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Administration of Microencapsulated Probiotic at Different Doses to Control Streptococcosis in Tilapia (*Oreochromis niloticus*)

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This study aimed to produce microencapsulated probiotic and determine its optimal dose to control streptococcosis in tilapia (*Oreochromis niloticus*). The probiotic used in this study was *Bacillus* sp. NP5 Rf^R that was encapsulated by sterile 10% maltodextrin solution and dried by spray dryer. The experimental fish were reared 28 d and fed by the administration of microencapsulated probiotic in feed with different doses (0.5% (A), 1% (B), and 2% (C)) which were 10^{10} CFU g^{-1} as the concentration and control without administration of microencapsulated probiotic, including negative (K-) and positive (K+) control. On day 30, all of the fish except K- were challenged by injecting 0.1 ml $fish^{-1}$ of *Streptococcus agalactiae* (10^5 CFU ml^{-1}) by intra-peritoneal (IP) route. This study showed that administration of 0.5% microencapsulated probiotic was effective to control streptococcosis in tilapia with higher post-challenge survival rate, better hematological parameter values, and could inhibit *S. agalactiae* growth in the host target organs.

Key words: probiotic, microencapsulated, streptococcosis, tilapia

Penelitian ini bertujuan untuk menghasilkan mikrokapsul probiotik dan menentukan dosis terbaik untuk pengendalian streptococcosis pada ikan nila (*Oreochromis niloticus*). Probiotik yang digunakan pada penelitian ini adalah *Bacillus* sp. NP5 Rf^R yang disalut dengan larutan maltodekstrin 10% steril dan dikeringkan dengan *spray dryer*. Ikan uji dipelihara selama 28 hari dan diberi pakan dengan pemberian mikrokapsul probiotik pada pakan dengan dosis yang berbeda 0,5% (A), 1% (B), dan 2% (C) dengan kepadatan 10^{10} CFU g^{-1} dan kontrol tanpa pemberian mikrokapsul probiotik meliputi kontrol negatif (K-) dan positif (K+). Pada hari ke-30, semua ikan kecuali K- diuji tantang dengan *Streptococcus agalactiae* melalui injeksi 0.1 ml $ekor^{-1}$ (10^5 CFU ml^{-1}) secara intra-peritoneal (IP). Penelitian ini menunjukkan bahwa pemberian 0.5% mikrokapsul probiotik efektif untuk mengendalikan streptococcosis pada ikan nila dengan tingkat kelangsungan hidup yang lebih tinggi setelah uji tantang, nilai parameter gambaran darah yang lebih baik, dan dapat menghambat pertumbuhan *S. agalactiae* pada organ target inang.

Kata kunci : ikan nila, mikrokapsul, probiotik, streptococcosis

Tilapia (*Oreochromis niloticus*) is a commodity that is widely cultivated in the world. Developing countries, especially Asia such as China, Indonesia, Philippines, and Thailand is a major producer of tilapia (Josupeit 2005). In 2012, the production of tilapia by the aquaculture sector in Asia increased to 3.3 million tonnes (FAO 2014).

Intensive culture is conducted to fulfill high demand of tilapia. Otherwise, intensive culture has some risks include disease emergencies. One of the diseases that attack of tilapia is streptococcosis caused by *Streptococcus agalactiae*. In experimental infection, *S. agalactiae* could cause mortality up to 90% at 6 days after injection (Evans *et al*. 2006b).

Some antibiotics such as erythromycin, doxycycline, kitasamycin, and lincomycin are effective to control streptococcosis. Currently, fish disease control through the use of antibiotics has been prohibited, because it requires a long time for antibiotic residues withdrawal from fish body (Iregui *et al*. 2014). The presence of antibiotics in water and sediment can affect normal flora, plankton, and animals, which can induce changes in microbiota diversity and ecological balance (Cabello 2006). The use of antibiotics can initiate bacteria resistance. Some strains of *Streptococcus* have been known able to develop resistance properties to an antibiotic (Darwish and Hobbs 2005). Furthermore, its resistance properties will lead to the ineffectiveness of antibiotics to control the disease, although it is given in high concentration (Yanong 2013).

Probiotics are live or dead microbes or microbial components that give some benefits to the host (Lazado and Caipang 2014). Based on study by Tanbiyaskur

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(2011), oral application of 1% fresh culture *Bacillus* sp. NP5 in tilapia that challenged by *S. agalactiae* had higher survival rate (80.56%) than positive control (13.89%). *Bacillus* sp. NP5 is a probiotic that was isolated from tilapia's digestive tract (Putra 2010).

The main aspects that must be considered in probiotic preparation are the viability of probiotic during preparation and storage, whereas those are the weakness of the use of fresh culture probiotic (Wang *et al*. 2008). Therefore, it needs a method that can protect and maintain cell viability in a long time. Microencapsulation has been applied in many food products to extend the storage time of probiotic and its cell viability in the digestive tract of the host through the protection of probiotic cells in microcapsule beads (Krasaekoopt *et al*. 2003). Based on the previous study, administration of microencapsulated *Bacillus* showed better growth and survival rate than control in white shrimp larvae (Nimrat *et al*. 2011). One of the factors that affect probiotic performance is dose (Nayak 2010). In addition, application of microencapsulated probiotic in aquaculture has not been studied widely, especially for streptococcosis control in tilapia. The fresh cultre dose applicated for tilapia still using 1%, so the aims of this study were to produce microencapsulated probiotic and determine the best dose to control streptococcosis in tilapia.

MATERIALS AND METHODS

Preparation of Microencapsulated Probiotic. Probiotic used in this study was *Bacillus* sp. NP5. The probiotic was given antibiotic rifampicin resistant marker (*Bacillus* sp. NP5 Rf^R). Probiotic cells were cultured in LBB (Luria Bertani Broth) and incubated in water bath shaker (140 rpm; 29 °C) for 18 h. Fresh culture was harvested by centrifugation (6000 - 7000 rpm) for 20 min to get probiotic biomass and encapsulated by sterile 10% maltodextrin solution. The mixture was homogenized, and then dried in BUCHI mini spray dryer at inlet temperature of 120 °C and an outlet temperature of 70 °C (Utami *et al.* 2015). The viability of product after drying was monitored to ensure probiotic count within the product. Viability test was carried out by the spread plate technique using LBA (Luria Bertani Agar) that was supplemented with 50 mg mL^{-1} rifampicin (LBA + Rf).

Preparation of Pathogen for the Challenge Test. Pathogen used for the challenge test was *S. agalactiae* NK1 that was obtained from Instalation of Research and Fish Diseases Control Development (IRFDCD) Depok, West Java, Indonesia. This pathogen is non-hemolytic bacterium that has characteristics include thin growth on solid medium, white to yellowish, slimy liquid, and hard to be taken (Hardi 2011). Bacteria stock was cultured in BHIA (Brain Heart Infusion Agar). Bacteria cells were cultured in 5 mL BHIB (Brain Heart Infusion Broth), then incubated in waterbath shaker at 29 °C with a speed of 140 rpm for 24 h, so that would be obtained *S. agalactiae* inoculant with concentration of 10^7 CFU mL^{-1}. Serial dilution was conducted to get the inoculant concentration of 10^5 CFU mL^{-1} which is the LD50 of *S. agalactiae* NK1 infection in tilapia (Aryanto 2011) and would be used in the challenge test.

Experimental Design. The fish strain used in this study was tilapia nirwana strain that was obtained from Center of Fish Breeding Research (CFBR) Sukamandi, West Java, Indonesia. The 6.38±0.05 g fish were reared in aquarium sized 60x30x30 cm^3 at a density of 10 fish per aquarium.

This study was conducted in CRD (completely randomized design) consist of 5 treatments with 5 replications, with administration of microencapsulated probiotic in diet with different doses (0.5% (A), 1% (B), and 2% (C)) which were 10^{10} CFU g^{-1} as the concentration and control without administration of microencapsulated probiotic (Utami *et al*. 2015), including negative (K-) and positive (K+) control. The test feed was commercial pellet (Hi-Provite 781-1) with 30.18% protein, 5.25% fat, 52.53% carbohydrate, 9.12% ash, and 2.92% crude fiber contents. The microencapsulated probiotic added to the diet and mixed with 2% egg white as a binder. The fish were fed three times a day (08:00, 12:00, 16:00) by at satiation for 28 d. On day 30, 8 fish from each treatment except K- challenged by injecting 0.1 mL per fish of *S. agalactiae* (10^5 CFU mL^{-1}) using a sterile syringe by intra-peritoneal (IP) route. Furthermore, fish were reared for 10 d. Replacement of water in the rearing tanks as much as 80% of total volume were conducted every 4 d to maintain water quality, whereas the water quality was maintained in the normal range for freshwater culture, according to Boyd (1990) that dissolved oxygen > 5 mg L^{-1}, temperature at 24-30 °C, pH of 6.5 - 9.5, and total ammonia nitrogen (TAN) < 0.52 ppm.

Survival Rate Observation. Survival Rate (SR) was calculated after the challenge test with the following equation:

$SR (\%) = [(Nt/N0) \times 100\%]$

Where: Nt is the number of fish that live at the end of

the study (individual), N0 is the number of fish at the beginning of the study (individual).

Measurement of Hematological Parameters. Blood samplings were done on day 0, 4, 8, and 10 of the challenge test. The fish were anaesthetized using MS222 and then 0.2 ml blood of the fish was drawn using a sterile syringe which previously had been rinsed by anticoagulant (Na citrate). Blood samples were used for measurement of hematocrit (Ht), hemoglobin (Hb), erythrocyte count (EC), leukocyte count (LC), and phagocytic activity (PA). Hematocrit was measured by taking blood sample using microhematocrit tube and centrifuged at the speed of 5000 rpm for 5 min. Hematocrit was determined by comparing the length of blood cells and the length of total blood volume in microhematocrit tube. Hemoglobin was measured by Sahli method using sahlinometer (Wedemeyer and Yasutake 1977), EC and LC following the procedure by Blaxhall and Daisley (1973). Phagocytic activity was assessed by making blood smear slide from the blood sample that was mixed with the *Staphylococcus aureus* suspension (10^7 CFU mL^{-1}) and incubated for 20 min. The slide was dried, fixed with methanol for 5 min and dried again, then stained by immersion in giemsa for 20 min. The slide was observed using a microscope with 400x magnification to determine PA based on percentage of 100 phagocyte cells which showed phagocytosis.

Enumeration of Total *S. agalactiae* in Target Organs. The enumeration was carried out by the spread plate technique using BHIA with target organ samples of streptococcosis (liver, kidneys, eyes, and brain). Colonies of *S. agalactiae* were distinguished with other bacteria based on the characteristics which are spherical, small with a thin growth on solid medium, white to yellowish and eradiate bluish color when exposed to light. Enumerations of total *S. agalactiae* in target organs were done on day 0, 7, and 10 of the challenge test to know the infection peak and recovery period in week 1 and week 2.

Statistical Analysis. All data were tabulated using Microsoft Excel 2007 and analyzed using one way-ANOVA, then continued by Duncan Multiple Range Test with a 0.05 significance using SPSS 20.

RESULTS

The highest survival rate (SR) after the challenge test with *S. agalactiae* was shown by treatment A (75±12.5%) that was not significantly different ($p>0.05$) with K- (87.5±12.5%), but was significantly different ($p<0.05$) with K+, B, and C (Fig 1).

A showed the highest Hb, Ht, and EC before the challenge test (Fig 2a, b, c.), where Hb was (7.20±0.20 g%) followed by B (6.47±0.31 g%), K- (6.00±0.80 g%), C (5.80±0.20 g%), and K+ (5.47±0.31 g%). A also showed the highest Ht (22.90±0.10%) that was

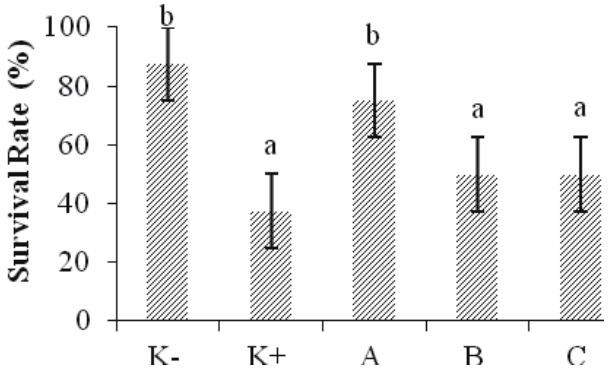

Fig 1 Survival rate of tilapia after the challenge test. Different letters on each bar (mean±SD) indicated significant differences (Duncan; $p<0.05$). Negative (K-); positive (K+) control; administration of microencapsulated probiotic in feed at a dose of 0.5% (A); 1% (B); 2% (C).

significantly different ($p<0.05$) with K-, K+, and C, but was not significantly different ($p>0.05$) with B. This was also reflected in EC, where A showed the highest of EC before the challenge test ($2.75\pm0.08\times10^6$ cells mm^{-3}) that was significantly different ($p<0.05$) with K-, K+, and C, but was not significantly different ($p>0.05$) with B.

A also showed the highest of Hb after challenge test, whereas Hb of A after challenge test on day 4, 8, and 10 were, 5.33±0.42; 5.40±0.20; 6.60±0.20 g%, respectively. Ht levels also declined and the peak occurred on day 8, where Ht was in the range of 10.83±1.47-18.13±1.90%. A also showed the highest of Ht on post-challenge test that was not significantly different ($p>0.05$) with K-, but significantly different ($p<0.05$) with K+. This is also reflected in EC, where EC declined on day 4 of the challenge test, then increased again on day 10, where the highest EC of the fish that were challenged by *S. agalactiae* was shown by A was, 1.26±0.10; 1.41±0.08; $1.55\pm0.04\times10^6$ cells mm^{-3}, respectively.

Otherwise, the administration of microencapsulated probiotic was not significantly different ($p>0.05$) in tilapia's LC and PA before the challenge test (Fig 2d, e.). A showed the increasing of LC and the peak occurred on day 8 ($0.95\pm0.06\times10^5$ cells mm^{-3}) that

was significantly different ($p<0.05$) with K+, B, and C, and then decreased on day 10. PA increased on day 8 and decreased again on day 10. A showed the highest of PA after challenge test was, 74.74±3.18; 67.14±6.23; 65.56±5.09%, respectively.

The lowest total *S. agalactiae* in almost target organs after the challenge test was shown by A (Fig 3a, b, c, d.). On day 7 and 10, the lowest total *S. agalactiae* in the liver was shown by A (5.80±0.02; 4.90±0.03 log CFU g^{-1}) was significantly different ($p<0.05$) with K+, B, and C. The lowest total *S. agalactiae* in the kidneys on day 7 was shown by A (5.16±0.01 log CFU g^{-1}). The lowest total of *S. agalactiae* in the eyes showed in A (5.83±0.02; 4.79±0.02 log CFU g^{-1}). The highest total of *S. agalactiae* in the brain on day 7 was shown by K+ (6.46±0.01 log CFU g^{-1}) was significantly different ($p<0.05$) with A, B, and C, while the lowest total of *S. agalactiae* in the brain on day 7 was shown by A (5.85±0.02 log CFU g^{-1}). Total *S. agalactiae* in target organs almost decreased on day 10.

DISCUSSION

Administration of probiotic is able to increase the host resistance against pathogen as reported by Brunt and Austin (2005) showed that *A. sobria* GC2 could improve the resistance of rainbow trout to

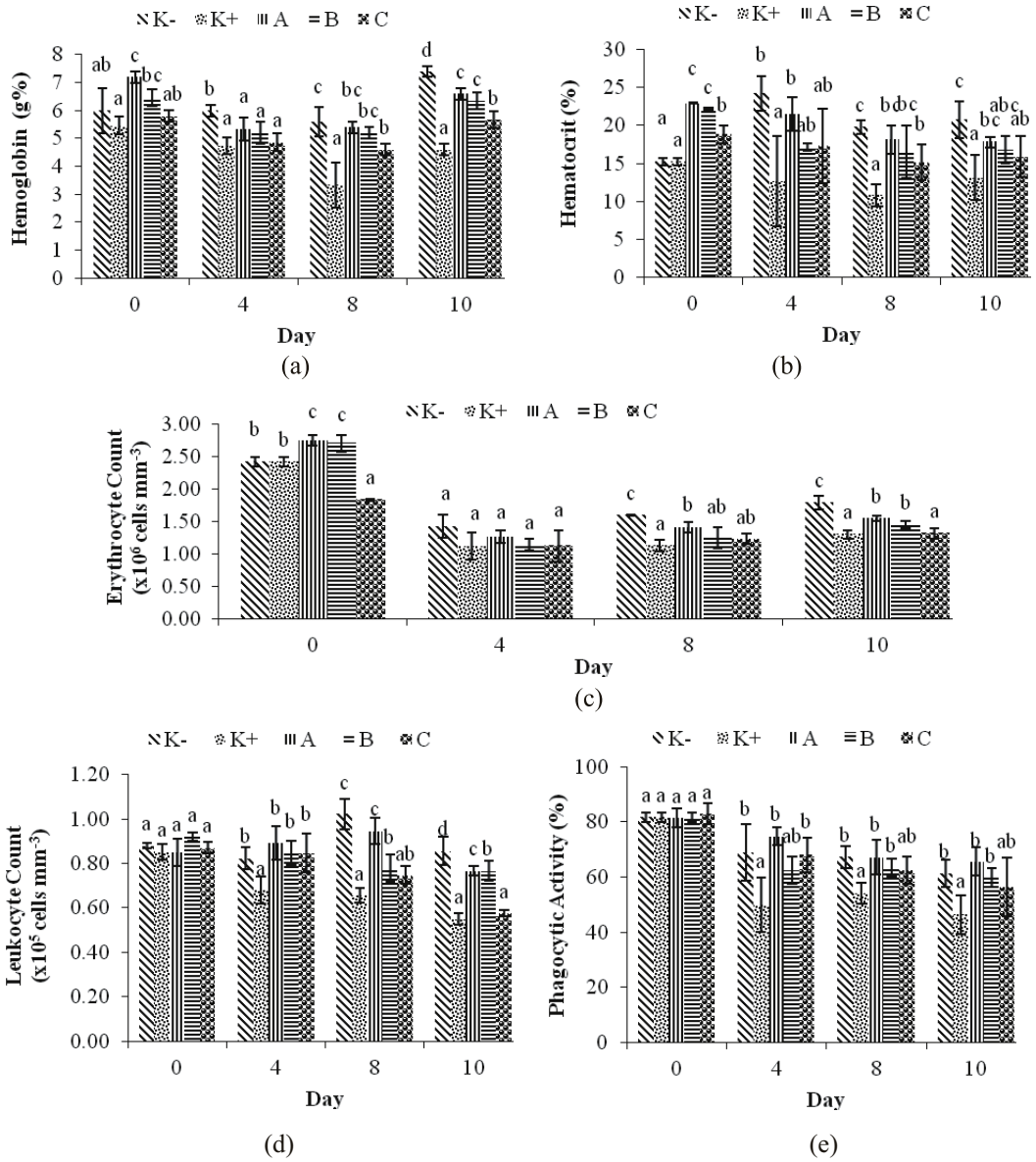

Fig 2 Hemoglobin (a); hematocrit (b); erythrocyte count (c); leukocyte count (d); phagocytic activity (e) of tilapia during the challenge test. Different letters on each bar on the same day (mean±SD) indicated significant differences (Duncan; $p<0.05$). Negative (K-); positive (K+) control; administration of microencapsulated probiotic in feed at a dose of 0.5% (A); 1% (B); 2% (C).

lactococcosis and streptococcosis compared to controls. It was similar to the results of this study, in which administration 0.5% of microencapsulated *Bacillus* sp. NP5 Rf^R improved tilapia resistance against *S. agalactiae*. It happened because one of probiotic modes action is to inhibit pathogen infection by enhancing the host immune response (Denev *et al.* 2009) through non-specific and cellular immune stimulation (Fyzul *et al.* 2014). Furthermore, it also happened because the probiotic and its components or products interact with gut associated lymphoid tissue (GALT) to induce the host immune response (Dimitroglou *et al.* 2011).

However, better results did not occur in administration of the higher doses of micro-encapsulated probiotic. Dose is one of the limiting factors to achieve the optimum effect of probiotics on the host immune system, whereas the dose of probiotics used in aquaculture vary from 10^6-10^{10} CFU g^{-1} (Nayak 2010). 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, in which it has been reported that administration of *L. rhamnosus* in high dose (10^{12} CFU g^{-1} feed) in *O. mykiss* showed higher mortality than lower dose (10^9 CFU g^{-1} feed).

In this study, administration of microencapsulated probiotic affected Hb, Ht, and EC before the challenge test. There is a link between Hb, Ht, and EC, where the higher EC, will show the higher Hb and Ht. Blaxhall and Daisley (1973) stated that EC, Hb, and Ht are the parameters that indicate anemia in fish, where EC in this study was in the normal range, which is 10-30x10^5 cells mm^{-3} (Takashima and Hibiya 1995). The higher Ht in the administration of microencapsulated probiotic treatments indicated that microencapsulated probiotic was safe and could improve fish health status as reported by Aly *et al.* (2008), where the administration of *Bacillus subtilis* and *Lactobacillus acidophilus* could improve Ht in tilapia. A positive result from the administration of probiotic on Hb, Ht, and EC also occurred in *Catla catla* (Hamilton) which were supplemented with *Lactobacillus acidophilus* in diet, and it was related to probiotic ability to improve hematological parameter values as a result of haemopoetic stimulation (Renuka *et al.* 2014).

Fluctuation of Hb, Ht, and EC occurred after the challenge test, where Hb decreased on day 4 and 8 of the challenge test, then increased again on day 10. The decreasing of Hb, Ht, and EC indicated that fish were anemic. Symptoms of anemia in fish can be assessed by packed cell volume (PCV) value or Ht, where Ht is less than 20% is a sign of anemia in teleostei (Clauss *et al.* 2008). *S. agalactiae* is a Gram-positive bacteria that cause septicemia, where the suffering organs due to *S. agalactiae* infection are brain, kidneys, and eyes (Abdullah *et al.* 2013). Anterior portion of the kidney is the main organ of blood production in teleostei (Arnold 2009). Problems in the kidneys will cause blood production disorders which lead to decrease of erythrocyte production, then it will cause fluctuations of Hb and Ht.

LC and PA values were not significantly different between treatments before the challenge test indicated that fish were not stress and exposed to infection. The differences between LC and PA occurred after the challenge test. Leukocytes play a major role in nonspecific immunity during inflammation and the amount can be an indicator of fish health status (Secombes 1996). LC improvement after infection associated with inflammatory response mediated by leukocytes against bacterial infections (Roberts 1978). Based on the results, it could be concluded that the administration of microencapsulated probiotic help to recover the hematological parameter changes due to infection, improved nonspecific immune response and resistance to disease, especially streptococcosis. The higher LC in the administration of microencapsulated probiotic treatments showed that leukocytes were produced in great numbers against *S. agalactiae* infection. Leukocytes are blood cells that play major role in phagocytosis process. Its role is played by monocytes and neutrophils that exhibited by PA. Monocytes and neutrophils are the components that produce superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and hydroxyl radical (OH^-) that have a very high microbiocidal ability (Ellis 2001; Secombes 1996).

The ability of probiotic to inhibit *S. agalactiae* growth in tilapia could be determined by total of *S. agalactiae* in target organs (liver, kidneys, eyes, and brain). On day 0 (before the challenge test), colonies of *S. agalactiae* were not found in target organs, as well as the negative control after the challenge test. It indicated that the fish were free from *S. agalactiae* infection. The highest total of *S. agalactiae* in all target organs on day 7 after the challenge test associated with fish mortality, where in this study mortality begun to occur on day 3 and reached the peak on day 8. It was related to Yanong and

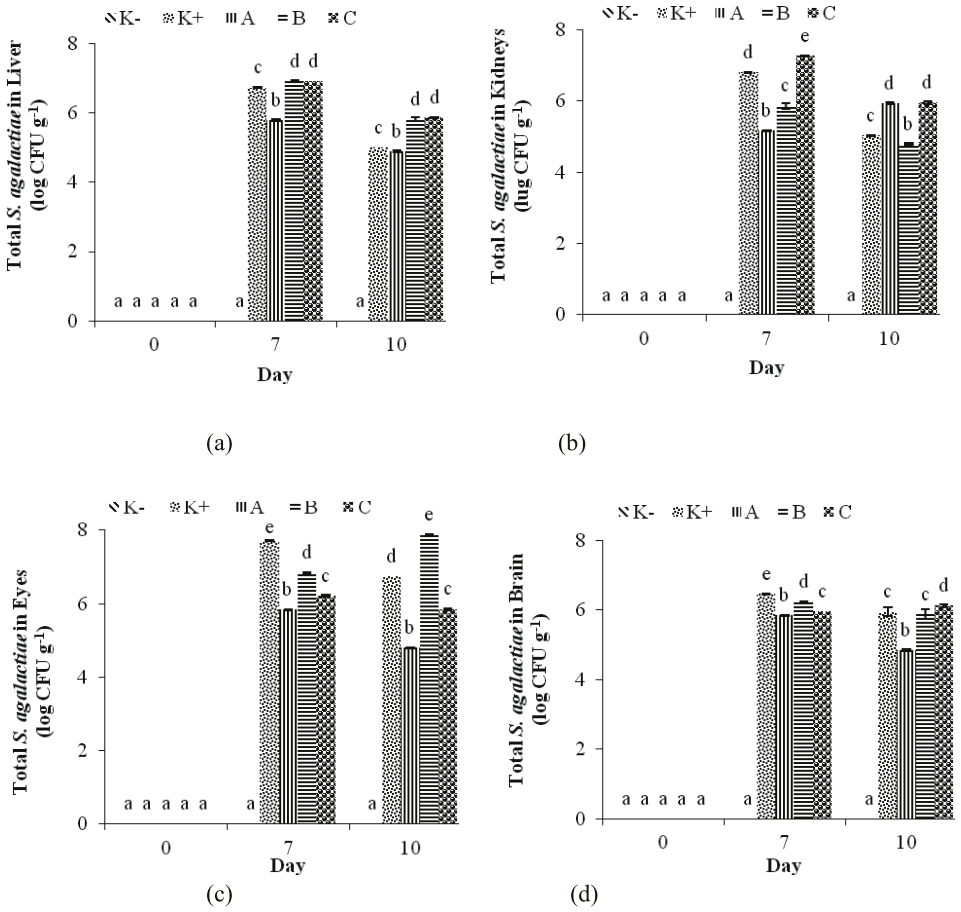

Fig 3 Total *S. agalactiae* in the liver (a); kidneys (b); eyes (c); brain (d) of tilapia during the challenge test. Different letters on each bar on the same day (mean±SD) indicated significant differences (Duncan; $p<0.05$). Negative (K-); positive (K+) control; administration of microencapsulated probiotic in feed at a dose of 0.5% (A); 1% (B); 2% (C).

Floyd (2002) statement that *Streptococcus* infection in fish can cause high mortality (> 50%) on day 3 to day 7 post-infection. The presence of *S. agalactiae* in the eyes caused some clinical signs such as purulens, opacity, and exopthalmia, while the presence of *S. agalactiae* in the brain caused abnormal swimming like gasping, tilted swimming even whirling (Hardi *et al.* 2011). These changes can lead to death, especially changes that show damage in the structure and function of organs which play a role in physiological processes in fish body, such as the liver, kidneys, and brain, while the symptoms that exhibited in the eyes are symptoms that occur in chronic phase (Evans *et al.* 2006a). Furthermore, the mortality was found in the fish that had shown abnormal swimming and drastic reduction in hematological parameters, while the experimental fish that showed symptoms in the eyes still looked active. Total of *S. agalactiae* in target organs were lower in the administration of microencapsulated probiotic treatments than K+ and total of *S. agalactiae* in target organs decreased on day 10 associated with phagocytic activity and incubation period of *S. agalactiae* in fish body, which is that time the body did homeostasis activity as indicated by Hb, Ht, and EC improvement, LC and PA reduction. At that moment, the infection rate also reduced.

In conclusion, administration of 0.5% micro-encapsulated probiotic was effective to control streptococcosis in tilapia after the challenge test with higher survival rate, better hematological parameter values, and could inhibit *S. agalactiae* growth in the host target organs.

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