Effect of dietary chromium to improve immune response in red tilapia (*Oreochromis* sp.)

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Abstract. This study was conducted to evaluate the effect of dietary chromium to improve immune response in red tilapia (*Oreochromis* sp.) against *Streptococcus agalactiae* infection. Three isonitrogenous and isocaloric experimental feeds were prepared. These diets were chromium picolinat (CrPic) 1 mg kg^{-1}, chromium yeast (CrYst) 2 mg kg^{-1} feed supplementation, and without chromium supplementation with three replications. Fish were reared for 50 days with feeding three times daily at satiation. During this rearing, all fish except negative control were challenged after 40 days with *S. agalactiae* via intraperitoneal route injection in the amount of 0.1 mL (10^7 CFU mL^{-1}). Survival rate and immune parameters (leukocytes, erythrocytes, hemoglobin, hematocrit, phagocytic index, respiratory burst and lysozyme activity) were observed during the 10 days of challenge test. The results showed that survival and immune response parameters of the fish fed CrPic supplementation were higher than other treatments after the challenge test. In conclusion, dietary CrPic 1 mg kg^{-1} improved immune response in red tilapia against *S. agalactiae* infection.

Key Words: chromium, immune response, red tilapia, *Streptococcus agalactiae*.

Introduction. Tilapia are highly susceptible to *Streptococcus agalactiae* infection (Chen et al 2012; Iregui et al 2016). The infection of *S. agalactiae* results in extremely high mortality that caused serious problems in wild and cultured fish species including freshwater and marine fish (Amal et al 2012). Streptococcosis in aquaculture was reported causing severe economic losses in aquaculture industry (Zamri-Saad et al 2014). The efforts to improve fish immunity by adding essential nutrients such as chromium to the diet can be done to prevent high mortality.

Chromium (Cr) is an essential mineral for humans and animals (Bhattacharya et al 2016). Chromium increases insulin activity through the formation of glucose tolerance factor (GTF) (Boyd 2013). Chromium also positively affects to glucose, lipids, proteins and nucleic acids metabolism (Amata 2013). In addition, Khan et al (2014) argued that chromium plays a role in supporting health, including antioxidant, anticholesteremic and hypolipidemic supporters, and affects stress and immunity.

Several studies in fish have shown that dietary chromium in the diet increased growth as did the work done by Liu et al (2010), Ahmed et al (2012), Giri et al (2014), and Abeer et al (2015). It was also added that dietary of chromium picolinate (CrPic) 1 mg kg^{-1} and chromium yeast (CrYst) 2 mg kg^{-1} had increased specific growth rate, feed efficiency ratio, liver glycogen and insulin sensitivity significantly in red tilapia (Rakhmawati et al 2018a). Furthermore, the dietary CrPic and CrYst improved protection of red tilapia by decreasing the negative effect of stress with reducing cortisol concentration and blood glucose during transport, and increasing superoxide dismustase enzyme concentration after 30 days rearing (Rakhmawati et al 2018b). Until recently, data showing the effect of dietary chromium on tilapia immunity is still limited. Thus, this study was conducted to evaluate the effect of dietary chromium to improve the immune response to *S. agalactiae* infection in red tilapia.

Material and Method

***Experimental diets**.* Ingredients and proximate composition of the experimental diets refer to Rakhmawati et al (2018a). Three isonitrogenous and isocaloric (approximately 29% and 3900 kcal kg^{-1}) experimental diets were prepared. Two types of organic chromium used were chromium picolinate (CrPic) and chromium yeast (CrYst). These diets were chromium picolinat (CrPic) 1 mg kg^{-1}, chromium yeast (CrYst) 2 mg kg^{-1} feed supplementation, and without chromium supplementation, with three replications each. Experimental feed was given to the experimental fish for 50 days. The diet without chromium supplementation (control) was divided into two during rearing, namely positive control and negative control. Positive control (PC) is the treatment of fish that is injected *S. agalactiae* bacterial infection while negative control (NC) is the treatment without bacterial infection.

***Experimental animals and rearing activity**.* In all, 320 male monosex red tilapia fingerlings with uniform size (10.48±0.16 g) were selected from 1000 male monosex fingerlings that were reared for one week and fed commercial feed (approximately protein 32%) to acclimate to experimental conditions. Red tilapia fingerlings were obtained from Aquaculture Department, Bogor Agricultural University. Futhermore, the fingerlings were selected and then randomly distributed into 16 rectangular aquaria (35 x 45 x 90 cm^3) with density of 150 fish m^{-3}. Satiation feeding was done three times a day (at 08.00, 12.00 and 16.00).

The experimental fish were reared for 50 days overall. Continuos aeration was provided along with 25% replacement of water every day and fish feaces were siphoned out of the aquaria on a daily basis too. Temperature and DO were measured using DO meter (HANNA Instrument), whereas pH and TAN (Total Ammonioc Nitrogen) were determined following APHA (2005). These water quality parameters were mesuared at the beginning and end of rearing.

***Challenge test of red tilapia with S. agalactiae**.* This challenge test was conducted to assess immune responses of red tilapia against bacterial infection. The bacterial isolate used was *S. agalactiae* that was obtained from the Research Institute for Freshwater Aquaculture, Bogor, Indonesia. After 40 days of feeding trial, the experimental animals were challenged by *S. agalactiae* via intraperitoneal route injection at a dose of 0.1 mL (10^7 CFU mL^{-1}) except for negative control which was injected with phosphat buffer salin (PBS) for 10 days. Mortality were monitored every day for 10 days and dead fish were removed from each aquarium. At the end of the challange trial, mean percent survival rate of each treatment was determined.

***Evaluation of immune responses**.* Immune response parameters observed included leukocyte and erythrocytes count (Blaxhall & Daisley 1973), hemoglobin (Wedemeyer & Yasutake 1977), hematocrit and phagocytic index (Anderson & Siwicki 1995), respiratory burst (Kumar et al 2013) and lysozyme activity (Hanif et al 2004). Blood was taken from a fish in one replication (three per treatment). The observations were performed on days 0, 40, 43, 46 and 50.

***Statistical analysis**.* All data are presented as mean±standard deviations of three replications for each treatment. Data were analyzed by one-way ANOVA and posthoc Duncan test using the software IBM SPSS Statistic 22. Differences were considered significant at $p < 0.05$.

Results

***Survival of red tilapia after challenge test with S. agalactiae**.* Survival rate of red tilapia fed experimental diets during the first 40 days was no significantly different, all treated fish had a survival rate of 100%. Negative control was the normal standard for other treatments. In this treatment the survival rate is 100%. While after the challenge test with *S. agalactiae*, survival ranged from 46 to 63%. Fish fed with CrPic 1 mg kg^{-1} supplemented feed had significantly higher survival rate than fish fed with CrYst 2 mg kg^{-1} and fish without chromium supplementation (Figure 1).

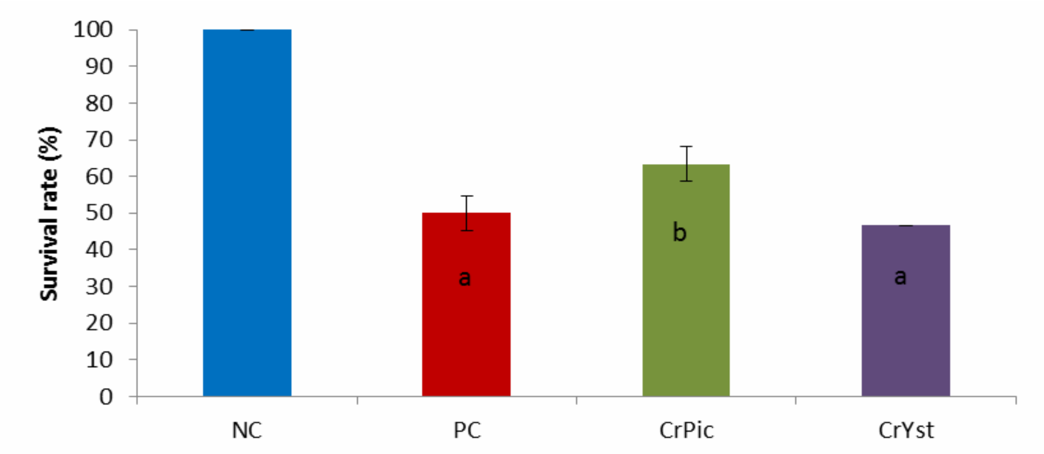

Figure 1. Survival of red tilapia after challenge test with *S. agalactiae*, fish fed experimental diets included feed without chromium supplementation (negative and positive control: NC and PC), and feed with chromium picolinate (CrPic) and chromium yeast (CrYst) supplementation.

Immune responses of red tilapia after challenge test with S. agalactiae

Leukocytes count (LC). Figure 2 shows leucocytes count of red tilapia at the beginning of feeding trial (D0), on day 40 (D40) of feeding trial and middle of the challenge test. The LC of red tilapia on D0 was not significantly different in all treatments. However, on day 40 of rearing, then 43, 46 and 50 (3, 6 and 10 days after the challenge test), there were significant differences between treatments. On day 40, the highest LC was obtained by fish fed with CrPic supplementation and lowest in fish fed with CrYst supplementation. While on days 43, 46 and 50 the LC in the treatment of CrPic supplementation feed remains the highest compared with other treatments.

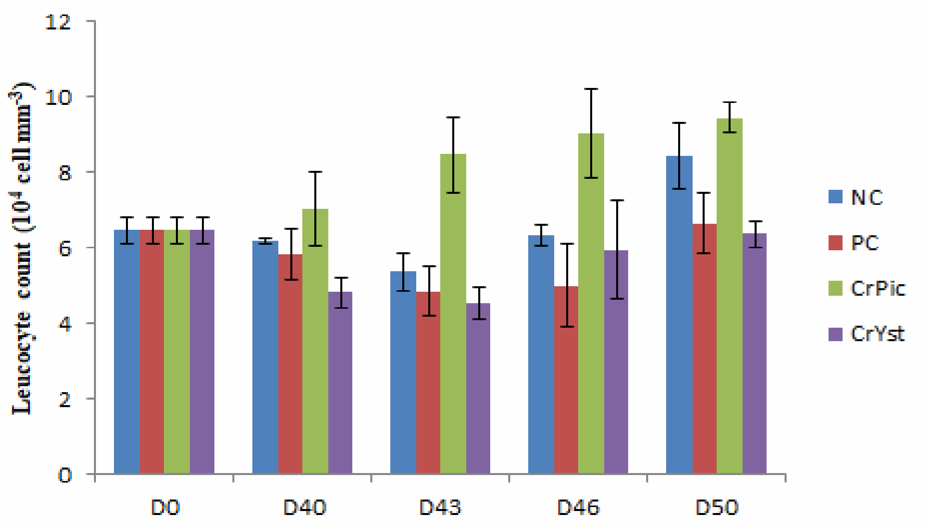

Figure 2. Leucocyte count of red tilapia before feeding trial (D0), on 40 days feeding trial (D40), 3 days (D43), 6 days (D46) and 10 days (D50) after the challenge test with *S.agalactiae* given feed without chromium supplementation (negative and positive control: NC and PC), and feed with chromium picolinate (CrPic) and chromium yeast (CrYst) supplementation.

Erythrocytes count, hemoglobin and hematocrit. The NC has generally higher immune response parameter values compared to other treatments because NC is not given the injection of *S. agalactiae* bacteria. NC is used as a standard value of the immune response parameter. Figure 3 shows erythrocytes count (EC), hemoglobin (Hb) and hematocrit (Ht) of red tilapia at the beginning of feeding trial, (D0), on day 40 (D40), and days 43, 46, 50 (3, 6 and 10 days after the challenge test).

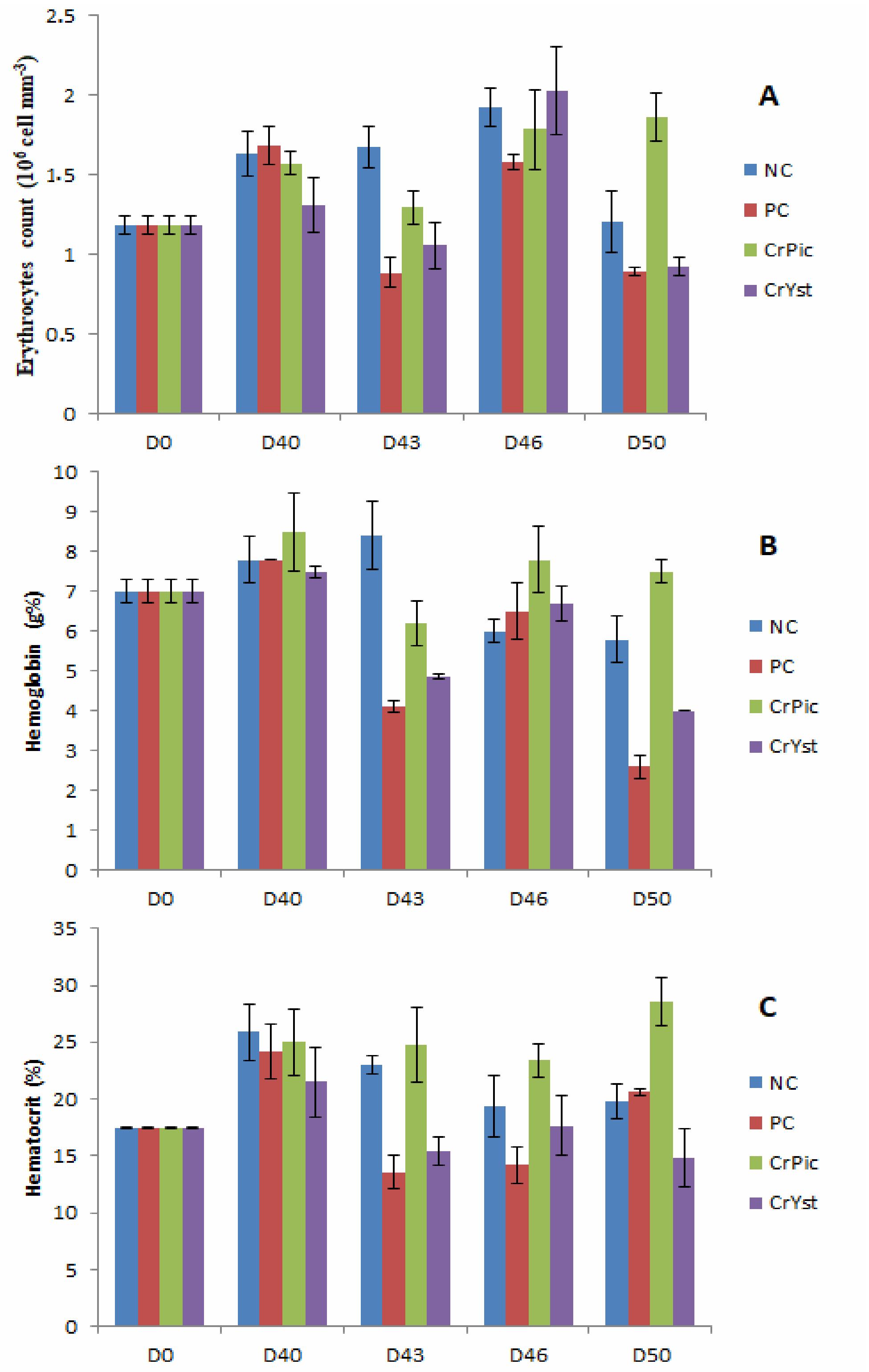

Figure 3. Erythrocytes count (A), hemoglobin (B) and hematocrit (C) of red tilapia before feeding trial (D0), on 40 days feeding trial (D40), 3 days (D43), 6 days (D46) and 10 days (D50) after the challenge test with *S. agalactiae* given feed without chromium supplementation (negative and positive control: NC and PC), and feed with chromium picolinate (CrPic) and chromium yeast (CrYst) supplementation.

The EC on days 0, 40 and 46 were not significantly different in all treatments. However, on days 43 and 50 there were significant differences between treatments. On day 43 the highest EC was obtained in fish fed with CrPic, followed by fish fed with CrYst supplementation, but the positive control obtained was the lowest. On day 50, the EC of fish fed with CrPic supplementation was higher than fish fed with CrYst supplementation or positive control.

Hemoglobin level of red tilapia in days 0 and 40 feeding trial was not significantly different in all treatments. However, on days 43, 46 and 50 there were significant differences between treatments. On day 43 the highest hemoglobin level was obtained in fish feed containing CrPic, followed by fish feed with CrYst and the lowest hemoglobin was found in positive control treatment. On day 46, the *S. agalactiae* infected fish hemoglobin level increased, fish fed with CrPic supplementation was higher than fish fed containing CrYst in the diet or positive control. Furthermore, hemoglobin level on day 50 was decreased in all treatments, but fish fed with CrPic supplementation had the highest level.

Hematocrit level of red tilapia on days 0 and 40 of feeding trial was also not significantly different in all treatments. However, on days 43, 46 and 50 there were significant differences between treatments. On day 43, Ht decreased in all *S. agalactiae* infection treatments, but fish fed with CrPic supplementation was higher than fish fed supplemented with CrYst and fish fed with no Cr supplementation in the feed. On day 46 it still decreased, but the highest Ht was in fish fed with CrPic supplementation compared to other treatments, followed by fish fed with CrYst supplementation and the lowest Ht was on positive control. While on day 50, Ht in fish fed with CrPic supplementation had the highest increase, while fish fed with CrYst supplementation had decreased.

Phagocytic index, respiratory burst and lysozyme activity. Figure 4 shows phagocytic index, respiratory burst and lysozyme activity of red tilapia on days 0 and 40 feeding trial and after the challenge test. The phagocytic index of red tilapia on days 0 and 40 was not significantly different in all treatments. However, on days 43, 46 and 50 there were significant differences between treatments. On day 43, the phagocytic index of fish fed with CrPic supplementation resulted in the highest increase compared to other treatments. On day 46, fish fed CrPic supplements still increased, as did fish with no dietary chromium supplementation, but fish fed with CrYst supplements decreased. While on day 50, the phagocytic index of fish fed with CrPic supplements obtained the highest increase, followed by fish with CrYst supplementary feed, while in the control treatment decreased.

Respiratory burst of red tilapia before feeding trial was not significantly different in all treatments. However, on days 40, 43, 46 and 50 obtained significant differences between treatments. On day 40, respiratory burst of fish fed supplemented CrPic and CrYst was higher than control. But on day 43, respiratory burst in fish fed with CrPic supplements decreased, whereas fish with CrYst supplements increased. Respiratory burst activity on 46 days overall increased and did not differ significantly in all treatments. While on day 50, respiratory burst activity in fish fed with CrPic supplements increased, while fish with CrYst supplements decreased.

Lysozyme activity of red tilapia on days 0, 40 feeding trial and after the challenge test, was not significantly different in all treatments. However, on days 43, 46 and 50 there were significant differences between treatments. On day 43, the activity of lysozyme fish infected by *S. agalactiae* was increased, the highest lysozyme activity was in fish fed with negative control but among the treatments in the challenge test, CrPic supplementation was higher and the lowest was in fish fed with CrYst supplementation. On day 46, lysozyme activity in fish with CrPic supplemented feed had the highest increase compared to other treatments. While on day 50, the activity of lysozyme in fish fed with CrPic supplementation decreased compared to day 46, but still higher than control, while fish fed with CrYst supplementation increased.

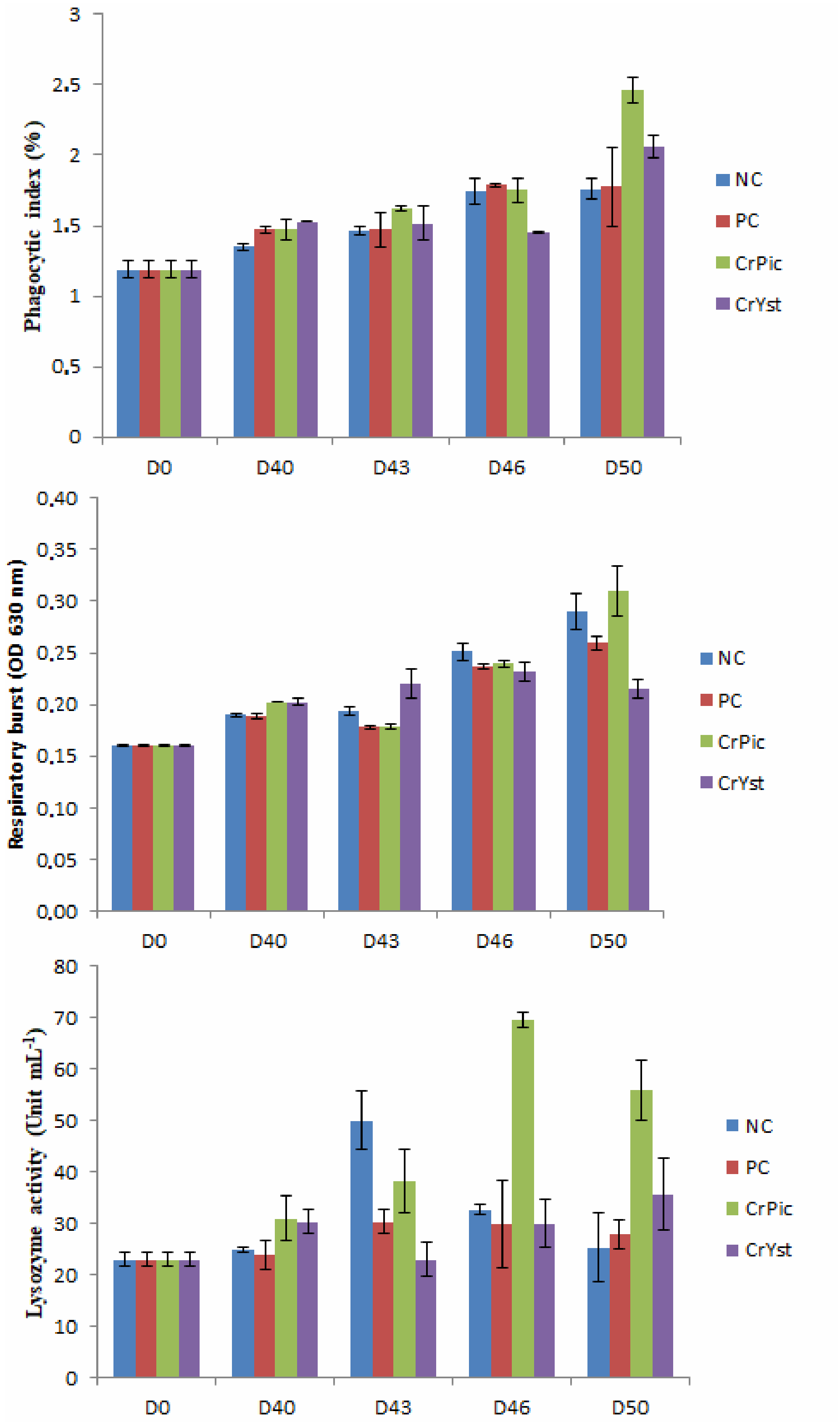

Figure 4. Phagocytic index (A), respiratory burst (B) and lysozyme activity (C) of red tilapia before feeding trial (D0), on 40 days feeding trial (D40), 3 days (D43), 6 days (D46) and 10 days (D50) after the challenge test with *S. agalactiae* given feed without chromium supplementation (negative and positive control: NC and PC), and feed with chromium picolinate (CrPic) and chromium yeast (CrYst) supplementation.

Discussion. The water quality parameters were in normal range during experimental period (temperature 27-28^{o}C, dissolved oxygen 5.8-7.6 mg L^{-1}, pH 6.4-7.4 and TAN 0.097-0.332 mg L^{-1}). This study showed that survival rate of red tilapia fed with CrPic and CrYst supplementation was significantly different after being tested against *S. agalactiae*. The fish fed supplemented CrPic 1 mg kg^{-1} obtained higher survival rate (63%) compared with fish fed with CrYst supplementation 2 mg kg^{-1} and without chromium supplementation (Figure 1). These results suggested that red tilapia also required dietary chromium at certain type and concentration to improve the survival of the fish which were infected by the bacteria. In this study, CrPic supplementation of 1 mg kg^{-1} in the diet increased fish survival when infected *S. agalactiae*, whereas in juvenile large yellow croaker (*Larimichthys crocea*) when infected with *C. irritans* parasite it was required CrNic 5 mg kg^{-1} supplementation (Wang et al 2014).

The high survival of fish fed with CrPic supplementation in the diet is supported by increased immune responses of fish bodies. In general, immune system performance (leukocyte count, erythrocytes count, hemoglobin, hematocrit, phagocytic index, respiratory burst and lisozyme activity) of fish fed with CrPic supplementation was higher than other infected fish. The leukocytes count in CrPic supplementation treatment increased after the challenge test (Figure 2), an increased leukocytes count during the acute phase of disease infection is a leading defense response in the face of pathogen attack (Hardi et al 2017).

Increases in erythrocytes count, hematocrit and hemoglobin levels were obtained after the challenge test for treatment with CrPic supplementation in the diet (Figure 3). These increases indicated that health status and recovery efforts in fish fed with CrPic supplementation after infection with *S. agalactiae* was higher than other treatments. Erythrocytes play an important role in the transport of oxygen. This carrying ability is due to erythrocytes cells containing hemoglobin. Hemoglobin has a function to bind oxygen to be used in the process of catabolism that produces energy. Hemoglobin is composed of proteins (globin) and pigments containing iron (hemme) (Kraemer et al 2011). Thus, the greater the hemoglobin level in the blood, the greater the ability to carry oxygen. On the other hand, the hematocrit is the ratio of solids to fluid in the blood. Hematocrit and blood flow velocity are the two most important variables associated with oxygen transport in the circulation (Lücker et al 2017).

Phagocytic index, respiratory burst and lysozyme activity in fish fed with CrPic supplementation were also higher than other treatments after the challenge test. This increase was in line with an increase of phagocytosis process to fight antigen (pathogenic bacteria) that enter into fish body, it suggested a non-specific immune system reaction obtained from CrPic supplementation in the diet. Fish have an immune system to anticipate infection of microorganisms through populations of B and T cells. These cells play a significant role in both cellular and humoral immune response (Salinas 2015).

Phagocytes in fish are the primary mechanism of non-specific immune response to pathogenic microorganisms. Safratilofa et al (2015) suggested that increased phagocytic processes are indicated by increased phagocytic and respiratory burst activity in catfish which added cinnamon extract in the diet after challenged with *Aeromonas hydrophilla*. In addition, lysozyme activity increased in tilapia fed with brewer's yeast after challenged with *S. agalactiae* (Amin et al 2015). Sahan et al (2015) found that respiratory burst activity increased significantly in the treatment of 5 g kg^{-1} spirulina supplementation in tilapia indicating an increase in non-specific immune responses.

CrPic supplementation 1 mg kg^{-1} in diet improved the performance of non-specific immune system of red tilapia on leukocytes count, erythrocytes count, hemoglobin, hematocrit level, phagocytic index, respiratory burst and lysozyme activity. In *Piaractus mesopotamicus*, Cr carboxylic supplementation of 18 and 36 mg kg^{-1} in the diets increased inflammatory response as a defense against infectious agents during acute inflammation (De Castro et al 2014). In livestock, Sirirat et al (2012) founded that 3 mg kg^{-1} nanoCrPic supplementation increased the retention of Zn, Fe, Ca and increased lymphocytes in broiler chickens. Bhagat et al (2008) reported that CrPic supplementation through the diet or beverages enhanced the immune response by increasing the expression of interferon-gamma regulation (IFN-$_{\gamma}$) after being vaccinated against new castle disease in broiler chickens. It was also added that CrPic 1 and 1.5 mg kg^{-1} supplementation in diet significantly increased antibody titre to new castle disease in broiler chickens (Toghyani et al 2007).

Supplementation with CrYst 2 mg kg^{-1} in red tilapia feed in this study has not given significant effect on immune system performance against *S. agalactiae* infection. However, the fish had also made efforts to maintain their immunity. In this treatment, hematocrit and hemoglobin level on days 3 and 6 after challenged were higher than the positive controls as same as a phagocytic index on day 10 after the challenge test. It may be the concentration that fish required to fight the infection was still not optimum. Gatta et al (2001) suggested that the rainbow trout (*Oncorhynchus mykiss*) immune response may be enhanced by CrYst supplementation at higher concentrations (2.34 and 4.11 mg kg^{-1} of diets) increased lysozyme, phagocytic activity and respiratory burst.

Chromium supplementation in the diet affects the immune system directly or indirectly as it affects carbohydrate metabolism (Gultepe et al 2017). Lee et al (2000) found that CrCl3 and CrPic supplementation at certain doses had an influence on intracellular glucose intake, O_2 production, glucose-6-phosphate dehydrogenase production and phagocytosin production of *Escherichia coli* macrophages. Other work said that chromium modulates the immune response through its effect on cytokine release (Rautenschlein et al 1999). Jain & Kannan (2001) added that chromium supplementation inhibits TNF-ɑ exposure, which has a chemostatic effect on macrophages, but produces insulin resistance in normal cells. In addition, Chang & Mowat (1992) suggested that chromium enhances synthesis of immune proteins by affecting enzyme activity or metabolism of Cu and Zn. Also Sahin & Sahin (2002) said that supplementation of CrPic 400 μg kg^{-1} in the diet increased mineral retention and decreased excretion of Ca, P, Cr, N, Zn and Fe in laying hens. More over, supplementation CrPic in the diet improved mineral retention ratio of Zn, Fe, Mn, Ca, and P in broiler chicken (Sirirat et al 2012).

Conclusions. Dietary CrPic 1 mg kg^{-1} improved immune response in red tilapia against *S. agalactiae* infection. The survival rate of fish infected by *S. agalactiae* in fish given dietary CrPic 1 mg kg^{-1} was higher compared to other treatments, also immune response parameters (leukocytes, erythrocytes, hemoglobin, hematocrit, phagocytic index, respiratory burst and lysozyme activity) were higher compared to other treatments. Further research is needed to evaluate the immunity and productivity improvement of economically important fish on a field scale with more complex factors.

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