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## The effect of induction of bacteria *Bacillus subtilis* in feed on the immune system of carp (*Cyprinus carpio* Linnaeus, 1758)

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### ABSTRACT

The induction of bacteria *Bacillus subtilis* into carp feed to improve the immune system has been widely carried out, but no one has discussed the blood picture and the resulting gene expression. This study aims to determine the effect of the induction of bacteria *Bacillus subtilis* on carp feed to improve the immune system. The method used is the exploration method with 3 treatments, namely Treatment A (addition of bacteria *Bacillus subtilis* 10 ml/kg feed with a density of  $10^8$  CFU/mL), Treatment B (addition of *Bacillus subtilis* bacteria 20 ml/kg feed with a density of  $10^8$  CFU/mL), and Treatment C (Control). After rearing fish for 14 days, gene expression analysis was performed. RNA taken from the kidneys and liver of carp was extracted using the phenol-chloroform method. RNA isolates were amplified using the semi-quantitative RT-PCR method. Observation parameters in the form of fish hematology consisting of total white blood cells and total red blood cells, as well as water quality. The results showed that the highest increase in gene expression was in treatment A, namely, liver samples (AH) of 19.76% and kidney samples (AG) of 16.04% which was in line with an increase in white blood cells by 29% and an increase in red blood cells by 21%.

**Keywords:** Carp, *Bacillus subtilis*, TLR 2, Immune System, *Cyprinus carpio*, *Aeromonas hydrophila*

## 1. INTRODUCTION

Freshwater fish has a high economic value because it is favored by the community. One of them is carp (*Cyprinus carpio* L.) which has a fairly high protein content and a high selling price compared to other freshwater fish such as catfish and Bonylip barb. Therefore, carp have great prospects for cultivation. However, the problem that is often faced by farmers is that fish are attacked by diseases that can reduce fish quality, decrease total production, and even mass death.

Countermeasures can be done by giving probiotics. Probiotics can increase the host response to disease and can be used as biocontrol agents to reduce disease attacks. One of the probiotics that can be used is *Bacillus subtilis*. *B. subtilis* is a gram-positive bacterium that can control and inhibit the growth of pathogens, produce toxic antibiotics, and enhance the non-specific immune system which is characterized by an increase in total leukocytes. The addition of *Bacillus subtilis* to feed as a probiotic can increase the body's resistance to bacterial attack that is expressed in the TLR 2 gene. TLR (*toll-like receptors*) 2 are membrane proteins that are expressed on the surface of certain cells and recognize foreign substances and transmit signals to cells. Immune system cells, so TLR 2 can activate white blood cells against pathogens.

Several studies have shown that bacteria are *B. subtilis* able to increase the immune system of carp and become a biological control agent for carp to protect carp from *Aeromonas* and *Vibrio* bacterial infections. However, the effect of bacterial induction of *B. subtilis* on feed which is reflected in the blood picture and gene expression has not been widely studied, so research has been carried out on the effect of bacterial induction of *Bacillus subtilis* in feed on the immune system of carp.

## 2. METHODS

The research was conducted from September 2019 to July 2021 at the Biotechnology Laboratory Building 3, Faculty of Fisheries and Marine Sciences, Aquaculture Laboratory, Building 4, Faculty of Fisheries and Marine Sciences, and the Central Laboratory of the University of Padjadjaran.

### 2. 1. Methods

This study used carp seeds measuring 8-10 cm which were placed in an aquarium measuring 40×25×25 cm<sup>3</sup>. Carp were reared for 14 days with commercial feed added with bacteria *B. subtilis* 10 ml/kg feed (Treatment A) and 20 ml/kg feed (Treatment B), as well as controls (Treatment C). Feeding was carried out 2 times a day using the method *ad satiation*. Bacterial culture of *B. subtilis* was carried out on NA using the scratch method and NB. The results of bacterial culture were calculated using a spectrophotometer with a standard McFarland turbidity 0.5 wavelengths 600nm.

After 14 days of rearing, RNA was isolated from the kidneys and liver of carp using the Phenol-Chloroform method. The concentration of the isolated RNA was calculated using a *microplate reader*. The results of the isolation of RNA whose purity <1.8 were purified. Purification was carried out by adding 202 L of CH<sub>3</sub>COONa to the RNA isolate, vortexing, and then adding 660 L of 100% ice-cold ethanol. The RNA pellets were centrifuged at a speed of 13,000 rpm for 30 minutes and a temperature of 4 °C. The RNA pellets were washed twice

with 500 L ice-cold ethanol 75% and centrifuged for 10 minutes at a speed of 13,000 rpm and a temperature of 4 °C.

The RNA isolates were amplified using the *Sensoquest Thermal Cycler* with the RT-PCR method. The primers used for gene expression were  $\beta$ -actin as an internal control and TLR 2 primer for gene expression. The PCR program is shown in Table 2.

**Table 1.** Primers.

Primer	Sequence (5'-3')
$\beta$ -actin	F: CCCTGGCCCCCAGCACAATG
	R: TCTGCGCAGTTGAGTCGGCG
TLR 2	F: TCAACACTCTTAATGTGAGCCA
	R: TGTGCTGGAAAGGTTTCAGAAA

**Table 2.** Program settings PCR.

Cycles	Temperature (°C)	Time	Number of Cycles
Reverse transcription	45	20 minutes	1
Inisialisasi Denaturation	95	1 minutes	1
Denaturation	95	10 seconds	37
Anneling	50 ( $\beta$ -actin) 55 (TLR 2)	40 seconds	37
Extension	72	30 seconds	37
Final extension	72	5 minutes	1

After amplification, then electrophoresis was carried out with a ladder of 100 bp for 30 minutes and a voltage of 100.

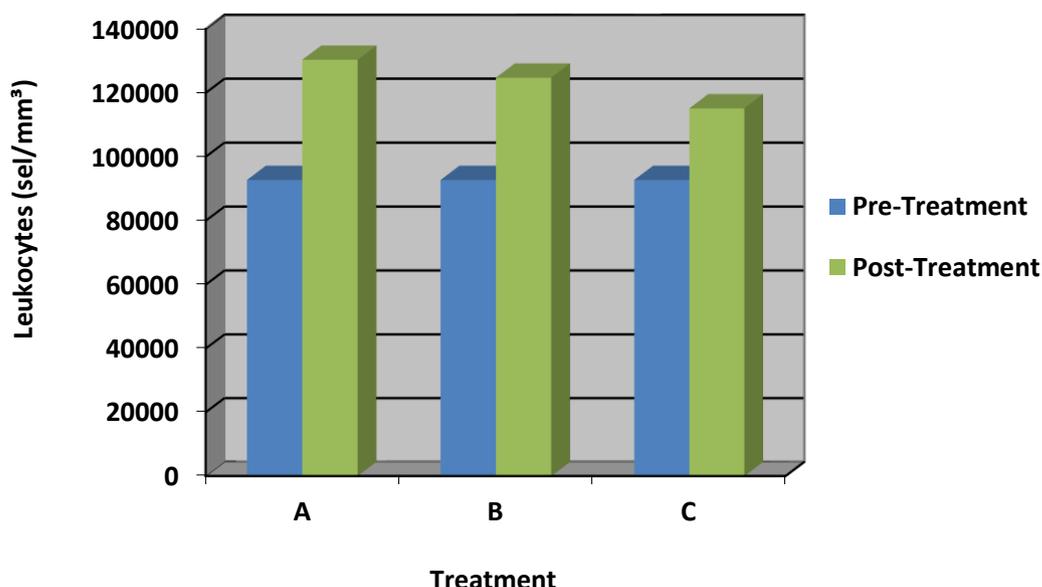
## 2. 2. Data Analysis

The results of electrophoresis from TLR 2 were analyzed using ImageJ software. The effect of treatment on TLR gene expression in carp was analyzed descriptively by comparing between treatments. Fish hematology test which includes red blood cells and white blood cells were analyzed using analysis of variance F ANOVA test (Analysis of Variance).

### 3. RESULT

#### 3. 1. Carp White Blood Cell Count

Observation of white blood cell count was carried out to determine changes in the white blood cell count of carp supplemented with bacteria *Bacillus subtilis* with different concentrations. A graph of the average white blood cells of carp can be seen in Figure 1.



**Figure 1.** Mean Number of White Blood Cells

Based on Figure 1, the average number of white blood cells of carp in the early treatment of 92 800 cells / mm<sup>3</sup>. After 14 days of being fed with supplementation *Bacillus subtilis*, the average white blood cell count of carp increased, the highest was in treatment A of 130,400 cells/mm<sup>3</sup> and the lowest in treatment C of 115,200 cells/mm<sup>3</sup>. While the average number of white blood cells in treatment B was 124,800 cells/mm<sup>3</sup>.

The number of white blood cells is a determinant of fish health because white blood cells help protect the fish body from foreign objects that can infect the body, such as attacks by pathogenic bacteria that attack the immune system. The number of white blood cells in carp during the study was still considered normal because it ranged from 32,000-146,000 cells/mm<sup>3</sup>. White blood cells are a reflection of the success of the fish's immune system in developing a cellular (non-specific) immune response as a trigger for the immune response. The results showed that the white blood cells in the treatment given supplementation *Bacillus subtilis* increased compared to before the treatment.

In addition, the total white blood cell can increase because the fish consume feed containing probiotics which are recognized as immunogenetic materials. The strengthening of the non-specific immune system as a result of consuming the probiotic *Bacillus subtilis* has also been shown to increase the total leukocytes of *major carp* and *rainbow trout*.

**Table 3.** Number of White Blood Cells Mean Post-Treatment.

Treatment	Mean White Blood Cell Count (sel/mm <sup>3</sup> )		Changes in leukocytes (%)
	Pre-Treatment	Post-Perlakuan	
A (10 ml/kg)	92.800	130.400	(+) 29 <sup>b</sup>
B (20 ml/kg)	92.800	124.800	(+) 26 <sup>ab</sup>
C (Kontrol)	92.800	115.200	(+) 19 <sup>a</sup>

Description: (+) increase

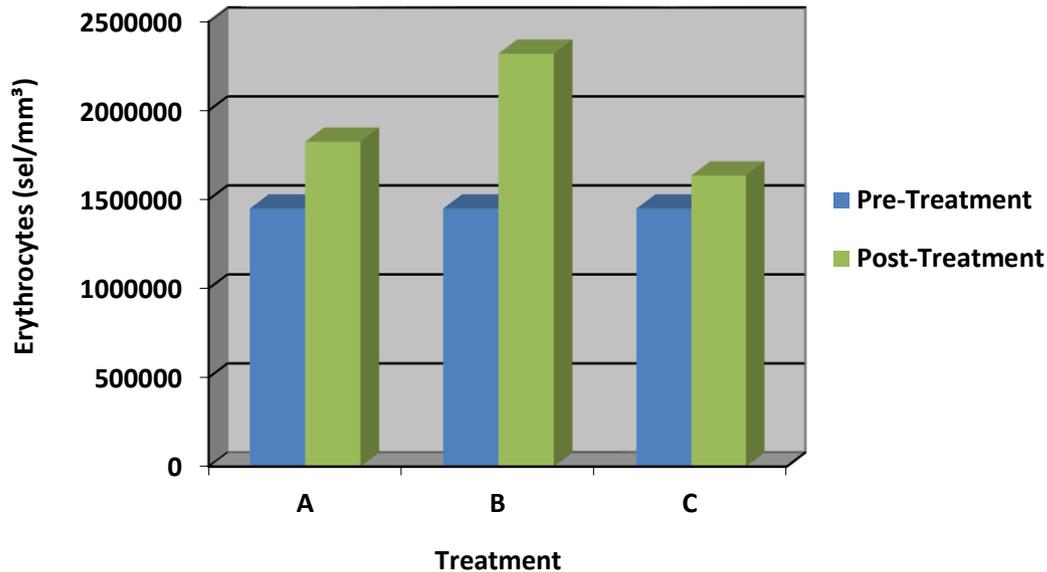
Based on Table 3 the number of white blood cells in each treatment has increased. Treatment A has increased by 29%, treatment B has increased by 26%, and treatment C has increased by 19%. Based on statistical tests, the addition of bacteria *Bacillus subtilis* into the feed affected the white blood cell count in carp. This can be seen from the results of Duncan's test in treatment A that was significantly different ( $p < 0.05$ ) with treatment C. While treatment B was not significantly different ( $p > 0.05$ ) with treatment C. So based on Duncan's test the difference in the mean white blood cell count of treatment A was the best treatment than the other treatments.

Supplementation *Bacillus subtilis* can affect increasing the immune system which can be seen from the increase in the number of white blood cells so that they can phagocytose pathogenic bacteria. Probiotic bacteria, one of which is bacteria, *Bacillus subtilis* functions to increase immune cells by stimulating the production of immune cells by inducing leukocyte-forming cells to produce more leukocyte cells such as lymphocytes, neutrophils, and monocytes. Probiotic bacteria can stimulate immunity through increased levels of macrophages and antibodies, thereby suppressing the population of pathogenic microbes in the body by producing antimicrobial compounds.

Treatment B increase the number of white blood cells which was lower than treatment A, presumably due to the presence of immunosuppressive factors. Immunosuppression occurs because the density of probiotics is too high, so the fish body is unable to respond to antigenic stimuli that enter the fish body. Another study regarding the administration of the vaccine whole-cell *Aeromonas hydrophilla* to gourami for 14 days also experienced immunosuppression which resulted in a decrease in the ability of the fish body to form an immune response as indicated by low total leukocytes. These results had similar results because on day 14 the fish were immunosuppressed.

### 3. 2. Carp Red Blood Cell Count

Observation of red blood cell count was carried out to determine changes in the number of carp red blood cells supplemented with bacteria *Bacillus subtilis* with different concentrations. Graphs of the average number of red blood cells of carp can be seen in Figure 2.



**Figure 2.** Mean Red Blood Cells

Based on Figure 2 the average number of red blood cells of carp at the beginning of treatment is equal to 1,450,000 cells / mm. After 14 days of being feed supplementation *Bacillus subtilis*, the average number of red blood cells of carp increased, the highest was in treatment B of 2,320,000 cells/mm<sup>3</sup> and the lowest was in treatment C of 1,635,000 cells/mm<sup>3</sup>. While the average number of white blood cells in treatment A was 1.825.000 cells/mm<sup>3</sup>.

The number of red blood cells in fish can indicate the health of the fish and its physiological condition. The number of red blood cells in carp during the study was still considered normal because it ranged from 1,050,000 – 3,000,000 cells/mm<sup>3</sup>. Bacteria *Bacillus subtilis* supplemented in feed can maintain the number of red blood cells in the fish's body within normal limits. This happens because of the homeostatic efforts in the fish body so that the fish body produces more blood cells to replace erythrocytes that experience lysis due to infection.

The number of red blood cells in the fish body is influenced by species, differences in the parent (genetic), nutritional conditions, physical activity, age, anemia, kidney damage, stress, and water temperature. Another study revealed that the addition of *Bacillus* sp. in the feed affected increasing the total erythrocytes of catfish, fish *Labeo rohita*, and rainbow trout fed the probiotic *Bacillus subtilis*.

Based on Table 4, the number of red blood cells in each treatment increased. Treatment A has increased by 21%, treatment B has increased by 38%, and treatment C has increased by 11%. Based on statistical tests, the addition of bacteria *Bacillus subtilis* into the feed affected the number of red blood cells in carp. This can be seen from the Duncan test results in treatment B that were significantly different ( $p < 0.05$ ) from treatment C. While treatment A was not significantly different ( $p > 0.05$ ) from treatment C. So based on Duncan's test the difference in the average number of red blood cells in treatment B was the best treatment than the other treatments.

**Table 4.** Number of Red Blood Cells Mean Post-Treatment.

Treatment	Mean Red Blood Cell Count (sel/mm <sup>3</sup> )		Changes in Erythrocytes (%)
	Pre-Treatment	Post-Treatment	
A (10 ml/kg)	1.450.000	1.825.000	(+) 21 <sup>ab</sup>
B (20 ml/kg)	1.450.000	2.320.000	(+) 38 <sup>c</sup>
C (Kontrol)	1.450.000	1.635.000	(+) 11 <sup>a</sup>

Description: (+) increase

Treatment A increased in the number of red blood cells which was lower than treatment B, presumably due to the anoxia factor. Red blood cells contain hemoglobin which functions to carry oxygen to all body tissues. A low number of red blood cells can result in fish not being able to take in large amounts of oxygen even though the availability of oxygen in the waters is sufficient. So that fish can experience anoxia (lack of oxygen).

Low erythrocyte levels indicate anemia, while high erythrocyte levels indicate that the fish are under stress. This is consistent with the assumption that fish in treatment B were immunosuppressed so that fish were stressed and red blood cell levels increased compared to other treatments. Fish under stress conditions will experience increased respiratory rate and blood pressure, the supply of red blood cells will be released into the blood circulation system, and the inflammatory response will decrease due to pressure from hormones from the adrenal glands.

Another study revealed that the addition of probiotic *Bacillus* sp. has a very significant effect on the total erythrocyte value of tilapia, and has an effect on maintaining and increasing the total erythrocytes of carp fry. This is in line with research conducted that supplementation with bacteria *Bacillus subtilis* can increase the total red blood cells of carp.

### 3. 3. TLR 2 Gene Expression

Observation of carp gene expression was carried out to determine the expression of the TLR 2 gene after being fed bacteria *Bacillus subtilis*. The results of RNA isolation using the Phenol Chloroform method were calculated to determine the level of purity and contaminants in the RNA. The results of the calculation of the concentration and purity of RNA can be seen in Table 5.

**Table 5.** Concentration and Purity of RNA.

Sample Code	A260	A280	Concentration (ng/μL)	Purity (Ratio A260/A280)
AG	1.7583	0.9387	1406.64	1.87

AH	3.2324	2.0545	2585.92	1.57
BG	2.5470	1.6188	2037.60	1.57
BH	1.8614	1.0125	1489.12	1.84
CG	2.2527	1.2890	1802.16	1.75
CH	2.5203	1.8255	2016.24	1.38

The concentration of RNA (Table 5) ranged between 1406.64 to 2585.92 ng / uL. The highest RNA purity in the AG sample (Treatment A kidney sample) was 1.87 and the lowest was CH sample (Treatment C liver sample) was 1.38. While the purity of the BH sample (Treatment B liver sample) was 1.84, the CG sample (Treatment C kidney sample) was 1.75, and the AH sample (Treatment A was a liver sample) and BG (Treatment B was kidney sample) were 1.57. The AG, BH, and CG samples showed good purity because the ratio of the total RNA samples was 1.8 so that they could be continued in the next process. The purity levels of AG, BG, and CH samples obtained were low, meaning that there were many contaminants in the sample in the form of excess phenol, chloroform, or salt. So that the purification process for the three samples was carried out. The results of RNA washing can be seen in Table 6.

**Table 6.** Concentration and Purity of RNA after Purification.

Sample Code	A260	A280	Concentration (ng/μL)	Purity (Ratio A260/A280)
AG	1.7583	0.9387	1406.64	1.87
AH	2.2545	1.0441	1803.60	2.16*
BG	0.0667	0.0324	53.36	2.06*
BH	1.8614	1.0125	1489.12	1.84
CG	2.2527	1.2890	1802.16	1.75
CH	0.0960	0.0503	76.80	1.91*

Description : (\*) After purification

Samples AH, BG, and CH that has been purified to increase the purity levels of RNA (Table 6). This happens because the RNA washing process uses 75% ethanol. Good purity values and meet the requirements needed for molecular analysis range from 1.8 to 2.0. The CH sample has a purity value of 1.91, meaning that the CH sample belongs to the ideal sample because it meets the requirements needed for molecular analysis. The AH sample has a purity value of 2.16 and the BG sample has a purity value of 2.06.

This means that the AH and BG samples exceed the ideal purity of RNA. The value of the purity of the extraction results of more than 2.0 indicates that the extraction results still contain contaminants from protein compounds. In addition to the purity and concentration of RNA, the requirements for RT-PCR molecular analysis are minimum degradation, concentration >50 ng/μL, and no contamination of gDNA and protein.

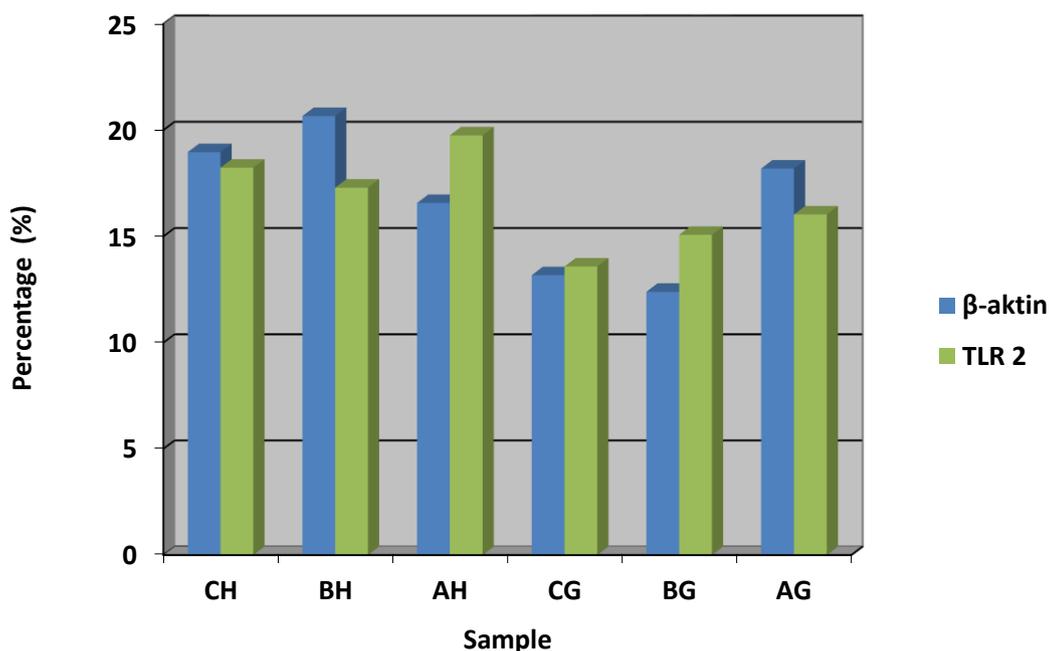
RNA samples that have met the requirements are then amplified and electrophoresis. The amplification results can be used to measure the RNA expression of the intensity of the amplified product in the gel semi-quantitatively. Band intensity or thickness was then quantified and compared with *housekeeping genes* to estimate the relative expression level of the amplified target. The expression level of each gene was observed through differences in band thickness produced by each sample on agarose gel by calculating the area and percentage produced. This was done to ensure the quality of gene expression produced by each sample. The results of measurements showing the area or thickness of the band and the percentage produced by each sample can be seen in Table 7.

**Table 7.** Results of Measurement of Area and Percentage of Band Thickness.

No	Sample	Area (pixel)	Percentage (%)
		TLR 2	TLR 2
1	CH	25859.86	18.24
2	BH	24530.15	17.30
3	AH	28016.30	19.76
4	CG	19267.40	13.59
5	BG	21383.47	15.08
6	AG	22739.00	16.04

The area of the primary TLR 2 (Table 7) is the highest for the AH sample of 28016.30 pixels, then the sample of CH30. the AG sample is 22739.00 pixels, the BG sample is 21383.47 pixels, and the lowest in the CG sample is 19267.40 pixels. The band resulting from the amplification process with the area of the analysis results has a directly proportional relationship. Samples that have a thick band will give a larger area so that the resulting percentage is also greater. The percentage of the thickness of the electrophoresis visualization band can be seen in Figure 3.

The percentage of TLR 2 gene expression (Figure 3) in the liver sample of treatment A was 18.24% followed by an increase in -actin gene expression by 18.97%, treatment B by 17.30% followed by an increase in -actin gene expression by 20.67%, while in treatment C the percentage of TLR 2 gene expression was 19.76% but there was a decrease in -actin gene expression by 16.58%. This indicates that the highest gene expression level in the liver sample was in treatment B.



**Figure 3.** Percentage of Band Thickness

The percentage of TLR 2 gene expression in the kidney sample in treatment C was 13.59% with decreased expression of the  $\beta$ -actin gene by 13.18%, treatment B by 15.08% with a decrease in gene expression of  $\beta$ -actin by 12.40%, while in treatment A the percentage of TLR 2 gene expression was 16.04% followed by an increase in gene expression.  $\beta$ -actin by 18.20%. This indicates that the highest level of gene expression in kidney samples was found in treatment A.

The percentage of gene expression in liver samples was higher than in kidney samples. This can happen because of the purity level of RNA used, so it can cause the dilution process and the composition of the ingredients to be inaccurate. Most expression levels are thought to be strongly related to the number of *copies* of transgene in each cell.

Supplementation of bacteria *Bacillus subtilis* into feed for 14 days was shown to increase the expression of the TLR 2 gene. This is because the TLR increased TLR 2 expression by bacteria *Bacillus subtilis* can make macrophages more sensitive to pathogens and enable more effective eradication of pathogens. The increase in TLR 2 gene expression varies depending on the ability of the inducing bacteria to control body homeostasis. In addition, the immune response by the addition of probiotics is more expressed in TLR 2. Other studies have revealed that the immune response due to probiotics is more related to TLR 2. This is thought to be related to the ability of macrophages to differentiate between pathogenic and commensal microbes. In addition, the administration of probiotics for 7 days in mice showed an increase in TLR 2 expression compared to the control treatment and the LPS group.

The administration of probiotic *Lactobacillus casei* to examine the level of TLR 2 immune response in the lamina propria and Peyer results in increased TLR 2 expression in monocular cells of the gastrointestinal mucosa compared to other groups. An increase in TLR 2 expression was seen after 7 days of probiotic administration.

Treatment A can increase the number of white blood cells in the body so that it has the best body defense against invading pathogens. This is by the role of white blood cells as a non-specific defense of the body. In addition, TLR 2 plays a very important role as a functional receptor that activates leukocytes to induce an innate immune response against pathogens. So that the PCR result from treatment A is the best treatment which is in line with the increase in white blood cells in the fish body, meaning that TLR 2 is well expressed in treatment A.

The criteria that need to be considered to get an efficient probiotic with a positive effect on the host is with a total density of around  $10^7$  cfu/mL –  $10^9$  CFU/mL. Treatment B with a higher concentration of bacteria than treatment A did not provide a good body defense and did not provide better body resistance and even caused more mortality. The number of bacteria in the digestive tract that is getting denser can cause competition between bacteria so that the activity of bacteria in digesting nutrients will be hampered and cause the death of these bacteria, and disrupt the balance of microflora in the maintenance medium and the host's body. Factors that influence the host's response to probiotics include microflora composition, host intestine, the dose used, age, and quality of probiotics. The use of probiotics in high doses did not guarantee better protection against the host.

In addition, the purity and concentration of RNA used during the amplification process can affect the results. The AG sample is the concentration and purity of RNA obtained by a one-time washing process or not through the purification process so that the AG sample is thought to be better than the other kidney samples. While the AH sample is a sample with the highest level of purity compared to other liver samples.

#### 4. CONCLUSIONS

Based on the research that has been done, it can be concluded that the addition of bacteria *Bacillus subtilis* into carp feed can improve the immune system seen from the level of gene expression and fish hematology. Increased gene expression highest in treatment A (Addition of *Bacillus subtilis* 10ml / kg of feed at a density of  $10^8$  CFU / mL), namely, the sample of the liver (AH) of 19.76% and a sample of the kidney (AG) of 16.04% which is in line with an increase in white blood cells by 29% and an increase in red blood cells by 21%.

#### References

- [1] Zhao. S, Chen. Z, Zheng. J, Dai. J, Ou. W, Xu. W, Ai. Q, Zhang. W, Niu. J, Mai. K, Zhang. Y, Citric acid mitigates soybean meal induced inflammatory response and tight junction disruption by altering TLR signal transduction in the intestine of turbot, *Scophthalmus maximus* L. *Journal Fish and Selfish Immunology* 92 (2019) 181-187
- [2] Kot, K., Kosik-Bogacka, D., Wojtkowiak-Giera, A. *et al.* The expression of TLR2 and TLR4 in the kidneys and heart of mice infected with *Acanthamoeba* spp.. *Parasites Vectors* 13, 480 (2020). <https://doi.org/10.1186/s13071-020-04351-4>
- [3] Yauch. L, Michael. K, Mansour. Shmuel, S. James, B. Rottman, and Stuart M, Involvement of CD14, Toll-Like Receptors 2 and 4, and MyD88 in the Host Response

- to the Fungal Patogen *Cryptococcus neoformans* In vivo. *Infection and Immunity* 72 (9) (2004) 5373-5382
- [4] Mohapatra. S, T. Chakraborty, V. Kumar, G. DeBoeck, KN. Mohanta, Aquaculture and stress management: a review of probiotic intervention. *Journal of Animal Physiology and Animal Nutrition* 97 (2013) 405–430
- [5] Dammermann. W, Wollenberg. L, Bentzien. F, Lohse. A, Luth. S, Toll Like Receptor 2 Agonists Lipoteichoic Acid and Peptidoglycan are Able to Enhance Antigen Specific If Release in Whole Blood During Recall Antigen Responses. *Journal of Immunological Methods* 396 (1-2) (2013) 107-115
- [6] Mulyani. Y, Aryantha. INP, Suhandono. S, Pancoro. A, Intestinal Bacteria of Common Carp (*Cyprinus carpio* L.) as a Biological Control Agent for *Aeromonas*. *Journal of Pure and Applied Microbiology* 12 (2) (2018) 601-610
- [7] Ribeiro CM, Hermsen T, Taverne-Thiele AJ, Savelkoul HF, Wiegertjes GF, Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. *J Immunol* 5 (184) (2010) 2355-2368
- [8] Aly S. M, AG Ahmed, AA Ghareeb, MF Mohamed, Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica* (*Oreochromis niloticus*) to challenge infections. *Fish Shellfish Immunol* 25 (1-2) (2008) 128-136.
- [9] Nayak. SK, Probiotics and Immunity: A Fish Perspective [review]. *Fish and Shellfish Immunology* 29 (2010) 2-14
- [10] Ellis. A. E, Innate Host Defense Mechanism of Fish Against Viruses and Bacteria. *J Developmental and Comparative Immunology* 25 (2001) 827-839
- [11] Lukistyowati. I, Kurniasih, Survival of carp (*Cyprinus carpio* L) fed garlic extract (*Allium sativum*) and infection with *Aeromonas hydrophila*. *Journal of Fisheries and Marine Affairs* 16 (1) (2011) 144-160
- [12] Hardi. E, Handayani, Sukenda, E. Harris, AM Lusiastuti, Characteristics and Pathogenicity *Streptococcus agalactiae* of -Hemolytic and Non-Hemolytic in *Tilapia*. *Veterinary Journal* 12 (2) (2011) 152-164
- [13] Rotondo. F, Manoranjan. B, Salehi. F, Scheithauer. B, W. Kovacs, K. Cusimano. M. D, Estrogen receptors  $\alpha$  and  $\beta$  immunohistochemical expression: Clinicopathological correlations in pituitary adenomas. *Anticancer Research* 30 (7) (2010) 2897-2904
- [14] Kumar. M. P, Ramulu. KS, Haematological changes in *Pangasius hypophthalmus* infected with *Aeromonas hydrophila*. *International Journal of Food, Agriculture, and Veterinary Sciences* 3 (1) (2013) 70–75
- [15] Mocanu. M, V. Cristea. L, Dediu, E. Bocioc, RI Grecu, S. Ion, I. Vasilean, The Effect of Probiotic Diet on Growth and Hematology Parameters of Rainbow Trout *Oncorhynchus mykiss* (Walbaum 1792). *Lucrari Stiintifice Sera Zooteniie* 59 (2010) 258–263

- [16] Morihito. S, Hitomi. S, Daisuke. I, Kazumi. K, Masanori. K, Hiroki. T, Toru. T, Hitoshi. I, Yasufumi. A, Yoshiro. K, Direct binding of Toll-Like Receptors 2 to Zymosan and TNF  $\alpha$  Secretion Are Down-Regulated by Lung Collectin Surfactan Protein A. *The Journal of Immunology* 171 (2003) 417-425
- [17] Tang. R, Wang. S, Han. P, Zhang. Q, Zhang. S, Xing. X, Shao. R, Xu. W, Xu. Q, Wei. Q, Qi. Z, Toll-like receptor (TLR) 2 and TLR13 from the endangered primitive-ray finned fish Dabry's sturgeon (*Acipenser dabryanus*) and their expression profiling upon immune stimulation. *Aquaculture Reports* 16 (2020) 1-10
- [18] Wedemeyer Sartijo, R. S. A. Harjuno, C. Haditomo, Effect of Soaking Ceremai Leaf Extract (*Phyllanthus acidus* [L] skeels) on Total Erythrocytes and Lifespan of Carp (*Cyprinus carpio*) Infected with *Aeromonas hydrophila* Bacteria. *Journal of Aquaculture Management and Technology* 7 (1) (2018) 114-119
- [19] Fink IR, Pietretti D, Voogdt CGP, Westphal AH, Savelkoul HFJ, Forlenza M, Wiegertjes GF, Molecular and functional characterization of Toll-like receptor (Tlr)1 and Tlr2 in common carp (*Cyprinus carpio*). *Fish Shellfish Immunol* 56 (2016) 70-83
- [20] Shoemaker CA, Klesius PH, Evans JJ, Prevalence of *Streptococcus iniae* in tilapia, hybrid striped bass, and channel catfish on commercial fish farms in the United States. *Am J Vet Res* 2 (62) (2001) 174-177
- [21] Lee S. Toni, Anastacia M. Garcia, Danielle A. Jeffrey, Xuan Jiang, Brian L. Stauffer, Shelley D. Miyamoto, Carmen C. Sucharov. Optimization of phenol-chloroform RNA extraction. *MethodsX* 5 (2018) 599-608
- [22] Tesena. P, Korchunjit. W, Taylor. J, Wongtawan. T, Comparison of commercial RNA extraction kits and qPCR master mixes for studying gene expression in small biopsy tissue samples from the equine gastric epithelium. *J Equine Sci* 28 (2017) 135-141
- [23] Lebeer. S, Vanderleyden. J, Keersmaecker. SCJD, Genes and Molecules of Lactobacilli Supporting Probiotic Action. *Microbiology and Molecular Biology Review* 4 (72) (2008) 728-764
- [24] Chang-Sheng Zhao, Di-An Fang, Dong-Po Xu, Toll-like receptors (TLRs) respond to tributyltin chloride (TBT-Cl) exposure in the river pufferfish (*Takifugu obscurus*): Evidences for its toxic injury function. *Fish & Shellfish Immunology* 99 (2020) 526-534
- [25] Hwang GL, Azizur Rahman M, Abdul Razak S, Sohm F, Farahmand H, Smith A, Brooks C, Maclean N, Isolation and characterisation of tilapia beta-actin promoter and comparison of its activity with carp beta-actin promoter. *Biochim Biophys Acta* 1625 (1) (2003) 11-18
- [26] Miettinen. M, Veckman. V, Latvala. S, Sereneva. T, Matikainen. S, Julkunen. I, Live *Lactobacillus rhamnosus* and *Streptococcus pyogenes* Differentially Regulate Toll-like Receptor (TLR) Genes Expression in Human Primary Macrophages. *Journal of Leukocyte Biology* 84 (2008) 1092-1100
- [27] Alexander. C, Rietschel. E.T, Bacterial Lipopolysaccharides and Innate Immunity. *Journal of Endotoxin Research* 3 (7) (2001) 167-2004

- [28] Galdeano. CM, Perdigon. G, The Probiotic Bacterium *Lactobacillus casei* Induces Activation of the Gut Mucosal Immune System Through Innate Immunity. *Clinical and Vaccine Immunology* 2 (13) (2006) 219-226.
- [29] Sumaraw. JT, H. Manoppo, RA Tumbol, IFM Rumengan, HA Dien, DA Sumilat, Study of Probiotic Bacteria to Improve Growth Performance and Survival of Carp (*Cyprinus carpio*). *Platax Scientific Journal* 7 (1) (2019) 243-255
- [30] Nikoskelane. S, Ouwehand. A, Salminen. S, Bylund. G, Protection of Rainbow Trout *Onchorhynchus mykiss* from furunculosis by *Lactobacillus rhamnosus*. *Aquaculture* 198 (2001) 229-236
- [31] Alejo A, Tafalla C, Chemokines in teleost fish species. *Dev Comp Immunol* 12 (35) (2011) 1215-1222
- [32] Woitke. M, H. Junge, W. H. Schnitzler, *Bacillus subtilis* as Growth Promotor in Hydroponically Grown Tomatoes Under Saline Conditions. *Acta Hort* 48 (659) (2004) 363-369
- [33] Ibnu Bangkit Bioshina Suryadi, Regan Hanifelian Yusa, Grandiosa H. Roffi, Zahidah, The performance of Koi (*Cyprinus carpio* L.) in a circular current container tested against the *Aeromonas hydrophila* bacteria. *World Scientific News* 159 (2021) 1-19
- [34] Ayi Yustiati, Sadra Muhammad Haydar Ali Saefulhadjar, Ibnu Bangkit Bioshina Suryadi, Iskandar, Hematological Performance and Survival of Common Carp (*Cyprinus carpio* Linnaeus, 1758) Strain Majalaya Fingerlings Against Temperature Stress. *World Scientific News* 150 (2020) 92-104
- [35] Raden Ahmad Sholahudin Fauzi, Ayi Yustiati, Eddy Afrianto, Ibnu Bangkit, Growth and sustainability performance of common carp seed (*Cyprinus carpio* Linnaeus, 1758) in round water flowing container. *World Scientific News* 141 (2020) 132-144
- [36] Zulfahana Amatulloh, Yuniar Mulyani, Lantun Paradhita Dewanti, Kiki Haetami, Growth Characteristics and Tracing Antagonistic Properties of *Bacillus flexus* and *Bacillus subtilis* as Antibacterials to Overcome the Attack of *Aeromonas hydrophila* Bacteria on Fish. *World Scientific News* 158 (2021) 91-104
- [37] Rosidah, Maria Dewi Yunita, Isni Nurruhwati, Achmad Rizal, Histopathological changes in gold fish (*Carassius auratus* (Linnaeus, 1758)) infected by *Aeromonas hydrophila* bacteria with various densities. *World Scientific News* 142 (2020) 150-168
- [38] Auryn Ramadhany Geraldine, Rosidah, Heti Herawati, Ibnu Bangkit Bioshina, Isolation and Identification of Potential Pathogenic Bacteria in Living Carp (*Cyprinus carpio* Linnaeus, 1758) Sold in Supermarkets in Cimahi City, Java. *World News of Natural Sciences* 32 (2020) 21-35
- [39] Afifah Shabirah, Rosidah, Yuniar Mulyani, Walim Lili, Effect of Types Isolated Lactic Acid Bacteria on Hematocrit and Differential Leukocytes Fingerling Common Carp (*Cyprinus carpio* L.) Infected with *Aeromonas hydrophila* bacteria. *World News of Natural Sciences* 24 (2019) 22-35