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# The dietary effect of medicinal herbs extract and multiple probiotics mixture on the growth performance, innate immune response and antibacterial activity of nile tilapia *Oreochromis niloticus*

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The study investigated the dietary effects of medicinal herbs extract and multiple probiotics mixture on the growth performance, innate immune response and antibacterial activity of nile tilapia *Oreochromis niloticus*. Tilapia were divided in four groups. The first is a fish group fed a basal diet added with 40% medicinal herbs extract (MHE). The second is a fish group fed a basal diet supplied with $2\times10^8$ CFU/g of 2 *Bacillus* sp, 2 *Lactobacillus* sp and 2 *Yeast* sp, respectively (PB). The third group was fed with a mixture of probiotics (2 *Bacillus* sp, 2 *Lactobacillus* sp and 2 *Yeast* sp) with the medicinal herbs extract added in basal diet (MHE+PB). The fourth group was fed only a basal diet (C). In a non-specific immune parameters analysis, respiratory burst activity, lysozyme activity, phagocytic activity (PA), alternative complement pathway activity ($ACH_{50}$) and superoxide dismutase (SOD) activity were significantly ($p<0.05$) increased in the group MHE+PB compared to other groups. Both PB and MHE groups showed a significant ($p<0.05$) increase in respiratory burst activity, lysozyme activity compared to the control C group, whereas no significant differences were observed in PA, $ACH_{50}$ and SOD activity compared to the control group. In challenging test, fish were administered with *Edwardsiella tarda* (*E. tarda*) on 30 days after feeding with each experimental diet and viable *E. tarda* cell reduction was checked over 21 days post injection. MHE+PB group showed a significantly ($p<0.05$) reduced *E. tarda* cells compared to other groups. No significant antibacterial difference ($p>0.05$) was observed between PB and MHE only treated group. Compared to the control, a significant antibacterial difference ($p<0.05$) appeared in PB but not in MHE ($p>0.05$). The results suggest that the probiotics and MHE mixture could be utilized as an alternative to antibiotics in the control of fish diseases caused by *E. tarda*.

**Key words:** Medicinal herbs extract, Probiotics, Innate immune response, Antibacterial activity

## Introduction

Fish is often the cheapest and most commonly consumed animal source food in countries with food and nutrition insecurity (FAO, 2014a, 2014b; World Bank, 2006). Globally, since 1961, total fish consumption has grown at an annual rate of 3.6% while population has grown at 1.8%, enabling a near doubling of per capita annual fish consumption (FAO, 2014a, 2014b). This transformation has been spearheaded by aquaculture – the farming of fish and oth-

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er aquatic organisms – which has become the most rapidly growing global food sector. Since the 1970s, aquaculture has grown at more than 8% annually.

Asia accounts for 88% of global aquaculture production, and it has seen, by far, the most dramatic growth in aquaculture. However, disease has been a primary constraint to the aquaculture industry, imposing severe losses on farming facilities all over the world. Antibiotic drugs have the capacity to kill or inhibit the growth of micro-organisms. However, the use of antibiotics to control fish disease needs to be restricted due to the emergence of drug-resistant bacteria and concerns about environmental hazards and food safety (Hernández Serrano, 2005). The use of antimicrobial agents in aquaculture has resulted in the increase in the frequency of strains resistant to these agents. Potentially these resistant strains can have an impact on the therapy of fish diseases, the therapy of human diseases, or the environment of the fish farms. The future development of aquaculture greatly depends on the development of alternative feed ingredients that can provide higher resistance against pathogens.

Recently, the health benefits of probiotics have attracted increased attention. Probiotics are defined as live microorganisms which play an important role in health and disease. Probiotics are well studied for their health benefits in improving immune system function. Probiotics are good or friendly bacteria that exert beneficial effects on the body by regulating the host's gut microflora balance. Nowadays, concern is increasing regarding the use of probiotics as a functional feed in the farming fishes industry. During the past several decades, antibiotics are increasingly widely employed, owing to their therapeutic effects against disease. However, as antibiotics ultimately cause antibiotic resistance, a number of countries have recently placed restrictions on the subtherapeutic use of antibiotics. The application of probiotics constitutes a potential alternative to antibiotic treatment. A large amount of studies related to probiotics have been conducted on humans, animals, fishes and probiotic treatments have been associated with the beneficial effects, including improved growth and disease prevention.

Meanwhile, medicinal herbs, as food or medicine, can strengthen the body and increase its resistance to disease by acting on various components of the immune system. Medicinal herbs have been known as immunostimulants for thousands of years. The application of medicinal herbs as natural and innocuous compounds has potential in aquaculture as an alternative to antibiotics and immunoprophylactics. The growing interest in these plants has increased worldwide because they are easy to prepare, cheap, and have few side effects on animals and the environment.

For this reasons, it is becoming increasingly imperative to identify suitable antibiotic alternatives. While various feed additives have been investigated for use in aquaculture feeds, organic acids are receiving increasing attention due to their strong antimicrobial and prophylactic properties against various pathogenic bacteria (Defoirdt *et al.*, 2006; Ng and Koh, 2011; Da silva *et al.*, 2013).

To develop a new feed additives, the study investigated a dietary effects of medicinal herbs extract and multiple probiotics mixture on the growth performance, innate immune response and antibacterial activity of nile tilapia *Oreochromis niloticus*.

## Materials and Methods

### Isolation and identification of probiotics (PB)

To isolate PB, various samples such as cabbage kimchi, young radish kimchi, green onion kimchi, pineapple and excreta were collected. Selective medium was used for isolating PB in the samples. Used selective medium was Luria Bertani broth (LB, Difco) for isolating *Bacillus*, *Lactobacilli* MRS broth (MRS, Difco) for *Lactobacillus* and YM broth & Potato dextrose broth (PDB, Difco) for *Yeast*. At first according to characteristic morphological feature of

*Bacillus* sp, *Lactobacillus* sp and *Yeast* sp, each PB was respectively isolated. It was being passaged culture and then pure isolation. The sequence of the individual clone was analyzed using universal primers (Table 1).

### Medicinal herbs extract (MHE)

MHE was obtained by overnight boiling water with the by-product of a digestive medicine manufactured from Hanpoong pharmaceutical companies.

### Pathogenic strains

*Edwardsiella tarda* (KCTC 12267) was purchased from Korean Collection for Type Culture (KCTC) and passaged culture by using brain heart infusion (BHI) broth and *Salmonella shigella* (SS) agar.

### Experimental diets and design

Basic folder was purchased at the (C) JEILFEED. Co.,Ltd. Four experimental diets were formulated to be isonitrogenous isocaloric [1%/g(fish)]. A fish meal based diet was formulated and regarded as a only a basal diet (control) and three other experimental diets were prepared by dietary 40% medicinal herbs extract (MHE), $2\times10^8$ CFU/g of 2 *Bacillus* sp, 2 *Lactobacillus* sp and 2 *Yeast* sp, respectively (PB) and a mixture of probiotics (2 *Bacillus* sp, 2 *Lactobacillus* sp and 2 *Yeast* sp) with the medicinal herbs extract added in basal diet (MHE+PB) of liquid. All diets were thoroughly mixed with 40% distilled water, 40% MHE, PB ($2\times10^8$ CFU/g) and MHE+PB (being formented mixture probiotics in MHE for 3 days). The mixed feed were subsequently dried at 25ºC and stored at 4ºC until use. Tilapia was divided in four groups. The first is a fish group fed a basal diet added with MHE. The second is a fish group fed a basal diet supplied with PB. The third fish group was fed with MHE+PB. The fourth fish group was fed control. In order to investigate growth performance and innate immune response, the fish were weighed after feeding 7, 14, 21 and 28 days and then collected samples. In order to investigate antibacterial activity, after feeding for 30 days we injected intraperitoneally with *E. tarda*($1\times10^8$ CFU/ml) and then collected samples after 0, 7, 21 and 28 days. Four experimental diets were subsequently fed after injection alike.

### Growth performance

Total three groups were respectively divided as C, MHE, PB and MHE+PB (6 fish/group). The body weight (g) of group tilapia was measured before and after feeding on weekly basis for 1 month. Specific growth parameters were measured as follows:

Weight gain (g) = Final weight (g) – Initial weight (g)

Percent weight gain (PWG) = [100 × (Final weight – Initial weight)/ Initial weight]

Specific growth rate (SGR) = [{LNFinal weight (g)-LNInitial weight (g)}/DAY]×100

Feed conversation (FCR) = Total feed given (g)/ Weight gain (g)

### Sample collection

At the end of the weekly feeding trial, four fish were randomly selected from each tank and anesthetized with 2-Phenoxyethanol solution (200 ppm). And then blood samples were collected from caudal

Table 1. Universal primers for probiotics 16S rRNA

| Primer name | Objects | Primer sequence 5′ to 3′ |
|---|---|---|
| 27F | 16S rRNA sequence amplification | AGAGTTTGATCCTGGCTCAG |
| 1492R | 16S rRNA sequence amplification | GGTTACCTTGTTACGACTT |
| ITS1 | 18S rRNA sequence amplification | TCCGTAGGTGAACCTGCGG |
| ITS4 | 18S rRNA sequence amplification | TCCTCCGCTTATTGATATGC |

veins with syringes to determine lysozyme, $ACH_{50}$ and superoxide dismutase (SOD) activities. Serum was separated from whole blood samples by centrifugation at 1200 × g for 5 min at 4 ℃. Leukocyts were collected according to the method described by Santarern *et al*. (1997). Harvested cells ($10^6$ cell/ml) were counted using counting chamber and utilized to determine respiratory burst activity and phagocytic activity.

### Respiratory burst activity

Respiratory burst activity was determined according to Secombes *et al*. (1988). Collected tilapia leukocytes were washed with PBS and the viable cell number ($1\times10^6$ cell /ml) was adjusted. The 200 μl cells were placed 96 well plate and then centrifuged at 30 × g for 5 min. After washing using PBS twice the 100 μl phorbol myristate acetate (PMA, 1 μg/ml) in nitroblue tetrazolium (NBT, 1μg/ml) was added and then incubated in the darkroom for 60 min. After incubation 96 well plate was centrifuged at 30 × g for 5 min and then the supernatant removed followed by washing with PBS. Washed leukocytes were fixed using 70% methanol and then washed twice followed by adding 120 μl KOH and 140 μl dimethyl sulfoxide (DMSO, Sigma). The optical density was measured at 620 nm with spectrophotometer.

### Phagocytosis activity

Phagocytosis activity (PA) was determined according to the method of Pulsford *et al*. (2012) with a slight modification. The harvested tilapia leukocytes 200 μl were placed in the chamber slide (Thermo scientific, Nunc) and then incubated in darkroom for 12 hr. After incubation 10 μl zymosan ($1\times10^6$ cell/ml, Sigma) was added and then incubated in darkroom for 1 hr. After incubation chamber slide was centrifuged at 30 × g for 5 min and then the supernatant removed after then washed using PBS. Washed leukocytes were fixed using 70% methanol and then washed using PBS. Cover and well of chamber slide were removed and then dried at room temperature. After air-drying, the slide stained using wright Giemsa stain method. After staining, one hundred leukocytes were randomly counted for each sample under the microscope. Phagocytosis activity defined as percentage phagocytosis was expressed as

PA = phagocytic leukocytes / total leukocytes
PI = zymosan in the phagocytic leukocytes / phagocytic leukocytes

### Lysozyme activity

Serum lysozyme activity was measured according to the method described by Sheikazadeh *et al*. (2012). One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of $.001^{-1}$ min $ml^{-1}$ serum

### Superoxide dismutase (SOD) activity

SOD activity was measured by its ability to inhibit superoxide anion generated by xanthine and xanthine oxidase reaction system according to Wang and Chen (2005) using and SOD detection kit (Biovision co, Korea). The optical density was measured at 405 nm. SOD activity defined as percentage was expressed as

$$\text{SOD Activity (inhibition rate \%)} = \frac{(\text{A blank1-A blank3}) - (\text{A sample-A blank2})}{(\text{A blank1-A blank3})} \times 100$$

### $ACH_{50}$

$ACH_{50}$ activity was measured according to the method described by Yano (1992). $ACH_{50}$ activity was usually assayed by using RaRBC as target cells in the presence of EGTA and $Mg^{2+}$ (Inai *et al*., 1982). EGTA is a chelating agent for $Ca^{2+}$ and was used to block the classical pathway. Different volumes of the diluted serum (1:15) ranging from 75 μl to 200 μl were dispensed into 96 well plate and total volume was made up to 200 μl with EGTA-Mg-GVB and then to each well is then added

100 μl of RaRBC suspension ($1\times10^8$ cell/ml). A 100% hemolysis control and a cell blank were included. The 96 well plate were incubated at 20℃ for 90 min and then centrifuged at 30 × g for 20 min. After centrifugation, the supernatants were transferred to a new 96 well plate and then absorbance of the supernatants was measured at 405 nm. The degree of hemolysis (y) was calculated by dividing the corrected $A_{405}$ value by the corrected $A_{405}$ of the 100% hemolysis control. $ACH_{50}$ activity defined as unit/ ml was expressed as

(Unit/ ml) = 1/K × (reciprocal of the serum dilution) × 0.5

### Antibacterial activity

Antibacterial activity tests were conducted in triplicate with 24 fish per group. After feeding each experimental diet for 30 days each tilapia was injected intraperitoneally with 200 μl PBS containing $1\times10^8$ CFU/ml live *E. tarda* from a 24 hr culture in SS medium at 36℃. Each experimental diet was given to the tilapia during the test. All organs were removed on day 0, 7, 14 and 21 post injection. Removed organs were put in the 10 ml PBS and homogenized and then centrifuged passing gauze at 1000 × g for 20 min. Organs suspension was diluted using serial dilution method and then total viable cells were counted using streak plate method on the SS medium.

### Statistical analysis

All data were subjected to one way ANOVA (analysis of variance) using SPSS 12.0 for windows. Differences between the means were tested by Duncan′s multiple range tests. Overall significance level $P < 0.05$ and the results were presented as means ± SEM (standard error of the mean).

## Results

### Isolation and identification of probiotics (PB)

Identification result of PB was shown in Table 2. As a sequence analysis result, 2 *bacillus* spp. were identified with accession number and 16S ribosomal RNA gene as *B. subtilis* and *B. licheniformis*. Two *Lactobacillus* spp. were identified with accession number and 16S ribosomal RNA gene as *L. plantarum* and *L. paracasei*, and 2 *Yeast* spp. were identified with accession number and 18S ribosomal RNA gene as *S. cerevisiae* and *P. anomala.*

### Growth performance

The effect of diets containing MHE, PB and MHE +PB on growth performance was established in this study. Analyzed data on the growth performance of

Table 2. Output of BLAST for probiotics supplemented in fish diets

| Strains | Accession | Description | Score (bits) | Identities (%) | E value |
|---|---|---|---|---|---|
| B1 | KC757129.1 | Bacillus subtilis strain GRSW1_B1 16S ribosomal RNA gene, partial sequence | 2575 | 100 | 0 |
| B-5 | AB743877.1 | Bacillus licheniformis gene for 16S rRNA, partial sequence, strain: BCJ | 2556 | 100 | 0 |
| L-1 | JX426117.1 | Lactobacillus plantarum strain CTBRBL22 16S ribosomal RNA gene, partial sequence | 2582 | 100 | 0 |
| L-2 | JF965377.1 | Lactobacillus paracasei strain BIM B-552D 16S ribosomal RNA gene, partial sequence | 2623 | 100 | 0 |
| Y-1 | JN887919.1 | Saccharomyces cerevisiae strain Wu-Y2 18S ribosomal RNA gene, partial sequence | 1353 | 99 | 0 |
| Y-2 | FJ865436.1 | Pichia anomala isolate M10 18S ribosomal RNA gene, partial sequence; | 1029 | 100 | 0 |

Table 3. Effects of dietary medicinal herbs extract and probiotics on the weight gain, percent weight gain, specific growth rate and feed conversion ratio in tilapia. Data represent the mean ± S.D. (n=6). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point

| | DAYS | Treatments | | | |
|---|---|---|---|---|---|
| | | Control | MHE | PB | MHE+PB |
| Initial weight (g) | 0 | 116.67±1.67$^{a}$ | 116.67±1.67$^{a}$ | 116.67±1.67$^{a}$ | 116.67±1.67$^{a}$ |
| Final weight (g) | 7 | 120.83±0.01$^{a}$ | 122.22±2.41$^{a}$ | 123.61±2.41$^{ab}$ | 126.39±2.41$^{b}$ |
| | 14 | 127.78±2.41$^{a}$ | 129.17±4.17$^{a}$ | 131.93±2.41$^{ab}$ | 136.11±2.41$^{b}$ |
| | 21 | 132.5±4.33$^{a}$ | 137.5±4.17$^{a}$ | 140.28±2.41$^{a}$ | 148.61±4.81$^{b}$ |
| | 28 | 138.89±4.81$^{a}$ | 143.06±4.81$^{a}$ | 145.83±4.17$^{a}$ | 156.94±4.81$^{b}$ |
| weight gain (g) | 7 | 4.17±0.01$^{a}$ | 5.56±2.41$^{ab}$ | 6.94±2.41$^{ab}$ | 9.72±2.41$^{b}$ |
| | 14 | 11.11±2.41$^{a}$ | 12.5±4.17$^{a}$ | 15.28±2.41$^{ab}$ | 19.44±2.41$^{b}$ |
| | 21 | 15.83±4.33$^{a}$ | 16.67±4.17$^{a}$ | 19.44±6.36$^{ab}$ | 26.39±4.81$^{b}$ |
| | 28 | 22.22±4.81$^{a}$ | 26.39±4.81$^{a}$ | 29.17±4.17$^{a}$ | 40.28±4.81$^{b}$ |
| PWG (%) | 7 | 3.57±0.01$^{a}$ | 4.76±2.06$^{ab}$ | 5.95±2.06$^{ab}$ | 8.33±2.06$^{b}$ |
| | 14 | 9.52±2.06$^{a}$ | 10.71±3.57$^{a}$ | 13.10±2.06$^{ab}$ | 16.67±2.06$^{b}$ |
| | 21 | 13.57±3.71$^{a}$ | 17.86±3.57$^{a}$ | 20.24±2.06$^{ab}$ | 27.38±4.12$^{b}$ |
| | 28 | 19.05±4.12$^{a}$ | 22.62±4.12$^{a}$ | 25.00±3.57$^{a}$ | 34.52±4.12$^{b}$ |
| SGR (%) | 7 | 0.5±0.01$^{a}$ | 0.66±0.28$^{ab}$ | 0.82±0.28$^{ab}$ | 1.14±0.27$^{b}$ |
| | 14 | 0.65±0.14$^{a}$ | 0.72±0.23$^{a}$ | 0.88±0.13$^{ab}$ | 1.10±0.13$^{b}$ |
| | 21 | 0.60±0.15$^{a}$ | 0.78±0.14$^{ab}$ | 0.88±0.08$^{b}$ | 1.15±0.15$^{c}$ |
| | 28 | 0.62±0.13$^{a}$ | 0.73±0.12$^{a}$ | 0.80±0.1$^{a}$ | 1.06±0.11$^{b}$ |
| FCR | 7 | 1.68±0.01$^{a}$ | 1.4±0.48$^{ab}$ | 1.12±0.48$^{ab}$ | 0.75±0.16$^{b}$ |
| | 14 | 1.31±0.32$^{a}$ | 1.21±0.43$^{ab}$ | 0.93±0.16$^{ab}$ | 0.73±0.1$^{b}$ |
| | 21 | 1.39±0.33$^{a}$ | 1.32±0.34$^{a}$ | 1.18±0.44$^{a}$ | 0.82±0.17$^{a}$ |
| | 28 | 1.31±0.32$^{a}$ | 1.09±0.22$^{ab}$ | 0.97±0.14$^{ab}$ | 0.70±0.08$^{b}$ |

tilapia in different treatments and control, including initial weight, final weight gain, PWG, SGR and FCR are shown in Table 3). No significant differences were observed for initial weight between three other experimental diets and control at the start of the experiment. At the end of the experiment, statistical analysis showed that tilapia fed MHE+PB diets grew significantly faster than the control, MHE and PB group. Final weight, weight gain, PWG, SGR and FCR were significantly different ($P < 0.05$) for MHE +PB compared to the control, MHE and PB.

### Respiratory burst activity

The effect of diets containing MHE, PB and MHE +PB on respiratory burst activity was shown in Fig. 1. Statistical analysis showed that tilapia fed MHE+ PB diets increased significantly ($P < 0.05$) than all other

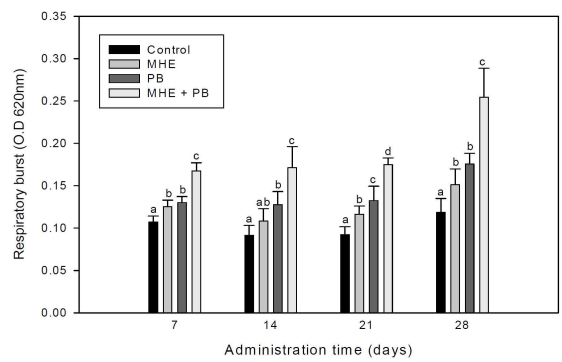


Fig. 1. Respiratory burst activity of HK leukocytes from tilapia fed the control diet (control), medicinal herbs extract diet (MHE), probiotics diet (PB), and medicinal herbs extract and probiotics mixture diet (MHE+PB) for 28 days. The absorbance was measured by micro plate reader at 620nm. Data represent the mean ± S.D. (n=6). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point.

groups throughout the experiment period. In addition significant differences ($P < 0.05$) were showed for PB and MHE in comparison with control.

### Phagocytosis activity

The effect of diets containing MHE, PB and MHE +PB on PA and PI was shown in Fig. 2 A & B. PA was shown that MHE+PB group increased significantly ($P < 0.05$) than the control group (Fig. 2 A). However, there was no significant difference ($P > 0.05$) in PI among the experimental groups (Fig. 2 B).

### Lysozyme activity

Fig. 3 shows the effect of diets containing MHE, PB and MHE+PB on lysozyme activity. In group of tilapia fed MHE+PB diets lysozyme activity was increased significantly ($P < 0.05$) compared to other groups throughout the experimental period. Compared to a control, significant differences ($P < 0.05$) were observed in each group of PB and MHE.

### Superoxide dismutase (SOD) activity

The effect of diets containing MHE, PB or MHE +PB on SOD activity was shown in Fig. 4. SOD activity was significantly ($P < 0.05$) enhanced in the group of MHE+PB compared to a control group, whereas no significant difference was observed in the group of MHE or PB.

### $ACH_{50}$

Fig. 5 shows the effect of diets containing MHE, PB or MHE+PB on $ACH_{50}$. No significant differences were observed among the experimental groups on day 7. However, on day 14, 21 and 28 post feeding, $ACH_{50}$ activity was significantly ($P < 0.05$) increased in the group of MHE+PB compared to a control.

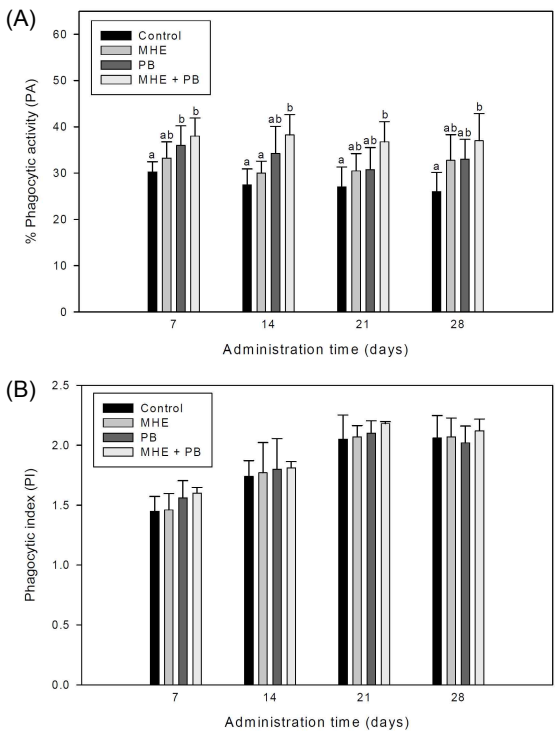


Fig. 2. Phagocytic activity (A) and phagocytic index (B) of HK leukocytes from tilapia fed the control diet (control), medicinal herbs extract diet (MHE), probiotics diet (PB), and medicinal herbs extract and probiotics mixture diet (MHE+PB) for 28 days. Data represent the mean ± S.D. (n=6). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point.

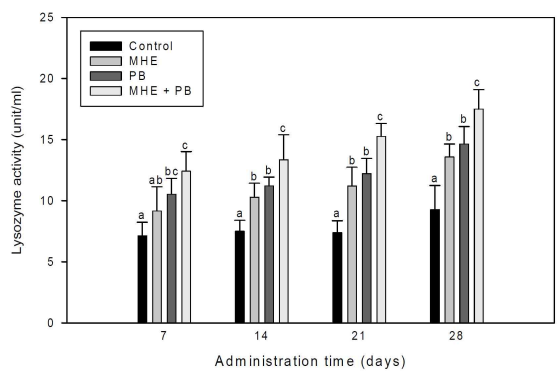


Fig. 3. Lysozyme activity in serum of tilapia fed the control diet (control), medicinal herbs extract diet (MHE), probiotics diet (PB), and medicinal herbs extract and probiotics mixture diet (MHE+PB) for 28 days. The absorbance was measured by micro plate reader at 405 nm. Data represent the mean ± S.D. (n=6). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point

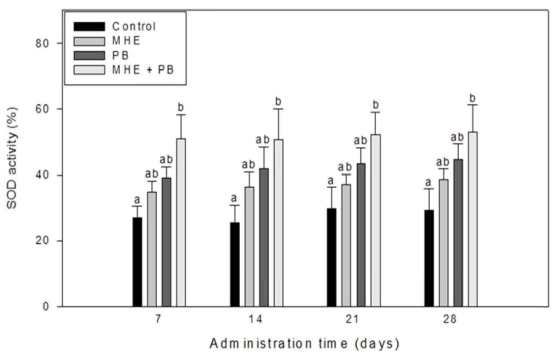


Fig. 4. SOD activity in serum of tilapia fed the control diet (control), medicinal herbs extract diet (MHE), probiotics diet (PB), and medicinal herbs extract and probiotics mixture diet (MHE+PB) for 28 days. The absorbance was measured by micro plate reader at 405 nm. Data represent the mean ± S.D. (n=6). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point.

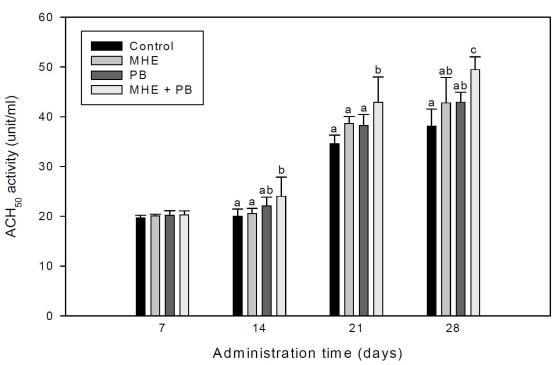


Fig. 5. $ACH_{50}$ activity in the serum of tilapia fed the control diet (control), medicinal herbs extract diet (MHE), probiotics diet (PB), and medicinal herbs extract and probiotics mixture diet (MHE+PB) for 28 days. The absorbance was measured by micro plate reader at 405 nm. Data represent the mean ± S.D. (n=6). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point.

### Antibacterial activity

The effect of diets containing MHE, PB and MHE +PB on antibacterial activity was shown in Fig. 6. There were no significant differences of antibacterial activity in between three other experimental diets and control on day 0. However, antibacterial activity

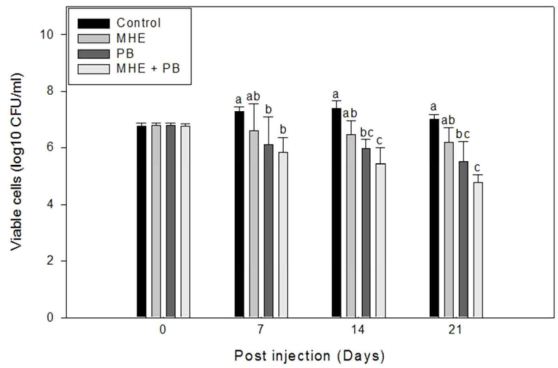


Fig. 6. Antibacterial effect of tilapia fed the control diet (control), medicinal herbs extract diet (MHE), probiotics diet (PB), and medicinal herbs extract and probiotics mixture diet (MHE+PB) for 30 days. Data represent the mean ± S.D. (n=5). Different letters above the bars indicate significant differences ($p < 0.05$) in different groups of the same time point.

was significantly ($P < 0.05$) enhanced in MHE+PB and PB group compared to a control on day 14, 21 and 28 post feeding. No significant difference, however, was observed in MHE group compared to a control.

## Discussion

The immune system of fish can be influenced by a wide range of factors including diseases, pollutants, hormones and feed (nutrition). Feed formulation of fish culture is largely depending on additive ingredients. Manipulation if microbiota using probiotics have been reported as a worthy practice for aquaculture in order to control or inhibit the pathogen bacteria, improve the growth performances and digestive enzymes, and enhance the immune responses of the host against pathogens or physical stress (Verschuere *et al*., 2000; Balcazar *et al*., 2006; Perez *et al*., 2010).

Medicinal herbs have been known as immunostimulants for thousands of years. The application of medicinal herbs as natural and innocuous compounds has potential in aquaculture as an alternative

to antibiotics and immunoprophylactics. The growing interest in these plants has increased world-wide because they are easy to prepare, cheap, and have few side effects on animals and the environment. The goal of this study was to determine the dietary effects of medicinal herbs extract and multiple probiotics mixture on the growth performance, innate immune response and antibacterial activity of tilapia.

In the study, MHE+PB supplemented diet fed tilapia resulted in significant increase ($P < 0.05$) of final weight, weight gain, PWG, SGR and FCR (Table 3). This indicates that the MHE and PB addition was required to attain superior growth performance in tilapia. Similar results have been reported in aquacultured fishes, *Paralichthys olivaceus* fed with probiotics and herbal mixtures supplemented diets had enhanced the growth performance (Harikrishnan *et al*., 2011). Although several studies have demonstrated the beneficial effects of probiotics and medicinal herbs on the growth performance in animals and aquacultured fishes (Aly *et al*., 2008; Kim *et al*., 2008; Chiu *et al*., 2010; Zokaeifar *et al*., 2012), the exact mechanism of action is not well understood. The one possible explanation could be related to the action of competitive exclusion, by which probiotics may create a hostile environment for pathogen colonization. Another possible explanation for the improvement of the Tilapia growth factors by probiotics may be due to the induction of digestive enzymes, including protease and amylase, which consequently stimulate the natural digestive enzyme activity of the host (Wang, 2007; Liu *et al*., 2009).

In addition, it is important to mention that a better appetite was observed in tilapia fed diets containing MHE+PB than the control, MHE and PB group during the feeding period because of undigested feed residues were not found. A better feed digestion may be related to an increase of the digestive enzyme activity and subsequently increased the appetite in tilapia. In a world, MHE+PB diets could be made increase growth performance and reduce feed cost by improving the appetite of aquacultured fishes.

Non-specific mechanism is so important in aquacultured fishes. Fishes rely on both specific and non-specific mechanisms to protect themselves against invading pathogens. The non-specific defenses include physical barriers, such as epithelial shield of scales, skin and mucus. Once a pathogen has managed to penetrate these initial barriers, chemical defenses such as serum lysozyme, lectins and complement components may coat the pathogen and opsonize them for further destruction (Fletcher, 1981).

Other components of non-specific immune system are also activated by invading stimuli (Ellis, 1981). Also, phagocytic cells play an important role in the defense mechanisms of the host by adhering to and engulfing invading particles. Such cells include tissue macrophages, circulatory monocytes and neutrophils. In this study, respiratory burst activity and phagocytosis activity were measured as an indicator of the non-specific cell-mediated immunity and lysozyme activity and $ACH_{50}$ activity were measured an indicator of the non-specific humoral immunity.

In this study, the non-specific immune response in fish was measured on respiratory burst activity and phagocytosis activity to be a function of macrophage activity. As a result, tilapia fed MHE+PB diet was significantly increased on respiratory burst activity compared with a control, MHE or PB group. Similar results have been reported in aquacultured fishes, probiotics and herbal mixtures supplemented diets had increased the respiratory burst activity (Jhon *et al*., 2009; Harikrishnan *et al*., 2011). These cells become more adherent to tissue cell surfaces by the production of adhesion proteins, which facilitate their migration from the capillaries to the sites of injury (Kishimoto *et al*., 1985). They also exhibit increased production of oxygen radicals ($O_2^-$, $OH^-$) during the oxidative burst process. These reactive species are capable of destroying pathogens (Hassett and Cohen, 1989). The ability of neutrophils to adhere to glass allows NBT to be used as a differential staining to

measure the level of reactive oxygen species in the cytoplasm of cells. In the presence of oxygen radicals, the soluble dye changes from yellow to the insoluble dark blue of formazan (Anderson, 1992). Also, phagocytosis activity was significantly increased similar with respiratory burst activity. Respiratory burst activity and phagocytosis activity can indicate the health status of fish. Phagocytosis activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defense. The highest phagocytic activity was recorded in MHE+PB diet fishes groups. These could be increasing their destructive and killing ability. The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against diseases among the fishes. The oxygen radicals and nitric oxide are the most destructive products produced by activated macrophages. Increase of respiratory burst activity and reactive nitrogen species can be correlated with increase of killing and nitric oxide radicals production and increase of killing activity.

Serum complement and lysozyme activity are important components of the humoral innate immune system, protecting fish from potentially invasive organisms. Lysozyme is considered as one of the important bactericidal enzymes and an indispensable tool of fish to fight infectious agents. Our research showed that the use of diets containing MHE+PB has significant increase on the lysozyme activity. Also, many reliable literatures have demonstrated that alternative complement pathway (ACP) is a suitable indicator for disease resistance in fish. The statistical analysis of our achieved results in relation to ACP revealed a significant increase of $ACH_{50}$ in the serum samples collected from MHE+PB group. Similar results have been reported in aquacultured fishes, probiotics and herbal mixtures supplemented diets had increased the lysozyme activity and $ACH_{50}$ activity (Jhon *et al*., 2009; Harikrishnan *et al*., 2011). Also, other study results in relation to medicinal herbs and probiotics had showed that treated groups followed by medicinal herbs and probiotics increased the lysozyme activity and $ACH_{50}$ activity (Wang *et al*., 2008; Aly *et al*., 2008; Chiu *et al*., 2010).

SOD is one of the most important antioxidative enzymes that alternately catalyzes the dismutation of the superoxide ($O_2^-$) radical into either ordinary molecular oxygen ($O_2$) or hydrogen peroxide ($H_2O_2$). Superoxide is produced as a by-product of oxygen metabolism and if not regulated causes many types of cell damage. Hydrogen peroxide is also damaging but less so and is degraded by other enzymes such as catalase. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. Our results showed that the use of diets containing MHE+PB has significant increase on the SOD activity. Similar results have been reported in aquacultured fishes (Chiu *et al*., 2010; Liu *et al*., 2012).

In this study, tilapia fed diets supplemented with MHE+PB significantly reduced viable cells of *E. tarda* after being challenged with *E. tarda* than MHE and control group. Also, PB group decreased for viable cells significantly than the control group. Similar results have been reported in post larvae, *M. rosenbergii* fed with Binifit$^{TM}$, *Lactobacillus sporogenes*, *B. subtilis* and *Saccharomyces cerevisiae*, supplemented diets had improved the survival performance (Seenivasan *et al*., 2011). Improvement in survival has been reported in juveniles of *P. indicus* fed with Latic acid bacteria (*Lactobacillus acidophilus, Saccharomyces cremoris, Lactobacillus bulgaricus*-56 and L. *bulgaricus*-57) supplemented diets (Fernandez *et al*., 2011). It has been has been reported that *Bacillus* supplemented diets improved the survival in black tiger shrimp, *penaeus monodon* (Wang, 2007; Boonthai *et al*., 2011) in rainbow trout, *onchorhynchus mykiss* (Bagheri *et al*., 2008). The improved resistance of *E. tarda* after challenge may be partly attributable to the increased innate immune system. By taking into account of the enzyme activity and appetite stim-

ulation, together with the colonization of probiotics in the tilapia fed MHE+PB diets, healthier tilapia and higher survival rate were resulted. Therefore, the findings indicated that the increased resistance to *E. tarda* was related to the enhanced immune status. More studies are needed to find the reasons for it. To the best of our knowledge, the exact mechanisms for these findings were not clear yet.

In conclusion, tilapia fed diets supplemented with MHE+PB showed significant differences in growth performance, innate immune responses and anti-bacterial effect compared to the control. The results suggested that diets supplemented with MHE+PB can play a crucial role in improving a natural immunity and preventing bacterial diseases for various fishes.

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