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Rhodobacter kunshanensis sp. nov., a Novel Bacterium Isolated from Activated Sludge

Junwei Liu[1] · Yixuan Bao[1] · Xuan Zhang[1] · Siqiong Xu[1] · Jiguo Qiu[1] · Jian He[1]

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Abstract

Strain HX-7-19^T was isolated from the activated sludge collected from an abandoned herbicide manufacturing plant in Kunshan, China. Cells were Gram-reaction-negative, rod-shaped, and non-motile. The phylogenetic analysis based on 16S rRNA gene indicated that strain HX-7-19^T formed a clade with *Rhodobacter blasticus* CGMCC 1.3365^T (96.3% sequence similarity). The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values between strain HX-7-19^T and *R. blasticus* CGMCC 1.3365^T were 76.2% and 20.3%, respectively. The genomic DNA G + C content of strain HX-7-19^T was 65.9%. The major fatty acids (> 10% of the total fatty acids) were $C_{18:1}$ ω7c and $C_{18:1}$ ω7c 11-methyl. The major respiratory quinone was quinone Q-10. The major polar lipid profile consists of phosphatidylglycerol (PG), diphosphatidyl-glycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). Photosynthesis pigments bacteriochlorophyll a and carotenoids were formed and photosynthesis genes *pufL* and *pufM* were detected. On the basis of phenotypic and phylogenetic evidences, strain HX-7-19^T is considered as a novel species in the genus *Rhodobacter*, for which the name *Rhodobacter kunshanensis* sp. nov. is proposed. The type strain is HX-7-19^T (= KCTC 72471^T = CCTCC AB 2020148^T).

Introduction

The family *Rhodobacteraceae*, which belongs to the class *Alphaproteobacteria* and represents a phenotypically, metabolically, ecologically diverse group of bacteria, was initially proposed by Garrity et al. [1]. The family *Rhodobacteraceae* contains 174 genera with validly published names at the time of writing (www.bacterio.net/rhodobacteraceae.html). Some bacteria of this family such as genera *Rhodobacter* and *Rhodovulum* are able to perform anoxygenic photosynthesis [2]. Phototrophy is recognized as being specific for certain genera of this family and is an important characteristic in differentiating the genera of phototrophs from those of chemotrophs [3, 4]. Species of genus *Rhodobacter* containing bacteriochlorophyll a are phototrophs. The genus *Rhodobacter* embrace 16 validly named species at the time of writing [5]. The species of this genus have been mostly isolated from various aquatic environments, such as hot spring sediment [5], semiarid tropical soil [6], marine habitats [7], and pond [8]. The aim of this study is to investigate the taxonomic position of a *Rhodobacter*-like strain using a polyphasic taxonomic approach.

Materials and Methods

Bacterial Isolation

The activated sludge was collected from an abandoned herbicide manufacturing plant (E120°56′38″, N31°22′05″) in Kunshan City, Jiangsu Province, China. The sludge sample was streaked on R2A agar. A yellowish colony, designated strain HX-7-19^T, was picked and purified after incubation for 7 days at 30 °C and stored at − 70 °C in R2A broth supplemented with 20% (v/v) glycerol. *Rhodobacter blasticus* CGMCC 1.3365^T was used as a reference strain and was cultured in R2A at 30 °C.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences and the whole genome of strains HX-7-19^T are MT101853 and JAALFE000000000, respectively.

✉ Jiguo Qiu
qiujiguo@njau.edu.cn

[1] Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, Jiangsu, People's Republic of China

PCR Assay

Genomic DNA of HX-7-19^T was extracted using a bacterial genomic kit according to the manufacturers' protocols (TIANamp Bacteria DNA Kit, Tiangen). The DNA was used as a template to amplification of 16S rRNA gene and photosynthetic genes (*pufL* and *pufM*). The bacterial universal primers 27F and 1492R were used to amplify the 16S rRNA gene sequence [9]. The amplified product was purified with an agarose gel DNA extraction Kit (Shanghai Sangon biotech, China), and then ligated into pMD-18T vector (Takara Biotechnology), and sequenced by an automated sequencer (model 3730, Applied Biosystems). The 16S rRNA gene sequence was compared with known sequences found in GenBank using the BLAST program of the NCBI (www.ncbi.nlm.nih.gov/BLAST/) and also identified in EzBioCloud's Identify service (www.ezbiocloud.net/identify). Sequence alignment was performed using the CLUSTAL_W program. Phylogenetic trees were reconstructed based on the neighbor-joining (NJ) algorithms [10], maximum-likelihood (ML) algorithms [11] and minimum-evolution (ME) algorithms [12], and were carried out by the MEGA software (version 7.0) according to Kimura's two-parameter calculation model [13], and were assessed using bootstrap analysis of 1000 replications [14]. The *pufL* and *pufM* genes were amplified by using the primer pair *pufLM*-67F (5′-TTCGACTTYTGG RTNGG NCC-3′) and *pufLM*-781R (5′-CCAKSGTCCAG CGCCAGAANA-3′) [15]. The expected length of the amplified *pufLM* genes fragment is about 1.5 kb. *R. blasticus* CGMCC 1.3365^T was used as the positive control.

Chemotaxonomic Characterization

For chemotaxonomic analysis, strain HX-7-19^T and the closest phylogenetically related strain *R. blasticus* CGMCC 1.3365^T were cultured in R2A on a rotary shaker (180 rpm) at 30 °C for 7 days and 5 days, respectively. The cells were harvested by centrifugation at exponential growth phases, and then washed with distilled water and freeze-dried. Cellular fatty acid were saponified, methylated, extracted, and analyzed according to the standard protocol of the Sherlock MIS (MIDI) system. The respiratory quinones were tested according to the method as described previously [16]. Polar lipids were extracted using a chloroform/methanol system [17, 18] and analyzed according to the method as described previously [19].

Genomic DNA of strain HX-7-19^T was extracted and purified according to standard procedures, and then sequenced and assembled by Illumina Hiseq 4000 platform at Shanghai Biozeron Biotechnology Co., Ltd, China. The assembled genomes were annotated with the Rapid Annotation with Subsystem Technology (RAST) server (https://rast.nmpdr.org/). The average nucleotide identity (ANI) between strain HX-7-19^T and *R. blasticus* CGMCC 1.3365^T were calculated using a tool of OrthoANIu algorithm (www.ezbiocloud.net/tools/ani) [20]. The digital DNA–DNA hybridization (dDDH) were calculated by the genome-to-genome distance calculator (http://ggdc.dsmz.de/ggdc.php/) [21]. The DNA G + C content was determined from the genome sequence.

Morphological, Physiological, and Biochemical Characterization

Cells of strain HX-7-19^T (exponential growth phase) were observed using a transmission electron microscopy (H-7650; Hitachi) to determinate the morphological characterization. The characterization of motility was observed using an optical microscope (BX40; Olympus) according to the hanging-drop method [22]. Gram staining was tested using a Gram-stain kit (Difco) according to the instructions of the manufacturer. The temperature range (4, 10, 15, 18, 20, 25, 30, 37, 42, and 47 °C) and pH range (pH 3.0–9.0 using increments of 0.5 pH units) for growth were determined by incubating the isolate for one week in R2A broth. The pH range was adjusted by using buffer system according to method by Xu et al. [5]. Growth in various NaCl concentrations (0–5%, w/v, at 0.5% interval) was evaluated in R2A broth. Growth was monitored by measuring OD_{600} nm by a UV-1800 spectrometer (Shimadzu Corp.; Japan).

Photo-organoheterotrophic growth of HX-7-19^T was investigated in Pfennig medium containing sodium pyruvate (0.3%, w/v) and NH_4Cl as the carbon and nitrogen source under light exposure (2400 lx) and anaerobic conditions at 30 °C [23]. Chemo-organoheterotrophic growth was determined in Pfennig medium containing sodium pyruvate (0.3%, w/v) as the only carbon source under dark and aerobic conditions. The chemo-lithoautotrophically growth was tested under aerobic and dark conditions with Na_2S (0.5 mM), $Na_2S_2O_3$ (0.5 mM) as electron donors and $NaHCO_3$ (0.1%, w/v) as carbon source, and fermentative growth was investigated at anaerobic and dark conditions with pyruvate (0.3%, w/v) as fermentable substrate. Vitamin (vitamin B12, biotin, niacin, paminobenzoic acid, and thiamine) requirement was tested by replacing yeast extract with single and also combinations of vitamins as growth factors. Biochemical characters of strain HX-7-19^T were studied using commercial identification kits (API 20NE, API 50CH, and API ZYM) and Biolog

GENIII MocroPlate according to the manufacturers' protocols (bioMérieux).

Results and Discussion

Phylogenetic and Genomic Analysis

The sequenced length of 16S rRNA gene of strain HX-7- was 1453 bp. Comparative analysis of 16S rRNA gene sequences indicated that strain HX-7-19^T was closely related to those of *R. blasticus* CGMCC 1.3365^T (96.3% sequence similarity), and were lower than 97% with other type strains of family *Rhodobacteraceae*. The phylogenetic tree showed that strain HX-7-19^T formed a distinct clade with the related type strain *R. blasticus* CGMCC 1.3365^T according to NJ algorithm (Fig. 1). The affiliation result was consistent with the ME algorithm (Fig. S6) and ML algorithm (Fig. S7).

The draft genome size of strain HX-7-19 ^{T} (4.46 Mb) was much bigger than that of *R. blasticus* CGMCC 1.3365^T (3.59 Mb). The predicted number CDSs and RNA genes of strain HX-7-19 which were higher than those determined in the genome of *R. blasticus* CGMCC 1.3365^T. The G + C content of the genome (65.9 mol%) was a slightly smaller than that of *R. blasticus* CGMCC 1.3365^T (66.5 mol%) (Table 1). In the subsystem features (subsystem coverage and subsystem category distribution), some differences were observed at the two genomes (Table 1). For example, the numbers of genes putatively involved in cofactors, vitamins, prosthetic groups, pigments, cell wall and capsule, and metabolisms of RNA, DNA, phosphorus, sulfur, and aromatic compounds in genome of HX-7-19^T were higher than those of *R. blasticus* CGMCC 1.3365^T. However, the numbers of genes putatively in genome of HX-7-19^T such as photosynthesis and metabolisms of potassium, iron, and nitrogen were lower than those of *R. blasticus* CGMCC 1.3365^T.

In addition, the ANI and dDDH values between strain HX-7-19^T and *R. blasticus* CGMCC 1.3365^T were 76.2% and 20.3%, respectively. Previous study indicated that the threshold values of ANI and dDDH were 95% and 70% generally accepted for bacterial species delineation, respectively [24]. Thus, strain HX-7-19^T should represent a novel species according to the results in ANI and dDDH.

Phenotypic and Physiological Characteristics

Individual cell of strain HX-7-19^T were rod-shaped with size 0.6–0.8 μm wide and 1.5–3.2 μm long (Fig. S1), and was non-motile and Gram-staining-negative. The colonies were yellowish. The growth of strain HX-7-19^T was observed at

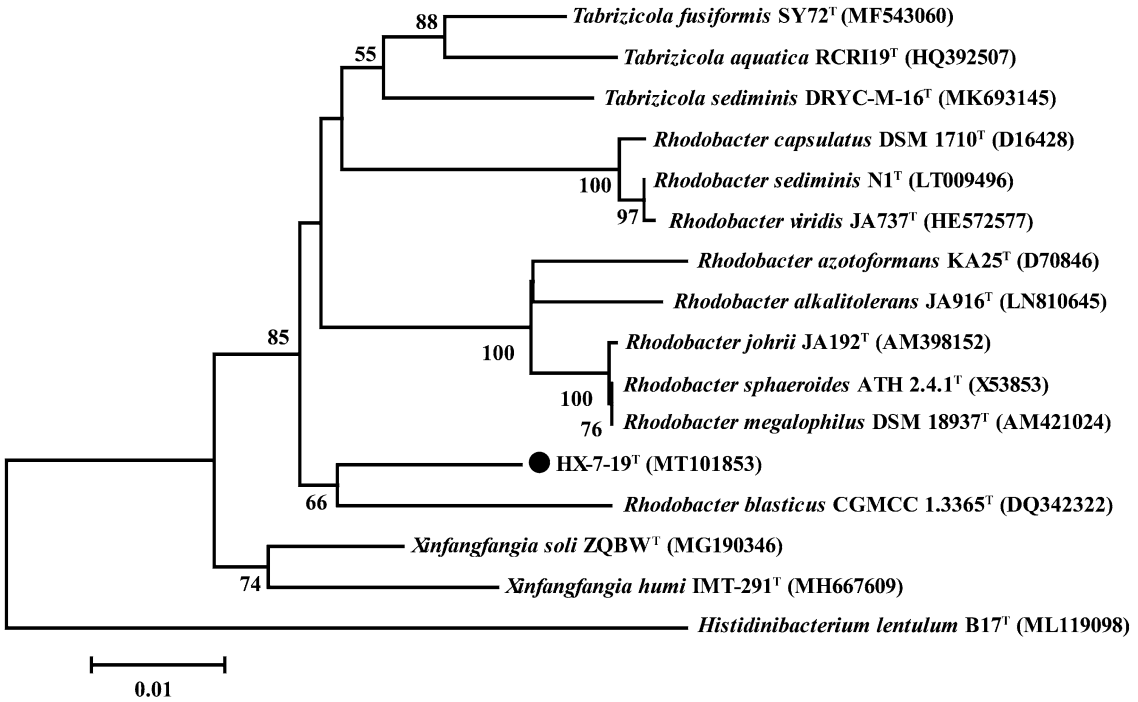

Fig. 1 Neighbor-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences, showing the taxonomic position of strain HX-7-19^T and related taxa. Bootstrap values were calculated from 1000 replications and values below 50% are not indicated at branch points. Bar, 0.01 substitutions per nucleotide position

Table 1 Genome characteristics of strain HX-7-19^T and *Rhodobacter blasticus* CGMCC 1.3365^T

Characteristics	1	2
Number of contigs	117	69
N50 value (Mb)	1.63	2.03
Genome size (Mb)	4.46	3.59
DNA G + C content (mol%)	65.9	66.5
Protein-coding genes	4432	3608
RNAs	52	48
Accession numbers	JAALFE010000000	CP020470
Genes of different functional categories		
Cofactors, vitamins, prosthetic groups, pigments	157	136
Cell wall and capsule	38	27
Photosynthesis	2	9
Potassium metabolism	4	7
Iron acquisition and metabolism	7	16
RNA metabolism	43	41
Protein metabolism	198	199
Secondary metabolism	5	5
DNA metabolism	88	79
Nitrogen metabolism	26	46
Phosphorus metabolism	46	34
Sulfur metabolism	23	7
Metabolism of aromatic compounds	36	9

lower than 1.0% of NaCl (optimum 0%, w/v), temperature at 15–42 °C (optimum at 30 °C), and pH 6.0–8.5 (optimum at 7.0–7.5) (Table 2). The phenotypic and physiological characteristics comparison between strain HX-7-19^T and *R. blasticus* CGMCC 1.3365^T were presented at Table 2.

The strain HX-7-19^T was able to grow chemo-organoheterophically and photo-organoheterotrophically; whereas it could not grow chemo-lithoautotrophically and fermentative. These nutritional characteristics of strain HX-7-19^T was consistent with that of genus *Rhodobacter*. The photosynthetically grown cell suspension was yellowish brown. The whole-cell absorption spectrum of strain HX-7-19^T in sucrose solution (60%, w/v) gave absorption maxima at 376, 485, 683, 755, and 848 nm (Fig. S2). This result indicated that cells of strain HX-7-19^T contained bacteriochlorophyll a and pigments [6]. Previous study indicated that the major carotenoids of *R. blasticus* CGMCC 1.3365^T were spheroidene and spheroidenone [5]. HPLC analysis indicated that strains HX-7-19^T and the closely related species *R. blasticus* CGMCC 1.3365^T showed the same pattern for major carotenoids (Fig. S3) implying the presence of spheroidene and spheroidenone as major cartenoids [6]. In addition, both strains have the photosynthetic genes *pufL* and *pufM* (Fig. S4 and S5), whereas other genus of the family *Rhodobacteraceae* have not genes *pufL* and *pufM*. Their presence confirm the affiliation of strain HX-7-19^T to *Rhodobacter*.

Chemotaxonomic Analysis

As shown in Table 3, the major fatty acids (> 5%) of strain HX-7-19^T were $C_{18:1}$ ω7c (65.98%), $C_{18:1}$ ω7c 11-methyl (16.69%), and $C_{18:0}$ (6.25%), while those of *R. blasticus* CGMCC 1.3365^T were $C_{18:1}$ ω7c (74.92%), $C_{18:0}$ 3–OH (7.21%), and $C_{16:0}$ (6.55%). On the whole, the fatty acids profile of strain HX-7-19^T was similar to that of *R. blasticus* CGMCC 1.3365^T. However, strain HX-7-19^T possessed relatively higher amounts of $C_{18:0}$ and $C_{18:1}$ ω7c 11-methyl, and lower amounts of $C_{18:0}$, $C_{18:0}$ 3–OH, and $C_{16:0}$. The polar lipid profile of strain HX-7-19^T is composed of phosphatidylglycerol (PG), diphosphatidyl-glycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), one unknown aminolipid, one unidentified phospholipids, and one unidentified lipids (Fig. S8). The major respiratory quinone of strain HX-7-19^T was quinone Q-10, which was coincident with other type strains in the genus *Rhodobacter*.

Taxonomic Conclusion

Strain HX-7-19^T represents a novel species within the genus *Rhodobacter* based on the distinct above phenotype and genotype analysis, for which the name *Rhodobacter kunshanensis* sp. nov. is proposed.

Table 2 Different phenotypic characteristics between strains HX-7-19^T and *Rhodobacter blasticus* CGMCC 1.3365^T

Characteristic	1	2
Colony color (R2A, 30 °C)	Translucent yellow	Orange brown
Cell shape	Rod-shaped	Ovoid to rod-shaped
Cell size	0.6–0.8 × 1.5–3.0	0.6–0.8 × 1.0–2.5
Motility	−	+
NaCl concentration (%, w/v)	0–1%	0–2%
Optimum temperature for growth (°C)	30–37	30–35
Optimum pH for growth	7.0–7.5	6.5–7.5
Reduction of nitrate	−	−
pufL and *pufM* genes	+	+
Phototrophic growth	+	+
Photosynthetic pigments	+	+
Utilization of:		
D-Glucose	+	+
Trisodium citrate	+	−
D-mannose	+	−
Capric acid	+	−
D-Sorbitol	−	+
D-Mannitol	−	+
Dulcitol	−	+
Maltose	−	+
Glycerol	−	+
Rhamnose	−	+
Fermentation of glucose	−	+
Arabinose	−	+
Vitamins required	−	+
Valine arylamidase	+	−
Cystine arylamidase	−	+
β-glucosidase	−	+

All data were obtained in this study unless otherwise indicated. + positive; − negative

Strains: *1* HX-7-19^T; *2 R. blasticus* CGMCC 1.3365^T

Description of *Rhodobacter kunshanensis* sp. nov.

Rhodobacter kunshanensis (kun.shan.en'sis. N.L. masc. adj. kunshanensis of or pertaining to Kunshan city, Jiangsu province, China, from where the type strain was isolated).

Cells are non-motile, Gram-staining-negative, and rod-shaped (0.6–0.8 × 1.5–3.2 μm). The colony is yellowish. The growth of strain HX-7-19^T was observed at lower than 1.0% of NaCl (optimum 0%, w/v), temperature at 15–42 °C (optimum at 30 °C), and pH 6.0–8.5 (optimum at 7.0–7.5). Growth occurs under anaerobic conditions in the light (photo-organoheterotrophy) or under aerobic conditions in the dark (chemo-organoheterotrophy). The color of phototrophic culture is yellowish brown. Bacteriochlorophyll a, spheroidene and spheroidenone are the photosynthetic pigments. The genomic DNA contains *pufL* and *pufM* genes. In the API ZYM system, positive for activities of alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. In addition, esterase lipase (C8), lipase (C14), and α-glucosidase are weakly positive. In API 20NE system, 4-nitrophenyl β-D-galactopyranoside and hydrolysis of esculin are positive. D-glucose, D-mannose, capric acid, and trisodium citrate are assimilated. In API 50CH system, acid produced from erythritol, D-ribose, L-xylose, D-glucose, D-fructose, methyl *α*-D-glucopyranoside, arbutin, inulin, and potassium 5-ketogluconate. In Biolog GENIII tests, positive for α-D-glucose, D-melibiose, L-galactonic acid lactone, D-glucuronic acid, gentiobiose, D-fructose-6-PO_4 and tetrazolium violet, negative for D-raffinose, L-arginine, L-lactic acid, L-aspartic acid, and L-glutamic. The polar lipid profile consists of phosphatidylglycerol (PG), diphosphatidyl-glycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), one unknown aminolipid, one unidentified phospholipids, and one unidentified lipids. The major fatty acids are $C_{18:1}$ *ω*7c, and $C_{18:1}$

Table 3 Cellular fatty acid compositions (percentages) of strain HX-7-19^T and related type strain *Rhodobacter blasticus* CGMCC 1.3365^T

Fatty acid	1	2
$C_{10:0}$ 3–OH	1.44	1.97
$C_{12:0}$	0.23	ND
$C_{14:0}$	ND	0.07
$C_{16:0}$	3.31	6.55
$C_{16:0}$ 3–OH	0.35	0.32
$C_{17:0}$	0.45	0.06
$C_{17:0}$ 10-methyl	ND	0.07
$C_{17:1}$ ω8c	0.22	ND
$C_{17:1}$ anteiso ω9c	0.09	ND
$C_{18:0}$	6.25	3.26
$C_{18:0}$ 3–OH	3.82	7.21
$C_{18:1}$ ω5c	ND	ND
$C_{18:1}$ ω7c	**65.98**	**74.92**
$C_{18:1}$ ω7c 11-methyl	**16.69**	1.42
$C_{19:0}$ 10-methyl	0.42	0.78
$C_{20:1}$ ω7c	0.21	ND
Summed features 2*	ND	0.06
Summed features 3*	0.29	2.24
Summed features 5*	0.26	ND
Summed features 7*	ND	1.08

1 HX-7-19^T (this study); *2 R. blasticus* CGMCC 1.3365^T (this study). Fatty acids representing to 10% or more of the total fatty acids are in bold

ND not detected

*Summed features represent the integration of two or three fatty acids which cannot be separated by the MIDI system. Summed feature 2 included $C_{12:0}$ aldehyde and/or unknown fatty acid with an equivalent chain length of 10.928; summed feature 3 comprised $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; summed feature 5 comprised $C_{18:2}$ ω6c and/or $C_{18:0}$ ante; summed feature 7 comprised $C_{19:1}$ ω6c and/or unknown fatty acid with an equivalent chain length of 18.846

ω7c 11-methyl. The major respiratory quinone is quinone Q-10. The DNA G + C content is 65.9 mol%.

The type strain HX-7-19^T (= KCTC 72471^T = CCTCC AB 2020148^T) was isolated from activated sludge of an abandoned herbicide manufacturing plant (Kunshan city, Jiangsu province, China).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-021-02628-0.

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Author Contributions JQ, JL, and JH carried out the concepts. JL and YB participated in the research and analyzed the data. XZ and SX provided assistances for literature search and data acquisition. JL drafted the manuscript. JQ and JH performed manuscript review. All authors read and approved the final manuscript.

Data Availability All authors have declared that all data are available.

Declarations

Conflict of interest The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All the authors declare that they have no conflict of interest.

Ethical Approval The authors have declared that no ethical issues exist.

Consent to Participate All authors agree to have participated in the research proposed to be published and agree to be published in the journal.

Research Involving Human and Animal Participants This article does not contain any studies with human participants or animals performed by any of the author.

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