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The Evaluation of the Addition of Commercial Yeast with β -Glucan Content in Feed on the Immunity of Snakehead Fish *Channa striata* Infected by *Aeromonas hydrophila* Bacteria

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Abstract

Snakehead fish *Channa striata* is a high commercial freshwater fish commodity. It has the potential as a pharmaceutical ingredient. Intensive snakehead fish cultivation starts to experience a problem, namely Motile Aeromonas Septicemia (MAS) caused by *Aeromonas hydrophila*. The purpose of this study is to evaluate the effect of the addition of commercial yeast in feed to improve snakehead fish immunity. This study was designed with a completely randomized design (CRD) with 5 treatments with 3 replications (reared in the net) and 6 treatments with 3 replications (reared in the aquarium). The treatments were K (feed without yeast), F5 (feed with the addition of 5 g/kg of cake yeast), R3 (feed with the addition of 3 g/kg of tempeh yeast, R5(feed with the addition of 5 g/kg of tempeh yeast), and R7 (feed with the addition of 7 g/kg of tempeh yeast). The results show that the survival rate after 30 days is 88.89-92.22%. The best treatment after A. hydrophila infection was found in fish fed with 3 g/kg of tempeh yeast with a survival rate of 56.67%, total erythrocytes of 4.07x 10^6 mm^{-3} cells, hemoglobin of 7.40 g% of total leukocytes 4.97x 10^4 mm^{-3} cells and phagocytic activity of 33.67. In conclusion, the addition of tempeh yeast at a dose of 3 g/kg could be used as an alternative to prevent the effect of *A. hydrophila* pathogen infection in snakehead fish.

INTRODUCTION

Snakehead fish *Channa striata* is a freshwater fish commodity that has economic value due to its high demand. This is because snakehead fish contains albumin which is beneficial for human health (Listyanto and Andriyanto, 2009; Chasanah *et al*., 2015). Albumin is a protein that is useful in the healing of the postoperative wound (Mustafa *et al*., 2012). The high demand for snakehead fish encourages some efforts to increase the production of the snakehead fish cultivation sector every year. FAO statistical data (2018) reported that snakehead fish production increased from 2010 to 2016, accounting for 377 tons, 481 tons, 511 tons, 518 tons. Nevertheless, several obstacles in snakehead fish farming activities begin to occur, one of which is caused by the factor of diseases (Olga, 2012).

One of the common diseases that often infect snakehead fish is Motile Aeromonas septicemia (MAS), which is caused by the *Aeromonas hydrophila* bacteria (KKP BPBAT, 2014). MAS disease is characterized by bleeding in the gills, kidney damage, and dropsy. This disease can cause death in snakehead fish from 23.30% to 100% (Olga, 2012). A series of efforts to overcome MAS have been carried out immunostimulants, one of which is the provision of vitamin C (Dwinanti *et al*., 2019). However, vitamin C has a high price, so it can increase production costs.

One of the immunostimulants that can increase immunity is yeast. Research on the use of cake yeast as an immunostimulant in fish has been widely reported, including the addition of 5 g/kg of cake yeast to the feed which can increase the survival rate after *Aeromonas hydrophila injection* and can reduce the number of bacteria (Abdel-Tawwab *et al*., 2008; Manoppo *et al*., 2015). The addition of 5 g/kg of cake yeast to the feed of rohu fish can increase the immunity (Pal *et al*., 2007), and the addition of yeast as much as 1.5, 10 g/kg to the feed of snapper can increase the immune response (Ortuño *et al*., 2002). The addition of cake yeast to the feed can increase the survival rate of the fish against MAS disease infection, increase hemoglobin, and increase phagocytic activity. Research on the use of 5 g/kg of yeast to feed as an immunostimulant in fish has been widely conducted. However, information regarding the use of tempeh yeast, which is cheaper, easy to obtain, and easy to apply as an immunostimulant in fish is still very limited.

Tempeh yeast is a *Rhizopus oligosporus* culture that contains 8% β-glucan (Purwijantiningsih *et al*., 2005). The β-glucan content which enters the body can induce phagocytic activity and lysozyme activity (Stier *et al*., 2014). This content will activate the lysozyme gene so that the innate body defenses will be stronger against bacterial diseases. The use of tempeh yeast in increasing fish immunity has never been done before. Therefore, there is a need for research on the addition of tempeh yeast to the feed in snakehead fish. This study aims to evaluate and analyze the effectiveness of commercial yeast as an immunostimulant for snakehead fish (*C. striata*).

METHODOLOGY

Place and Time

This research was conducted from January to August 2019 at the Sukamandi Fish Breeding Research Institute (BRPI), Subang West Java, Fish Health Laboratory, Department of Aquaculture, FPIK, IPB, and several analyzes were carried out at the Station for Investigation of Fish Health and Environment (LP2IL), Serang, Banten.

Research Materials

The yeasts used in this research were cake yeast and commercial tempeh yeast. The fish used were snakehead fish measuring 7.37 ± 0.12 cm, coming from BRPI Sukamandi, Subang. The bacterial isolate used was *A. hydrophila* coming from the collection of the Sukamandi BRPI Microbiology Laboratory. The bacteria were cultured on Rhimler Shotts (RS) medium and were incubated for 24 hours at a temperature of 37 ° C. The bacteria were then tested for their virulence and were identified biochemically using KIT API 20 NE to determine the type of isolate.

Research Design

The design used in this study was a completely randomized design (CRD) with five treatments (in a net) and six treatments (in an aquarium) with three replications. Net treatment was done by giving feed without yeast (K), feed with cake yeast of 5 g/kg (F5), feed with tempeh yeast of 3 g/kg (R3), feed with yeast tempeh of 5 g/kg (R5), and feed with yeast tempeh of 7 g/kg (R7). Meanwhile, the aquarium treatment included feed without the addition of yeast and fish injected with *Aeromonas hydrophila* (K+), feed without the

addition of yeast and fish injected with *phosphate buffer saline* (PBS) (K-), feed with cake yeast of 5 g/kg and fish injected with *A. hydrophila* (F5), feed with tempeh yeast of 3 g/kg and fish injected with *A. hydrophila* (R3), feed with tempeh yeast of 5 g/kg and fish injected with *A. hydrophila* (R5), and feed with tempeh yeast of 7 g/kg of tempeh yeast and fish injected with *A. hydrophila* (R7).

Work Procedures

Feed Manufacturing and Testing of β-glucans

The feed used was commercial feed with a protein content of 40%. The feed was floured, and yeast was added according to the treatment, 2% of eggs, and 100 mL of water. The feed was then added with flour (repeletting) and heated in the oven (50 °C) for six hours. Afterward, the feed was cooled and stored at room temperature. Moreover, 90 mg of feed samples were taken for the analysis of β-glucan content (Table 1). Samples were analyzed in LP2IL Serang using the Megazyme K-YBGL Kit (USA) and read using a UV-Visible GBC 911 machine (USA).

Table 1. β -glucan content in pure yeast and yeast mixture in feed.

Sample	Percentage (%)
Cake yeast	36.04
Tempeh yeast	41.89
Feed with yeast tempeh of 3 g Kg-1 (R3)	16.10
Feed with yeast tempeh of 7 g Kg-1 (R7)	18.49
Feed with cake yeast of 5 g Kg-1 (F5)	16.56

Fish Farming

The seeds of snakehead fish were reared in a net measuring 2x2x1 m for 30 days with a density of 30 fish per net. Feed was given twice a day ad satiation. During maintenance, the quality of the water was made to be suitable for the life of snakehead fish, by adding new water of around 10% of the pond volume per day and giving aeration.

Challenge Test on Fish

The challenge test was carried out on snakehead fish seeds that had been treated with different β-glucan doses for 30 days. The fish were put into an aquarium measuring 60x60x40 cm and then acclimatized for seven days. The challenge test was carried out by injecting 0.1 mL of the *A. hydrophila* bacteria culture (106 cfu/mL) intramuscularly. Afterward, the snakehead fish mortality was observed every day for 18 days, and the cause of death of the fish was identified. Moreover, bacteria isolated from dead fish were identified biochemically using KIT API 20 NE.

Research Parameters

Parameters observed included fish growth, survival rate, blood picture, lysozyme activity and the G-type lysozyme gene expression. Specifically, blood picture parameters included total erythrocytes, total leukocytes (Blaxhall and Daisley, 1973), and hemoglobin (Wedemeyer and Yasatuke, 1977). Phagocytic activity (Anderson and Siwicki, 1995) was observed at the pre-challenge test (H-30 and H-1) and post-challenge test (H + 2, H + 4, and H + 6). Lysozyme activity (Hanif *et al*., 2004) and G-type lysozyme gene expression (Kumaresan *et al*., 2015) were observed in the pre-challenge test (H-1) and post-challenge test (H + 2).

Data Analysis

The research data were tabulated using Ms. program. Office Excel 2010. Data analysis was performed using one-way ANOVA through the SPSS program version 20. The results which were significantly different were further tested with the Duncan test at a 95% confidence interval.

RESULTS AND DISCUSSION

Growth Performance

In the cultivation of snakehead fish for 30 days in a net, feeding enriched with yeast has no significant effect (P> 0.05) on the survival rate (TKH), total feed consumption (JKP), and feed conversion ratio (FCR) (Table 2) for 30 days of cultivation. This is because, during fish rearing, the optimal conditions for snakehead fish life were maintained, without any treatment that triggered stress on the fish. The results obtained are in accordance with the results of research by Adloo *et al*. (2015), which states that giving yeast to feed does not have a significant effect on either TKH, JKP, or FCR on catfish that are kept in normal conditions, and there is no trigger of stress on fish. Meanwhile, Misra *et al*. (2006) reported that the addition of feed with yeast containing glucans can increase immunity, growth, and TKH of *Labeo rohita* fish infected with *A. hydrophila* bacteria. The differences in the results obtained in these previous studies are due to differences in the treatments given. According to Pilarski *et al*. (2017) and Whittington *et al*. (2005), the effect of the addition of immunostimulants on TKH, JKP, and RKP is influenced by environmental conditions, types of feed, length of fish maintenance, and types of fish cultivated.

Table 2. Total feed consumption (JKP), specific growth rate (LPS), feed conversion ratio (FCR), and survival rate (TKH) of snakehead fish during 30 days of cultivation.

Parameter	Treatment				
	K	F5	R3	R5	R7
JKP (g)	128.68±1.33^{a}	132.81±2.01^{a}	131.92±1.27^{a}	140.12±1.40^{b}	132.63±1.42^{a}
LPS (g/day)	4.78±0.08^{abc}	4.57±0.17^{ab}	5.08±0.12^{c}	4.51±0.20^{bc}	4.96±0.38^{a}
FCR	0.70±0.06^{a}	0.86±0.11^{a}	0.74±0.16^{a}	0.95±0.01^{a}	0.78±0.20^{a}
TKH (%)	88.33±1.58^{a}	92.22±1.18^{a}	88.89±1.70^{a}	91.11±1.62^{a}	90.00±1.61^{a}

*different notations on the lines indicate significant differences at the 5% test level (Duncan's multiple interval test). Control (K), cake yeast of 5 g/Kg (F5), tempeh yeast of 3 g/Kg (R3), tempeh yeast of 5 g/Kg (R5), and tempeh yeast of 7g/Kg (R7).

Post-Challenge Test Survival Rates

The effect of giving yeast which contains β-glucan on the survival rate of the fish is very significant in the post-challenge test with pathogenic bacteria *A. hydrophila*. The percentage of TKH of post-challenge test with *A. hydrophila* shows a difference (P <0.05) between K+ and K-, F5, R3, while other treatments were not significantly different (P> 0.05) (Figure 1). TKH in the R3 treatment (56.67%) is higher than that in K+ (13.33%) (Figure 1). These results indicate that the administration of β-glucan in the appropriate amount is effective in increasing the immunity of snakehead fish. These results are consistent with the results of research by Kumari and Sahoo (2006) and Domenico *et al*. (2017) which states that giving yeast containing β-glucan increases the immunity of fish after pathogenic bacterial infection, by activating macrophages so that they can fight these bacterial infections. The mechanism of β-glucan in increasing fish immunity is β-glucan which binds to the toll-like receptor (TLR) and Complement receptor 3 (CR 3) will activate macrophages, further increasing the activity of hydrolytic enzymes and signaling processes that lead to the activation of phagocytosis and secretion of cytokines (Vetvicka and Vetvickova, 2016; Kumar *et al*., 2013). However, giving β-glucan in excessive amounts will inhibit the fish's immune mechanism, which can lead to death. The β-glucan levels in the body will stimulate the phagocytic cells of fish that exceed their ability, thereby reducing the activity of these phagocytic cells in killing bacteria (Vechklang *et al*., 2011).

These results indicate that in the application of immunostimulants, the dose

and length of time of administration need to be given attention because prolonged administration with high doses can suppress the immune system (immunosuppression) of fish (Sakai, 1999). Sajeevan *et al*. (2009) further stated that the dose and duration of immunostimulant administration are very important in fish health management.

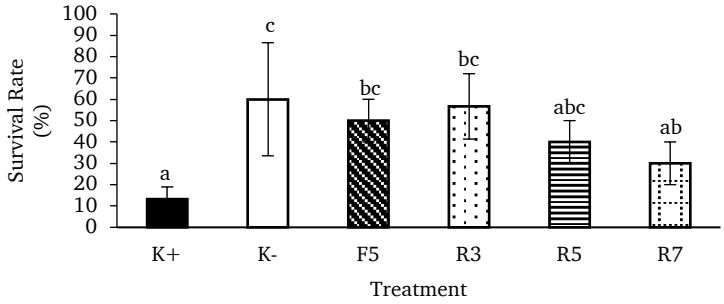

Figure 1. The survival rate of snakehead fish after A. hydrophila bacteria injection at the end of treatment. Different letters in each treatment show significantly different results (P <0.05). Positive control (K+), negative control (K-), cake yeast of 5 g/Kg (F5), tempeh yeast of 3 g/Kg (R3), tempeh yeast of 5 g/Kg (R5), and tempeh yeast of 7g/Kg (R7).

Blood Picture

Immunostimulants affect the parameters of the blood picture, and several blood image parameters show fluctuation values. Total erythrocytes on the second day of post-challenge test (H + 2) decreases and is significantly different (P <0.05) with the K+ treatment. Meanwhile, total erythrocytes in treatment R3 have a higher value compared to those in treatment K+ (Table 3). Snakehead fish fed with yeast content have a higher survival rate after *A. hydrophila* infection because β-glucan stimulates the immune system to work faster. Erythrocytes in the post-challenge test of snakehead fish tend to decrease due to pathogenic bacteria which lysate erythrocytes, so after infection the number decreases. The normal number of erythrocytes in snakehead fish is 2.17-2.47 x 106 cells/mm (Wahyu, 2015), while the number of erythrocytes in snakehead fish at K+ is below 2.0 x 106 cells/mm. The total number of erythrocytes of snakehead fish decreases in the post-challenge test with *A. hydrophila* bacteria. The decrease in the total number of erythrocytes is caused by the infection of *A. hydrophila*, which causes hemolytic activity. Ray *et al*. (2016) further state that *A. hydrophila* produces exotoxin in the form of hemolysin, which is an enzyme that can lysate erythrocytes and free hemoglobin so that the erythrocytes of the infected fish decrease. Moreover, the total number of erythrocytes in K+ treatment is lower than that in K- and other treatments. This shows that the immune system in snakehead fish that is given yeast works faster so that the impact of bacterial infection on erythrocyte lysis is lower.

Hemoglobin is the red pigment that carries oxygen in erythrocytes. Hemoglobin in snakehead fish infected with pathogens decreases, presumably because the amount of oxygen in the fish's body reduces due to the decrease in the number of erythrocytes. However, hemoglobin on the fourth day of the post-challenge test (H + 4) has an increase and is significantly different (P <0.05). As stated by Ray *et al*. (2016), *A. hydrophila* infection causes erythrocytes to experience lysis and hemoglobin levels to fall. The relationship between the total number of erythrocytes and hemoglobin level is stated by Harikrishnan *et al*.

(2003), who states that pathogenic infections cause erythrocytes to be damaged so that oxygen entering cells reduces.

Table 3. Total number of erythrocytes, hemoglobin (hb), total leukocytes (leu) and phagocytic activity (AF), and lysozyme activity (AL) of snakeheadfish during pre-challenge and posy-challenge tests with *Aeromonas hydrophila* bacteria.

Treatment		Treatment					
		K+	K-	F5	R3	R5	R7
Erit (x 10^6 cell mm $^{-3}$)	H-30	1.11±0.83	1.11±0.83	1.11±0.83	1.11±0.83	1.11±0.83	1.11±0.83
	H-1	1.65±0.60^{a}	1.65±0.60^{a}	3.21±0.70^{d}	2.10±0.94^{c}	1.95±0.87^{b}	3.23±1.28^{d}
	H+2	1.21±0.46^{a}	1.54±1.17^{d}	1.29±0.53^{b}	1.77±0.23^{e}	1.36±0.12^{c}	1.27±1.01^{ab}
	H+4	2.96±0.53^{c}	2.35±0.12^{a}	3.63±1.11^{e}	4.07±0.76^{f}	3.17±0.76^{d}	2.56±0.31^{b}
	H+6	2.53±0.64^{d}	2.54±0.12^{d}	2.27±0.31^{c}	2.85±0.53^{e}	2.15±1.25^{b}	2.02±0.95^{a}
Hb (g %)	H-30	6.07±0.12	6.07±0.12	6.07±0.12	6.07±0.12	6.07±0.12	6.07±0.12
	H-1	6.40±0.40^{a}	6.40±0.40^{a}	8.13±0.31^{b}	7.87±0.42^{b}	7.80±0.20^{b}	7.47±0.31^{b}
	H+2	4.73±0.31^{a}	6.07±0.12^{d}	5.13±0.23^{ab}	5.80±0.20^{cd}	4.87±0.12^{a}	5.40±0.40^{bc}
	H+4	5.47±0.60^{a}	7.20±0.20^{b}	5.60±0.20^{a}	7.00±0.92^{b}	5.40±0.40^{a}	7.27±0.46^{b}
	H+6	5.53±0.50^{b}	5.47±0.50^{b}	4.07±0.12^{a}	7.40±0.40^{c}	6.60±0.53^{c}	7.40±0.40^{c}
Leu (x 10^4 cell mm $^{-3}$)	H-30	1.97±0.70	1.97±0.70	1.97±0.70	1.97±0.70	1.97±0.70	1.97±0.70
	H-1	2.40±1.00^{b}	2.40±0.20^{b}	2.27±0.20^{b}	1.60±0.60^{a}	1.67±0.50^{a}	1.90±0.52^{a}
	H+2	2.37±0.31^{a}	2.50±0.35^{ab}	3.43±0.83^{bc}	3.67±0.83^{b}	3.03±0.8^{abc}	4.03±0.61^{b}
	H+4	2.57±0.6a^{b}	1.77±0.70^{a}	2.10±0.20^{a}	4.97±0.46^{c}	3.33±0.92^{b}	1.90±0.87^{a}
	H+6	2.47±0.90^{b}	1.17±0.30^{a}	2.97±0.46^{bc}	1.57±0.64^{a}	2.40±0.20^{b}	3.47±0.57^{c}
AF (%)	H-30	14.67±5.67	14.67±5.67	14.67±5.67	14.67±5.67	14.67±5.67	14.67±5.67
	H-1	19.33±2.00^{b}	19.33±2.08^{b}	14.67±4.04^{ab}	9.67±1.57^{ab}	15.00±4.35^{ab}	13.33±3.51^{ab}
	H+2	27.33±6.10^{bc}	17.33±2.50^{a}	35.00±5.29^{c}	21.33±.50^{ab}	32.67±3.20^{c}	36.00±6.20^{c}
	H+4	21.00±3.00^{bc}	12.33±2.50^{a}	21.67±3.00^{bc}	33.67±3.00^{d}	27.67±4.00^{cd}	17.33±3.00^{ab}
	H+6	20.60±3.20^{b}	7.67±2.51^{a}	24.00±3.08^{b}	17.70±2.51^{b}	20.00±1.00^{b}	16.00±2.57^{ab}
AL (IU/mL/ minute)	H-1	25.33±4.00 ^{bc}	25.33±4.00 ^{bc}	34.17±0.17^{cd}	17.00±1.00^{b}	12.67±2.33^{a}	38.00±1.67^{c}
	H+2	1.67±1.00^{a}	8.00±3.00^{a}	36.67±3.33^{c}	20.83±6.50 ^{ab}	28.83±3.17 ^{bc}	35.00±1.67 ^{cd}

* different notations on the lines indicate significant differences at the 5% test level (Duncan's multiple interval test). Control (K), cake yeast of 5 g/Kg (F5), tempeh yeast of 3 g/Kg (R3), tempeh yeast of 5 g/Kg (R5), and tempeh yeast of 7g/Kg (R7).

The high survival rate of snakehead fish is closely related to the effectiveness of the fish immune system, which is indicated by the number of leukocytes, phagocytic activity, and lysozyme activity. The total number of leukocytes on the second day of the post-challenge test (H + 2) is not significantly different (P> 0.05) between treatments. The highest total number of leukocytes at H + 2 is observed in treatment R3 (Table 3). The total number of leukocytes in the body reflects the health status of the fish, especially when there is a pathogenic infection. Specifically, the total number of leukocytes of snakehead fish in the post-challenge test increases, as a result of infection with A. *hydrophila* bacteria. The pathogenic infection causes inflammation, which is a form of fish immune response to the infection, resulting in an increase in body immunity through the production of leukocytes which are responsible for phagocytosis of bacteria and synthesis of antibodies (Lagler *et al*., 1977; Moyle and Cech, 2004). The total number of leukocytes in the post-challenge test increases to more than 3.0 x 10^5 mm^{-3} cells, almost double the total number of leukocytes of snakehead fish under normal conditions, which is around 1.86 x 10^5 mm^{-3} cells (Wahyu, 2015).

In line with the total number of leukocytes, the phagocytic activity of snakehead fish also increases on the fourth day of the post-challenge test (H + 4). Phagocytic activity is the ability of the non-specific immune system to phagocytose foreign bodies. In the R3 treatment, phagocytic activity is observed on the fourth day of the post-challenge test (H + 4), with a higher value compared to that

in other treatments (Table 3). Phagocytic activity in snakehead fish increases due to the infection with *A. hydrophila* bacteria. Cells that work to phagocytose foreign bodies are macrophages and monocytes. The mechanism of macrophages as a form of phagocytes in killing foreign antigens is oxidative. This mechanism is also employed by β-glucans, which kill bacteria by oxidative means. In this mechanism, phagocytic cells produce excess reactive oxygen (ROS) so that it can kill germs (Amin, 2016).

Lysozymes are widely distributed in fish bodies and are part of the non-specific immune system (Uribe *et al*., 2011), which are bactericidal enzymes, by breaking the β-1.4-glycoside bond between acid-N-acetyl-glucosamine and acid-N-acetylmuramate in peptidoglycan so that it can damage bacteria (Kusumaningrum *et al*., 2018). Lysozyme activity in the post-challenge test of snakehead fish fed with the addition of yeast also increases in a higher value than that in the K+ and K- treatments (Table 3). The highest lysozyme activity in the pre-challenge test in the R7 treatment, while in the post-challenge test it is observed in the F5 treatment. In short, lysozyme activity in snakehead fish fed with the addition of yeast increases, both in the pre-challenge and post-challenge tests. This is assumed to occur because the β-glucan content activates phagocytic cells. This is in accordance with the research results of Paulsen *et al*. (2003), stating that the addition of β-glucan in salmon will increase neutrophils and macrophages which are the main sources of lysozyme so that their activity will increase.

G-Type Lysozyme Expression

The expression of G-type lysozyme genes in almost all treatments increases from pre-challenge test to post-challenge test. The G-type lysozyme gene in the R3 treatment is expressed 1.5 times compared to that in K, while in the R5 treatment it is expressed 4 times more than that in K (Figure 2). In short, the expression of the G-type lysozyme gene and serum lysozyme in snakehead fish generally increases in both pre-test and post-challenge tests. This is because β-glucan increases the non-specific immune response of fish, one of which is by synthesizing lysozyme. As stated by Sirimanapong *et al*. (2015), β-glucan will stimulate the expression of immune genes in most of the important organs of fish.

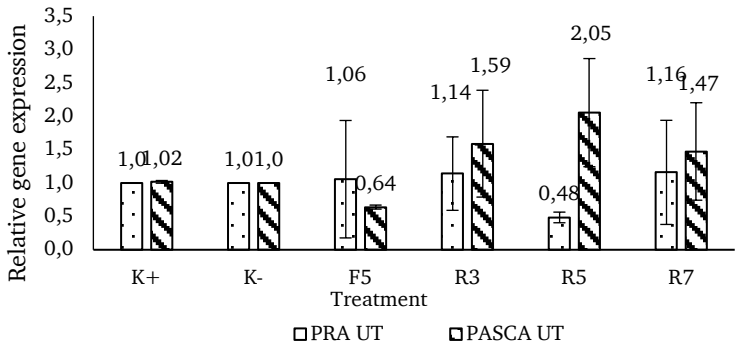

Figure 2. Expression of the lysozyme genes of snakeheadfish in pre-challenge test (H-1) and post-challenge test (H + 2), Control (K), cake yeast 5 g Kg-1 (F5), tempeh yeast of 3 g Kg-1 (R3), tempeh yeast of 5 g Kg-1 (R5), and tempeh yeast of 7g Kg-1 (R7).

CONCLUSION

The addition of 3 g Kg^{-1} of tempeh yeast to the feed has a positive effect on snakehead fish immunity, which is marked by an increase in blood picture (leukocytes, erythrocytes, and phagocytic activity) and the survival rate of

snakeheadfish in the post-challenge test with *Aeromonas hydrophila* bacteria.

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