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PROFILE ERYTHROCYTES OF *Oreochromis niloticus* FED WITH HERBS FERMENTED AND INFECTED WITH *Aeromonas hydrophila*

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ABSTRACT

Herbs Fermented consist of *Kaempferia galanga* L, *Curcuma xanthorrhiza* Roxb, and *Curcuma domestica* Val, which are environmentally friendly disease prevention alternatives that can be added to feed because they have several advantages, such as increasing appetite, digestion, growth, disease resistance, and reducing stress levels in fish. This research was carried out from August to December 2021 at the Marine Science Biotechnology Laboratory and Fish Parasites and Diseases Laboratory, Faculty of Fisheries and Marine, Universitas Riau. This study aimed to obtain the best dose from adding fermented herbs to increase the immune system of *Oreochromis niloticus* and prevent diseases caused by *Aeromonas hydrophila* bacteria in terms of profile erythrocytes. The method used was an experimental method using a Completely Randomized Design (CRD) with one factor, five levels of treatment, and three replications so that 15 experimental units were obtained. The challenge test was carried out on the 32nd day using *A.hydrophila* with a density of 10^8 CFU/mL. The best dose was found in P_3 with a dose of 200 mL/kg of feed, which showed a total erythrocyte of 2.09x10^6 cells/mm^3, hemoglobin level of 7.70 g/dL, hematocrit value of 37.70%, and a normal erythrocyte morphology. Water quality ranged from temperature 27-29^{o}C, pH 6.9-7.2, DO 3.1-4.4 mg/L, and Ammonia 0.016-0.024 mg/L. Based on the data obtained, it can be concluded that fermented herbs can increase the immunity of *O. niloticus* to diseases caused by *A.hydrophila*.

Keywords: *Oreochromis niloticus*, Erythrocytes, Herbs Fermented, *Aeromonas hydrophila*

1. INTRODUCTION

Oreochromis niloticus is one of the freshwater consumption fish commodities with many enthusiasts; therefore, this fish has high economic value, so many farmers cultivate this fish. According to Dailami et al.[1], fish farming cannot be separated from various disturbances, including disease. Several things, such as the cultivation environment, techniques, and attacks by pathogenic microorganisms, can cause diseases that interfere with fish farming. One of the disease-causing pathogens that often attack freshwater fish is bacteria.

Bacterial attack is one factor that influences failure in aquaculture, which can cause losses to fish farmers. One type of disease often found in fish is *Motile Aeromonas Septicemia* (MAS), caused by *Aeromonas* sp. The resulting symptoms are wound-like and hemorrhagic (bleeding)[2]. This bacterial attack is latent, so it does not show disease symptoms even though it has been found in the fish's body. This bacterial attack is only seen when the fish's body resistance decreases due to stress caused by decreased water quality, lack of feed, or poor handling[3].

One of the efforts to prevent bacterial infections is by using natural ingredients. Disease prevention is carried out to increase the body's immunity so that fish can resist

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various types of pathogens that attack. One alternative for disease prevention that is environmentally friendly, easy to obtain, safe to use, and relatively inexpensive is fermented herbs. Herbs Fermented consists of a mixture of natural ingredients, including *Curcuma domestica* Val, *Kaempferia galanga* L, and *Curcuma xanthorrhiza* Roxb, which is given to the feed and can be an alternative to increase the body's defenses of fish. According to Syawal et al.[4], giving herbal supplements that have been fermented and added to feed can increase fish appetite fast growth, immunity against disease, and reduce fish stress levels against environmental changes. Food ingredients will experience beneficial physical and chemical changes through fermentation, such as the formation of preferred flavors and aromas. The presence of active substances contained in herbal supplements can improve the body's defense system, growth, and health of fish[5].

Blood must be observed to determine the health condition of fish infected with *A.hydrophila*. Observing blood cells can provide information about the presence of a disease in the organism's body. The blood profile plays a crucial role in the body's metabolic and physiological activities and is a component of defense against disease attacks that enter the fish's body[6]. This study aims to obtain the best-fermented herbs added to feed to increase tilapia body resistance and prevent *A.hydrophila* attacks.

2. RESEARCH METHOD

Time and Place

This research was carried out from August to December 2021 at the Marine Science Biotechnology Laboratory and the Laboratory of Parasites and Fish Diseases, Faculty of Fisheries and Marine, Universitas Riau, Pekanbaru.

Method

The method used in this study is an experimental method that applies a one-factor, Completely Randomized Design (CRD) with five treatment levels, and three repetitions were carried out so that 15 experimental units were needed. The treatment used in the study refers to Syawal et al.[7]:

Kn : Negative control (Feed without herbs fermented and without being challenged with *A.hydrophila*

Kp : Positive control (feed without herbs fermented and challenged with *A.hydrophila*

P_1 : Feed containing herbs fermented at a dose of 150 mL/kg of feed and challenge tested with *A. hydrophila*

P_2 : Feed containing herbs fermented at a dose of 175 mL/kg of feed and challenge tested with *A. hydrophila*

P_3 : Feed containing herbs fermented at a dose of 200 mL/kg of feed and challenge tested with *A.hydrophila*.

Procedure

Container Preparation

The containers used are fiber tubs and 15 units of buckets with a volume of 100 L. Preparing the container starts with cleaning the fiber tub and bucket, which is washed, rinsed, and then filled with water. The container containing 25 ppm of $KMnO_4$ was added to the water, aerated for 24 hours, then rinsed and dried for one day. Then, each container was filled with water with a volume of 80 L, and fish with a size of 5-6 cm with a stocking density of 1 fish/4 L were added.

Preparation of Herbs Fermented

The composition of these fermented herbs refers to Syawal et al.[7]. *C.domestica* Val, *K.galanga* L, and *C.xanthorrhiza* Roxb were peeled, washed, weighed as much as 100 g each, then sliced thin and blended until smooth. These materials are filtered and discarded dregs. The filter results were put into the pan, clean water was added up to volume 3 L, boiled until boiling, and waited until it cooled down. After cooling, 65 mL of probiotic drink, 175 g of molasses, and 50 mg of tape yeast were added, then stirred until homogeneous.

Then, the herbs are put into a closed storage container like a jerry can.

Furthermore, the herbs are stored at room temperature and protected from direct sunlight. The fermentation process was carried out for ±7 days. The success of the fermentation is indicated by the change in the aromas, which became more fragrant and not overpowering, and the absence of gas formation.

Adaptation and Maintenance of Test Fish

500 tilapia fish measuring 5-6 cm, obtained from Kampar Regency, Riau Province, were used. The tilapia seeds were adapted for seven days, and during the adaptation process, the fish were fed F999 pellets with a frequency of feeding three times a day. The maintenance of the test fish was carried out for 45 days, and the test fish seeds were fed according to the treatment. A feeding frequency of 3 times daily is as much as 10% of the fish's body weight. Feeding is done thrice daily, namely at 07.00, 12.00 and 17.00 WIB. Sampling is carried out every 10 days; the length and weight of the test fish are measured to determine the amount of feed to be given in subsequent rearing.

Provision of Isolate *A. hydrophila*

The *A.hydrophila* isolates were obtained from collections held at the Laboratory of Parasites and Fish Diseases, Universitas Riau. The isolates were then cultured onto GSP media and incubated in an incubator for 18-24 hours at 28-30^{o}C. Bacterial cultures on GSP media were cultured back into TSB liquid media and incubated in an incubator for 18-24 hours at 28-30^{o}C. After 24 hours, the bacteria can be used for a challenge test.

Challenge Test

After 32 days of rearing the test fish, the challenge test was carried out. Fish were injected with *A.hydrophila* at a 10^8 CFU/mL density of as much as 0.1 mL. Before the fish are infected, they are first anesthetized using clove oil as much as 0.1 mL of water to reduce stress on the fish. After infection, the fish were kept for 14 days, and clinical symptoms were observed. After the infection, the fish are still fed with added fermented herbs.

Fish Blood Sampling

Fish were anesthetized with 0.1 mL/L of clove oil for ±5 minutes in 5 L of water: 1 mL syringe and Eppendorf tube moistened with 10% EDTA before blood collection to prevent blood clots. Blood collection was carried out in the caudal vein. Then, the blood that was in the syringe was put into the Ependorf tube. Blood sampling for the test fish was carried out in the morning. Blood sampling of test fish for observation was carried out three times, namely immediately before treatment, the second on the 30th day of rearing, and the third on the 14th day after the challenge test with *A. hydrophila*.

Parameters Measured

Observation of Clinical Symptoms

Observations of clinical symptoms were seen after the challenge test, including changes in behavior and physical condition (morphology). Changes in fish behavior include not swimming actively, changing to irregular swimming ways, and decreasing response to food. Meanwhile, changes in physical conditions occur, such as chipped fins, bleeding, excessive mucus, and followed by ulcers.

Total Erythrocytes

Formulas and the procedure for calculating the number of erythrocytes are measured according to Blaxhall & Daisley[8]:

$$\sum \text{Erythrocytes} = \sum n \times 10^4 \text{ cells/mm}^3$$

Information:

$\sum n$ = Number of counted erythrocytes in 5 visual fields

10^4 = Dilution factor

Hematocrit Levels

Measurement of hematocrit levels refers to Anderson & Siwicki[9]. Hematocrit

levels are expressed in percent as % blood volume.

Hemoglobin

The hemoglobin level procedure was followed[10]. Hemoglobin levels are expressed in g/dL or g%.

Water Quality

Parameters measured in water quality are temperature, pH, DO, and ammonia.

3. RESULT AND DISCUSSION

Clinical Symptoms

Based on the results of the study, the negative control (Kn) tilapia was under normal conditions; this was because, in this treatment, no challenge test was carried out with *A.hydrophila* while the positive control (Kp), P_1, P_2, and P_3 were challenged with *A. hydrophila* thus showing clinical symptoms such as excess mucus, flaky scales, chipped fins, ulcers at the injection site, and bulging eyes. This is supported by the opinion of Rosidah et al.[11] that the clinical symptoms of fish infected with *A.hydrophila* are damage to the surface of the fish's body in the form of red spots (hemorrhagic), darkening of the fish's body color, distended abdomen (dropsy), and protruding eyes (exophthalmia).

P_3 treatment showed the fewest clinical symptoms from day 1 to day 14 compared to the Kp, P_1 treatment, and P_2. This shows increased body resistance to tilapia from administering herbs fermented added to fish feed. This is because herbs fermented from *C.domestica* Val, *K.galanga* L, and *C.xanthorrhiza* Roxb contain secondary metabolites with a yellow substance or curcumin that acts as an anti-inflammatory or anti-inflammatory[12]. Herbs Fermented also contain secondary metabolites, such as curcuminoids, vitamin C, essential oils, tannins, and flavonoids, which trigger blood-producing organs, such as the spleen and kidneys, to produce more blood to repair damaged cells and form the immune system.

The content of flavonoids can increase the work of blood-producing organs (lymphomyeloids) so that blood production can increase[13]. Meanwhile, Vitamin C plays a role in normalizing immune function, reducing stress, and accelerating wound healing in fish[14]. Vitamin C is a nutrient the body needs and can promote average growth, seed health or reduce stress, accelerate wound healing, and increase defense or immunity[15]. In addition, Vitamin C plays an essential role in osmoregulation, protection against pathogens, and antioxidative agents[16].

Total Erythrocytes

Total erythrocytes were counted to see changes in total erythrocytes that occurred at the beginning of maintenance, the 30th day of rearing, and the 14th day after the challenge test with *A.hydrophila*. The results of observations of the total erythrocytes of tilapia in more detail can be seen in Table 1.

Table 1. Total erythrocytes of tilapia during the study

Treatment	Total Erythrocytes (10^6 cells/ mm^3)		
	Day 0	30th day	14th day (Post-test challenge)
Kn	1,13	1.23±0.02^a	1.31± 0.02^b
Kp	1,12	1.24±0.02^a	0.97±0.01^a
P1	1,13	1.81± 0.01^b	1.96± 0.03^c
P2	1,12	1.84± 0.01^b	2.05 ± 0.00^d
P3	1,13	1.92± 0.02^c	2.09± 0.01^e

Information:*Different superscripts indicate that there is a significant difference between treatments ($P<0.05$); ± Standard Deviation (SD).

Table 1 shows that the addition of herbs fermented to feed with different doses had a significant effect on total erythrocytes on the 30th day, ranging from 1.81 to 1.92 $x10^6$ cells/mm^3 and post-challenge testing ranging from 1.96 to 2.09 $x10^6$ cells/mm^3. The total height of the erythrocytes is still within the normal range; this follows the opinion of Royan et al.[17], in teleost fish, the standard number of erythrocytes is 1.05-3.0 x 10^6 cells/mm^3.

The study's results on the 30th day showed that the total erythrocytes of tilapia in each treatment increased compared to the initial rearing conditions. Giving herbs fermented to treatments P_1, P_2, and P_3 can increase the total erythrocytes of tilapia because the herbs fermented added to the feed contain *C.domestica* Val, *K.galanga* L, and *C.xanthorrhiza* Roxb, all three of which contain curcumin which can be beneficial for fish to increase appetite. Improve digestibility and improve health[7].

The results of the post-challenge study of tilapia had total erythrocyte values ranging from 0.97 to 2.09 $x10^6$ cells/mm^3. The Student Newman-Keuls post-challenge test results showed that each treatment was significantly different. Based on the statistical test of analysis of variance (ANOVA), the feeding given with herbs fermented affected the total erythrocytes ($P<0.05$). The highest total erythrocyte value was in the P_3 treatment, while the lowest was in the Kp treatment. Total erythrocytes that decreased in Kp treatment were caused by an attack of *A.hydrophila* during the challenge test, which produced hemolysin, which can lyse erythrocytes so that erythrocytes cells tend to decrease so that blood-producing organs cannot produce more blood cells to replace blood that comes out due to wounds in infected parts[18].

Several factors influence the increase in total erythrocytes at P1, P2, and P3. According to Zissalwa et al.[19], species, age, feed nutrition, parent, size, physical activity, and environmental conditions affect the number of erythrocytes. Elevated erythrocyte levels in the blood indicate increased oxygen content and indicate that they are starting to adapt to the environment[20]. The provision of fermented herbs can improve fish health. This is presumably due to the content of secondary metabolites, such as curcuminoids, vitamin C, essential oils, tannins, and flavonoids, which can trigger blood-producing organs, such as the spleen and kidneys, to produce more blood in order to repair damaged cells and form the immune system[21].

Hematocrit

Hematocrit levels were calculated to see changes in hematocrit that occurred after being reared for 30 days with fermented feed containing herbs and after being infected with *A.hydrophila*. The results of observations of hematocrit levels in more detail can be seen in Table 2.

Hematocrit is the percentage of erythrocyte volume in fish blood[12]. According to Sarjito & Haditomo[22], normal fish hematocrit values range from 28-40%. Based on the results of the analysis of variance (ANOVA) showed that the addition of herbs fermented to the feed had a significant effect on the hematocrit value in the blood of tilapia after 30 days of rearing ($P<0.05$), ranging from 30.00 to 36.66%, where the highest value was found in the P_3 treatment of 36.66%.

The hematocrit value of tilapia on the 14^{th} day after the challenge test ranged from 23.00-37.33%. The highest value was found in the P_3 treatment, 37.33%, while the Kp treatment had the lowest value, namely 23.00%, because the Kp treatment was not given feed containing fermented herbs. This follows the statement of Hasibuan et al.[18], which states that there is an imbalance between the activity of bacteria and the increase in fish immunity. Hence, the activity of bacteria is more potent and faster than the activity of increasing the fish's natural immunity so that the defense of the fish's body is weak after being infected with *A.hydrophila*. In addition, the appetite of fish attacked by bacteria decreases and

results in a lack of nutrients entering the body, so the erythrocyte level decreases because nutrition is essential to help the process of forming erythrocyte cells in the body; if the erythrocyte level decreases, the hematocrit level also decreases.

Table 2. Hematocrit values of tilapia

Treatment	Hematocrit (%)		
	Day 0	30th day	14th day (Post-test challenge)
Kn	26	30.00 ± 1.00^{a}	34.33 ± 0.57^{a}
Kp	26	30.33 ± 0.57^{a}	23.00 ± 2.00^{b}
P1	27	32.33 ± 1.15^{b}	35.00 ± 1.00^{bc}
P2	27	35.00 ± 1.00^{c}	35.33 ± 0.57^{bc}
P3	26	36.66 ± 0.57^{d}	37.33 ± 0.57^{c}

Information:*Different superscripts indicate that there is a significant difference between treatments (P<0.05); ± Standard Deviation (SD).

The hematocrit values in the P_1, P_2, and P_3 treatments added to the fermented herbs increased. This is due to the presence of secondary metabolites found in *C.domestica* Val, *K.galanga* L, and *C.xanthorrhiza* Roxb contained in fermented herbs, such as curcumin, flavonoids, tannins, quinones, and minerals, thus triggering blood-producing organs, such as the kidneys to produce erythrocytes cells. Nursatia et al.[23] stated that the hematocrit value can change depending on the season, temperature, feeding, and the impact of immunostimulants. Hematocrit values vary depending on nutritional factors, fish age, sex, body size, and spawning period.

The essential oil in *C.domestica* Val prevents excessive gastric acid from coming out so that the stomach is not too acidic and facilitates the absorption of nutrients by the small intestine. Curcumin has a function that can stimulate the gallbladder's walls to secrete bile into the small intestine to increase the digestion of fats, proteins, and carbohydrates so that the activity of absorption of food substances increases[24]. According to Syawal et al.[4], giving herbal supplements with a fermentation process in feed can stimulate fish appetite due to physical and chemical changes such as the formation of flavors and aromas that make fish have an appetite, increase fish immunity to disease, and reduce fish stress levels against environmental changes.

Hemoglobin levels

Calculation of hemoglobin levels was carried out to see changes in the hemoglobin of tilapia from the start of rearing, the 30th day of rearing, and the 14th day after the challenge test with *A.hydrophila*. The results of tilapia hemoglobin measurements during the study are presented in Table 3.

Table 3. Hemoglobin levels of tilapia

Treatment	Rate Hemoglobin (g/dL)		
	Day 0	30th day	14th day (Post-test challenge)
Kn	6	6.10 ± 0.20^{a}	6.43 ± 0.15^{a}
Kp	6	6.36 ± 0.11^{b}	4.26 ± 0.05^{b}
P1	6	7.16 ± 0.11^{c}	7.30 ± 0.10^{c}
P2	6	7.36 ± 0.11^{c}	7.46 ± 0.05^{c}
P3	6	7.63 ± 0.11^{s}	7.70 ± 0.10^{d}

Information:*Different superscripts indicate that there is a significant difference between treatments (P<0.05); ± Standard Deviation (SD).

Based on Table 3, the hemoglobin levels of tilapia after 30 days of rearing ranged from 6.10 to 7.63 g/dL. The results of the analysis of variance (ANOVA) showed that the addition of feed containing fermented herbs had a significant effect on hemoglobin levels in the blood of *O. niloticus* after 30 days of rearing ($P<0.05$). According to Salasia et al.[25] normal hemoglobin levels in tilapia range from 5.05-8.33 g/dL.

The hemoglobin of tilapia after the challenge test with *A. hydrophila* ranged from 4.26-7.70 g/dL. The highest hemoglobin level was found at a 200 mL/kg dose, which was 7.70 g/dL. Meanwhile, the lowest hemoglobin level was found in the positive control (Kp) without fermented herbs and infected with A. *hydrophila* 4.26 g/dL. The results of the analysis of variance (ANOVA) showed that the addition of feed containing fermented herbs had a significant effect on hemoglobin levels in the blood of tilapia after the 14^{th} day challenge test ($P<0.05$). Treatment of Kn, P_1, P_2, and P_3 in the blood increased, while the treatment of Kp decreased. Decreased hemoglobin levels in Kp due to attacks from *A. hydrophila* can cause death because it infects the entire body of the fish, accompanied by bleeding in the body's internal organs. This bacterium can spread rapidly and cause seed death of up to 90%[26].

The hemoglobin level of tilapia in this study increased after adding herbs to fermented feed; this following the opinion of Wahjuningrum et al.[27] that increased hemoglobin levels in fish can be due to the activity of flavonoids in the content of active plant compounds used in herbs fermented, Flavonoid activity What is in the *K.galanga* L plant used in herbs fermented can improve the work of blood-producing organs so that blood production increases.

Curcuma domestica Val and *K.galanga* L contained in herbs fermented contain protein. Protein is vital as a functional and structural component in all body cells[28]. Protein also plays a role in the transport of nutrients. Nutrients that have been digested must be carried to the body's cells to be utilized. Most of these nutrients are transported by proteins, such as lipoproteins, transporting lipids and lipid-like materials, as well as iron and manganese[29]. In addition to protein, iron is also found in fermented herbs. Iron is an essential element for forming hemoglobin (Hb). Iron functions include transporting, storing, and utilizing oxygen as hemoglobin, myoglobin, or cytochrome. To meet the needs for the formation of hemoglobin,

The number of erythrocytes and hematocrit influences hemoglobin levels. The correlation between hemoglobin and hematocrit is that erythrocytes contain Hb, which binds oxygen and is used for catabolic processes so that energy is produced. The lower the number of erythrocyte cells, the lower the hemoglobin level in the blood[20]. Giving herbs fermented at a dose of 200 mL/kg (P_3) added to feed containing herbs fermented is the optimum dose in improving the health of tilapia, as seen from the high levels of hemoglobin in the P_3 treatment.

Water Quality

Water quality can affect the movement and behavior of tilapia if it is in conditions that do not follow water quality standards. Water quality parameters measured include temperature, pH, dissolved oxygen (DO), and ammonia (NH_3). The average measurement results for each water quality parameter during the study can be seen in Table 4.

Based on Table 4, it can be seen that the results obtained when measuring water quality during the study for each treatment were still categorized as quite good. Water quality is a very important factor in fish farming, because it will determine the results obtained. Some physical factors that become water quality parameters in freshwater fish farming include temperature, pH, DO, and ammonia.

Table 4. Water quality during the study

Parameter	Treatment				
	Kn	Kp	P_1 (150mL/kg)	P_2 (175mL/kg)	P3 (200mL/kg)
Temperature (^{O}C)	27-29	27-28	27-28	28-29	28-29
pH	7-7,1	6,9-7	7-7,2	7,1-7,2	7-1
DO (mg/L)	4.0-4.2	3,1-3,5	3,9-4,2	3,9-4,4	4.0-4.3
NH_3 (mg/L)	0.016-0.023	0.016-0.024	0.016-0.024	0.016-0.023	0.016-0.022

Temperature dramatically influences fish survival, and water temperature during the study is considered suitable for tilapia growth. The optimal temperature range in freshwater fish farming is 28-32˚C[30], whereas according to Gupta & Acosta[31], a suitable temperature range for tilapia cultivation is 25-30˚C, the optimum temperature for tilapia growth is in the range of 27-30°C so that the water temperature in the test study during the study was still relatively safe and could be tolerated by tilapia seeds.

The results of observing the pH during the study show a good range of 6.9-7.2. The pH value of the water is still suitable for use in maintaining tilapia. Kordi[32] stated that a suitable pH for rearing tilapia is 6-8.5, but optimal growth occurs at a pH of 7-8. The pH value that tilapia can tolerate is between 5 and 11.

Dissolved oxygen is one of the parameters that can be used as the primary choice to determine the suitability of water for fish farming [33]. The results of DO measurements (mg/L) during the study were 2.9-3.2 mg/L. According to Effendi[34], the ideal DO level for the growth and development of aquatic organisms being kept is >3 mg/L. This range already meets the oxygen demand for tilapia maintenance. Syawal et al.[21] stated that optimal growth of tilapia requires waters with a minimum oxygen content of 3 mg/L.

Ammonia in water comes from the decomposition process of organic matter, which contains many nitrogen compounds (proteins) from leftover feed and fertilization[35]. The results showed that the total levels during rearing ranged from 0.016–0.024 mg/L, which fish could tolerate at this value[36].

4. CONCLUSION

Based on the research results, it can be concluded that feeding containing fermented herbs affects the appearance of erythrocytes of tilapia challenged with *A.hydrophila*. The best dose of adding herbs fermented to feed is 200 mL/kg (P3), with total erythrocytes of $2.09x10^6$ cells/mm^3, hemoglobin level of 7.70 g/dL, hematocrit level of 37.33%, and showing normal erythrocyte morphology. Water quality during the study: Temperature 27-29˚C, pH 6.9-7.2, DO 3.1-4.4 mg/L, and Ammonia 0.016-0.024 mg/L.

Based on the results of the research that has been carried out, it can be suggested to examine the effect of herbs fermented in improving fish health in different fish and given at a distance (not every day) as seen from hematology.

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