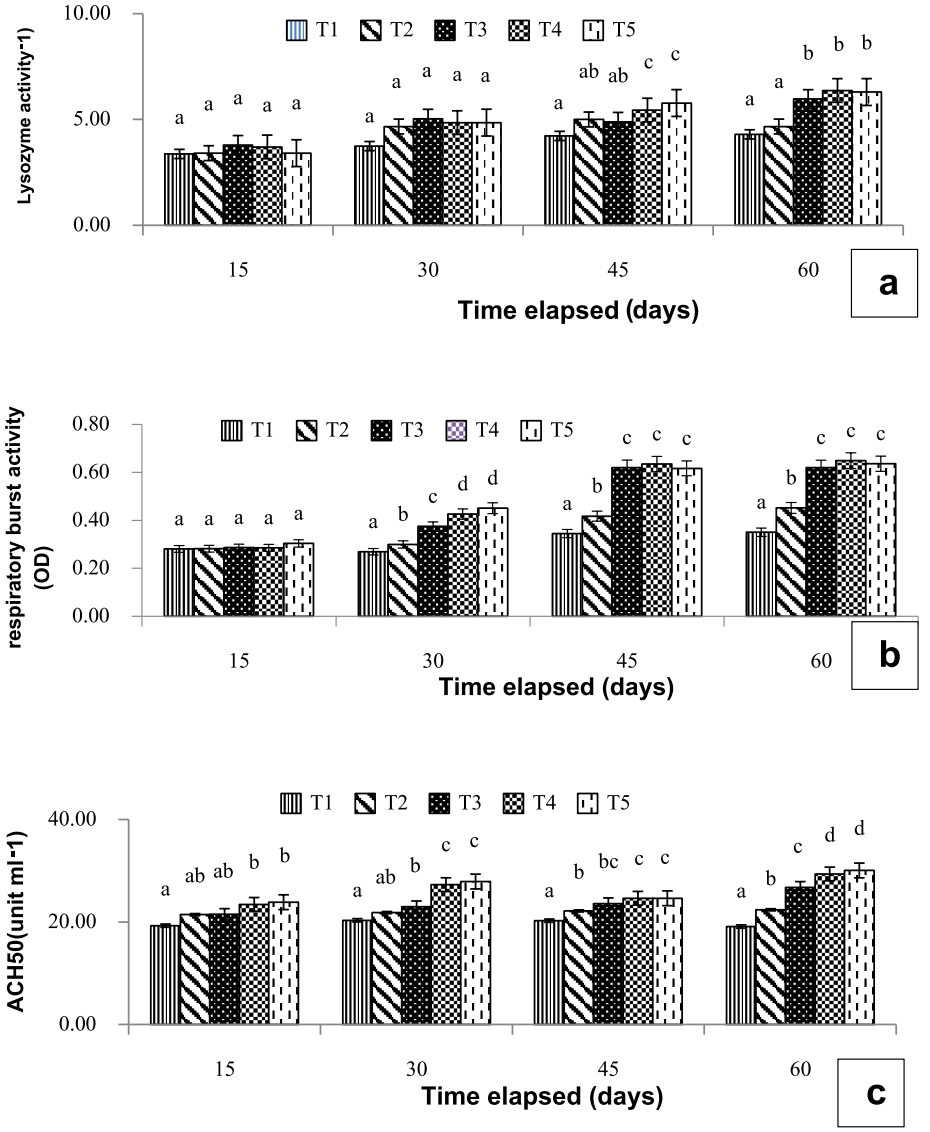

Fig. 1. Lysozyme activity;(a), respiratory burst activity; (b) and alternative complement activity; (c) of *P. larnaudii* fed with *L. plantarum* CR1T5 at 0(T1), 10^6(T2), 10^7(T3), 10^8(T4) 10^9(T5) cfug^{-1}. Data represent the mean (±S.E.). Different letters indicate a significant difference (P<0.05) between values when compared by Duncan's multiple range test.

Lactobacillus acidophilus and *Streptococcus faecium* showed better growth and feed utilization than fish that fed control diet. (Lara-Flores *et al*., 2003).[12] Grouper (*Epinephelus-coioides*) fed with *L. plantarum* dietary supplement especially at 10^8cfu kg^{-1} for 4 weeks had a significantly increased percentage of weight gain, and feed efficiency (Son *et. al.* 2009).[13] Dietary administration of *L. plantarum* VSG3 at 0, 10^6, 10^8 and 10^{10}cfu g^{-1} had significant effects on the specific growth rate and feed utilization efficiency of *Labiorohita* (Giri *et. al.* 2013)[14]**.**

Either monospecies or multiple speciesof probiotics, including *Lactobacillus, Lactococcus, Enterococcus, Carnobacterium, Bacillus, Enterobacter, Pseudomonas, Clostridium,* and *Saccharomyces* species, were used in aquaculture practices for improving growth and disease resistance (Nayak, 2010)[15]. Fish defenses against pathogens via modulation of immune system which

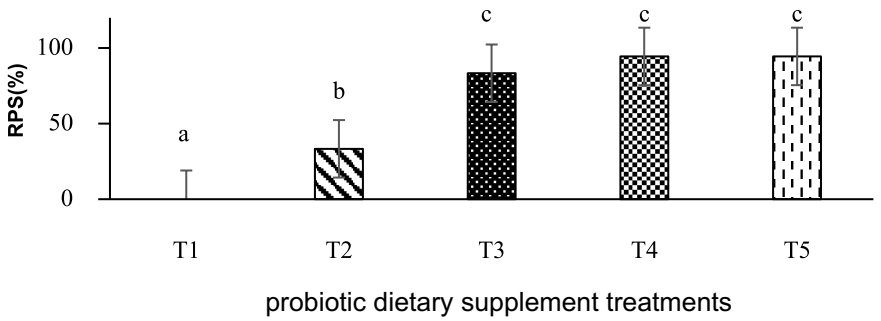

Fig. 2. Relative percent survival of *P. larnaudii* fed with *L. plantarum* CR1T5 at 0(T1), 10^6(T2), 10^7(T3), 10^8(T4) 10^9(T5) cfu g^{-1}. Data represent the mean (±S.E.). Different letters indicate a significant difference (P<0.05) between values when compared by Duncan's multiple range test.

consists of physical barriers, humoral and cellular components. Innate humoral immunity includes lysozyme, complement, transferrin, lectins and antiproteases while innate cellular immunity is comprised of phagocytes, including macrophage and neutrophils. These components can be used as parameter to determine status of innate immune response of fish (Magnadottir, 2006)[16]. Lysozyme is an important enzyme in non-specific immune, which protects fish from invading pathogenic bacteria by splitting the ʎ-1-4- linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial cell walls peptidoglycan (Saurabh and Sahoo, 2008)[17]. Lysozyme itself is known to attack gram positive bacteria directly while lysozyme in conjugation with complement can attack gram negative bacteria(Paulsen *et al*., 2001)[18]. Complement may eliminate pathogens via cell membranelysis and activation of non-specific mediators in inflammation process. Complement system has facilitate schemotoxis, opsonisation and pathogen destruction (Holland and Lambris, 2002)[19]. Innate cellular components include various types of white blood cells, e.g. macrophages and neutrophils. These white blood cells directly eliminate bacterial cells by phagocytosis. Macrophages and neutrophils can also induce respiratory burst by producing reactive oxygen species compounds. The superoxide anion (O_2^-) initiates the respiratory burst and yields antimicrobial compounds, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), hypochlorous acid (OCl^-) and peroxynitrite ($ONOO^-$) (Gomez and Balcazar, 2008)[20].

Lactobacillus is a lactic acid bacterium which is used as a biological control agent in aquaculture practices (Balcazar *et al.*, 2006)[21]. *Lactobacillus* ferment carbohydrates mainly into lactate and acetate. It can also produce bacteriocin which inhibits the growth of Gram-positive and Gram-negative bacteria (Lash *et al*., 2005)[22]. *Lactobacillus* is beneficial flora of gastrointestinal tract of fish (Galindo, 2004).[23] Nowadays, *Lactobacillus* spp. were used as probiotic dietary supplements to improve innate immune and disease resistance in various fish (Genge*t al.,* 2012)[24]. The use of *Lactobacillus* in many reports showed positive results in fish. The results from this study indicated that dietary *L. plantarum* CR1T5 supplements at $10^8$and 10^9cfu g^{-1} were the optimum concentration which led to increase of immune parameters at different time of feeding. The immune parameter in fish has been reported to increase after feeding with *Lactobacillus* sp. dietary supplement and in response to pathogens. In a previous study, lysozyme activity and complement activity in rainbow trout (*Oncorhynchus mykiss*) were greater in fish fed 10^{11}cfu g^{-1} of *L. rhamnosus* JCM 1136 (Panigrahi *et al*., 2004)[25]. The similar results reported by Abumourad *et. al*.(2013)[26], that the diet containing *L. plantarum* at 10^6cfu g^{-1} showed significantly improvement of growth rate, protection against *Pseudomonas fluorescens* in *Oreochromis niloticus*. In contrast, using *L. plantarum* dietary supplements on nursing of *Macrobrachium roenbergii* showed no significant differences in terms of growth, specific growth rate and survival rate (Sangpara and Doolginda-chabaporn, 2010)[27]. Dietary *L. plantarum* administration for 4 weeks revealed resistance to *Streptococcus* sp. and irido virus

infection and had significantly increase the lysozyme activity, glutathione peroxidase (GPx) activity complement activity and respiratory burst activity in *Epinephelus coioides* (Son *et.al*., 2009)[13]. The study in kelp grouper (*Epinephelus bruneus*) fed with *L.sakei* BK19 at 10^8 cells g^{-1} supplementary diet for 2 weeks showed significantly enhancement of head kidney macrophage phagocytic and peroxidase activities, serum lysozyme activity, and total protein levels. The result of *L. sakei* BK19 enriched diet revealed the higher level of disease protection in streptococcus-infected kelp grouper (Harikrishnan *et. al*., 2010)[28]. Further more, Giri *et al*. (2013)[14] reported that the tropical fresh water fish, *Labeorohita* fed dietary administration of *L*. *plantarum* VSG3 at 0, 10^6, 10^8, 10^{10}cfu g^{-1} had significantly increased the serum lysozyme and alternative complement pathway (ACP) activities, phagocytosis and respiratory burst activity after 30 and 60 days of post-feeding.

The challenge test revealed that all fish fed probiotic *L. plantarum* CR1T5 had significantly increased survival rate compared to the control. Carnevali *et al*. (2004)[29] reported that *L. plantarum* (906) dietary supplement had significantly decreased larvae and fry mortality of sea bream (*Sparus aurata*). Rainbow trout (*Onchorhynchus mykis*) fed with *L. plantarum* CLFP 238 at 10^7cfu g^{-1} for 30 days showed reduced mortality after challenged with *Lactococ-cusgarvieae*. (Vendrell *et al*., 2008)[30]. Hybrid catfish (*Clariasgarie pinus* x *Clariasma-croceplalus*) fed diet supplement with *L. plantarum* C014 at 10^7 cfu g^{-1} had lysozyme activity increased. The fish challenged with *A. hydrophila* showed reduction of mortality rate after 45 days of feeding (Butprom *et al*., 2013)[31]. *Labeorohita* administerd with the diets containing of *L. plantarum* VSG3 at 10^8 and 10^{10}cfu g^{-1}had significantly higher on disease resistance against *Aeromonas hydrophila* infection (Giri *et al.,* 2013)[14]. Administration of *L. rhamnosus* at 10^9 and 10^{12}cfu g^{-1} for 51 days increased the survival rate of rainbow trout after challenged with *Aeromonas-salmonicida* (Nikoskelainen *et al.,* 2001)[32]. Therefore, using probiotic *Lactobacillus* spp. can increase disease resistance in fish. Probiotic dietary supplement in fish improved growth performance, immune parameter and pathogen resistance. However, the effects of probiotic bacteria on growth performance and immune parameter may be affected by feeding duration, the concentration of probiotic, the species of fish, environmental conditions and the quality and quantity of food dietary ingredients (Ayoola *et al.,* 2013 and son *et.al*., 2009)[33,13]**.**

CONCLUSIONS

Dietary *L. plantarum* CR1T5 supplements at 10^8 and 10^9cfu g^{-1} can improve the best growth performance and complement activity at 15 days post-feed while, probiotic diets at 10^8cfu g^{-1} stimulated lysozyme activity and respiratory burst activity at 45 and 30 days of feeding. Defending against infection in fish was found in fish all of group probiotic supplements.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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