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Isolation and Characterization of Arylamidase-Producing *Pseudomonas Alcaligenes* from the Water of Al-Asfar Lake, Al Ahsa Oasis, Saudi Arabia

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Abstract

Al-Asfar Lake is an important shallow lake that suffered greatly from anthropogenic eutrophication; therefore, it received considerable interest for economic and environmental purposes. Therefore, the current study aimed to investigate the presence of P. alcaligenes based on culture-dependent methods and detect this enzyme activity by the VITEK biochemical tests. The water sample was collected from the lake, serially diluted through sterile saline, and then inoculated and spread onto CHROMagar media and Mueller Hinton agar media. Then purified by the single selection of all different types of colonies by streaking to obtain well-isolated colonies The P. alcaligenes and the enzyme has been successfully identified with a probability of 95% by VITEK 2® COMPACT automated system after determining the Gram stain characteristic for the isolate under a microscope This study proved the existence of P. alcaligenes species that have never been reported previously in Al-Asfar Lake. Therefore, P.alcaligenes species can be an ideal candidate to take advantage of in pollutant biodegradation and to be invested in bioremediation applications to enhance environmental sustainability, in the future.

Keywords: *Pseudomonas alcaligenes;* Al-asfar lake; Isolation; Characterization.

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1. Introduction

Wetland ecosystems are regarded as the most significant ecosystem in the world due to their pivotal role in protecting the ecological balance and possessing various functions, such as biodiversity conservation and climate regulation, in addition, due to their consideration as an unrivaled habitat and shelter for many wild animals and plants [1, 2].

Lakes are a biodiverse environment where bacteria are deemed to be the most valuable component due to their extraordinary metabolic activities in comparison with any other group of organisms in the lake community [3, 4]. Bacteria represent fundamental roles in degradation processes and decomposition of organic matter as well as nutrient cycling [5, 6]. Furthermore, play a crucial role in human subsistence and economics including drinking water supply, food, recreation, and tourism [7-9]

Al-Asfar Lake (yellow lake) is an important shallow wetland lake located in the world's largest oasis about 70 km to the west of the Arabian Gulf [10, 11]. The lake is an enormous man-made lake that suffered greatly from anthropogenic eutrophication created by the drainage of the earthen agriculture and livestock waste in addition to treated and untreated sewage water through a water network system that was built in 1971 [12]

The *Pseudomonas* genus was described in 1894 [13]. The group of this genus is considered one of the most significant and ecologically diverse groups of bacteria [14]. *Pseudomonas* members are ubiquitous and found in big numbers in all kinds of environments that have been isolated from plant and plant rhizosphere, soil, sediment, deserts, fungi, and water [13, 14]. Furthermore, *Pseudomonas* species form a friendly association with animals and plants. This global distribution proposes a remarkable grade of genetic and physiological adaptability for this genus. In addition, strains of this genus own an extraordinary capability to degrade a vast range of substrates such as halogenated derivatives, aromatic compounds, and stubborn organic residues [14]. *Pseudomonas alcaligenes* is a gram-negative, aerobic rod-shaped widespread inhabitant bacterium of water and soil belonging to the bacterial family *Pseudomonadaceae* which can be exploited beneficially in many fields as reported in many studies and has scarcely been confirmed as a human pathogen [15-17].

Several studies confirmed the significance of the bacterial arylamidase enzyme in that has a great role in the mineralization of nitrogen in soil by accelerating the hydrolysis of N-terminal amino acids from arylamides, peptides, or amides [18, 19]. The potency of the use of *P. alcaligenes* in the removal of toxic aromatic hydrocarbons from contaminated soil has been reported in many studies [20]. While another study in 2006 pointed to the potential ability of *P. alcaligenes* isolated from kallar grass in nitrogen fixation and promoting the growth of rice plants [21]. In China in 2015, a research study implemented the isolation of the *P. Alcaligenes* strain from hospital-polluted water that was able to degrade dexamethasone [22]. A study in 2021 conducted on the Medellin River in Colombia which receives metal-contaminated industrial waste, able to isolate *P. alcaligenes* that own a high capability of tolerating lead by biosorption and exopolysaccharide production and thus highlighted the possibility of using this genus in bioremediation of heavy metals [23].

However, none of any previous studies have addressed the isolation and identification of the *Pseudomonas alcaligenes* species from Al-Asfar Lake. Therefore, this research aimed to investigate the presence of *P. alcaligenes* in the lake based on culture-dependent methods to be invested in bioremediation applications to enhance environmental sustainability.

2. Material and Methods

2.1. Study Area

The lake is about 13 km east of Al-Ahsa and extends between Latitudes 25^0 05ʾ and 25^0 40ʾ north and between Longitudes 49^0 10ʾ and 49^0 55ʾ east and rises about 109 m above sea level, Located about 60 km inland from the coast of the Arabian Gulf (Fig.1). It has vast expanses of open water sand dunes making it somewhat challenging to reach. There is a widespread growth of common reed occurs around the lake, which is also known as *Phragmites australis* (Fig.2).

Figure-1. Map of the Al-Asfar Lake study area in Al Ahsa Oasis, Saudi Arabia

Figure-2. An image shows the expanses of open water, sand dunes, and vegetation pattern around the Al- Asfar Lake

2.2. Samples Collection and Physiochemical Characteristics Measurements

Water samples have been collected during the summer period on August 4, 2022, from the lake (Fig.3). All samples were labeled and abbreviated with the following code (WAT). Each collected sample has been retained in a clean and sterilized container and then was cooled and preserved immediately in an icebox at 4°C to be transferred into the laboratory under refrigerated conditions for further analysis.

Electrical conductivity (EC) and pH were measured at the study site. The pH values of samples were measured directly by pH Meter (sensION^{+} PH31, HACH) while the Electrical conductivity was measured by a conductivity Meter (sensION^{+} EC71, HACH). The readings were calculated as mean values. Statistical analysis was implemented to determine whether there was a significant difference in pH and EC values of the water samples of the lake. The statistical significance of the differences was determined using t-tests. (Table 1).

Figure-3. The sampling location of water from Al-Asfar Lake site.

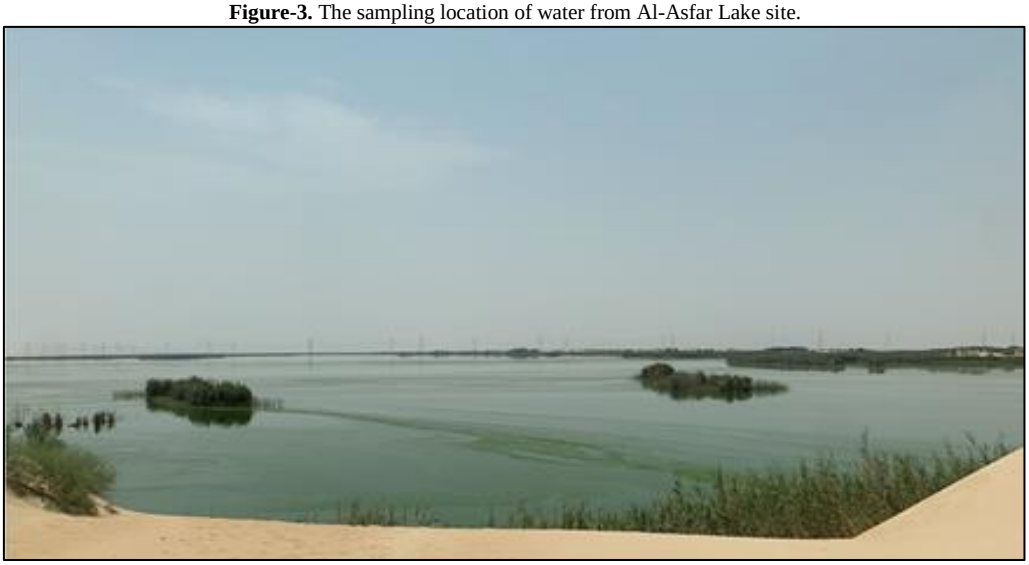


Table-1. Location from the site of collection of water samples from Al-Asfar Lake physiochemical charsraristic

Sample code	Longitude	Latitude	pH	EC (mmohs)
WAT	49°48'00.1"E	25°32'10.5"N	7.6	11.6

3. Isolation of Bacteria

3.1. Media Preparation

Different kinds of media have been prepared for isolation such as Mueller Hinton agar media and the CHROMagar™ Orientation media that facilitate the recognition and differentiation of the bacterial colonies based on color. All medias were heated on a hot plate with a magnetic stirrer until boiling then sterilized in an autoclave at 121 degrees C for 15 minutes.

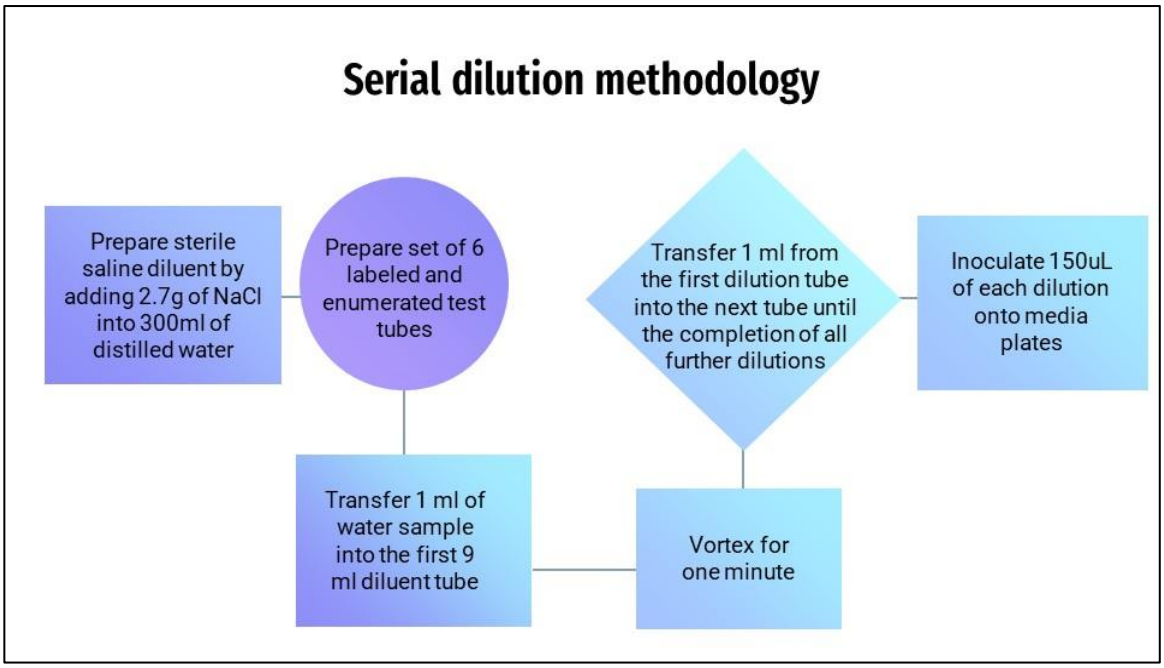
3.2. Serial Dilution Method

All samples were diluted through 0.9% sterile saline diluent prepared by adding 2.7 g of NaCl into 300 ml of DW. A set of six capped glass test tubes have been labeled and numerated for all waters samples as follows: WAT (10^{-1}), WAT (10^{-2}), WAT (10^{-3}), WAT (10^{-4}), WAT(10^{-5}), WAT(10^{-6}). 1 ml the water sample was transferred into the first 9 ml diluent tubes WAT (10^{-1}) and then mixed by using a vortex for one minute. After that, transfer 1 ml from first sample dilution tube (10^{-1}) into the next tube until the completion of all further dilutions.

All samples were diluted through 0.9% sterile saline diluent prepared by adding 2.7 g of NaCl into 300 ml of DW. A set of six capped glass test tubes have been labeled and numerated for all waters samples as follows: WAT (10-1), WAT (10-2), WAT (10-3), WAT (10-4), WAT(10-5), WAT(10-6). 1 ml the water sample was transferred into the first 9 ml diluent tubes WAT (10-1) and then mixed by using a vortex for one minute. After that, transfer 1 ml from first sample dilution tube (10-1) into the next tube until the completion of all further dilutions.

After performing the dilution, inoculate 150uL of each dilution onto the media plates by spreading the diluted sample with a sterilized L-shaped rod. Then, incubate all plates at 38 °C for 24–72 (Fig.4).

Figure-4. Flowchart diagram explaining the methodology of the serial dilution steps



3.3. Purification of Bacterial Culture

Obtaining pure bacterial culture is a must for successful identification. Therefore, a single selection of all different types of colonies has been performed under sterilized conditions by using a sterile disposable loop and streaking out the inoculant on different kinds of media plates to get single pure colonies cultures. Then all the streaked plates were incubated at 38 °C for 24–72.

A subculture procedure has been performed almost three times under the same conditions to maintain the viability of bacterial cells by transferring them into fresh nutritive media to be suitable for the next identification step.

3.4. Preservation of Bacterial Isolates

For preservation, all the pure obtained species have been inoculated into coded and enumerated Tryptic Soy Agar slant and Tryptic Soy broth tubes stored with glycerol and then kept in a freezer immediately.

4. Characterization of Bacteria

4.1. Gram Stain Characterization

The bacterial isolates have been characterized by Gram staining reaction test by using a Gram-staining kit following the instructions of the manufacturer. The gram stain begins by adding one drop of sterile saline onto the center of the slide then takes a sterile loop and touches the edge of an isolated colony on the media plate then place the loop into the drop on the slide and mixes to distribute the cells evenly then heat it on flame carefully to dray before using the stains. Place the slide on a staining rack then apply the crystal violet stain for one minute then gently wash it with tap water after that apply the Gram's iodine stain for one minute and then wash it with tap water next add a drop by drop the 95% alcohols until becomes almost clear then rinse it with tap water then add the safranin stain for 45 seconds then wash with tap water and leave it to dry before the microscopic examination.

5. Identification of Bacteria

5.1. VITEK 2® Compact Identification

The VITEK 2® COMPACT system (BioMérieux) is an automated bacterial identification system that uses fluorescence-based technology. After knowing the isolate gram stain characteristic. The isolate was identified through this system by using a gram-negative reagent card. The reagent card has 64 wells that contain different substrates to measure various metabolic activities.

Before utilizing the machine for identification, an isolate suspension should be prepared as follows:

Pour 3 ml of saline into a sterilized tube. Then with a sterile applicator stick, an amount of a bacterial colony is picked to be transferred into the tube and stirred until the inoculant is dissolved. After that, the optical density of the suspension will be measured by DensiCHEK. The acceptable turbidity range for the inoculum suspension must be between 0.5-0.63.

After that, the suspension is placed into a special rack and the identification testing card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. After that, the racks are placed into a vacuum chamber station to transfer the suspension into micro-channels that fill all the wells. **Results:**

5.2. Measurements of Physiochemical Characteristics Results

The obtained data shows that all water samples have neutral pH with mean values ranging between 7.5 and 7.6. For Electrical conductivity measurements, and the EC mean values for all samples ranged was 11.6 (Table.1)

5.3. Gram Staining Characterization and VITEK 2® Compact Identification Result

The *Pseudomonas alcaligenes* species has been successfully identified with a probability of 95% by the VITEK 2® COMPACT automated system after determining the Gram stain characteristic for the isolate under a microscope that has been isolated from the Lake Mueller Hinton agar media and on the chromogenic culture medium that is intended to use for qualitative direct detection. This genus has been characterized as rod-shaped, gram-negative bacteria, and the colonies showed a greenish-blue pigmentation appearance in the media plate (Fig.5).

Figure-5. (A) Electron microscopy photo demonstrating the morphology of *P. alcaligenes*, **(B)** Photo of *P.alcaligenes* isolated onto CHROMagar

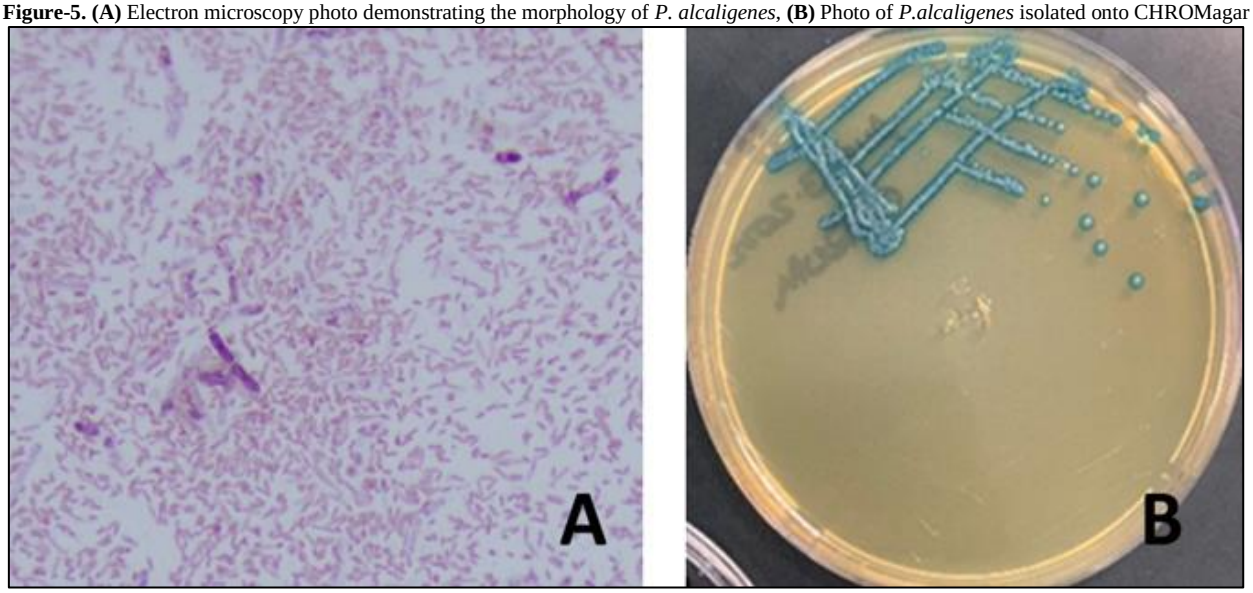


Table-2. The morphological characteristic of *P. alcaligenes* colonies.

Colony color	Margin	Elevation	Texture	Shape
greenish blue	Entire (even)	convex	moist	Circular

Biochemical tests by VITEK are conducted to determine the type of bacteria isolates. The Biochemical test identification for *P. alcaligenes* indicated a positive result for L-Proline ARYLAMIDASE (ProA), L-MALTASE assimilation (lMLTa), Tryosine ARYLAMIDASE (TyrA), COUMARATE (CMT), and L-LACTATE assimilation (lLATa). Examination by VITEK 2® COMPACT can be seen in (Table 3).

The detected activity of ARYLAMIDASE enzyme by the VITEK has been reported in several studies as a significant bioindicator for pollutants that has a great role in the mineralization of nitrogen in soil by accelerating the hydrolysis of N-terminal amino acids from arylamides, peptides, or amides.

Table-3. Biochemical test results for *P.alcaligenes* using VITEK 2® COMPACT automated system

Well	Test	Reaction
2	Ala-Phe-Pro-ARYLAMIDASE	-
3	ADONITOL	-
4	L-Pyrrolydonyl- ARYLAMIDASE	-
5	L-ARABITOL	-
7	D-CELLOBIOSE	-
9	BETA-GALACTOSIDASE	-
10	H2S PRODUCTION	-
11	BETA-N-ACETYL GLUCOSAMINIDASE	-
12	Glutamyl Arylamidase pNA	-
13	D-GLUCOSE	-
14	GAMMA-GLUTAMYL-TRANSFERASE	-
15	FERMENTATION/GLUCOSE	-
17	BETA-GLUCOSIDASE	-
18	D-MALTOSE	-
19	D-MANNITOL	-
20	D-MANNOSE	-
21	BETA-XYLOSIDASE	-
22	BETA-Alanine arylamidase pNA	-

23	L-Proline ARYLAMIDASE	+
26	LIPASE	-
27	PALATINOSE	-
29	Tyrosine ARYLAMIDASE	+
31	UREASE	-
32	D-SORBITOL	-
33	SACCHAROSE/SUCROSE	-
34	D-TAGATOSE	-
35	D-TREHALOSE	-
36	CITRATE(SODIUM)	-
37	MALONATE	-
39	5-KETO-D-GLUCONATE	-
40	L-LACTATE alkalinisation	-
41	ALPHA-GLUCOSIDASE	-
42	SUCCINATE alkalinisation	-
43	Beta-N-ACETYL-GALACTOSAMINISADE	-
44	ALPHA-GALACTOSIDASE	-
45	PHOSPHATE	-
46	Glycine ARYLAMIDASE	-
47	ORNITHINE DECARBOXYLASE	-
48	LYSINE DECARBOXYLASE	-
53	L-HISTIDINE assimilation	-
56	COUMARATE	+
57	BETA-GLUCORONIDASE	-
58	O/129 RESISTANE (comp.vibrio)	-
59	Glu-Gly-Arg- ARYLAMIDASE	-
61	L-MALATE assimilation	+
62	ELLMAN	-
64	L-LACTATE assimilation	+

6. Discussion

Al-Asfar Lake is a significant shallow lake that suffered greatly from anthropogenic eutrophication. Governmental authorities have given this lake a considerable interest for economical and agricultural purposes. In line with this general trend, this study sought to determine the presence of of arylamidase-producing *P. alcaligenes* using culture-dependent methods. Arylamidase enzyme plays a significant role in the degradation of arylamides, peptides, or amides, highlighting the emerging biotechnological applications for bioremediation purposes. Moreover, *P. alcaligenes* possess remarkable genetic and physiological adaptability and have an extraordinary capacity to degrade a vast range of substrates.

The results of the physiochemical characteristics of Al Al-Asfar Lake reveal that water has a neutral pH value. The neutrality of water possibly due to a large amount of inflowing drained water into the lake. It can be noticed that the results indicated narrow pH ranges as reported in a previous study in 2008 where the samples pH values ranged from 7 to 8.3 [24]. While EC values were noticed to be slightly high which could be referred to the accumulation of salts in addition to the high evaporation rate. As stated in a previous study the salinity level reached 102.3 and thus could be referred to as the shallow depth, the closed nature of the lake, restricted circumstances, and minor connection with continuous freshwater sources [24].

The VITEK analysis test of Al-Asfar Lake water indicated the presence of *Pseudomonas alcaligenes* species with a probability of 95% without having been previously reported in this area. In other studies, *P. alcaligenes* has been broadly associated with harsh and hazardous environments such as toxic aromatic hydrocarbons contaminated soil [20], hospital-polluted water [22], and metal-contaminated industrial wastewater [23], which proves the potency and the adaptation ability of *P.alcaligenes* to live and survive in such environments. According to a conducted that aimed to assess the pollution degree of Al-Asfar Lake where the level of heavy metals was found relatively high and didn't match the legal international limit where the maximum content of Cu was (2.62 ppm), Zn (2.6 ppm) and Cd (0.5 ppm) [25]. Therefore, the *P.alcaligenes* species is an ideal candidate to take advantage of in pollutant bioremediation.

Many studies confirmed the significance of the amino acid arylamidase enzyme that has been detected in the VITEK biochemical test. As reported in earlier research, the Arylamidase enzyme has a great role in the mineralization of nitrogen in soil by accelerating the hydrolysis of N-terminal amino acids from arylamides, peptides, or amides [18, 19]. While another study that have been conducted in Danish lakes stated that the proteolytic activity of bacteria can be measured by the level of leucine arylaminase activity [26]. Furthermore, a French study assessed the possibility of using the arylamidase enzyme activity as an adequate bioindicator of pesticides contaminated soil [27]. In addition, some other Studies showed the ability of *Paracoccus* sp. strain FLN-7 in the hydrolysis of amide pesticides like propanil by the activity of arylamidase enzyme through amide bond

cleavage in the amide pesticides [28]. Therefore, based on the abovementioned studies this study suggests *P.alcaligenes* is a good candidate for amide pesticides bioremediation.

7. Conclusion

This study proved the existence of *Pseudomonas alcaligenes* species without having been previously reported in Al-Asfar Lake which has been assessed in earlier studies for its relatively high pollution degree that didn't match the legal international limits. Therefore, the detected activity of arylamidase enzyme in *P.alcaligenes* species makes it an ideal candidate to take advantage of in pollutant biodegradation and to be invested in bioremediation applications to enhance environmental sustainability. in the future, further studies for the sequence-based identification of the 16S rRNA gene are suggested.

Declarations

Acknowledgments

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Conflict of Interest

All authors declare that there is no conflict of interest.

Authors' Contribution

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Ethics Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of Data

All datasets generated or analysed during this study are included in the manuscript.

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