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Molecular Diversity and Compositional Analysis of Microbiota in Aged Refuse Biofilter Revealed by Amplicon Sequencing

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Abstract: The increasing significance of aged refuse biofilters for biodegradation processes in wastewater treatment necessitated the constant search for its bacterial properties. This study focused on the diversity of the bacteria present in aged refuse by DNA extraction. The PCR amplifier was used for DNA sequencing using DNA polymerase primer. For PCR amplicons purification, amplicons were extracted from 2% agarose gels and purified using DNA Gel Extraction Kit while quantification was done using QuantiFluor™ -ST. Sample libraries were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform while Raw fastq files were demultiplexed, quality-filtered using QIIME. The taxonomy of each 16S rRNA gene sequence was evaluated by RDP using confidence threshold of 0.7 while operational Units (OTUs) were clustered with 97% similarity cut-off using UPARSE. The result revealed twelve phyla clusters which include; Firmicutes, *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Planctomycetes*, *Nitrospirae*, *Cyanobacteria*, *Latescibacteria* and *Gemmatimonadetes*. Furthermore, additional group classification revealed up to 513 reads of bacteria kingdom with dominant family classification of Anaerolineaceae, Ruminococcaceae, *Nitrosomonadaceae*, *Planctomycetaceae*, *Clostridiaceae*, *Beijerinkiaceae*, *Xanthobacteracaea*, *Veillonellacaea*, *Cemmatimonadacaea*. The presence of such diverse organisms in the aged refuse biofilter, show that the aged refuse biofilter has biodegradation potentials when treatment conditions are efficiently optimized.

Keywords: Diversity, microbial community, aged refuse biofilter, *Commamox*, nitrification, metagenomics.

Introduction

The effective management of wastewater has become a major risk to both physical environment and human/animal life. However, biological treatment methods are widely used as they are cheap, efficient, simple in application, requires less energy and chemicals and also environmental friendly (Anijiofor *et al.*, 2017). Bacterial metabolism determines the efficiency of most organic wastewater treatment, and so it is necessary to define the relationships between the species structure and the performance of full-scale installations (Cydzik-Kwiatkowska *et al*., 2016). Micro-organisms are small in size and therefore

frequently overlooked due to varying methodologies which has limited its characterization and analysis of species composition, species diversity and structure of microbial communities (Prosser, 2002). Biological wastewater treatment systems are usually designed from an engineering perspective, in most cases neglecting many aspects of microbial communities' ecology and dynamics (Cydzik-Kwiatkowska *et al*., 2016). In recent studies on microbial diversity, there is the need for an integration of theoretical ecology in the design and operation of WWTPs for better prediction of microbial community assembly and possible variations in community structure and function in response to the environmental changes and disturbances.

The fundamental role of microorganisms in biodegradation is very important and this compelled several methods for assessing bacterial diversity. Such methods include, direct observation for groups with distinct morphological physiognomies. However, such approach may be restricted and incomplete for analysis of complex diversities in soil. In recent times, laboratory cultivation of organisms from samples are preferred and this approach provides information on which species are present, while the relative abundance of different organisms can be used to determine measures of diversity, e.g. diversity indices, evenness and dominance. Various procedures have been applied in the study of microbial communities of environmental samples such as denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1993), terminal restriction fragment length polymorphism (T-RFLP) (Osborn *et al.*, 2000), sequencing technology (Sogin *et al*., 2006), which has influenced better knowledge of complex microbial communities present in soil, feces and even in palm oil mill effluent (Mohammadi *et al.*, 2012). Furthermore, since 2006, the second generation sequencing technology has been used to evaluate the microbial community of environmental samples and has led to significant understanding of complex microbial communities present in soil (Jones *et al.*, 2009). However, recent studies which include (McLellan *et al.*, 2010; Lykidis *et al*., 2010; Xie *et al.*, 2012) have shown that pyrosequencing of partial 16S rRNA gene is effective in comparing the microbial community structure of different environment.

The aged refuse has soil like appearance, according to Anijiofor *et al*. (2018), and characteristics such as; organic content of 9.90 %, porosity 51%, bulk density 1.23g/cm^3. Soil microbial communities are potential reservoirs of complex genetic diversity although such studies are limited and diversity, distribution and origins of resistance genes are not fully maximized (Allen *et al.*, 2009). Soil bacteria have intrinsic genetic diversity which usually predicts the presence of resistance genes. D'costa *et al*. (2006) studied *Streptomyces* isolates from diverse soils and discovered that 480 strains tested were resistant to multiple antibiotics. Culture-based analysis excludes most of the microbial diversity (Kuske *et al.*, 2002; Liles *at al.*, 2008), therefore, functional metagenomics, enables discovery of resistant genes from organisms that may or may not be readily culturable (Justin *et al.*, 2010). Such resistance genes are abundant in soil, since some soils have low concentrations of compounds that select for resistance but rich with microorganisms that produce b-lactam antibiotics, such as penicillins and cephalosporins (Martín & Liras, 1989). The microbial community could be used to compare similarity or dissimilarity between different sample groups, analyses the relationship between microbial community

and environmental factors, phylogenetic analysis, and other statistical analysis. Meanwhile, limited data is available for microbial community and configuration for aged refuse, while several studies prove that microorganism in the aged refuse are responsible for pollutants decomposition using aged refuse biofilters, (Zhao *et al.*, 2002; Xie *et al.*, 2012) and many others. Aged Refuse (AR), has been cited as an alternative cost-effective media for wastewater treatment and has been pioneered for landfill leachate treatment using various column reactors sizes ranging from 80 to 150 and 20 to 80 cm in height and diameter respectively. Zhao *et al.* (2002), reported NH_3-N removal rates of up to 90 % from landfill leachate while Zhao *et al.* (2002), reported removal rates of 100 % for NH_3-N from sewage. Zhang *et al*. (2011), reported removal rates of (61.04 ± 6.75 %) TN and (69.14 ± 9.25 %) TP from domestic wastewater and Han *et al*. (2012), reported up to 95 % TN from leachate. The advantages include low investment and maintenance costs, simple construction and operational mechanisms, low sludge production, high pollutants removal rates and environmentally safe. This technology is auspicious in developing countries due to the high organic content of MSW generated when compared to the lower organic content of wastes generated in developed countries

Therefore, there is the need to understand the microbial structure of this growing technology. Furthermore, bacteria metabolism determine the effectiveness of biological wastewater treatment, therefore it becomes imperative to investigate the microbial community and structure of treatment media. This study investigates the functional microbial diversity in "Aged Refuse" suitable for biodegradation, as an approach towards maximizing such landfilled materials as biofilter media for wastewater treatment. Previous studies on the use of such biofilters for wastewater treatment focused mainly on the efficiency of the biofilter in pollutant reduction, and limited knowledge on microbial diversity for aged refuse is available. However, Xie *et al.* (2012) attempted using pyrosequencing technology for microbial diversity of aged refuse. Aged refuse differ from region to region depending on landfill characteristics, climatic, geographical and geological characteristics. Therefore, this study will enhance better understanding of different bioreactors due to structure and dynamics of bacterial communities of aged refuse, which will advance optimization of operating conditions.

Materials & Methods

Aged refuse sampling

The aged refuse samples were collected from Air Hitam Sanitary Landfill Site (AHSL), Selangor, Malaysia located at Longitude 101º 39′ 55″ E and Latitude 03º 0′ 10″ N. An excavator was used to collect the waste samples at a depth of 1.5m. The landfill comprises different segments and also solid waste materials deposited in the landfill differ in composition. Therefore, decomposition processes in the landfill depends on the composition of the wastes. In order to ascertain relative microbial distribution in the landfill, the samples were collected randomly from different points and labeled L1, L2, and L3. The samples were transported to the lab and prepared for analysis as previously stated in (Anijiofor *et al.*, 2018). The samples were all stored in cool temperature prior DNA extraction.

Culture procedure

Microbial DNA was extracted from the samples using E.Z.N.A.Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's procedures. The following was adopted for the DNA detection methods, DNA Purity: NanoDrop 2000, DNA Concentration: NanoDrop 2000, DNA Integrity: Agarose Gel Electrophoresis (AGE). The samples were fully mixed and centrifuged after melting on the ice, and then tested with 2ul (AGE). Agarose gel concentration of 1% was used for Electrophoresis Conditions at a Voltage of 5 V/cm for 30 min. After the DNA isolation, PCR reactions were performed by PCR amplifier (ABI GeneAmp® 9700) using TransStart Fastpfu DNA Polymerase primer in triplicates and 10ng of template DNA. The PCR amplification was carried out at different cycles and heated at temperature up to 95 ºC with different time interval using both forward and reverse primer sequences. For accuracy and reliability, the number of cycle was repeated and a level of consistency in cycle number for each sample maintained to enable denaturation of the DNA molecule and allow primers attach to DNA template and initiate polymerisation. The sequencing region of V3+ V4 was used at primers 338F (5'-ACTCCTACGGGAGGCAGCA - 3') forward primer and 806R reverse primer (5' - GGACTACHVGGGTWTCTAAT - 3'). For PCR amplicons purification and quantification**,** amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit and quantified using QuantiFluor™ -ST. Sample libraries were pooled in equimolar and paired-end sequenced (2 × 250/300 bp) on an Illumina MiSeq platform according to the standard protocols.

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.9.1), and paired-reads were merged into a single read based on overlap. The merged reads were used for OTU clustering, taxonomy classifying and community diversity assessment. Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARSE and chimeric sequences were identified and removed using UCHIME (Edgar *et al.,* 2011). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP (Cole *et al.*, 2009) classifier against the Silva (Quast *et al*., 2013), (SSU123) 16S rRNA database using confidence threshold of 0.7. Other databases for Bacteria and archaea 16S rRNA gene are Greengene (DeSantis *et al.,* 2006), while functional gene database include FGR (Fish *et al.,* 2013).

Result & Discussion

The microbial community could be used to compare similarity or dissimilarity between different sample groups, examines the relationship between microbial community and environmental factors, phylogenetic analysis and other statistical analysis. In phylogeny an operational taxonomic unit (OTU) is an operational definition of a specior group of species often used. The DNA extraction is summarized as in table (1)

Table (1): Amplicon sequencing detection result.

Sample	Type	Conc. (ng/uL)	OD260/280	OD260/230
LS1	DNA	15	0.7	0.22
LS2	DNA	15	0.8	0.3
LS3	DNA	16	0.7	0.24

The 16S rRNA gene amplification was conducted using samples of DNA extracted from the different samples. Moreover, the PCR amplification pre-experiment was conducted to ascertain accurate reaction conditions. The results indicates that samples LS1, LS2 and LS3 were graded A after 27 cycles, carried out at a temperature of 55 ºC with sequencing primer 338F- 806R, signifying excellent quality for the proposed experiment. However, the type of primers used determines the rRNA genes population, and this represents the natural microbial population. Furthermore, the PCR result show that the products had accurate concentration and strip size adequate for the experiment. The raw data was obtained by image data output from sequencing machine transformed by base calling into sequence data, which is called raw data or raw reads and stored in fastq format. The statistics of the valid sequences at 338_860R, insert size of 468 bp, sequencing length of PE300 shows raw reads of 160443*2 and total bases of 96586686 bp, correspondingly, to the trimmed sequence for the three samples as in table (2).

Table (2): Base-pairs of extracted samples.

Sample	Sequences	Bases (bp)	Average length (bp)
LS 1	52483	22702415	432.57
LS 2	60442	26462488	437.82
LS 3	47518	20804844	437.83

Compositional Analysis of Microbiota

The compositional analysis of bacteria community provides information on the population of different bacteria present in the samples. This data could be presented either in barplot, pieplot or using R programming language. The major phylum bacterial community in the samples include; *Firmicutes, Cloroflexi, Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Planctomycetes, Nitrospirae, Cyanobacteria, Latescibacteria,* and *Gemmatimonadetes.* The percentage distribution is shown in fig. (1), while

additional high abundant microorganisms present are classified in fig. (2).

Fig. (1) show a breakdown of bacteria classes present in 3 samples and their percentages. The bacteria composition in the different samples; LS1, LS2 and LS3 were relatively distributed. The composition of *Firmicutes* in LS1 and LS3 was high, up to 40% and 38%, respectively, but less than 5% in LS2. Also, the composition of *Proteobacteria* in LS3 was about 40% and 20 % in LS2 and less than 10% in LS1. The composition of *Actinobacteria* was less than 20 % in all samples while *Acidobacteria* was only visible in LS2. Similarly, the composition of *Chloroflexi* was visible in three samples but very high in LS1 up to 40%, 20% in LS2 and 10% in LS3. However, other classes are subdominant phyla (Fig.1) which include; *Bacteroidetes, Acidobacteria, Planctomycetes, Latescibacteria,* and others had lower percentages. As earlier explained, the various compositions of solid wastes deposited in the landfill and their biodegradability could be responsible for different bacteria composition in different samples. The classes of bacteria in the samples responsible for nitrogen cycle in wastewater treatment are the *Nitrospira*. The *Nitrospira* is a genus of bacteria, suitable for most wastewater treatment plants and is very significant during nitrification processes (Koch *et al.*, 2015).

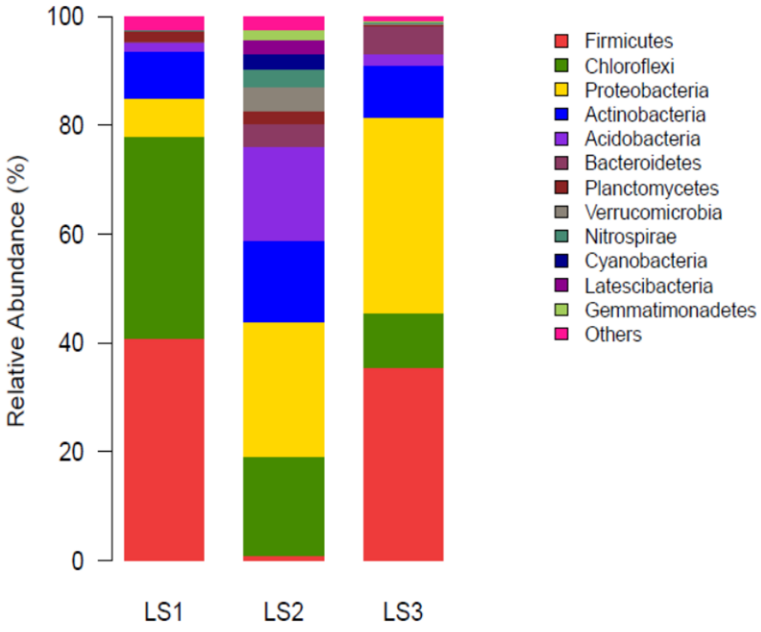

Fig. (1): Phylum bar plot of microbial community

Furthermore, members of this genre are said to be nitrite-oxidizers and have nitrite oxidoreductase genes (Pester *et al.*, 2014). According to Daims *et al.* (2015), Van Kessel *et al.* (2015) and some of such bacteria have capabilities to achieve thorough nitrification, which is principal to the discovery of *Commamox* organisms within *Nitrospirae*. Moreover, in engineered wastewater systems, such wide-ranging nitrification process lowers greenhouse gas emissions into the atmosphere. Also, the Nitrospira absorbs organic molecules and carbon in biofilms during such biological processes.

For organic and nutrient removal, *Proteobacteria, Firmicutes Actinobacteria,*

Nitrospirae are the most abundant class, while the *Betaproteobacteria* which consists mainly of facultative bacteria are extremely suitable for degradation but also contain *Nitrosomonas* which oxidizes ammonia to nitrite and suitable in sewage and industrial waste treatment. Further classification of bacteria is shown in fig. (2). The samples which were collected from different locations in the landfill show that each sample consists of different bacteria composition. This is attributed to different aged refuse composition and as such, there is need for characterization considering the effect of microbes in wastewater biodegradation. Nonetheless, all the samples had different groups of bacterial compositions sufficient for such biodegradation. The dominant family classification include *Anaerolineaceae, Ruminococcaceae, Nitrosomonadaceae, Planctomycetaceae, Clostridiaceae, Beijerinkiaceae, Xanthobacteracaea, Veillonellacaea, Cemmatimonadacaea* and others. Ammonia-oxidizing bacteria (AOB) diversity are mainly responsible for nitrification (Zhang *et al.*, 2011), and analysis of sequences of nitrification genes showed that AOB mainly belonged to *Nitrosomonas* sp. and *Nitrosospira* sp.

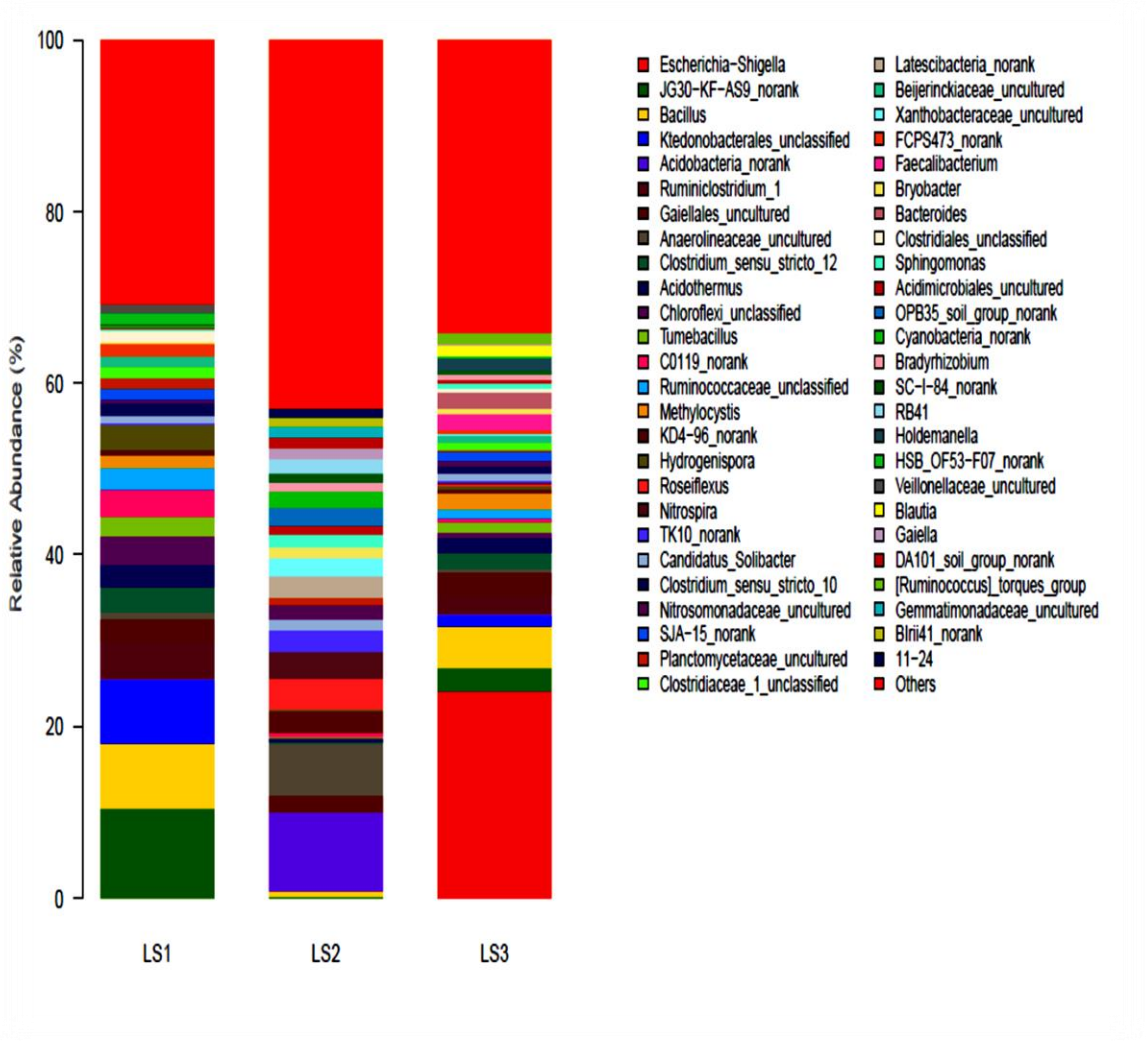

Fig. (2): Bar plot of high abundance microorganisms

Principal component analysis (PCA)

The Principal component analysis (PCA) according to Wang *et al.*, (2012), "is a statistical technique that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components". Since the data involved were limited, the basic steps were standardization of the variables to the same scale; covariance matrix computation to establish relationship between variables of input data set; then computation of eigenvectors and eigenvalues of covariance matrix; and lastly selection of feature vector. The result of PCA is shown in fig. (3).

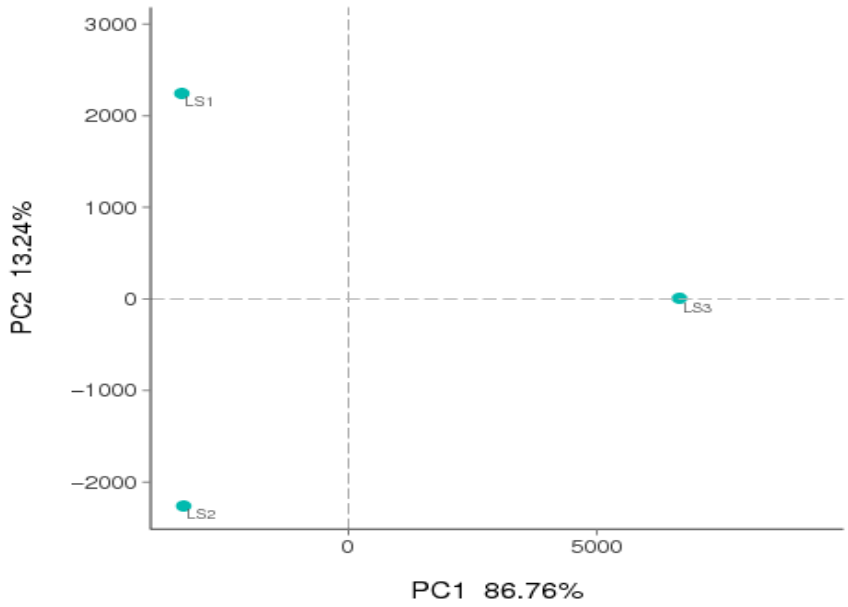

Fig. (3): Principal Component Analysis

The samples L1, L2 and L3 showed varying microbial community (Fig. 3), which is typical with samples of aged refuse collected from different points in the landfill. The number of principal components is less than or equal to the original variables, which is complex to its comparative measurement. Therefore, points of the samples are closer to one another once they have comparable bacterial community. The PC1 result (Fig. 3), shows more variability among the samples L1, L2 and L3, although the samples are composed of solid waste materials, they are collected from different locations which supposedly may possess varied characteristics. This explicates the inconsistent levels of biodegradation experienced in different biofilters. Therefore, in biological treatment of wastewaters, media characteristics play a significant role.

Conclusions

Microbial activities in biodegradation of pollutants and nutrient reduction using aged refuse biofilters vary due to different bacteria present. This study reveals the presence of abundant diverse groups of cultured, uncultured and unclassified bacteria. The class of *Firmicutes Chloroflexi, Proteobacteria* and *Actinobacteria* were highly dominant while

other classes are subdominant phyla which include; *Bacteroidetes, Acidobacteria, Planctomycetes, Latescibacteria*, *Nitrospirae* and others, which had lower percentages. The composition of these bacteria may relate to its important role in biodegradation, also could be differed according to the composition of solid waste in the region. Furthermore, the result of the principal component analysis (PCA) shows that the samples were distributed far from each other in different clusters, and this reveals diverse bacterial groups. Therefore, extensive characterization of aged refuse must be ensured for better optimization of operating conditions for effective performance of the aged refuse bioreactors during biological wastewater treatment.

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Conflicts of Interest

The authors declare no conflict of interest, and also the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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