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Interpreting Proteobacteria diversity through 16S rRNA analysis in Manasbal Lake, Kashmir

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ABSTRACT

Bacterial community composition in aquatic ecosystems is of renewed interest for its health as well as the people living in the adjoining areas, and the current work on a freshwater ecosystem in Kashmir Himalayas–the Manasbal Lake was carried out in this backdrop. Water samples were collected from various sampling stations, selected as the zones of special ecological interest and according to the degree of difference in the anthropogenic intrusions in the lake. For identification, the isolated bacterial colonies were subjected to morphological and biochemical characterization, which were further confirmed by targeting their 16S rRNA gene, using 27F and 1492R as the universal bacterial primers. Major bacterial phylum, thus isolated, was proteobacteria with 15 different bacterial species belonging to class alpha-proteobacteria, beta-proteobacteria, and gamma-proteobacteria. However, the most diverse class isolated was Alpha-proteobacteria comprising seven species followed by Beta-proteobacteria comprising six species and Gamma-proteobacteria comprising only two species. The distribution of the bacterial group was seen influenced on a Spatio-temporal scale with the maximum density observed during summer and minimum during winter, further the load was highest for littoral sampling stations in comparison to the open water sites. Proteobacteria are important since they perform basic functions in global transformations of elements. But at the same time, they show close interaction with eukaryotes, both as pathogens and as symbionts.

1. Introduction

Phylum proteobacteria representing various gram-negative bacteria comprises the largest cultured bacterial group within prokaryotes[1]. Using 16SrRNA gene sequence structures, taxonomic origin of proteobacteria as purple bacteriaw as defined to possessing four bacterial groups [2]. representing five classes including Alpha-proteobacteria, Beta-proteobacteria, and Gamma-proteobacteria Delta-proteobacteria and Epsilon-proteobacteria [3]. Gamma-proteobacteria is represented by purple sulfur bacteria whilealpha and beta–purple non-sulfur bacteria represent proteobacteria. Because of their pathogenicity to humans, animals and plants, the proteobacteria are of greater biological significance [4]. Additionally, the species of this group have contributions towards the origin of eukaryotic cells and their organelles[5]. Proteobacteria possess a high metabolic diversity including the chemoorganotrophic, chemoautotrophic, and phototrophic bacteria, which are of a greater significance in agricultural, medical, and industrial fields [6]. Further, different species have been placed in this bacterial group based on 16S rRNA oligonucleotide catalogs, rRNA cistron similarities, phylogenetic analysis, and DNA-rRNA

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hybridization [7]. Class Alpha-proteobacteria (α) includes most of the species that are capable of growing inoligotrophic environments. Members of this group, which include some human and plant pathogens as well as some symbionts have extraordinary morphologies, like stalk and prostheca. Class beta-proteobacteria (β) is similar to alpha-proteobacteria, i.e. they grow on very small amounts of nutrients, but the majority of organisms grow in anaerobic environments on the leached nutrients. Gamma-proteobacteria class (γ), characterized by exceptional diversity is considered as the broadest proteobacteria subgroup, with 14 orders and 25 families [8].

2. Materials and Methods

2.1 Description of study area:

Manasbal Lake, located at 34°14'40"-34°15'20"N and 74°39'00"-74°14'20"E, is the deepest of all freshwater lakes of Kashmir with a depth of around 12 meters [9]. While springs are the main water supply, it is also fed by a seasonal irrigation canal (Laar-Kul) on its eastern side. Since the lake water is used for various purposes like drinking, washing, swimming, leisure and agricultural activities, it acts as a natural water reserve for the locals in the area [10]. For the current study a total of ten sampling station as areas of special environmental interests and according to the human interference (Table 1; Fig. 1) were selected in the lake to collect the water samples for the isolation of proteobacterial community composition.

2.2 Sample Collection

Water samples were collected seasonally for a period of two years from the previously selected sites of the lake in polyethylene (PET) bottles, which were previously carefully cleaned three to four times by autoclaved water and 70 percent alcohol. The container was rinsed with lake water thrice at the time of collection, and the samples were collected.

2.3 Bacterial isolation

For the isolation of bacteria, the collected water samples were serially diluted four-folds using the normal saline solution (0.85%) followed by direct plating method [11], spreading 0.1ml inoculum from the serial dilution tubes (Clesceri,1998) on Petri dishes containing nutrient agar medium [12]. The inoculated plates were incubated at a temperature of 37 °C for 24–48 h.

For bacterial isolation the collected water samples were serially diluted four times by normal saline solution (0.85%). Using direct plating method [13] 0.1 ml of the serially diluted inoculum was spread on Petri plates containing a nutrient agar medium [14]. The inoculated petri plates were incubated at a temperature of 37°C for 24–48 hours for the proper isolation of bacteria.

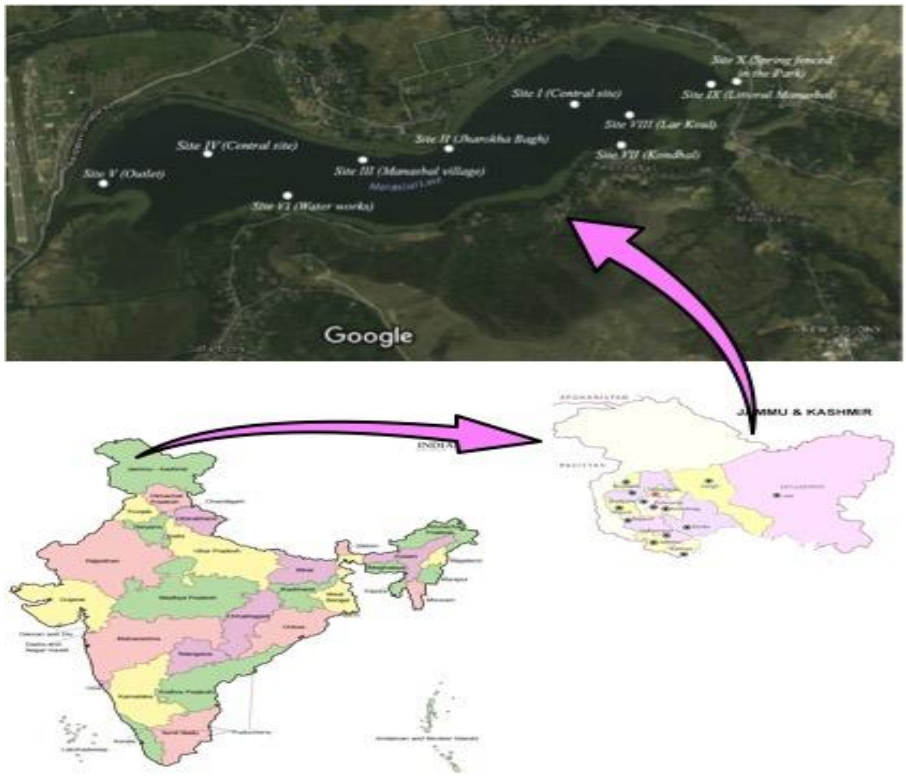

Fig. 1 Map of Study Area (Manasbal Lake J&K India)

Table 1. Study site description of Manasbal Lake

Site	Study Site location	Land Use Patterns	Geographical Coordinates
I	Central site	open water zone with highest depths	34°15′08.4″ N; 74° 40′59.6″ E; 1587-m elevation
II	Jharokha-Bagh	Urban and commercial runoff	34°15′00.7″ N; 74°40′20.2″ E;1600-m elevation
III	Manasbal village	Urban runoff	34°15′00.0″ N; 74°40′07.02″ E; 1605-m elevation
IV	Central site	Open water zone with highest depths	34°14′51.9″ N; 74°39′41.9″ E;1589-m elevation
V	Outlet	Urban runoff and agricultural runoff	34°14′49.4″N; 74°39′21.3″E; 1599- m elevation
VI	Water works		34°14′46.1″ N; 74°39′40.0″ E;1591-m elevation
VII	Kondbal	Urban runoff	34°14′58.2″ N; 74°40′59.5″ E;of 1580-melevation
VIII	Laar-kul	Agricultural runoff	34°15′09.2″ N; 74°41′00.2″ E; 1586-m elevation
IX	Littoral Manasbal	Urban runoff and commercial wash out	34°15′13.6″ N; 74°41′18.3″ E; of 1610-melevation
X	Spring fenced in the park	Natural site	34°15′14.7″ N; 74°41′19.2″ E; of 1594-melevation

2.4 Bacterial Identification by different approaches

2.4.1 Morphological

Tests were carried out to classify bacteria through direct microscopy and motility. Samples were stained and examined for the dimensions, shapes and arrangements etc. to study the micro- and macro-morphological characteristics of bacteria. In order to obtain pure cultures, all recognizable colonies were streaked and re-streaked on the required media. Motility testing of cultures was done by using motility agar media [15].

2.4.2 Biochemical

Every single strain to be used for biochemical profiling was first purified by the technique of pure culture. Each isolated strains were first cultivated in Nutrient Agar and then a well-isolated colony was taken in 10ml nutrient broth for the preparation of a homogenous suspension to be incubated for 4-6 hours at 35-37°C. The biochemical tests conducted included Indole, Nitrate and Hydrogen sulphide production testing, Voges-proskauer, Citrate, Catalase, Amylase and Nitrate[16].

2.4.3 Molecular

For the proper conduct of molecular operations on the isolated bacterial strains, each of them was grown afresh on nutrient

agar medium for 24 hours**.** The HiPurA bacterial and yeast genomic DNA mini-prep purification spin kit **(**Hi-Media, Lot MB505**)** was used for DNA extraction in accordance with the manufacturer**'**s instructions**.** Following the extraction of DNA, 16S rRNA gene was amplified using universal bacterial primers 27F**(**Forward 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse 1429R **(**5'GGTTACCTTGTTACGACTT 3'**).** Amplification was performed in a thermal cycler in PCR reaction tubes containing 50µl reaction mixture (table 2) with initial denaturation at 94°C for 5 minutes, 30 denaturation cycles at 94°C for 1 minute, annealing at 55°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 10 minutes. PCR products were sent for sequencing to SciGenom Laboratory Cochin, India.The sequenced data thus obtained BLAST searched using BLAST 2.0 program [17] and the sequences were compared to those already present in the GenBank/ NCBI database.

Table 2. Amount and concentration of reaction reagents used for amplification

Reagents	Final Concentration	Required volume
Taq Buffer	1 X	5.0 µl
$MgCl_2$	1**.**5mM	3µl
dNTP mix	0.2 Mm	5.0 µl
Forward Primer	0.5 pmoles/µl	1.0 µl
Reverse Primer	0.5 pmoles/µl	1.0 µl
Taq DNA Polymerase	0.03 units/ µl	0.3 µl
Genomic DNA	50-100ng	1.5 µl
MilliQ water		33.2 µl
Total volume		**50.0 µl**

3. Results

Based on the use of different approaches including the conventional, biochemical, and molecular methods, a total of fifteen proteobacteria bacterial species were identified from the Lake water. The macro morphological characteristics of all the isolates were quite different and varying. In this phylum, the most diverse class isolated was Alpha-Proteobacteria (Table 3) represented by two orders Rhizobiales and Caulobacterales comprising seven species, namely *Paenochrobactrum glaciei*, *Paenochrobactrum gallinarii*, *Pseudochrobactrumas accharolyticum*, *Pseudochrobactrum lubricantis*, *Amorphus orientalis*, *Brevundimonas versicularis*, and *Caulobacter daechungensi*. Class Beta-proteobacteria were represented by only one order Burkholderiales contributing six species *Pusillimonas soli*, *Alcaligenesfaecalis*, *A.aquatilis*, *Alcaligenes faecalis subspecie sparafaecalis*, *Alcaligenes faecalis subspecies phenolicus*, and *Advenella kashmirensis*. However, Gamma-Proteobacteria were represented by two orders Pseudomonadales and Xanthomonadales, contributing only two species viz. *Pseudomonas chengduensis* and *Stenotrophomonas rhizophila* thus making it the least diverse class of bacteria.

A gene bank similitude of 81 to 98% with a fragment size range from 1213bp to 1290bp was shown by molecular analysis of the isolated bacterial strains (Table 4). The biochemical analysis of the bacterial species showed that all but *Caulobacter daechungensis* were positive for catalases. The results obtained for the bacterial species were variable for all other biochemical tests. Classical analysis however showed the rod shape and Gram-negative nature in all bacterial species (Table 5).

The maximum number of ten bacterial species was detected at site V followed by nine at site I and VI, eight species at site IV, seven species each at site III and site X, six species each at site VIII and IX and a least five species at site II and VII **(**Table 6**).**

Comparative analysis of these bacterial species at the different sampling stations revealed a wide distribution, although some species exhibited a limited distribution in some sampling stations**.** Three species viz. *A.faecalis*, *A.aquatilis*, *A.faecalis subspecies parafaecalis* showed their presence at all sampling sites while *Paenochrobactrum gallinarii* and *Amorphus orientalis* were

present at only two sites showing a limited distribution.

Table 3. Isolated bacterial species and their taxonomic position

Species	Genus	Order	Class	Family	Phylum	Kingdom
Paenochrobactrum glaciei	Paenochrobactrum	Rhizobiales	Alpha Proteobacteria	Brucellaceae	Proteobacteria	Bacteria
Paenochrobactrum gallinarii	Paenochrobactrum	Rhizobiales	Alpha Proteobacteria	Brucellaceae	Proteobacteria	Bacteria
Pseudochrobactrum asaccharolyticum	Pseudochrobactrum	Rhizobiales	Alpha Proteobacteria	Brucellaceae	Proteobacteria	Bacteria
Pseudochrobactrum lubricantis	Pseudochrobactrum	Rhizobiales	Alpha Proteobacteria	Brucellaceae	Proteobacteria	Bacteria
Amorphus orientalis	Amorphus	Rhizobiales	Alpha Proteobacteria	Rhodobiaceae	Proteobacteria	Bacteria
Brevundimonas versicularis	Brevundimonas	Caulobacterales	Alpha Proteobacteria	Caulobacteraceae	Proteobacteria	Bacteria
Caulobacter daechungensis	Caulobacter	Caulobacterales	Alpha Proteobacteria	Caulobacteraceae	Proteobacteria	Bacteria
Pusillimonas soli	Pusillimonas	Burkholderiales	Beta proteobacteria	Alcaligenaceae	Proteobacteria	Bacteria
Alcaligenes faecalis	Alcaligenes	Burkholderiales	Beta Proteobacteria	Alcaligenaceae	Proteobacteria	Bacteria
Alcaligenes aquatilis	Alcaligenes	Burkholderiales	Beta Proteobacteria	Alcaligenaceae	Proteobacteria	Bacteria
Alcaligenes faecalis subspecies parafaecalis	Alcaligenes	Burkholderiales	Beta Proteobacteria	Alcaligenaceae	Proteobacteria	Bacteria
Alcaligenes faecalis subspecies phenolicus	Alcaligenes	Burkholderiales	Beta Proteobacteria	Alcaligenaceae	Proteobacteria	Bacteria
Advenella kashmirensis	Advenella	Burkholderiales	Beta Proteobacteria	Alcaligenaceae	Proteobacteria	Bacteria
Pseudomonas chengduensis	Pseudomonas	Pseudomonadales	Gamma Proteobacteria	Pseudomonadaceae	Proteobacteria	Bacteria
Stenotrophomonas rhizophila	Stenotrophomonas	Xanthomonadales	Gamma Proteobacteria	Xanthomonadaceae	Proteobacteria	Bacteria

Table 4. Molecular details of isolated bacterial species form Manasbal lake

Name	% Similarity	Gen Bank Acc. no.	Fragment size (bp)
Paenochrobactrum gallinarii	97	NR_116966.1	1224
Paenochrobactrum glaciei	97	NR_112750.1	1224
Pseudochrobactrum asaccharolyticum	98	NR_042474.1	1267
Pseudochrobactrum lubricantis	97	NR_104538.1	1267
Amorphus orientalis	90	NR_104491.1	1253
Brevundimonas versicularis	98	NR_113586.1	1253
Caulobacter daechungensis	95	NR_118485.1	1253
Pusillimonas soli	93	NR_117261.1	1242
Alcaligenes faecalis	96	NR_113606.1	1228
Alcaligenes aquatilis	98	NR_114959.1	1244
Alcaligenes faecalis subspecies Parafaecalis	96	NR_025357.1	1247
Alcaligenes faecalis subspecies phenolicus	95	NR_042830.1	1237
Advenella kashmirensis	92	NR_042360.1	1235
Pseudomonas chengduensis	81	NR_125523.1	1290
Stenotrophomonas rhizophila	97	NR_121739.1	1213

Table 5. Biochemical tests performed on isolated bacterial strains

Code	Isolate name	I	MR	VP	Ci	Ca	Am	H_2S	Ni	Mo	GT	CS	Species Identified
RWM	MB18	-	-	-	-	+	-	-		NM	-	Rods	*Paenochrobactrum gallinarii*
PLO	MB33	-	-	-	-	+	-	-	-	NM	-	Rods	*Paenochrobactrum glaciei*
WSC	MB43	-	-	-	-	+	-	-	-	NM	-	Rods	*Pseudochrobactrum asaccharolyticum*
YPIVC	MB11	-	+	-	-	+	-	-	-	NM	-	Rods	*Pseudochrobactrum lubricantis*
LYC	MB26	-	-	-	-	+	+	-	-	NM	-	Rods	*Amorphus orientalis*
IOC	MB41	-	-	-	-	+	NA	-	-	M	-	Rods	*Brevundimonas versicularis*
LYSC	MB36	-	-	-	-	-	NA	-	-	M	-	Rods	*Caulobacter daechungensis*
OPI	MB61	-	NA	NA	+	+	-	NA	+	M	-	Rods	*Pusillimonas soli*
WCS	MB14	-	-	+	+	+	+	+	+	M	-	Rods	*Alcaligenes faecalis*
WYC	MB52	+	+	-	+	+	-	-	+	M	-	Rods	*Alcaligenes aquatilis*
WOCL	MB57	-	-	-	+	+	-	-	+	M	-	Rods	*Alcaligenes faecalis subspecies phenolicus*
CCM	MB16	-	-	+	+	+	+	-	+	M	-	Rods	*Alcaligenes faecalis subspecies Parafaecalis*
WCO	MB21	-	-	+	+	+	+	-	+	M	-	Rods	*Advenella kashmirensis*
SWIB	MB02	+	-	-	+	+	-	-	+	M	-	Rods	*Pseudomonas chengduensis*
YRM	MB12	-	-	-	-	+	-	-	+	M	-	Rods	*Stenotrophomonas rhizophila*

Table 6. Site-specific variation in the lake bacterial community composition

Species	Site I	Site II	Site III	Site IV	Site V	Site VI	Site VII	Site VIII	Site IX	Site X
Paenochrobactrum gallinarii	-	-	-	+	+	-	-	-	-	-
Paenochrobactrum glaciei	+	+	-	-	+	+	-	-	-	-
Pseudochrobactrum asaccharolyticum	-	-	-	+	-	+	-	-	-	+
Pseudochrobactrum lubricantis	-	-	-	+	+	-	-	-	+	-
Amorphus orientalis	+	-	-	+	-	-	-	-	-	-
Brevundimonas versicularis	+	-	+	-	+	-	+	-	-	-
Caulobacter daechungensis	+	+	+	-	-	+	-	-	-	+
Pusillimonas soli	+	-	-	-	+	-	-	-	-	+
Alcaligenes faecalis	+	+	+	+	+	+	+	+	+	+
Alcaligenes aquatilis	+	+	+	+	+	+	+	+	+	+
Alcaligenes faecalis subspecies Parafaecalis	+	+	+	+	+	+	+	+	+	+
Alcaligenes faecalis subspecies phenolicus	+	-	+	-	-	-	-	+	+	-
Advenella kashmirensis	-	-	-	-	+	+	+	-	-	-
Pseudomonas chengduensis	-	-	+	-	+	+	-	+	+	-
Stenotrophomonas rhizophila	-	-	-	+	-	+	-	+	-	+

4. Discussion

By phenotypic analysis, biochemical and molecular characterization, we were able to differentiate bacteria up to species level [10]. Analysis of 16S rRNA genes of the isolated strains in the present study showed their

phylogenetic diversity with single major phylum Proteobacteria that were further classified into different families within classes α-proteobacteria, β-proteobacteria, and γ-proteobacteria which perform some basic functions like global transformation of elements in nature [18]. While analyzing the proteobacteria in Lake Martel obtained similar results with the dominance of α, β, and γ-Proteobacteria classes. This phylum shows close interaction with eukaryotes, both as pathogens and assymbionts [19].

Beta-proteobacteria (purple nonsulfur bacteria) is reported from industrial effluents [20, paddy soils , 21], and riverine ecosystems, while Gamma-proteobacteria (purple sulfur bacteria) were isolated from tropical waters of Pichavaram mangroves and other coastal ecosystems [22]. Order Rhizobales of alpha-Proteobacteria includes a variety of bacterial species advantageously significant for their diversity in function and niche occupancy. Therefore, the studies of this bacterial group are crucial because they comprise not only the symbiotic nitrogen fixers but also the obligate and facultative pathogens of plants and animals. Such specific species, are thus able to adapt to, and use various ecologically applicable niches [23].

Altogether, the genomic comparisons among pathogenic and symbiotic bacteria represented by this order may provide huge information about genome functionality, genetic variability and operon organization of related species. Genus Amorphus [24] belonging to the class Alpha-proteobacteria were initially isolated from the Lake water and was reported as a pathogen. However, the genus Pseudomonas is a genetically diverse bacterial community of more than hundred species [25] and has a significant role in the breakdown of organic matter and in denitrification in the nitrogen cycle and plant growth promotion, they can also act as plant and animal pathogens [26].

In Beta-proteobacteria, genus Alcaligenes the most abundant and ubiquitous is found in water, soil, rotten eggs, dairy products and the intestinal tract of vertebrates where they probably live saprophytically [27], belonging to the family Alcaligenaceae, which also include genus Pusillimonas [28]. Further, it has been reported that *Alcaligenes*spp. Occasionally causes opportunistic infections in humans [29] hence posing severe environmental and human health concerns. Endotoxins derived from *A. faecalis* represent a potential risk of inflammatory respiratory reaction and lung disease for persons working in agricultural fields where they inhale organic dust contaminated with these organisms [30]. In another study conducted by Gontang *et al*. [31] *Alcaligenes faecalis* have been used as nitrite reductase in a biosensor system for the measurement of nitrite in natural waters. Phylogenetically speaking, the Stenotrophomonas genus is found in the Proteobacterial γ-subclass [32] and are omnipresent in the ecosystems and play an important ecological function in nature's elemental cycle [33].

Species are distributed only in small numbers and completely absent from others, as the activities in the catchment have a profound impact on bacterial diversity in certain areas and their direct relation to their surroundings may be attributed to the reservoir itself. Our workwas in consonance with the results of other workers as for example, Saleem *et al.* [34] found the developmental activities and occupancy in the area exerting pressure on the water body. Three species viz., *Alcaligenes faecalis*, *Alcaligenes faecalis* sub-species *parafaecalis*, and *Alcaligenesaquatilis*showed wide distribution throughout the lake by showing their presence at every site. According to Alfreider *et al.* [35], Betaproteobacteria can be considered as ecological generalists in freshwater, while other groups are more specialized. Some workers have reported that bacteria dominate different freshwater microbial communities, phylogenetically affiliated to β-proteobacteria subclass of Proteobacteria [36].

The species of the class Gamma-Proteobacteria such as *Pseudomonas chengduensis* and *Stenotrophomonas rhizophila* were found at site VIII, where the nutrient concentration is high because of the agricultural runoff, our results are confirmed by the Pinhassi *et al.*[37] who found that γ-Proteobacteria have a preference for high

nutrient concentration because they are typical r-strategists and are hence able to rapidly exploit the extra nutrients [38]. Besides, Pomeroy *et al*. [39] suggested that the interaction of substrate concentrations and temperature should be considered simultaneously while evaluating bacteria plankton responses.

5. Conclusion

In this analysis, molecular techniques targeting the 16S rRNAgene were found to be the effective methods for clarifying the bacterial diversity of the lakes. The study also found that certain pathogenic bacteria are present in lake water. Anthropogenic impacts from catchment area were evident, especially in the nutrient status in Manasbal Lake.

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Conflict of interest

We declare that we don't have any conflict of interest in the current study.

Conflict of interest

Authors declares no conflict of interest

Ethical approval

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

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

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