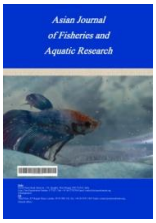

Asian Journal of Fisheries and Aquatic Research

7(4): 22-29, 2020; Article no.AJFAR.58674
ISSN: 2582-3760

Potassium Diformateas Feed Additive to Enhance the Performance of Immune System on Giant Gourami Fingerlings (*Osphronemus goramy* Lacepede, 1801)

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Authors' contributions

This work was carried out in collaboration among all authors. Author AY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SR and Rosidah managed the analyses of the study. Author IBBS managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI:10.9734/AJFAR/2020/v7i430126
Editor(s):
(1) Dr. Matheus Ramalho de Lima, Federal University of South of Bahia, Brazil.
Reviewers:
(1) Muhammad Abdurrahman Munir, National University of Malaysia, Malaysia.
(2) Beena Somanath, Manonmaniam Sundaranar University, India.
(3) Sushree Sangeeta Dey, Icar-Central Institute of Fisheries Education, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/58674

Original Research Article

Received 02 May 2020
Accepted 07 July 2020
Published 23 July 2020

ABSTRACT

This research aims to determine the optimum dose of potassium diformate (KDF) which is added to commercial feed to improve the immune performances of giant gourami fingerlings. The research was conducted in October – December 2019 in Aquaculture Laboratories and Biotechnology Laboratory of the Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. The method used in this research is Completely Randomized Design (CRD) with four treatments and four replications. The treatments are addition of potassium diformate in feed with a dose of 0% (A), 0,3% KDF (B), 0,5% KDF (C) and 0,8% KDF (D).The giant gouramimaintained for 40 days. The observed parameters are total leukocytescount, total erythrocytescount, differential leukocytes, and water quality. The data of total leukocytes, total erythrocytes were analyzed using ANOVA and followed by Duncan's Multiple Range Test, while differential leukocytes and water quality are analyzed descriptively. The results showed that the addition of 0.3% potassium

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diformate is the most effective to improve the immune performances of giant gourami fingerlings. During 40 days of treatments, total leukocytes count increased 25% from6.55×10^4 cells/mm^3 to 8.74×10^4 cells/mm^3, total erythrocytes countincreased 10.03% from 1.28 ×10^6 cells/mm^3 to 1.43×10^6 cells/mm^3. Potassium diformate treatment 0.3% was an effective dose to stimulate differential leukocytes of gourami fingerlings are triggered to enhance the immune system.

Keywords: Aeromonas hydrophila; giant gourami fingerlings; immune response; potassium diformate.

1. INTRODUCTION

Giant gourami fish (*Osphronemus goramy* Lacepède, 1801) is a type of potential fish in Indonesia [1]. Giant gourami has long been cultivated commercially but still facing obstacles in terms of cultivation is slow growth and low life resistance [2]. Low body resistance caused by disease attacks. One of the pathogenic bacteria that cause death in gourami is *Aeromonas hydrophila* [3]. Antibiotics are an option commonly used by farmers to overcome these problems. However, the negative effects of antibiotics can lead to bacterial resistance to such antibiotics. Resistance can arise through DNA mutation or by horizontal transfer between bacteria through conjugation resulting in the transfer of DNA between bacteria [4].

Restriction or ban on antibiotic administration in aquaculture encourages the development of environment-friendly feed additives as immunostimulants [5]. Considering the negative impacts raised by prophylactic and therapeutic use of antibiotics in aquaculture, the administration of dietary immunostimulant has been suggested as an alternative to antimicrobial agents [6]. One strategy that can be used to improve the health of aquaculture fish is the addition of organic acids (acidifier) as a feed additives. The use of acidifier-supplemented feeds has no negative effects on the farming environment, and then will lead to the formulation of eco-friendly aquafeeds [5] Organic acids can reduce the impact of bacterial infections, thereby preventing disease and therefore provide a higher survival rate [7] The potassium diformate as an organic acid that is already used widely. Potassium diformate is registered as a non-antibiotic growth promoter with the purpose to replace in-feed antibiotics ensuring safer products to the consumers, potassium diformate can be used as an alternative growth driver and health [8]. Potassium diformate can acidify the cytoplasm of gram-negative bacteria, such as *Aeromonas hydrophila* and *Vibrio anguillarum* which ultimately results in cell death [9].

This research aims to determine the effect of giving potassium diformate and determine the dose of adding potassium diformate in the commercial feed which is the most effective to increase the immunity of gourami fingerlings to the bacterial attack of *A. hydrophila.*

2. MATERIALS AND METHODS

The giant gourami fingerlings size 6-8 cm was procured from the branch of Marine Service and Fisheries of South Tasikmalaya, West Java. The 16 aquariums measuring 40 cm × 30 cm × 40 cm which was used as maintenance containers. Giant gourami fingerlings were kept in an aquarium as a rearing container with a density of 10 fish/aquarium. The test fish was fed with a mixture of potassium diformate for 40 days [10], with dosages according to the treatment control (non-KDF), 0.3% KDF, 0.5% KDF, and 0.8% KDF. The amount of feed given as much as 3% of body weight. The frequency of feeding was done twice daily at 08.00 West Indonesian Time and 16.00 West Indonesian Time. After the test challenge, the feed was given only commercial feed non-the added potassium diformate. The water was replaced by 10% to maintain water quality while the siphoning was done. Water quality parameters were recorded during the maintenance period in once a week. The observed water quality parameters include temperature, pH, and dissolved oxygen.

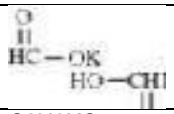
The experimental design carried out in this research was a Completely Randomized Design (CRD) consisting of four treatments and four replications, namely:

Treatment A: addition of potassium diformate as much as 0%.
Treatment B: addition of potassium diformate as much as 0.3%.
Treatment C: addition of potassium diformate as much as 0.5%.
Treatment D: addition of potassium diformate as much as 0.8%.

2.1 Mixing of Potassium Diformate in Feed

The feed used in this research was commercial feed in the form of floating pellets with a protein content of 31-33%, 4-6% fat, 3-5% fiber and 9-10% water content. The commercial feed was weighed as much as 100 grams, and potassium diformate was weighed according to the each treatment. Furthermore, potassium diformate was added into the feed and stirred it until homogeneous. The feed that has been mixed with water was 10% of the total feed and dried with aerated.

Physical and chemical properties of potassium diformate [11].

Structure	
Molecular formula	C2H3KO4
Typical Content	Potassium Diformate ≥ 97%
Loss on drying	≤ 3%
Appearance	White crystalline or slightly yellowish granular
Molecular weight	136.16
Solubility	Easily soluble in the water.

2.2 Cultures Isolates of *Aeromonas hydrophila* Bacteria

Isolate bacteria *A. hydrophila* derived from the Installation of Research and Development of Depok Fish Disease Control. The isolates of *Aeromonas hydrophila* were inoculated on NA (*Nutrient Agar*) media using the streaking bacterial plate to obtain a single colony, then incubated at 30°C for 24 hours. The results of the bacteria that were grown was taken by using one needle with as much as 1 ose and dissolved with NB (*Nutrient Broth*) media, then incubated for 24 hours in incubator shakerat a temperature of 37°C at a speed of 150 rpm. The results of the liquid culture was taken into 2 ml cuvette then checked the density using a Spectrophotometer Agilent Carry 60 to get a density of 10^8 CFU/ml.

2.3 Challenge Test

Infection of *A. hydrophila* against giant gourami fingerlings was done at intraperitoneally section as much as 0.1 mL/individual with a density of 10^8 cfu/mL. The infection of *A. hydrophila* was done only one time at the beginning of the test challenge. After being challenged with *A. hydrophila,* it was observed for 14 days.

2.4 Observation of Blood Cells

The observation of leukocytes, erythrocytes, and differential leukocytes performed six times, namely before the treatment of KDF, after the treatment of KDF 40 days, and after the test challenge the bacteria *A. hydrophila* on day 3, day 7, day 10, and day 14.The procedure of taking leukocytes and erythrocytes according to the [12] procedures, whereas the differential leukocyte cells of lymphocytes, monocytes, neutrophils are taken based on the [13] procedure.

2.5 Data Analysis

The leukocytes count, erythrocytes count, and survival rate was analyzed with the F test and Duncan's advanced test at a 95% confidence level.Moreover, the data differential leukocytes and water quality in this research was descriptive analysis.

3. RESULTS AND DISCUSSION

3.1 Leukocytes

The addition of KDF indicated an influence on leukocytes (Fig. 1). Based on the variation analysis after the KDF treatment for 40 days, it showed that the KDF treatment was 0.3% significantly different with the treatment non-KDF, KDF 0.5%,and KDF 0.8%. Potassium diformate treatment of 0.3% experienced elevated leukocytes with an increase of 25% from the value of 6.55×10^4 cells/mm^3 to 8.74×10^4 cells/mm^3. Post-Test challenge the number of leukocytes increased in all treatments. The 3rd day of post-trial challenge (CT-3) indicated that the treatment of KDF 0.3% significantly different with the treatment non-KDF, but it was non-significantly different with the treatment of KDF 0.5% and KDF 0.8%. Post-trial challenge day 7 to day 14 indicated that the treatment of KDF 0.8% was non-significant different with the control treatment (non-KDF).

Potassium diformate provided a positive effect on the immune response. Organic acids (KDF) give a eubiotic effect to the intestines thereby increasing the density of LAB which can inhibitthe attachment and invasion of pathogenic bacteria [9], thus supporting the natural body

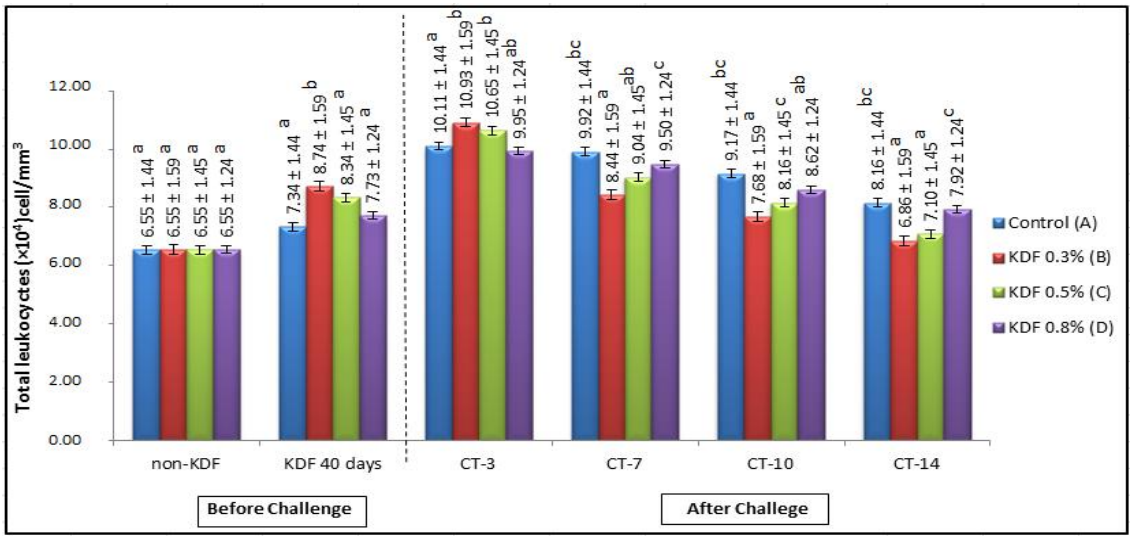


Fig. 1. Total leukocytes count

Description:Non-KDF: before treatment KDF, CT-7: Seventh day after challenge, KDF: KDF 40 Day, CT-10: Tenth day after challenge, CT-3: Third day after Challenge, CT-14: fourteenth day after challenge

defense mechanisms in fish which plays an important role in the activation of the immune response against diseases. An increase in the count of leukocytes after 40 days of administration of KDF in the fish occurs because giant gourami fingerlings has an induction process of an optimal immune system due to the influence of 0.3% KDF that entered the body as Immunostimulant. Immunostimulatory mechanisms will stimulate the immune system of the fish by increasing the number of leukocytes, which can eat foreign bodies in the body and form a body defense system to ward off pathogenic bacteria [14]. Day 3 post-trial challenge (Test Challenge-3) shows an increase in the number of leukocytes occurs due to increased attack of *A. hydrophila* bacteria. According to [15] the average count of leukocytes of fish increases along with increasing pathogenic bacterial infections. The highest value change was in the treatment of non-KDF which indicates that the number of leukocytes was not enough to fight the bacterial attack. The 0.3% KDF treatment showed the lowest value changes as the number of leukocytes available was sufficient to counter *A. hydrophila* bacterial attack. During the post-recovery period of 7 to 14 days challenge, indicating that the treatment of KDF 0.3% showed a lower number of leukocytes that has begun to return to normal conditions. It showed that the test fish has begun to withstand the attack of *A. hydrophila* bacteria**.** Potassium diformate treatment showed better results compared to treatment non-KDF, but the addition with high doses (0.8% KDF) was less effective in gourami fingerlings. In fighting bacterial attacks, the active ingredient in KDF was excessively less effective in increasing fish immunity [16] because the excess of double salt in KDF can interrupt the fish osmoregulation system.

3.2 Erythrocytes

The addition of KDF indicated an influence on erythrocytes (Fig. 2). Based on the variation analysis after the KDF treatment for 40 days, showed that the KDF treatment was 0.3% significantly different with the treatment non-KDF, KDF 0.5%, and KDF 0.8%. The highest average amount after the KDF treatment was found in the KDF 0.3% treatment of 1.43 × 10^6 cells/mm^3 and the lowest on treatment non-KDF of 1.27 × 10^6 cells/mm^3. KDF treatment of 0.3% showed the highest increase of 10.03% from 1,28 × 10^6cells/mm^3 to 1,43 × 10^6 cells/m^3. During The Post-Test challenge with bacteria*A. hydrophila*,all treatments showed a decrease in the number of erythrocytes. The Post-trial of the 3rd day (CT-3) indicate that the treatment of KDF 0.3% significantly different with the treatment non-KDF and KDF 0.8%, but it was non-significantly different with the treatment of KDF 0.5%**.**The Post-trial challenge Day 7 to day 14 indicated that the treatment of KDF 0.8% was non-significantly different with the control treatment (non-KDF).

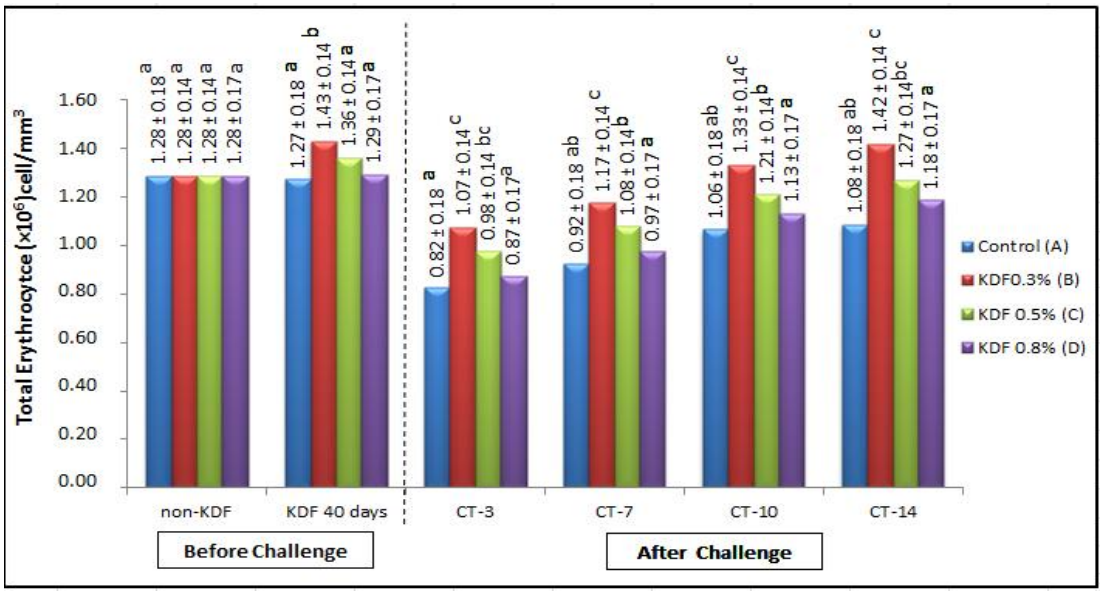


Fig. 2. Total erythrocytes count

Description:Non-KDF: before treatment KDF,CT-7: Seventh day after challenge, KDF: KDF 40 Day, CT-10: Tenth day after challenge, CT-3: Third day after Challenge, CT-14: Fourteenth day after challenge

The increased erythrocyte value after 40 days of administration of KDF in the feed occurs due to the effect of optimal dosing of KDF and the increase of fish age growth. The Post-Test challenge was the number of erythrocytes decreases. According to [17] erythrocytes have decreased allegedly due to the occurrence of incoming bacterial phagocytosis. The 0.3% KDF treatment showed the lowest value, it meant that KDF administration at optimal dosages may affect fish body resistance. The treatment non-KDF, KDF 0.5%, and KDF 0.8% showed low erythrocytes value that was below the normal fish condition range whichinfer that the fish was in a sick state due to bacterial attack and had anemia. The amount of erythrocytes has increased during the recovery period on all the treatments in the 7th to the 14th day.However, on the 7th day of erythrocytes in the treatment of non-KDF and KDF 0.8% still have not returned to the normal range of fish conditions, indicating that the fish is still suffering from anemia. The decrease in erythrocytes indicated the presence of anemia in the kidney organ of fish signed with bleeding [18]. Potassium diformate treatment 0.3% and KDF 0.5% showed the highest value of the rate, it showed that gourami already have a body resistance that begin to recover and produce more erythrocyte cells to replace the cells of erythrocytes that have been dilated by a hemolysin enzyme *A. hydrophila.*

3.3 Differential Leukocytes

The results of differential leukocytesshowed that there is the presence of varying values between treatments, it can be seen based on the value between before and after the challenge test. According to [19] that the stages of most cells number was lymphocytes, the neutrophil cell and the monocyte cell.

The differential leukocytes include the cell type lymphocytes, monocytes, neutrophils [20]. Blood screening is performed to see an increased pattern of immune response by calculating the total leukocytes and differential leukocytes in the blood [21]. The amount of lymphocytes in the blood decreases post-test challenge with *A. hydrophila* because of the activity of resistance from leukocytes to *A. hydrophila* (Fig. 3).The normal range of fish lymphocytes ranges from 74-86% [22]. The treatment non-KDF and 0.8% of KDF showed a value that was below the normal range of lymphocytes, treatment non-KDF of 71.25% and a 0.8% KDF treatment of 72.50% which meant fish in a sick state due to bacterial attack. The 0.3% KDF and 0.5% KDF treatment showed values that were still in the normal range. The presence of increasing intensity of infection by certain pathogens will be trigger the need for leukocytes and increase the need to result in a reduction in the cell number of the agent provider of the body immune cells

lymphocytes [23]. The percentage of post-test monocytes has increased in each treatment (Fig. 4). An increase in the number of monocyte cells occurs due to the presence of bacterial infections in fish, so that monocyte cells will develop into macrophages to the place of infection to eliminate foreign bodies and phagocytosis. The highest percentage of monocytes in the treatment of 0.3% KDF with a value of 10.50%, the increase indicated the presence of non-specific defense activity of fish in the form of increased blood monocytes to eliminate foreign agents. The percentage of post-test neutrophils has increased in each treatment (Fig. 5). The increased neutrophil cell count in all treatments indicated the presence of neutrophil cell activity in reaching and attacking antigens (foreign particles) entering into the body indicating the occurrence of phagocytosis. The differences in neutrophil percentage changed in each treatment were suspected due to a blood balance response to the increased proportion of other types of leukocytes cells namely lymphocytes and monocytes. The 0.3% KDF treatment showed the lowest percentage of 14.50%, but it has a higher increase compared to treatment non-KDF, it was suspected because the active ingredient in the KDF can improve response quickly responding to infectious diseases.

Based on the differential results of leukocytes, suggests that the KDF treatment can stimulate the immune system better than non-KDF, it is shown with higher percentage of lymphocytes (as a defense of the body-forming antibodies) and monocytes (as antigen phagocyte cells) on the treatment of KDF. Potassium diformate treatment 0.3% was an effective dose to stimulate differential leukocytes of gourami to enhance the immune system. Resistance to disease increases with the KDF of feed in a dose-dependent manner [9]. It seemed that adding of KDF on feed, has led to the activation of some blood parameters in giant gourami fingerlings.

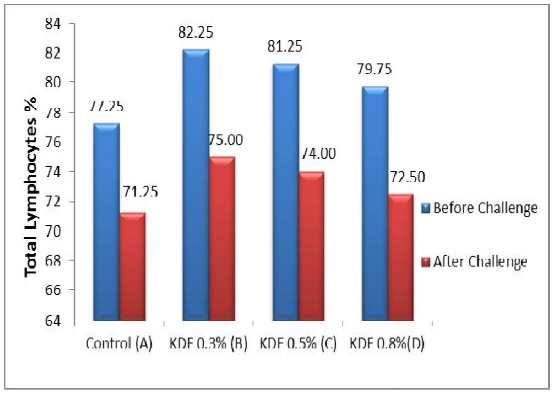


Fig. 3. Total lymphocytes

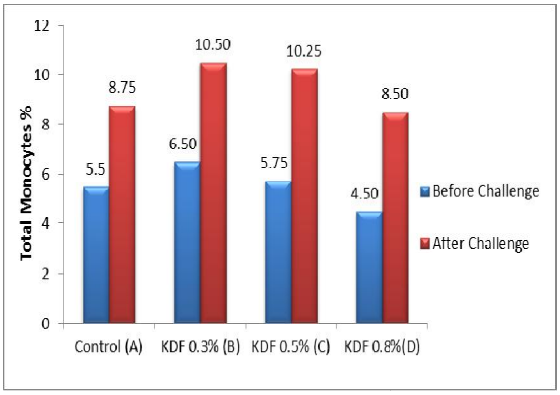


Fig. 4. Total monocytes

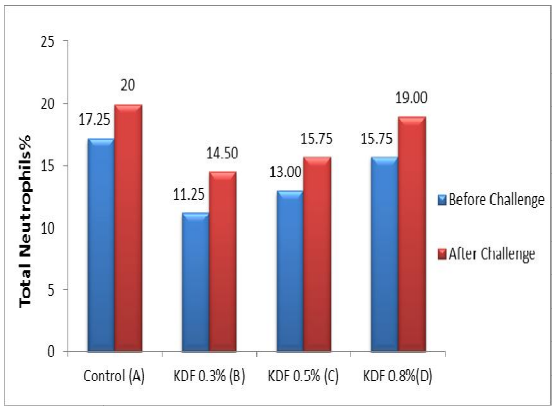


Fig. 5. Total neutrophils

Table 1. Water quality

Treatment	Water quality parameters		
	Temperature (°C)	pH	DO (mg/l)
A	26,75-28,00	6.66-7.11	6.68-6.88
B	27,00-28,00	6.71-7.09	6,80-6.95
C	27,00-28,25	6.78-7.12	6,70-6.85
D	27,00-28,25	6.72-7.09	6.68-6.83
Optimal (SNI 2000)	25-30 (SNI)	6.5-8.5 (SNI)	> 4 (SNI)

3.4 Water Quality

Water quality during the maintenance period was adjusted to the Indonesian National Standard (SNI 2000) of gourami.

Based on the results of water quality (Table 1) observations during research showed that KDF was not adversely affect water quality, it can be seen from the value of water quality range that does not differ considerably from treatment non-KDF. Potassium diformate (Formi) is odorless, low-corrosive and easy to handle [7]. The use of acidifier-supplemented feeds has no negative effects on the farming environment [5]. Water quality during the maintenance period includes the optimal range by Indonesian National Standard (SNI 2000) of gourami, becauseit wasalways carried out water change and siphoning to maintain water quality on the maintenance media.

4. CONCLUSION

The results showed that the addition of KDF provide a good effect on increasing the immune response of gourami, but the administration with high doses exceeding the optimum dose becomes less effective. The treatment 0.3% KDF was an effective dose for the gourami fingerlings, it was shown with no occurrence of anemia in the post-test fish challenge. The addition of KDF can affect the immune response through an increased percentage of leukocytes, erythrocytes, lymphocytes, and monocytes that provide a good effect on fish health.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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