Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2016.101.111

Research Article
Characterization of *Bacillus* sp. NP5 and its Application as Probiotic for Common Carp (*Cyprinus carpio*)

[1]Ricky Djauhari, [2]Widanarni, [2]Sukenda, [2]Muhammad Agus Suprayudi and [2]Muhammad Zairin Jr.

[1]Study Program of Aquaculture, Graduate School, Bogor Agricultural University, Indonesia
[2]Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Indonesia

Abstract
This study aimed to characterize *Bacillus* sp. NP5 and to evaluate the effectiveness of its application as a probiotic on growth performances and health status of common carp (*Cyprinus carpio*) before and after infected by *Aeromonas hydrophila*. *Bacillus* sp. NP5 cells were given to common carps through the feed for 30 days at doses of 10^6, 10^8 and 10^{10} CFU g^{-1} feed. On day 31, common carps were challenged with *A. hydrophila*, which was injected via intramuscular route. Result of phenotypic and genotypic identification of *Bacillus* sp. NP5 isolate showed that this isolate was *Bacillus cereus* with 99% of similarity index. *Bacillus* sp. NP5 was able to produce protease, amylase and lipase, it was also potential to inhibit *A. hydrophila, Streptococcus agalactiae* and *Mycobacterium fortuitum*. Supplementation of feed containing probiotic at a dose of 10^{10} CFU g^{-1} feed resulted a higher total bacterial count and a higher total probiotic count in the intestine, those followed with higher value of amylase, protease and lipase activity, along with the highest daily growth rate and the lowest feed conversion ratio ($p<0.05$). Total leukocytes and phagocytic activity in probiotic treatment with a dose of 10^{10} CFU g^{-1} feed was higher ($p<0.05$) compared to controls at the end of the rearing period over 30 days. Common carps fed probiotic showed survival rates after the challenge test with values ranging between 81-100%, while the survival rate of fish without probiotic supplementation was only 50%. The results of this study showed that probiotic supplementation on common carp could reduce the pathogenicity of disease caused by *A. hydrophila* infection, which indicated an improvement in cellular immune response.

Key words: *Bacillus* sp. NP5, growth, cellular immune response, MAS, *Cyprinus carpio*

Received: January 07, 2016 **Accepted:** March 08, 2016 **Published:** April 15, 2016

Citation: Ricky Djauhari, Widanarni, Sukenda, Muhammad Agus Suprayudi and Muhammad Zairin Jr., 2016. Characterization of *Bacillus* sp. NP5 and its application as probiotic for common carp (*Cyprinus carpio*). Res. J. Microbiol., 11: 101-111.

Corresponding Author: Widanarni, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the aquaculture industry of the world including Indonesia, common carp (*Cyprinus carpio*) is classified as the oldest aquaculture species (Balon, 2006) and it is one of the economically important species. As the increasing of animal protein source demands, aquaculture has emerged as one of the sustainable industries that can be relied to fulfill the nutritional requirements and to sustain the human food safety. However, most of the intensive farming centers face major obstacles caused by diseases outbreaks.

Motile Aeromonad Septicaemia (MAS) caused by *Aeromonas hydrophila* infection has become a common problem in the aquaculture industry, that attacks various fish species in the world. The MAS can spread very quickly, it generally causes mortality about 25%, but the mortality rate will be higher in high stocking density condition and poor water quality (Hoole *et al*., 2001). This pathogen has characteristics: Gram-negative, motile, short rod shape and it is commonly found in all aquatic environment types (Harikrishnan *et al*., 2003). The clinical symptoms of MAS include: Swelling tissues, dropsy, red spots on the body surface, necrosis, ulceration and haemorrhagic septicemia (Karunasagar *et al*., 1989). Some fish species infected by MAS include tilapia (Tellez-Banuelos *et al*., 2010), catfish (Ullal *et al*., 2008), goldfish (Harikrishnan *et al*., 2009), common carp (Jeney *et al*., 2009; Yin *et al*., 2009) and eel (Esteve *et al*., 1994). This pathogen generally acts as a secondary pathogen, but it is also proved as primary pathogen (Pridgeon and Klesius, 2011), it has acute mortality pattern that can cause a mortality less than 24 h.

Antibiotics and chemical drugs has commonly used to inhibit and to prevent pathogens growth because of growth promotion stimulation and chemotherapy (Jagoda *et al*., 2014). However, the use of those materials have high risk with the increasing of resistant bacterial strains, disruption of stability and balance of the intestinal microflora and the residue is accumulated in fish carcass and aquatic environment (Skjermo and Vadstein, 1999). The use of antibiotics also requires a long time for antibiotic residues withdrawal from fish body (Iregui *et al*., 2014), so now a days it has been prohibited to be used for fish diseases control. Probiotics are live or dead microbes or microbial components, which give some benefits to the host (Fuller, 1989; Lazado and Caipang, 2014). The use of probiotics is one of the eco-friendly alternative efforts to reduce the use of antibiotics, it improves fish appetite, it also maintains stability and the balance of intestinal microflora (Panigrahi *et al*., 2010). Probiotic has contribution in enzymatic digestion, because it can produce extracellular amylase, protease and lipase (Kuhlwein *et al*., 2014). Various probiotics which are commonly used are from Lactococcus genus (Sugita *et al*., 2009), *Bacillus* sp. (Kumar *et al*., 2008), Lactobacillus and *Saccharomyces cerevisiae* (Ramakrishnan *et al*., 2008). Probiotic derived from *Bacillus* sp. has several advantages, because it has spores, which are relatively stable to heat, some are able to survive at low pH (Barbosa *et al*., 2005) and its viability is relatively stable during long-term storage for the purpose of mass scale commercial feed production (Ringo *et al*., 2014).

Some probiotics generally show their dominance in the fish gastrointestinal tract only in treatment or administration period. Therefore, the main challenge to be achieved in the probiotics administration is to create bacterial colonization in the fish intestine in long term period, it is especially subjected to probiotic, which is sourced from different fish species. This research used *Bacillus* sp. NP5 isolated from the digestive tract of tilapia (Putra and Widanarni, 2015) and it has been proven being able to control streptococcosis on tilapia (Widanarni and Tanbiyaskur, 2015). One of the factors that affect probiotic performance is dose (Nayak, 2010), because probiotic which is given in sufficient quantities will produce the beneficial effect for growth and health on fish, so the dose of probiotic, which is given to the host must be carefully determined to avoid the overdose that can give unexpected side effects and lost in production cost (Dash *et al*., 2014). Nikoskelainen *et al*. (2001) stated that low doses of probiotic may fail to stimulate the fish immune system, but high doses of probiotic can cause interference in the host. Probiotic in the very high dose will cause imbalance of the microbiota in the digestive tract and interfere immune response that can cause the lost of energy, which used for the growth (Li *et al*., 2012; Ramos *et al*., 2013). To produce maximum therapeutic effect is required minimum concentration of 10^6 CFU mL^{-1} (Kurman and Rasic, 1991), the range of 10^7-10^8 CFU mL^{-1} or in higher number (Jayamanne and Adams, 2006). The administration of *Bacillus subtilis* and *Lactobacillus acidophilus* at a concentration of 10^7 CFU g^{-1} feed for 1 and 2 months in tilapia could increase the activity of lysozyme and bactericidal serum against *Aeromonas hydrophila* with post-challenge test survival of 43.52% (Aly *et al*., 2008).

Before it is applied to aquatic organisms, probiotic bacteria need to be characterized and identified to differentiate with other species, which are pathogenic potential species. Moreover, the characterization and identification of probiotic bacteria are also important for quality control and the patent purpose (Gomez-Gil *et al*., 2000; Romero and Navarrete, 2006). Based on the above

cases, this study aimed to characterize *Bacillus* sp. NP5 and to evaluate the effectiveness of its application on the growth performances and health status of common carp (*Cyprinus carpio*) before and after infected by *Aeromonas hydrophila.*

MATERIALS AND METHODS

Identification of *Bacillus* sp. NP5: Phenotypic character of *Bacillus* sp. NP5 was evaluated using api® 50 CHB V4.0 kit, bioMérieux, Marcy-l'Etoile, France. Genotypic character was evaluated through amplification of 16S rRNA encoding gene with 63 forward primer (5'-CAG GCC TAA CACATG CAA GTC-3') and 1387 reverse primer (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*., 1998). The composition of master mix PCR in each tube consisted of 25 µL GoTaq (Promega), 4 µL 63 forward primer, 4 µL 1387 reverse primer, 17 µL ddH_2O and DNA template, which was taken directly from *Bacillus* sp. NP5 isolate using a toothpick. The PCR conditions were as follow: Predenaturation at 95°C for 5 min, denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, elongation at 72°C for 1 min and primer extension at 72°C for 5 min. Amplification process on PCR machine consisted of 30 cycles. Termination reaction was carried out by temperature droping to 4°C. The PCR products were run for electrophoresis on agarose gel 1% in TAE buffer 1x at 80 V for 45 min, followed by visualization using UV transiluminator. Sequencing was done by sending amplified-DNA to sequencing service provider company. Sequence results were aligned with database in Gene Bank using BLAST-N online software program (www.ncbi.nlm.nih.gov).

Semi-quantitative test of amylolytic, proteolytic and lipolytic activity: This test aimed to measured amylolytic, proteolytic and lipolytic activity of probiotic bacteria through hydrolysis test of starch, skim milk and olive oil. Hydrolysis ability was characterized by clear zone around the bacterial colony. Amylolytic, proteolytic and lipolytic activity were evaluated by Amylolytic Index (AI), Proteolytic Index (PI) and Lipolytic Index (LI) that can be measured by the equation according to Lim *et al*. (1987) as follows:

$$\text{AI/PI/LI} = \frac{\text{Clear zone diameter-Bacterial colony diameter}}{\text{Bacterial colony diameter}}$$

Quantitative test of amylolytic, proteolytic and lipolytic activity: Enzymes were obtained from centrifugation of bacterial culture at a speed of 5000x g for 30 min. Amylase activity was measured according to Bernfeld (1955). Amylase activity of supernatant was measured quantitatively with a spectrophotometer at a wavelength of 550 nm. One unit of amylase activity is defined as the amount of enzyme that produces reductor sugar (maltose) as much as 1 µmol min^{-1} in test condition. Protease activity was measured according to Walter (1984). Absorbance was read at a wavelength of 578 nm with a standard used was L-tyrosine 5 mM. One unit of protease activity is defined as the amount of enzyme that is able to produce 1 µmol of tyrosine per minute. Lipase activity was measured according to Kwon and Rhee (1986). Measurement of lipase activity was performed using margarat acid (heptadecanoat acid) standard curve, which was measured its absorbance at 715 nm. One unit of lipase activity is defined as the amount of enzyme that liberates 1 µmol mL^{-1} min^{-1} of fatty acid at 60°C.

Inhibition test of *Bacillus* sp. NP5 against pathogenic bacteria: Inhbitory activity was evaluated by Kirby-Bauer method and competition test between *Bacillus* sp. NP5 with pathogenic bacteria (*Aeromonas hydrophila*, *Streptococcus agalactiae* and *Mycobacterium fortuitum*). Inhibition test with Kirby-Bauer method was performed using paper disc by measuring the inhibition zones, while competition method was performed by growing *Bacillus* sp. NP5 with each pathogen isolate, which has been given antibiotic resistant marker in Trypticase Soy Broth (TSB) medium, it was followed by enumerating the No. of pathogenic bacteria cells (CFU mL^{-1}) which grew on Trypticase Soy Agar (TSA) medium, which was supplemented with antibiotic marker (Widanarni *et al*., 2003). Result of growth inhibition of pathogenic bacteria in competition test was measured by the equation as follows:

$$\text{Growth inhibition} = \frac{\text{Pathogenic bacteria cells in mixed tube (probiotic and pathogen)} - \text{Pathogenic bacteria cells in control tube (pathogen)}}{\text{Pathogenic bacteria cells in control tube (pathogen)}}$$

Preparation of probiotic: Probiotic bacteria used as feed supplement in this study, was *Bacillus* sp. NP5 which was previously given antibiotic rifampicin marker (*Bacillus* sp. NP5 Rf^R) (Widanarni *et al*., 2003). Probiotic cells were cultured in TSB medium and incubated in a water bath shaker at a temperature of 29-30°C, 160 rpm for 24 h. The suspension of bacterial cells was then centrifuged and the bacterial cells pellet was rinsed twice using Phosphate Buffer Saline (PBS) solution.

Preparation of test feed: Preparation of test feed using commercial feed with a protein content of 31% was carried out by adding three probiotic doses (10^6, 10^8 and

10^{10} CFU g^{-1} feed) into the feed. Mixing feed and probiotic was done by adding 2% egg white as a binder, while the control feed was only added 2% egg white.

Rearing condition and experimental design: This study was conducted over 45 days, including 30 days of rearing period with feeding trial using test feed, one day of rest period before the challenge test and 14 days of the challenge test. Experimental animals used in this study were common carps with an average weight of 4.81±0.25 g, which have previously been adapted to the experimental medium for 2 weeks. Fish were stocked randomly in each treatment aquarium measuring 65×40×40 cm^3 with a density of 15 fish per aquarium. This study, used a completely randomized design, consisting of five treatments with three replicates, control (-) (feed without probiotic and without pathogen infection), control (+) (feed without probiotic and with pathogen infection), A (feed suplementation with probiotic at a dose of 10^6 CFU g^{-1} feed and with pathogen infection), B (feed supplementation with probiotic at a dose of 10^8 CFU g^{-1} feed and with pathogen infection) and C (feed supplementation with probiotic at a dose of 10^{10} CFU g^{-1} feed and with pathogen infection). Feeding was done by at satiation method, in which fish were fed three times a day (08.00, 12.00 and 16.00 Western Indonesia Time) for 30 days. Water quality was maintained by removing feces and water replacement in the rearing medium as much as 20% every day. Water quality was monitored during rearing period with the parameters and ranges were: Temperature at 28.0-28.5°C, DO at 7.2-8.1 mg L^{-1}, pH at 7.4-8.04 and TAN at 0.0032-0.0065 mg L^{-1}.

Observation of bacterial population in the intestine of common carp: The intestine of fish was weighed as much as 0.1 g and was homogenized in 0.9 mL PBS. Serial dilutions were performed in PBS, then each dilution (0.05 mL) was spread on TSA medium for total bacterial count and TSA with rifampicin suplementation for total *Bacillus* sp. NP5 Rf^R count. The No. of bacteria in the sample could be determined by enumerating the No. of colonies that grew on the medium multiplied by the dilution factor in CFU g^{-1} unit (Madigan *et al*., 2003).

Challenge test: After receiving test feed for 30 days, common carps from each treatment (except negative control) were challenged by *Aeromonas hydrophila*. The aim of the challenge test was to evaluate the probiotic performance in improving the resistance of common carp against *A. hydrophila* infection. Common carps were infected by *A. hydrophila* at a concentration of 10^7 CFU mL^{-1} (0.1 mL $individual^{-1}$). Infection was done by injection using a syringe via intramuscular route and negative control fish were injected with PBS. Clinical symptoms and mortality of common carp were observed for 14 days after the injection.

Experimental parameters: Experimental parameters observed consisted of bacterial population in the intestine, digestive enzymes activity included amylase, protease (Drapeau, 1974) and lipase (Debnath *et al*., 2007), growth performances, immune responses and the survival rate of common carp after the challenge test that described fish resistance to infection. Growth performance parameters observed were Daily Growth Rate (DGR) and Feed Conversion Ratio (FCR). The bacterial population in the intestine included total bacterial count and total *Bacillus* sp. NP5 Rf^R count. Growth performances and bacterial population in the intestine were evaluated immediately after 30 days of probiotic treatment. Immune responses parameters observed included total leukocytes, phagocytic activity (Balcazar *et al*., 2006), differential leukocytes (lymphocytes, monocytes and neutrophils), hematocrit, hemoglobin and total erythrocytes. Immune responses and survival rate of fish were measured twice: After 30 days of feeding trial (before the challenge test) and 14 days after the challenge test.

Statistical analysis: Data obtained were statistically analyzed using SPSS Statistics 17.0 software and the test was continued with Duncan test for significant difference test ($p<0.05$).

RESULTS

Identification of 16S rRNA: Result of identification of phenotypic character of *Bacillus* sp. NP5 using api® 50 CHB V4.0 kit showed that the species of *Bacillus* sp. NP5 was *Bacillus cereus* strain EM5. The identification based on the result of partial sequencing of 16S rRNA gene and BLAST-N analysis with 1250 bp alignment also showed that *Bacillus* sp. NP5 had closeness with *Bacillus cereus* strain EM5 on similarity index of 99%.

Amylolytic, proteolytic and lipolytic activity: The activity of amylase, protease and lipase of *Bacillus* sp. NP5 obtained the range of Amylolytic Index (AI), Proteolytic Index (PI) and Lipolytic Index (LI) were 0.50, 0.20 and 1.33, respectively (Table 1). The highest activity of amylase was achieved at 48 h of observation (0.044 U mg^{-1} protein), protease at 96 h (0.0030 U mg^{-1} protein) and lipase was observed only up to 24 h (0.062 U mg^{-1} protein) (Table 2).

Inhibitory ability of *Bacillus* sp. NP5 against pathogenic bacteria: The result of inhibition test of *Bacillus* sp., NP5 against pathogenic bacteria; *A. hydrophila*, *S. agalactiae* and *M. fortuitum* showed that *Bacillus* sp. NP5 had inhibitory activity against pathogenic bacteria with the inhibition index values were 0.44, 0.70 and 0.15, respectively (Table 3) and it was able to reduce those pathogens in competition test with the percentage values were 66.1, 98.81 and 30%, respectively (Table 4).

Bacterial population in the intestine of common carp: Probiotic supplementation through the feed on common carp for 30 days had significantly effect ($p<0.05$) on the bacterial population in the intestine of common carp. The highest value of total bacterial count in the fish intestine was found in C that was not significantly different ($p>0.05$) with B, but it was significantly different ($p<0.05$) with control and A. In addition, the highest total *Bacillus* sp. NP5 count in the fish intestine was also found in C that was significantly different ($p<0.05$) with all treatments (Table 5).

Growth performances of common carp: The digestive enzymes (amylase, protease and lipase) activity on probiotic treatment with a dose of 10^{10} CFU g^{-1} feed was higher ($p<0.05$) compared to control (Table 6). There was significant difference in daily growth rate between C with control and A ($p<0.05$), but it was not significantly different ($p>0.05$) with B. The value of FCR in the probiotic treatments were lower ($p<0.05$) compared to control, but there was no difference ($p>0.05$) between probiotic treatmeants (Table 7).

Immune responses: The value of total leukocytes, phagocytic activity, hemoglobin and total erythrocytes before the challenge test in C was higher ($p<0.05$) compared to A, B and controls. The value of total leukocytes after the challenge test in B and C was higher ($p<0.05$) compared to A and controls. Hemoglobin and total erythrocytes of fish in all treatments, which were injected with pathogen decreased at the end of the challenge test (Table 8 and 9).

Survival rate: The survival rate of fish before the challenge test ranged from 80-100%. However, the survival rate of fish

Table 1: Potential index of amylolytic, proteolytic and lipolytic activity of *Bacillus* sp. NP5

Parameters	Clear zone diameter (cm)	Bacterial colony diameter (cm)	Potential index value
Amylolytic	0.9	0.6	0.50
Proteolytic	1.2	1	0.20
Lipolytic	3.5	1.5	1.33

Table 2: Amylase, protease dan lipase activity produced by *Bacillus* sp. NP5 on different time

Time (h)	Amylase (U mg^{-1} protein)	Protease (U mg^{-1} protein)	Lipase (U mg^{-1} protein)
18	0.000	0.0009	0.054
21	0.001	0.0008	0.060
24	0.029	0.0012	0.062
48	0.044	0.0027	na
72	0.035	0.0014	na
96	0.024	0.0030	na

na: Not analyzed

Table 3: Inhibitory activity of *Bacillus* sp. NP5 against pathogenic bacteria evaluated by Kirby-Bauer method

Pathogen	Inhibition zone diameter (cm)	Bacterial colony diameter (cm)	Inhibition index
Aeromonas hydrophila	1.3	0.9	0.44
Streptococcus agalactiae	1.7	1.0	0.70
Mycobacterium fortuitum	1.5	1.3	0.15

Table 4: Inhibitory activity of *Bacillus* sp. NP5 against pathogenic bacteria evaluated by competition test

Pathogen	Total pathogenic bacterial count (log CFU mL^{-1})	Inhibition activity (%)
Aeromonas hydrophila (control tube)	9.37±0.12	
Aeromonas hydrophila (mixed tube)	8.90±0.15	66.1
Streptococcus agalactiae (control tube)	10.00±0.11	
Streptococcus agalactiae (mixed tube)	8.08±0.13	98.8
Mycobacterium fortuitum (control tube)	10.00±0.13	
Mycobacterium fortuitum (mixed tube)	9.85±0.16	30.0

Table 5: Total bacterial count and total *Bacillus* sp. NP5 count in the intestine of common carp (*Cyprinus carpio*) after 30 days of rearing period

Parameters	Control	A	B	C
Total Bacterial count on day 30 (log CFU g^{-1} intestine)	7.70 ± 0.41^{a}	8.85 ± 0.35^{b}	9.78 ± 0.32^{c}	10.28 ± 0.24^{c}
Total Probiotic count on day 30 (log CFU g^{-1} intestine)	nd^{a}	7.03 ± 0.24^{b}	8.38 ± 0.35^{c}	9.43 ± 0.10^{d}

nd: Not detected, different letters in the same row indicate significant differences (Duncan, $p<0.05$), the values shown are means and standard deviations

Table 6: Digestive enzymes activity (U mg^{-1} protein) of common carp (*Cyprinus carpio*) fed probiotic at a dose of 1% (10^{10} CFU g^{-1} feed)

Parameters	Probiotic	Control
Amylase	0.77 ± 0.06^{b}	0.50 ± 0.12^{a}
Protease	0.07 ± 0.01^{b}	0.04 ± 0.01^{a}
Lipase	0.80 ± 0.19^{b}	0.47 ± 0.01^{a}

Different letters in the same row indicate significant differences (Duncan, $p<0.05$). The values shown are means and standard deviations

Table 7: Survival Rate (SR), Daily Growth Rate (DGR) and Feed Conversion Ratio (FCR) of common carp (*Cyprinus carpio*) after 30 days of feeding trial

Parameters	Control	A	B	C
SR (%)	86.50 ± 7.51^{a}	90.00 ± 3.46^{ab}	96.50 ± 4.04^{bc}	100.00 ± 0.00^{c}
DGR (%)	1.40 ± 0.04^{a}	1.52 ± 0.30^{a}	2.33 ± 0.85^{b}	2.91 ± 0.19^{b}
FCR	2.55 ± 0.11^{a}	1.72 ± 0.13^{b}	1.66 ± 0.24^{b}	1.54 ± 0.01^{b}

Different letters in the same row indicate significant differences (Duncan, $p<0.05$). The values shown are means and standard deviations

Table 8: Immunity parameters of common carp (*Cyprinus carpio*) after 30 days of feeding trial

Parameters	Control (-)	Control (+)	A	B	C
Total leukocytes (x10^5 cells mm^{-3})	1.39±0.04^a	1.31±0.03^a	2.10±0.06^b	2.15±0.08^b	3.15±0.05^c
Total erythrocytes (x10^6 cells mm^{-3})	1.50±0.09^a	1.54±0.18^a	1.73±0.14^a	1.96±0.07^b	2.76±0.12^c
Hematocrit (%)	34.00±0.47^a	34.00±0.41^a	33.00±0.43^a	30.00±0.36^a	34.00±0.38^a
Hemoglobin (g%)	6.60±0.26^a	6.25±0.24^a	6.20±0.21^a	6.50±0.20^a	7.60±0.18^b
Lymphocytes (%)	92.00±0.19^a	90.00±0.20^a	92.00±0.14^a	91.00±0.12^a	92.00±0.17^a
Monocytes (%)	2.00±0.12^a	1.00±0.14^a	2.00±0.09^a	3.00±0.11^a	3.00±0.10^a
Neutrophils (%)	6.00±0.42^a	9.00±0.49^a	6.00±0.43^a	6.00±0.40^a	5.00±0.38^a
Phagocytic activity (%)	27.00±0.29^a	26.00±0.25^a	17.00±0.34^a	25.00±0.32^a	64.00±0.27^b

Different letters in the same row indicate significant differences (Duncan, $p<0.05$). The values shown are means and standard deviations

Table 9: Survival Rate (SR) and immunity parameters of common carp (*Cyprinus carpio*) after the challenge test with *Aeromonas hydrophila*

Parameters	Control (-)	Control (+)	A	B	C
SR (%)	94.00±6.00^{bc}	50.00±0.00^a	81.33±6.51^b	87.67±12.50^{bc}	100.00±0.00^c
Total leukocytes (x10^5 cells mm^{-3})	3.42±0.11^b	1.49±0.15^a	3.43±0.13^b	3.69±0.20^c	3.70±0.17^c
Total erythrocytes (x10^6 cells mm^{-3})	1.12±0.21^a	1.04±0.17^a	1.43±0.11^a	1.32±0.16^a	1.08±0.18^a
Hematocrit (%)	31.00±0.45^c	20.00±0.37^a	34.00±0.35^d	28.00±0.40^b	31.00±0.42^c
Hemoglobin (g%)	6.00±0.23^a	5.00±0.16^a	6.00±0.13^a	5.10±0.15^a	4.70±0.20^a
Lymphocytes (%)	97.00±0.15^a	82.00±0.17^a	97.00±0.23^a	95.00±0.18^a	94.00±0.20^a
Monocytes (%)	1.00±0.10^a	1.00±0.08^a	2.00±0.17^a	1.00±0.15^a	2.00±0.14^a
Neutrophils (%)	2.00±0.44^a	7.00±0.46^a	1.00±0.48^a	4.00±0.41^a	4.00±0.42^a
Phagocytic activity (%)	26.00±0.26^a	36.00±0.23^a	31.00±0.31^a	36.00±0.28^a	66.00±0.30^a

Different letters in the same row indicate significant differences (Duncan, $p<0.05$). The values shown are means and standard deviations

after the challenge test in all probiotic treatments and negative control were higher ($p<0.05$) compared to positive control. The survival rate obtained in negative control, A, B and C were 94.00, 81.33, 87.67 and 100.00%, respectively, while positive control only reached 50.00% (Table 9).

DISCUSSION

Identification of 16S rRNA gene is a molecular approach that was conducted to determine the bacteria species based on similarity of gene sequences with the data in Gene Bank. Based on the distribution of BLAST-N result, *Bacillus* sp. NP5 had a homology value of 99% with *Bacillus cereus.* Homology showed that those sequences having evolutionary relationship (Pertsemlidis and Fondon III, 2002). *Bacillus* sp. NP5 is used as probiotic associated with its ability to produce digestive enzymes such as amylase, protease and lipase. In addition, this probiotic also has inhibitory activity against several aquaculture pathogenic bacteria such as *A. hydrophila*, *S. agalactiae* and *M. fortuitum*.

Introduction of every types of bacteria into the intestine has a particular requirement, in which the bacteria must be able to compete with the resident microflora, which have been formed with the ecological niche (nutrients and attachment) in order to survive. Probiotic strains must be able to stick to the intestinal mucosa layer and utilize mucus as nutrients source for colonizing, it must be persistent and must be able to proliferate in the digestive tract of fish (Merrifield *et al*., 2010). The ability of *Bacillus* sp. NP5 to survive in the intestine of common carp was shown by the data of total probiotic count in the fish intestine, total probiotic count values were higher on the highest dose of probiotic treatment. Probiotic is an intestinal microflora, which plays a role in the mechanism of host resistance against stress conditions and pathogens; through immune response stimulation, production of specific antimicrobial substance (bacteriocin), release of metabolic product such as Short Chain Fatty Acid (SCFA) which causes the pH of intestinal fluid decreasing to a level under the optimum conditions for the pathogens growth, improvement of intestinal epithelial cells and action mechanism of imunity cells.

Probiotic produced significant effect on the digestive enzymes activity of common carp, because probiotic is able to improve digestibility of protein, carbohydrate and fat. This is further clarified by the better value of daily growth rate and FCR. Results of this study, showed that *Bacillus* sp. NP5 having an ability to assist the digestive process, it has been proven by the better growth of the fish, because it produces various extracellular enzymes, such as amylase, protease and lipase to facilitate the absorption and utilization of nutrients becoming more efficient. This is in line with the result of the study by Bairagi *et al.* (2002) which stated that *Bacillus* sp. isolated from the gut of *C. carpio* had high amount of extracellular amylolytic, proteolytic and lipolytic activity. The effects of probiotic supplementation on the digestibility improvement have also been observed in various fish species (Tovar-Ramirez *et al*., 2004). Protease secreted by probiotic serves to break down peptide bonds in protein stucture, then

break down into protein core elements as protein monomers and free amino acids, which are very useful for the fish nutritional status improvement. *Bacillus* sp. NP5 is also able to secrete lipase, which triggers the production and assimilation of essential fatty acids, resulting in higher growth and immunity of common carp. Essential fatty acids are not only become a booster for the immune system, but also promotes the growth (Sharma *et al*., 2010). Bacterial enzymatic hydrolysis promotes the growth of common carp that is supported by a lower value of FCR, it shows the increasing of protein and fat bioavailability. Amylase and lipase are the major enzymes associated with the carbohydrate and fat break down. The results showed that the amylase, protease and lipase activity were higher in fish fed probiotic compared to control. The activity of amylase, protease and lipase in the probiotic treatment were largely the result of probiotic stimulation, there by encouraging exoenzymes to synthesize digestive endoenzymes, which ultimately synergize to improve nutrient digestibility and growth performances.

Dose of probiotic is a limiting factor to achieve the optimum beneficial effects (Minelli and Benini, 2008). Dose of probiotic in aquaculture generally ranges from 10^6-10^{10} CFU g^{-1} feed with optimum dose varies depending on fish species and immunity parameters observed. The optimum concentration is not only indicated by the bacterial colonization and proliferation in the gut, but also by the growth, immune responses and protection of the host. Effective dose of *Bacillus* sp. is $2x10^8$ CFU g^{-1} feed for *Oncorhynchus mykiss* resulting in a low mortality percentage after the challenge test with pathogenic bacteria (Brunt *et al*., 2007). Phagocytic activity increased in higher value on *O. mykiss* given *Lactobacillus rhamnosus* at a dose of 10^{11} CFU g^{-1} feed for 30 days, but decreased at a dose of 10^9 CFU g^{-1} feed (Panigrahi *et al*., 2004). Best dose of *Lactobacillus plantarum* to increase growth, immune response and protection of grouper (*Epinephelus coioides*) is 10^8 CFU kg^{-1} feed, this dose showed better results than 10^6 and 10^{10} CFU kg^{-1} feed (Son *et al*., 2009). The administration of 0.5% dried *Bacillus* NP5 showed better results than higher doses (1 and 2%), this dose resulted the best growth performances in tilapia and was effective to control streptococcosis in tilapia with higher post-challenge test survival rate, better hematological parameter values and could inhibit *S. agalactiae* growth in the host target organs (Utami *et al*., 2015a, b). Low doses are not sufficient to stimulate maximum growth performance and cellular immune system related to the lack of colonization capacity, but high doses can cause high mortality, as happened on *O. mykiss* given *L. rhamnosus* at a dose of 10^{12} CFU g^{-1} feed, but it did not occur at a dose of 10^9 CFU g^{-1} feed (Nikoskelainen *et al*., 2001).

The addition of probiotic (10^6-10^{10} CFU g^{-1} feed) could reduce the amount of feed, which was required for the growth of common carp, resulting in reduction of production costs. The administration of *Lactobacillus casei* at a dose of 5×10^7 CFU g^{-1} for 30 days and 5×10^8 CFU g^{-1} for 60 days significantly improved the growth performances (daily growth rate and FCR) of *Barbus gryprus* (Vand *et al*., 2014). *Lactobacillus plantarum* at a dose of 10^8 and 10^{10} CFU g^{-1} feed given to *Labeo rohita* could improve daily body weight gain and FCR, it also showed an improvement in SGR (Giri *et al*., 2013). Similar results were also found in *Epinephelus coioides* (Son *et al*., 2009), tilapia nilotica (*Oreochromis niloticus*) (Aly *et al*., 2008), gilthead sea bream (*Sparus aurata*) (Suzer *et al*., 2008), *Clarias gariepinus* (Al-Dohail *et al*., 2009) and *Macrobrachium rosenbergii* (Venkat *et al*., 2004). Suplementation of commercial probiotic (*Streptococcus faecium*) was able to improve growth and feed efficiency on Israeli carp than antibiotics and yeast (*Saccharomyces cerevisiae*) (Bogut *et al*., 1998), it also occurred in terrestrial mammals, especially on pig (Bertin *et al*., 1997). The increasing doses of probiotic also showed the influence on the presence of bacteria in the intestine of common carp; the higher doses showed higher values on total bacterial count and total probiotic count. The high survival of Bacillus in intestinal mucosa cells can be caused by the competitive elimination mechanism against other bacteria, especially against pathogenic bacteria that has been demonstrated on the results of inhibition test (*in vitro*) of probiotic against some potential pathogens. This probiotic has antagonistic activity against *A. hydrophila* which is a resident pathogen of freshwater fish (Gonzalez *et al*., 1999), through the inhibitor compounds production. The increasing of probiotic population can produce some fermentative products such as lactic and acetic which cause a reduction in the pH of the intestinal fluid under optimum conditions for pathogens. Therefore, supplementation of *Bacillus* sp. NP5 could increase protection and resistance of common carp against a pathogen, which was showed by high survival rates after the challenge test. This is in line with the results of study by Tamamdusturi *et al.* (2016), which reported that the oral supplementation of microencapsulated probiotic *Bacillus* sp. NP5 showed higher survival rate of catfish (*Pangasianodon hypophthalmus*) after the challenge test with *A. hydrophila* compared to positive control. The oral supplementation of microencapsulated probiotic

Bacillus sp. NP5 also showed higher survival rate of tilapia after the challenge test with *Streptococcus agalactiae* compared to positive control (Agung *et al*., 2015).

Probiotic supplementation could improve the cellular immune responses of common carp to facilitate the elimination of potential pathogens in the intesinal tissue. A positive result from the administration of probiotic on hemoglobin, hematocrit and total erythrocytes also occurred in *Catla catla* (Hamilton), which were supplemented with *Lactobacillus acidophilus* in diet and it was related toprobiotic ability to improve hematological parameter values as a result of haemopoetic stimulation (Renuka *et al*., 2014). Cytotoxicity of *A. hydrophila* and accumulation of its extracellular products (α and β hemolysin, aerolisin, enterotoxin ACT, ALT and AST, protease and RNase) cause erythrocytes necrotic, hemolysis of erythrocytes and iron ions (Rey *et al*., 2009), thus causing a decreasing in total red blood cells of experimental fish. This is in line with the results of this study, total erythrocytes of infected common carp decreased from total erythrocytes of common carp before getting an infection. Probiotic interacts with mononuclear phagocytic cells (monocytes and macrophages) and polymorphonuclear leukocytes (neutrophils) and natural killer cells. Probiotic can act as an effective trigger for phagocytic cells, thus increasing phagocytic activity as happened on tilapia (*Oreochromis niloticus*) given feed containing *L. rhamnosus* for 2 weeks (Pirarat *et al*., 2006). An increasing in phagocytic activity also occurred in gilthead sea bream (*Sparus aurata* L.) given a diet containing *Lactobacillus delbrueckii* spp. *lactis* (CECT 287) at a dose of 10^7 CFU g^{-1} for 14 days (Salinas *et al*., 2005). Phagocytosis is responsible for initial activation of inflammatory response before antibodies production occured and it acts as mediator of phagocytic cells (neutrophils, monocytes and macrophages). *In vivo* activation of phagocytic cells by immunomodulator also causes secretion of a large number of active biological molecules such as inhibitor enzymes, cationic peptides, complement components, production of oxygen and nitrogen reactive (ROS and NOS), which are entirely involved in bactericidal activity (Kwak *et al*., 2003). *Bacillus* sp. NP5 could increase active phagocytic cells and total leukocytes. Stressors cause stress response in leukocytes on all vertebrates including fish (Davis *et al*., 2008). An increasing in total leukocytes could be caused by the experimental fish were in stress condition due to daily consumption of feed containing probiotic, which was recognized as foreign material. Along with the increasing of total leukocytes, fish mortality in probiotic treatment (in a dose of 10^{10} CFU g^{-1}) showed a lower value after the challenge test with pathogen compared to positive control, which indicated the improvement on health status of the fish, which consumed feed containing probiotic.

CONCLUSION

Result of phenotypic and genotipic identification of *Bacillus* sp. NP5 isolate showed that this isolate was *Bacillus cereus* with 99% of similarity index. Supplementation of feed containing probiotic at a dose of 10^{10} CFU g^{-1} feed resulted a higher total bacterial count and a higher total probiotic count in the intestine, those followed with higher value of amylase, protease and lipase activity, along with the highest daily growth rate and the lowest feed conversion ratio ($p<0.05$). This dose also showed higher value of total leukocytes and phagocytic activity ($p<0.05$) compared to controls at the end of the rearing period. Common carps fed probiotic showed survival rates after the challenge test with values ranging between 81-100%, while the survival rate of fish without probiotic supplementation was only 50%. This showed, that probiotic supplementation on common carp could reduce the pathogenicity of disease caused by *Aeromonas hydrophila* infection, which indicated an improvement in cellular immune response.

REFERENCES

Agung, L.A., Widanarni and M. Yuhana, 2015. Application of micro-encapsulated probiotic *Bacillus* NP5 and prebiotic Mannan Oligosaccharide (MOS) to prevent streptococcosis on Tilapia *Oreochromis niloticus*. Res. J. Microbiol., 10: 571-581.

Al-Dohail, M.A., R. Hashim and M. Aliyu-Paiko, 2009. Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (*Clarias gariepinus*, Burchell 1822) fingerling. Aquacult. Res., 40: 1642-1652.

Aly, S.M., Y.A.G. Ahmed, A.A.A. Ghareeb and M.F. Mohamed, 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immunol., 25: 128-136.

Bairagi, A., K.S. Ghosh, S.K. Sen and A.K. Ray, 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacult. Int., 10: 109-121.

Balcazar, J.L., D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, O. Girones and J.L. Muzquiz, 2006. Immune modulation by probiotic strains: Quantification of phagocytosis of *Aeromonas salmonicida* by leukocytes isolated from gut of rainbow trout (*Oncorhynchus mykiss*) using a radiolabelling assay. Comp. Immunol. Microbiol. Infect. Dis., 29: 335-343.

Balon, E.K., 2006. The oldest domesticated fishes and the consequences of an epigenetic dichotomy in fish culture. Int. J. Ichthyol., 11: 47-86.

Barbosa, T.M., C.R. Serra, R.M. La Ragione, M.J. Woodward and A.O. Henriques, 2005. Screening for Bacillus isolates in the broiler gastrointestinal tract. Applied Environ. Microbiol., 71: 968-978.

Bernfeld, P., 1955. Amylases, α and β. Methods Enzymol., 1: 149-158.

Bertin, G., M. Brault, M. Baud, M. Mercier and J. Tournut, 1997. *Saccharomyces cerevisiae* I-1079, microbial feed additive: Zootechnical effects on piglets. Proceeding of the 7th International Symposium on Digestive Physiology in Pigs, Volume 88, May 26-28, 1997, Saint Malo, pp: 446-449.

Bogut, I., Z. Milakovic, Z.I. Bukvic, S. Brkic and R. Zimmer, 1998. Influence of probiotic (*Streptococcus faecium* M74) on growth and content of intestinal microflora in carp (*Cyprinus carpio*). Czech J. Anim. Sci., 43: 231-235.

Brunt, J., A. Newaj-Fyzul and B. Austin, 2007. The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (*Walbaum*). J. Fish Dis., 30: 573-579.

Dash, G., R.P. Raman, K.P. Prasad, M. Makesh, M.A. Pradeep and S. Sen, 2014. Evaluation of *Lactobacillus plantarum* as feed supplement on host associated microflora, growth, feed efficiency, carcass biochemical composition and immune response of giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). Aquaculture, 432: 225-236.

Davis, A.K., D.L. Maney and J.C. Maerz, 2008. The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. Funct. Ecol., 22: 760-772.

Debnath, D., A.K. Pal, N.P. Sahu, S. Yengkokpam, K. Baruah, D. Choudhury and G. Venkateshwarlu, 2007. Digestive enzymes and metabolic profile of *Labeo rohita* finger lings fed diets with different crude protein levels. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol., 146: 107-114.

Drapeau, G.R., 1974. Protease from *Staphylococcus aureus*. In: Methods in Enzymology, Volume 45, Part B: Proteolytic Enzymes, Lorand, B.L. (Ed.). Chapter 38, Academic Press, New York, USA., ISBN: 978-0-12-181945-3, pp: 469-475.

Esteve, C., C. Amaro and A.E. Toranzo, 1994. O-Serogrouping and surface components of *Aeromonas hydrophila* and *Aeromonas jandaei* pathogenic for eels. FEMS Microbiol. Lett., 117: 85-90.

Fuller, R., 1989. Probiotics in man and animals. J. Applied Bacteriol., 66: 365-378.

Giri, S.S., V. Sukumaran and M. Oviya, 2013. Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity and disease resistance of tropical freshwater fish, *Labeo rohita*. Fish Shellfish Immunol., 34: 660-666.

Gomez-Gil, B., A. Roque and J.F. Turnbull, 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture, 191: 259-270.

Gonzalez, C.J., T.M. Lopez-Diaz, M.L. Garcia-Lopez, M. Prieto and A. Otero, 1999. Bacterial microflora of wild brown trout (*Salmo trutta*), wild pike (*Esox lucius*) and aquacultured rainbow trout (*Oncorhynchus mykiss*). J. Food Protect., 62: 1270-1277.

Harikrishnan, R., C. Balasundaram and M.S. Heo, 2009. Effect of chemotherapy, vaccines and immunostimulants on innate immunity of goldfish infected with *Aeromonas hydrophila*. Dis. Aquat. Organ., 88: 45-54.

Harikrishnan, R., M.N. Rani and C. Balasundaram, 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture, 222: 41-50.

Hoole, D., D. Bucke, P. Burgess and I. Wellby, 2001. Diseases of Carp and Other Cyprinid Fishes. 1st Edn., Wiley-Blackwell, New Jersey, USA., ISBN-13: 978-0852382523, Pages: 280.

Iregui, C., P. Barato, A. Rey, G. Vasquez and N. Verjan, 2014. Epidemiology of *Streptococcus agalactiae* and Streptococcosis in Tilapia Fish (*Oreochromis* sp.). In: Epidemiology I: Theory, Research and Practice, iConcept Press (Ed.). 1st Edn., Chapter 14, iConcept Press Ltd., USA., ISBN-13: 978-1922227829, pp: 251-268.

Jagoda, S.S.S.S., T.G. Wijewardana, A. Arulkanthan, Y. Igarashi and E. Tan *et al*., 2014. Characterization and antimicrobial susceptibility of motile aeromonads isolated from fresh water ornamental fish showing signs of septicaemia. Dis. Aquat. Organ., 109: 127-137.

Jayamanne, V.S. and M.R. Adams, 2006. Determination of survival, identity and stress resistance of probiotic bifidobacteria in bio-yoghurts. Lett. Applied Microbiol., 42: 189-194.

Jeney, Z., T. Racz, K.D. Thompson, S. Poobalane, L. Ardo, A. Adams and G. Jeney, 2009. Differences in the antibody response and survival of genetically different varieties of common carp (*Cyprinus carpio* L.) vaccinated with a commercial *Aeromonas salmonicida*/*A. hydrophila* vaccine and challenged with *A. hydrophila*. Fish Physiol. Biochem., 35: 677-682.

Karunasagar, I., G.M. Rosalind, I. Karunasagar and K.G. Rao, 1989. *Aeromonas hydrophila* septicaemia of Indian major carps in some commercial fish farms of west Godavari district and hra Pradesh. Curr. Sci., 58: 1044-1045.

Kuhlwein, H., D.L. Merrifield, M.D. Rawling, A.D. Foey and S.J. Davies, 2014. Effects of dietary β-(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.). J. Anim. Physiol. Anim. Nutr., 98: 279-289.

Kumar, R., S.C. Mukherjee, R. Ranjan and S.K. Nayak, 2008. Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. Fish Shellfish Immunol., 24: 168-172.

Kurman, J.A. and J.L. Rasic, 1991. The Health Potential of Products Containing Bifidobacteria. In: Therapeutic Properties of Fermented Milks, Robinson, R.K. (Ed.). Elsevier Applied Science, London, UK., ISBN-13: 978-1851665525, pp: 117-157.

Kwak, J.K., S.W. Park, J.G. Koo, M.G. Cho, R. Buchholz and P. Goetz, 2003. Enhancement of the non-specific defence activities in carp (*Cyprinus carpio*) and flounder (*Paralichthys olivcaces*) by oral administration of Schizophyllan. Acta Biotechnologica, 23: 359-371.

Kwon, D.Y. and J.S. Rhee, 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. J. Am. Oil Chem. Soc., 63: 89-92.

Lazado, C.C. and C.M.A. Caipang, 2014. Mucosal immunity and probiotics in fish. Fish Shellfish Immunol., 39: 78-89.

Li, X.Q., Y.H. Zhu, H.F. Zhang, Y. Yue and Z.X. Cai *et al*., 2012. Risks associated with high-dose *Lactobacillus rhamnosus* in an *Escherichia coli* model of piglet diarrhoea: Intestinal microbiota and immune imbalances. PLoS ONE, Vol. 7. 10.1371/journal.pone.0040666

Lim, G., T.K. Tan and N.A. Rahim, 1987. Variations in amylase and protease activities among *Rhizopus* isolates. MIRCEN J. Applied Microbiol. Biotechnol., 3: 319-322.

Madigan, T.M., M.J. Martinko and J. Parker, 2003. Brock Biology of Microorganisms. 10th Edn., Prentice Hall/Pearson Education, USA., ISBN-13: 9780130662712, pp: 620.

Marchesi, J.R., T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom and W.G. Wade, 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied Environ. Microbiol., 64: 795-799.

Merrifield, D.L., A. Dimitroglou, A. Foey, S J. Davies and R.T.M. Baker *et al*., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture, 302: 1-18.

Minelli, E.B. and A. Benini, 2008. Relationship between number of bacteria and their probiotic effects. Microb. Ecol. Health Dis., 20: 180-183.

Nayak, S.K., 2010. Probiotics and immunity: A fish perspective. Fish Shellfish Immunol., 29: 2-14.

Nikoskelainen, S., A. Ouwehand, S. Salminen and G. Bylund, 2001. Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. Aquaculture, 198: 229-236.

Panigrahi, A., V. Kiron, S. Satoh and T. Watanabe, 2010. Probiotic bacteria *Lactobacillus rhamnosus* influences the blood profile in rainbow trout *Oncorhynchus mykiss* (Walbaum). Fish Physiol. Biochem., 36: 969-977.

Panigrahi, A., V. Kiron, T. Kobayashi, J. Puangkaew, S. Satoh and H. Sugita, 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Vet. Immunol. Immunopathol., 102: 379-388.

Pertsemlidis, A. and J.W. Fondon III, 2002. Having a BLAST with bioinformatics (and avoiding BLASTphemy). Genome Biol., Vol. 2.

Pirarat, N., T. Kobayashi, T. Katagiri, M. Maita and M. Endo, 2006. Protective effects and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against experimental *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*). Vet. Immunol. Immunopathol., 113: 339-347.

Pridgeon, J.W. and P.H. Klesius, 2011. Molecular identification and virulence of three *Aeromonas hydrophila* isolates cultured from infected channel catfish during a disease outbreak in West Alabama (USA) in 2009. Dis. Aquat. Organ., 94: 249-253.

Putra, A.N. and Widanarni, 2015. Screening of amylolytic bacteria as candidates of probiotics in tilapia (*Oreochromis* sp.). Res. J. Mycrobiol., 10: 1-13.

Ramakrishnan, C.M., M.A. Haniffa, M. Manohar, M. Dhanaraj, A.J. Arockiaraj, S. Seetharaman and S.V. Arunsingh, 2008. Effects of probiotics and spirulina on survival and growth of juvenile common carp (*Cyprinus carpio*). Israeli J. Aquacult., 60: 128-133.

Ramos, M.A., B. Weber, J.F. Goncalves, G.A. Santos, P. Rema and R.O.A. Ozorio, 2013. Dietary probiotic supplementation modulated gut microbiota and improved growth of juvenile rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol., 166: 302-307.

Renuka, K.P., M. Venkateshwarlu and A.T.R. Naik, 2014. Effect of probiotic (*Lactobacillus acidophilus*) on haematological parameters of *Catla catla* (Hamilton). Int. J. Curr. Microbiol. Applied Sci., 3: 326-335.

Rey, A., N. Verjan, H.W. Ferguson and C. Iregui, 2009. Pathogenesis of *Aeromonas hydrophila* strain KJ99 infection and its extracellular products in two species of fish. Vet. Rec., 164: 493-499.

Ringo, E., A. Dimitroglou, S.H. Hoseinifar and S.J. Davies, 2014. Prebiotics in Finfish: An Update. In: Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics, Merrifield, D.L. and E. Ringo (Eds.). Chapter 14, Wiley-Blackwell, New Jersey, USA., ISBN-13: 9781118897270, pp: 360-400.

Romero, J. and P. Navarrete, 2006. 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). Microb. Ecol., 51: 422-430.

Salinas, I., A. Cuesta, M.A. Esteban and J. Meseguer, 2005. Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. Fish Shellfish Immunol., 19: 67-77.

Sharma, P., V. Kumar, A.K. Sinha, J. Ranjan, H.M.P. Kithsiri and G. Venkateshwarlu, 2010. Comparative fatty acid profiles of wild and farmed tropical freshwater fish rohu (*Labeo rohita*). Fish Physiol. Biochem., 36: 411-417.

Skjermo, J. and O. Vadstein, 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture, 177: 333-343.

Son, V.M., C. Changa, M.C. Wu, Y.K. Guu, C.H. Chiu and W. Cheng, 2009. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses and disease resistance of the grouper *Epinephelus coioides*. Fish Shellfish Immunol., 26: 691-698.

Sugita, H., T. Fujie, T. Sagesaka and S. Itoi, 2009. The effect of *Lactococcus lactis* on the abundance of aeromonads in the rearing water of the goldfish, *Carassius auratus* (Linnaeus). Aquacult. Res., 41: 153-156.

Suzer, C., D. Coban, H.O. Kamaci, S. Saka, K. Firat, O. Otgucuoglu and H. Kucuksari, 2008. *Lactobacillus* sp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. Aquaculture, 280: 140-145.

Tamamdusturi, R., Widanarni and M. Yuhana, 2016. Administration of microencapsulated probiotic *Bacillus* sp. NP5 and prebiotic mannan oligosaccharide for prevention of *Aeromonas hydrophila* infection on *Pangasianodon hypophthalmus*. J. Fish. Aquat. Sci., 11: 67-76.

Tellez-Banuelos, M.C., A. Santerre, J. Casas-Solis and G. Zaitseva, 2010. Endosulfan increases seric Interleukin-2 Like (IL-2L) factor and Immunoglobulin M (IgM) of Nile tilapia (*Oreochromis niloticus*) challenged with *Aeromona hydrophila*. Fish Shellfish Immunol., 28: 401-405.

Tovar-Ramirez, D., J.Z. Infante, C. Cahu, F.J. Gatesoupe and R. Vazquez-Juarez, 2004. Influence of dietary live yeast on European sea bass (*Dicentrarchus labrax*) larval development. Aquaculture, 234: 415-427.

Ullal, A.J., R.W. Litaker and E.J. Noga, 2008. Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). Dev. Comp. Immunol., 32: 1301-1312.

Utami, D.A.S., Widanarni and M.A. Suprayudi, 2015a. Administration of microencapsulated probiotic at different doses to control streptococcosis in Tilapia (*Oreochromis niloticus*). Microbiol. Indonesia, 9: 17-24.

Utami, D.A.S., Widanarni and M.A. Suprayudi, 2015b. Quality of dried *Bacillus* NP5 and its effect on growth performance of tilapia (*Oreochromis niloticus*). Pak. J. Biol. Sci., 18: 88-93.

Vand, Z.D.A., M. Alishahi and M.R. Tabande, 2014. Effects of different levels of *Lactobacillus casei* as probiotic on growth performance and digestive enzymes activity of *Barbus gryprus*. Int. J. Biosci., 4: 106-116.

Venkat, H.K., N.P. Sahu and K.K. Jain, 2004. Effect of feeding Lactobacillus-based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii* (de Man). Aquacult. Res., 35: 501-507.

Walter, H.E., 1984. Proteinases: Methods with Hemoglobin, Casein and Azocoll as Substrates. In: Methods of Enzymatic Analysis, Volume 5: Enzymes 3: Peptidases, Proteinases and their Inhibitors, Bergmeyer, Z.H.U. (Ed.). VCH Publishers, Weinheim, Germany, ISBN-13: 978-3527260454, pp: 270-277.

Widanarni and Tanbiyaskur, 2015. Application of probiotic, prebiotic and synbiotic for the control of streptococcosis in tilapia *Oreochromis niloticus*. Pak. J. Biol. Sci., 18: 59-66.

Widanarni, W., A. Suwanto, S. Sukenda and B.W. Lay, 2003. Potency of vibrio isolates for biocontrol of vibriosis in tiger shrimp (*Penaeus monodon*) larvae. J. Biotropia, 20: 11-23.

Yin, G., L. Ardo, K.D. Thompson, A. Adams, Z. Jeney and G. Jeney, 2009. Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio* and protection against *Aeromonas hydrophila*. Fish Shellfish Immunol., 26: 140-145.