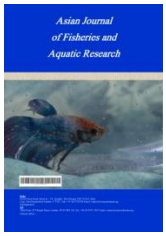

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Effectiveness of Adding Garlic Extract (*Allium sativum*) in Commercial Feed to the Resistance of Nilem Fish (*Osteochillus hasselti*) Infected with *Aeromonas hydrophila* Bacteria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Determining the concentration of garlic extract addition in commercial feeds that are effective for increasing the body's resistance and survival of nilem fish to the attack of *Aeromonas hydrophila* bacteria. This study was carried out at the Aquatic animal physiology laboratory of building 2 and the biotechnology laboratory, faculty of fisheries and marine sciences, Universitas Padjadjaran, between November and December 2021. This study used the Complete Randomized Design (CRD) method with five treatments and three tests. The treatment used is the addition of garlic extract to commercial feed at doses: 0 g / kg of feed (A), 10 g / kg of feed (B), 20 g / kg of feed (C), 30 g / kg of feed (D), and 40 g / kg of feed (E). The treatment period is carried out for 21 days. Furthermore, nilem fish were tested with *Aeromonas hydrophila* bacteria with a density of 10^8 CFU / ml by intramuscular injection. The results showed that the addition of garlic extract to the feed did not produce a noticeable influence on the survival value of fish. However, the administration of garlic extract into the feed of 20 g / kg affects the health of the fish body in the form of a white blood cell count of 12800 cells / mm^3.

Keywords: Nilem carp; Aeromonas hydrophila; extract garlic; immunostimulants.

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1. INTRODUCTION

Nilem carp (*Osteochilus hasselti*) is adored by most people. However, the cultivation of nilem carp is almost abandoned and has not been carried out intensively. The decreased cultivation of nilem carp is caused by the high mortality rate caused by diseases from bacteria and parasites [1].

MAS (Motile Aeromonas Septicemia) or red spot disease caused by the pathogenic bacterium Aeromonas hydrophila. This disease is one of the obstacles that hinder fish cultivation. The percentage of mortality due to *Aeromonas hydrophila* bacteria ranges from 80-100% [2].

One of the disease control attempts is prevention. Immunostimulant is a substance that can increase the body's immune system. The natural substance could be used as an immunostimulant. Garlic is one of the natural immunostimulants.

The use of garlic as the immunostimulant is based on the contained compounds in garlic named allicin. Allicin in garlic can significantly improve the fish's immune system [3].

2. MATERIALS AND METHODS

2.1 Time and Place

This research was carried out at the Aquatic animal physiology laboratory of building 2 and the biotechnology laboratory, faculty of fisheries and marine sciences, Universitas Padjadjaran. Data collection for this research was conducted from November – December 2021.

2.2 Material and Tools

The tools used in this research are aquarium used is 60×29.5×35.5 cm^3 of 15 pieces, aerator, heater, spray bottle, microscope, hemocytometer, and hand counter. The materials used in this study were nilem carp seeds with a size of 7-9 cm, commercial feed PF-1000, aquadest, Hayem's solution, Turk's solution, and garlic.

2.3 Research Procedure

The first thing to do in this research is feed test preparation, and garlic extract was weighed according to each treatment, then the extract was thawed with distilled water. Afterward, the thawed garlic extract was placed through the spray bottle. The next step is spraying the garlic extract into the feed distributed evenly, then drying the feed that has been sprayed with the garlic extract at room temperature. Dried feed mixed with the garlic extract is given to the fish as much as 5% of the fish's body weight and given 2 times a day at 08.00 am and 4.00 pm Indonesian Time Zone. Fish were reared with feed mixed with garlic extract for 21 days. Each aqurium is filled with 15 fish.

After 21 days of rearing, a challenge test with Aeromonas hydrophila with 10^8 CFU/ml density was carried out. The challenge test was carried out by injecting 0.1 ml of bacteria to each fish. Fish were injected intramuscularly [4].

Observations of clinical symptoms were carried out for 14 days after the challenge test. The visible clinical symptoms were damage to the fish's body, feed response, and shock response.

White blood cells number were observed by slashing the caudal vein of the fish until the blood came out. The blood was sucked with a Sahli pipette to 0,5 scale, followed by sucking the Turk's solution to 11 scales, then shaking it until homogeneous. Furthermore, it was dropped on a hemocytometer and observed under a microscope with ten times magnification while counting the number of white blood cells [5].

Red blood cells number were observed by slashing the caudal vein of the fish until the blood came out. The blood was sucked with a Sahli pipette to 0,5 scale, followed by sucking the hayem solution to 101 scales, then shaking it until homogeneous. Furthermore, it was dropped on a hemocytometer and observed under a microscope with ten times magnification while counting the number of red blood cells [5].

2.4 Method

The method used in this research is an experimental method using a completely randomized design (CRD) with five treatments and three replications, i.e., A (Control), B (10g/kg), C (20g/kg), D (30g/kg), and E (40g/kg). The used treatment is the garlic extract used in the mixed feed as the immunostimulant.

The general model of a completely randomized design is the linear model [6] as follows:

$$Y_{ij} = \mu_i + \tau_i + \varepsilon_i \text{ or } Y_{ij} = \mu_i + \varepsilon_i$$

Description:
i = 1,2, ..., t
j = 1, 2,..., r

Y_{ij} = observations on the i treatment and j repetition
μ = general average
τ_i = effect of the i treatment
ε_i = random effect on the i treatment and j repetition

2.5 Data Analysis

An increasing number of white blood cell and red blood cell data of nilem carp were analyzed by the F test, and if there was a difference between the treatments, it was analyzed by Duncan's test with a 95% confidence level. Clinical symptoms, water quality, and survival rate data were analyzed descriptively.

3. RESULTS AND DISCUSSION

3.1 White Blood Cell Number

Based on the results and examination of the health condition of the fish using white blood cells during rearing, it was seen that there were differences after 21 days of rearing and post-challenge test. The performance of white blood cells is shown in Figs. 1 and 2.

The number of white blood cells during the 21 days rearing period with garlic extract feed shows that the white blood cells were in the normal range. The number of white blood cells after adding garlic extract to the feed is shown in Fig. 1 in the range of 86833 – 12800 cells/mm^3, while the average number of white blood cells is 3390 – 14200 cells/mm^3 [7]. The increasing white blood cells can be an indicator of an increasing nonspecific immune system [8].

After the challenge test, the number of white blood cells in each treatment increased, especially in treatment A (control) (Fig. 2). An increase in white blood cells after a challenge test with Aeromonas hydrophila bacteria indicates a response to the body's resistance to disease-causing antigens. The number of white blood cells will increase when the fish is experiencing an infection because white blood cells are an active unit in the body's defense system. The white blood cells have a role in fighting against foreign objects in the body, one of which is bacteria [9].

There was an increase in the number of white blood cells at the B, C, D, and E treatment, which was higher than the control. The reason why in the control, the white blood cells are higher, because the bacterial infections still attack fish so that the body produces more leukocytes to defend itself. In the B, C, D, and E treatments after being treated with garlic extract, there was a higher increase in the number of white blood cells compared to the control, so that when infected with *A. hydrophila* bacteria were able to resist the attack of these bacteria, while in the control treatment the number of white blood cells that existed was unable to resist bacterial attacks, so to fight it by producing more white blood cells. This caused the white blood cell count in treatment A after being challenged to be higher than in other treatments [10]. The primary function of white blood cells is to fight and protect the body from infection by phagocytosing foreign organisms and producing and distributing antibodies throughout the body. Therefore, animals with low white blood cells have a high risk of attack by infectious diseases, while animals with high white blood cells can generate antibodies in the phagocytosis process and have high resistance to disease [11,12].

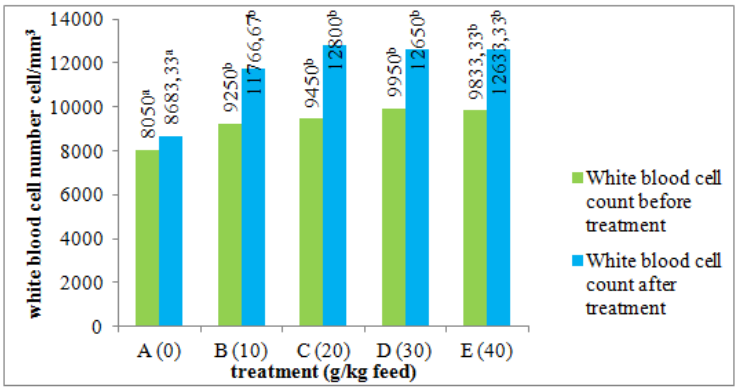

Fig. 1. White blood cell numbers before and after the treatment

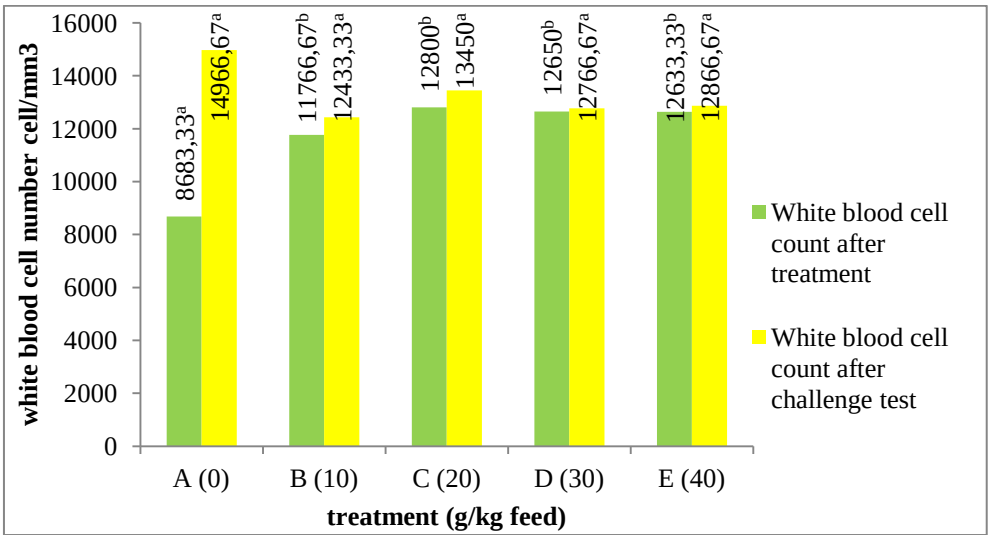

Fig. 2. White blood cell number after the challenge test

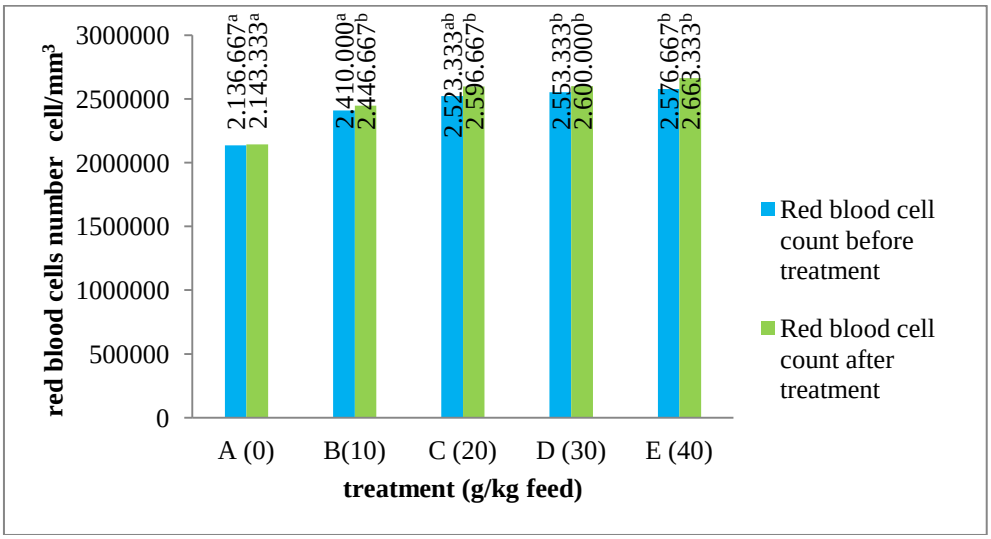

Fig. 3. The number of red blood cells before and after treatment

3.2 Red Blood Cell Number

Based on the results and examination of the health condition of the fish using red blood cells during rearing, it was seen that there was a difference in values after 21 days of rearing and post-challenge testing. The performance of red blood cells is shown in Figs. 3 and 4.

The number of red blood cells in the 21-day rearing period with adding garlic extract to the feed showed that it was in the normal range. The value shown after adding garlic extract to the feed in Fig. 3 is in the range (2.14 – 2.60)x106 cells/mm3, while the normal range of red blood cells for teleost fish is 1.05×106 ± 3.0× 106 cells/mm3 (Irianto 2005). This shows that feeding containing phytopharmaca does not interfere with the health of the test fish [13].

After the challenge test, the number of red blood cells in Fig. 2 seems to have decreased. This happens because the bacterial attack resulted in the rupture of the fish's blood vessels, decreasing the number of red blood cells. This decrease in red blood cells was caused by bleeding after being infected with A. hydrophila bacteria causing the fish to experience anemia [14]. In addition, it is also suspected that red blood cells undergo lysis caused by A. hydrophila bacteria. These bacteria have a toxic, namely hemolysin. Hemolysin in A. hydrophila bacteria can cause red blood cells to undergo lysis Haditomo [15]. While the decrease in the number

of red blood cells of fish given the addition of garlic extract was relatively low, this value indicated that the fish were undergoing healing. This is evidenced by the damage to the fish body that is not too severe. According to Feldberg et al. [16], allicin compounds are antibacterial so that they can fight pathogenic bacteria. In addition to garlic, allicin contains the enzyme gramanium. The enzyme gramanium is a substance that can prevent the destruction of red blood cells, so when the challenge test was carried out on treated nilem fish, there was no significant decrease in the number of red blood cells.

3.3 Macroscopic Clinical Symptoms

3.3.1 Damage to the body of the nilem fish

Clinical symptoms on the fish's body began to appear in the 5th hour after the challenge with Aeromonas hydrophila bacteria. Then during the 24-hour observation after the challenge test, the symptoms that appeared were peeling scales (Fig. 5a), abdominal bulge (Fig. 5b), bleeding (Fig. 5c), and inflammation (Fig. 5d). There is damage to the body of fish infected with Aeromonas hydrophila bacteria in the form of wounds and bleeding caused by extracellular

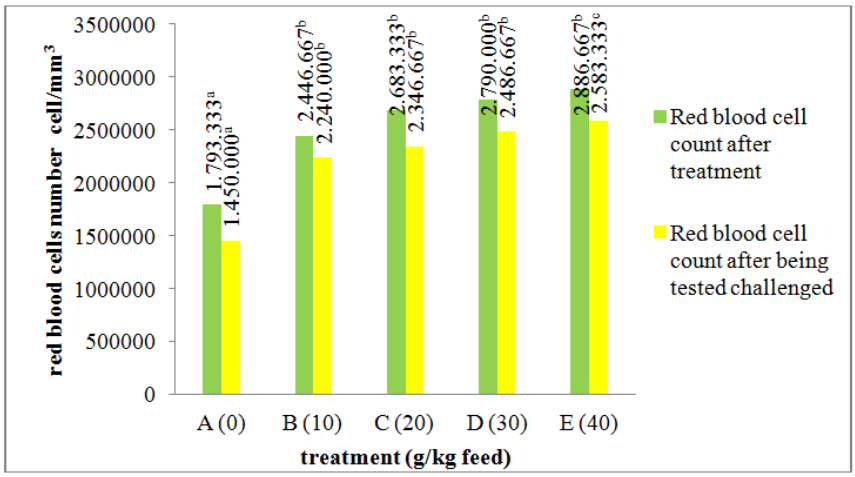

Fig. 4. The number of red blood cells before and after treatment

Table 1. Results of observation of responses to surprises

Days	Treatment														
	A			B			C			D			E		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+
3	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	M	+	+	+	+	+	+	+	+	+	M	M	+	M
7	+	M	+	+	+	+	+	+	+	+	M	M	M	M	M
8	+	M	M	+	+	++	+	++	M	M	M	M	M	M	M
9	+	M	M	+	+	++	+	++	M	M	M	M	M	M	M
10	M	M	M	M	M	++	++	++	M	M	M	M	M	M	M
11	M	M	M	M	M	++	++	++	M	M	M	M	M	M	M
12	M	M	M	M	M	++	++	++	M	M	M	M	M	M	M
13	M	M	M	M	M	++	++	++	M	M	M	M	M	M	M
14	M	M	M	M	M	++	++	++	M	M	M	M	M	M	M

Description: (-) No response; (+) Response to shock is low; (++) Response to shock is normal; (M) Dead

toxins that work in collaboration to damage the tissues in the fish body. Hemocillin produced by these bacteria works to break down and lyse red blood cells [17].

3.3.2 Shock response

Observation of fish response to shock was carried out 14 days after the challenge of A. hydrophila bacteria. The goal is to determine the reflexes of fish movement to stimuli in the form of surprise. One of the clinical symptoms of fish infected with A. hydrophila bacteria is behavioral changes, such as a decreased response to shock. Fish are more often clustered around aeration and become weak. Prayitno [18]. Based on the results of observations, the response of the test fish to shock varied for each treatment (Table 1).

Based on Table 1, it can be seen that on the first day of observation, the test fish in all treatments did not give a surprise response. The decrease in response occurred after the test fish were infected with A. hydrophila bacteria, which fish indicated at the bottom of the aquarium and clustered close to aeration. The fish swam abnormally, namely, swimming sideways.

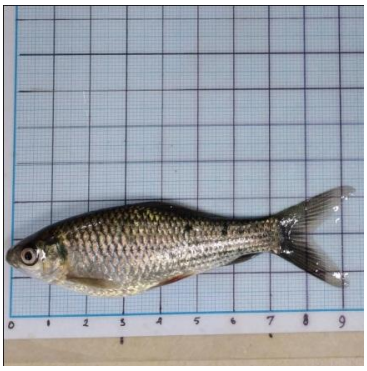

Fig. 5a. Healthy nilem fish

Fig. 5b. peeling scales

Fig. 5c. *dropsy*

Fig. 5d. *hemorrhagic*

Fig. 5e. inflammation

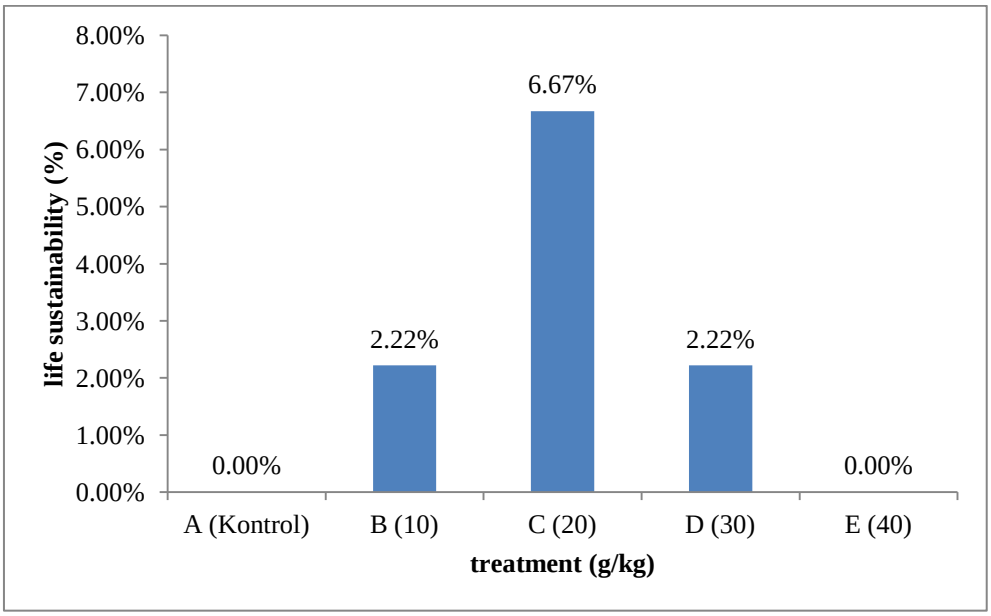

Fig. 6. Graph of nilem fish survival after challenge test

3.4 Life Sustainability

Observation of the survival of the test fish was carried out for 14 days after the challenge test with A. hydrophila bacteria. The results of the analysis of variance showed that the administration of garlic extract in the feed was not significantly different. Because the results showed no significant difference, it could not be continued with Duncan's follow-up test. However, it resulted in varying survival in each treatment (Fig. 6).

Based on Figure 6 nilem fish in treatment A (control) and E (40 g / kg of feed) showed a very low survival of 0%. This happens, because the nilem fish in treatment A was not induced by garlic extract, so when tested with *Aeromonas hydrophila* 10^8 CFU / ml bacteria was unable to fight, indicated by white blood cell levels that continue to increase as a sign that the fish is in a sick condition and there is a decrease in the number of red blood cells, a sign of red blood cell damage due to toxins released by *Aeromonas hydrophila* bacteria. The bacteria have a toxic, namely hemolysine. Hemolysine found in *A. hydrophila* bacteria can cause red blood cells to undergo lysis [15]. The survival value of nilem fish in the E treatment, is likely due to the concentration of garlic extract given too high, so the fish need more energy to carry out the process of decomposition and absorption of incoming garlic, so that it can suppress the body's defense mechanisms, as a result when tested challenge with *Aeromonas hydrophila* bacteria are unable to resist. The higher the dose of garlic given, the higher the emphasis of the defense mechanism and will be the surpressor [19]. Therefore the defense system of the body of the fish becomes less than optimal and even does not work at all. Nilem fish in treatment B, C and D produced a relatively low survival rate, since the highest survival reached only 6.67%. This condition shows that the defense of the nilem fish body formed from the results of garlic extract is not effective against the attack of *Aeromonas hydrophila* bacteria with a density of 10^8 CFU / ml. The low survival of nilem fish in this study is likely not only caused by too high a concentration, as in the E treatment, it is also likely caused by *Aeromonas hydrophila* bacteria that are given too pathogenic for nilem fish.

3.5 Water Quality

Observation of water quality was carried out for 21 days of maintenance period of feeding added with garlic extract. The measured water quality samples included temperature measurements, dissolved oxygen (DO), and pH. Water quality measurement data was used as a supporting parameter during the research. The results of water quality observations during the maintenance period are presented in Table 2.

Table 2. Results of observation of water quality during research

Treatment (mL/kg)	Water quality parameter		
	Temperature (^oC)	DO (mg/L)	pH
A (control)	26 – 27,20	5,70 – 7	6,70 – 6,80
B (10)	25,30 – 27,30	5,60 – 7,10	6,80 – 7
C (20)	27 – 29,50	5,10– 7,20	6,70 – 7,10
D (30)	26,20 – 27,40	5,40 – 7,90	6,80 – 7
E (40)	26,90 – 27,60	5,60 – 7	5,60 – 6
Quality Standard Parameters *	28 – 32^oC	$\geq$ 5 mg/L	6,8 – 8,5

** Government Regulation No. 82 (2001) for freshwater fish farming activities (class II)*

Table 2 shows that the results of water quality measurements in the form of dissolved oxygen (DO) have an average value in the range of 5 – 7 mg/L, pH ranges from 6 – 7, and an average temperature range of 25 – 29°C. Based on measurements and observations of water quality during the study, adding garlic extract to feed did not adversely affect water quality. This can be seen from the value of the water quality range, which does not exceed the quality standard limits according to PP No. 82 of 2001 [20].

4. CONCLUSION

Based on the research that has been carried out, it can be concluded that:

1. Addition of garlic extract to commercial feed is practical to increase fish's immune system in the form of white blood cells, which is 12800 cells/mm^3.
2. After the challenge test, the fish experienced high mortality, in this case, due to insufficient white blood cells to fight the bacterial attack. The energy generated from adding garlic extract was still used for adaptation, so the survival value produced was only 6,67%.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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