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Molecular Phylogenetics of Dominant Endophytic Fungi in Leaves of *Vitellaria Paradoxa* Using Internal Transcribed Spacer Gene Region

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Abstract

On a global scale, diverse fungal species live inside plant tissues in mutualistic association either as highly specific to single hosts or widespread in many species. Despite the huge importance of *Vitellaria paradoxa* (Shea Butter Tree) in Africa, the fungal endophytes associated with this plant have not been documented. In this study, we characterized and determined the phylogenetic relationship of the endophytic fungi in *V. paradoxa*. Fungal isolation was done from *V. paradoxa* between July and December, 2019 by cutting, sterilizing and inoculating the healthy leaves on Potato Dextrose Agar (PDA) and Water Agar (WA) for 7 and 21 days respectively. Result of the isolation showed a dominant fungal endophyte repeatedly isolated from the leaves of *V. paradoxa.* Morphological features of the isolates were described and the genomic DNA was extracted for molecular analyses. Accurate molecular phylogenetic analyses of the sequences of the Internal Transcribed Spacer (ITS) gene regions using maximum likelihood analysis revealed the isolate as *Lasiodiplodia theobromae*. This study therefore succinctly represents the first report of *L. theobromae* as an endophyte in leaves of *V. paradoxa* using a robust analysis of its phylogeny. The implication of the occurrence of this endophytic fungus from shea butter fruit is also discussed.

1. Introduction

Vitellaria paradoxa is a tree indigenous and endemic to Africa and is the only species in the genus *Vitellaria*, the Sapotaceae family. It grows perennially and deciduously throughout the Guinea Savannah to Sahel Savannah regions with a gestation period of 15-20 years [1]. *Vitellaria paradoxa* is highly valued both domestically and internationally and it is one of the most important sources of vegetable oil in rural areas of the Savanna zone of West Africa, in many parts of the Sahel Africa and particularly in Northern Nigeria [2, 3]. It also has the potential for improving nutrition and boosting food supply in the annual hunger season, promoting rural growth, and sustainable land care [4].

Endophytes form inconspicuous infections within tissues of healthy plants for all or nearly all their life cycle and their host tissues appear symptomless, and they remain asymptomatic for many years, only to become parasitic when their hosts are stressed [5, 6]. Global occurrence of endophytes have been carried out over

several years corroborating their roles in plant vigour, growth [7, 8, 9, 10], enhancing nutrient uptake, providing defence against pathogens or herbivores [11], and productivity as well as their secondary metabolites and their roles in disease occurrence in their host [8, 12, 13, 14]. Endophytes may produce a plethora of bioactive metabolites that may be involved in the host-endophyte relationship [10] and may serve as potential sources of novel natural products for exploitation in medicine, agriculture, and industry [15, 16].

Several studies have also explored relationships between endophytes and their role as pathogens and saprobes [17, 18]. The evidence is circumstantial; however, it seems likely that some saprobes are derived from endophytes [17, 18, 19]. Some endophytes are known to be latent pathogens and much has been written on the subject [14, 20, 21]. In fact numerous examples of endophytes becoming pathogens have been documented, spurring , a considerable interest in screening for endophytes and for their novel compounds [22, 23].

Endophytic fungi in African Sapotaceae species play critical roles in plant health and ecological adaptation. They are known to enhance stress tolerance and promote secondary metabolite production in various tropical fruit-bearing trees, suggesting potential similar roles in underexplored Sapotaceae species such as Pouteria alnifolia and Synsepalum dulcificum [24], and Vitellaria paradoxa. These associations highlight the potential of endophytes in enhancing the sustainability of African forest ecosystems. Vitellaria paradoxa, though highly valued for its ecological and economic benefits, remains poorly investigated for its fungal endophytes. This gap limits our understanding into its plant–microbe interactions that may influence host resilience, productivity, and adaptation.

Endophytes have further been linked with numerous functions, including them being able to produce several compounds that promote the growth of plants and help them adapt to their environment [25]. They are thus also recognized as an indissociable member of the plant microsystem [12]. Several studies have reported the occurrences of endophytic fungi from economically important forest trees. For example, *Lasiodiplodia theobromae* was isolated as dominant from *Adansonia gregorii* [26], *Cunnighamella* sp. from *Chrysophyllum albidum* [27], *Cenangium ferruginosum , Lophodermium pinastri, L. seditiosum,* and *L. conigenum* were all isolated from Scots pine (*Pinus sylvestris*) [28]. Also, [29] isolated antagonistic *Trichoderma koningiopsis* from a wild *Hevea guianensis* in the Peruvian Amazon. However, little is known about the fungal endophytes of *Vitellaria paradoxa*. Therefore, this study seeks to bring to light the endophytes present in the healthy leaf tissues of *Vitellaria paradoxa* using molecular identification techniques.

2. Materials and Methods

2.1 Collection of Samples

Green, healthy leaf samples were collected from *Vitellaria paradoxa* plants from different locations in the University of Ilorin (8.4910° N, 4.5952° E), Kwara State, Nigeria. Collection took place between July and December, 2019. The sample collections were done with sterile blades and kept in sterile polythene bags at the collection location before being taken to the laboratory for endophytic fungi isolation.

2.2 Isolation of Fungal Endophytes

The isolation process followed the method of [30] and [31] with modifications. The healthy plant leaves of *Vitellaria paradoxa* were cut by a sterilized scissors into 1.5 cm^2 segments, sterilized by first washing in sterile distilled water for 1 min, then in 70 % ethanol for 1 min, 1 % sodium hypochlorite (NaOCl) for 3 mins, 70 % ethanol for 30 s and finally rinsed in sterile distilled water. Sterilized filter papers were used to blot dry the leaves under aseptic condition before inoculation on Potato Dextrose Agar (PDA) containing 0.05 mg streptomycin. The inoculated plates were then incubated at 25 ± 2 °C for 7 days. The fungal isolates were then sub-cultured to obtain pure isolates.

2.3 Morphological Identification

The pure cultures were classified based on their morphological characters such as surface texture, surface topography, surface pigmentation, reverse pigmentation and growth rate. The identification of the isolates was performed using the relevant publications [32].

2.4 Fungal DNA Extraction, Amplification and Sequencing

Eight days old pure cultures of the morphologically similar isolates were used for genomic DNA extraction. DNA extraction was carried out with a commercial kit (Zymo research fungal mini prep) (USA) following the manufacturer's instructions. The Polymerase Chain Reaction (PCR) was then carried out using the primer pairs ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [33] , *EF1-983F* (5′-GCYCCYGGHCAYCGTGAYTTYAT-3′) and EF1-1567R (5′-ACHGTRCCRATACCACCRATCTT-3′) [34] and LR5 (5′-

TCCTGAGGGAAACTTCG-3′) and LR0R (5′-ACCCGCTGAACTTAAGC-3′) [35], [36] to amplify the internal transcribed spacer (ITS) region, the translation elongation factor (*tef1-α*) and the large sub unit (LSU) genes of the total genomic DNA. Polymerase Chain Reaction (PCR) and sequencing were carried out at Macrogen Europe B.V. (Netherlands) using the same forward and reverse primer set.

2.5 DNA Sequence Assembly and Alignment

After the sequencing results were received, BioEdit software [37] was used to obtain a consensus DNA sequence from both the forward and reverse sequences. Sequence similarity searches were performed using the consensus DNA sequence to compare with similar sequences in the GenBank database maintained by the National Center for Biotechnology Information (NCBI) using the on-line tool BLAST-n on the GenBank website (http://blast.ncbi.nlm.nih.gov). Sequences from the translation elongation factor (*tef1-α*) and the large sub unit (LSU) gene regions failed the similarity searches and they were subsequently removed from the analyses.

Similar sequences and outgroup species (*Diplodia corticola*) sequences of the ITS region from reputable sources were downloaded from GenBank (Table 1). AliView version 1.17-beta1 software [38] was used to do the alignment for the ITS gene region of our isolate. Afterwards the sequences for the ITS gene region were processed for the phylogenetic analysis.

Table 1 *Details of the DNA sequences of Lasiodiplodia spp. included in the phylogenetic study*

S/N	Species	Isolate	Host	Origin	GenBank accession number
1	*Diplodia corticola*	CBS112549	N/A	N/A	AY259100 (Outgroup)
2	*D. corticola*	CBS112546	N/A	N/A	AY259090 (Outgroup)
3	*Lasiodiplodia theobromae*	UIL99	*Vitellaria paradoxa*	Nigeria	OL414949 (this study)
4	*L. theobromae*	NAS-225	Strawberry	Pakistan	MF176233
5	*L. theobromae*	Eg36	*Eucalyptus grandis*	Kenya	FJ904839
6	*L. theobromae*	Xsd08008	N/A	N/A	EU918707
7	*L. theobromae*	CMW28571	*Terminalia ivorensis*	Cameroon	GQ469924
8	*L. theobromae*	C311	*Acacia* sp.	Thailand	MK347754
9	*L. theobromae*	L3	Mango	China	KR260793
10	*L. theobromae*	MFLUCC	*Celtis formosana*	Taiwan	MW063184
11	*L. theobromae*	CBS129754	N/A	N/A	JX545099
12	*L. theobromae*	L1	Mango	China	KR260791
13	*L. theobromae*	CERC3516	*Eucalyptus urophylla x Eucalyptus grandis*	China	KX278049
14	*L. pseudotheobromae*	Meyer129	N/A	N/A	KX244814
15	*L. pseudotheobromae*	CMM3887	*Jatropha curcas*	Brazil	KF234559
16	*L. pseudotheobromae*	CBS116459	*Gmelina arborea*	Costa Rica	EF622077
17	*L. pseudotheobromae*	LASOM2	*Mangifera indica*	Peru	KU507476
18	*L. venezuelensis*	CBS129755	N/A	N/A	JX545102

2.6 Phylogenetic Analysis

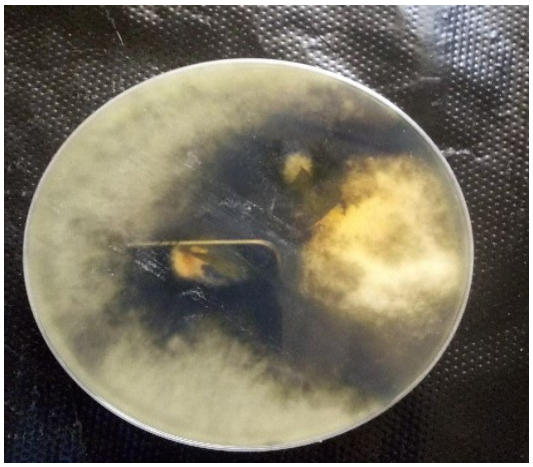
Maximum Likelihood (ML) analysis was done using MEGA-X (Molecular Evolutionary Genetics Analysis) software [39] and phylogenetic tree reconstruction pipelines available on the Phylogeny.fr platform (http://www.phylogeny.fr). For the phylogenetic tree created using the maximum likelihood approach, the Nearest-Neighbour-Interchange (NNI) heuristic search option was used with all characters equally weighted and partial deletion approach used for treating gaps at 90% cut off.

3. Results

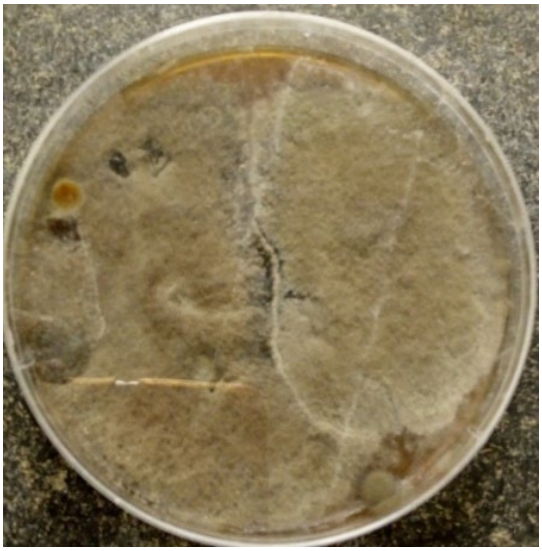
3.1 Isolation and Morphology of the Endophyte

Several morphological similar fungal endophyte strains were dominantly observed growing from the leaves of *Vitellaria paradoxa* grown on PDA. These fungal strains were initially identified as *Lasiodiplodia* sp. The morphological characters of the pure cultures of *Lasiodiplodia* at room temperature after 7 days showed fast growing white aerial mycelia (Figure 1A-D) which turns dark olivaecious, with the reverse side of the colony black at the centre and a butter-like color towards the margin (Figure 1E). Microscopic examination of the colonies at 100× magnification further showed the intricate network of the mycelia (Figure 1F).

(a)

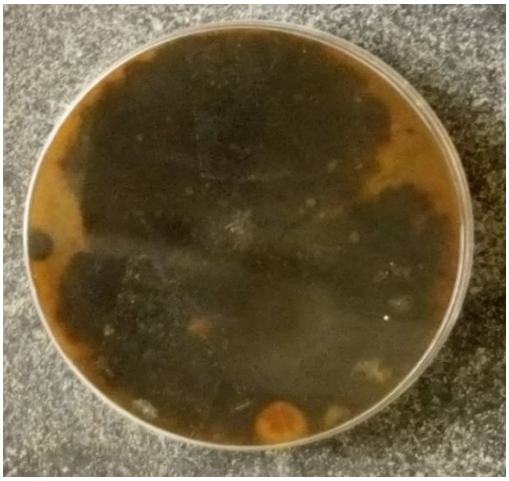


(b)

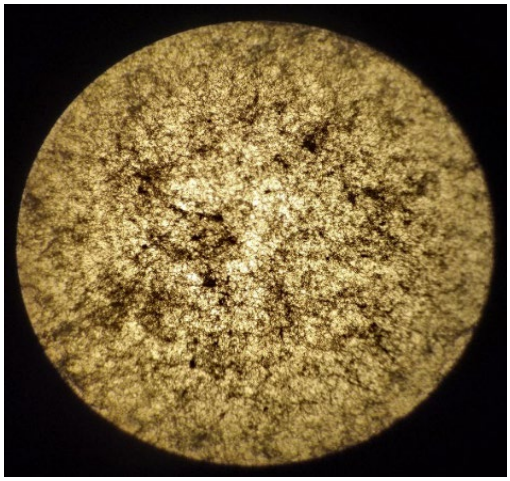


(c)

(d)



(e)



(f)

Fig. 1 *L. theobromae isolated from Vitellaria paradoxa. (a) Front view of the covered petri dish showing the growth on potato dextrose agar (PDA) after 8 days; (b) Reverse view of the covered petri dish showing the growth on potato dextrose agar (PDA) after 8 days; (c - d) Front view of the covered and uncovered petri dish showing the growth on PDA after 14 days ; (e) Reverse view of the growth on PDA after 14 days; (f) Microscopic view of the aerial mycelium at 100x magnification after 8 days*

3.2 Molecular Identification and Phylogenetic Analysis of the Isolate

Molecular characterization of *Lasiodiplodia* was done to confirm the identity of the isolate to species level using the DNA sequence of the ITS region. The sequence length of the ITS region was 560 bp. A BLAST-n search using the consensus ITS sequence showed over 99% identity (Table 2) with several sequence of *Lasiodiplodia theobromae*, *L. pseudotheobromae, L. brasiliensis, L. venezuelensis, L. parva* and other unspecified fungal species. *L. theobromae* had the highest number of hits (60 out of the identified 100 hits). The molecular phylogenetic relationship of the isolate was further inferred with the phylogenetic analysis software (MEGA). Eighteen (18) sequence datasets were analyzed for the ITS regions (Table 1) (including the isolate and two outgroup sequences) which comprised 2410 characters. Based on the molecular phylogenetic analysis of the ITS region, the isolate was identified as *Lasiodiplodia theobromae* (Figure 2).

Table 2 *Details of the similarity indices based on BLAST result on the GenBank website*

S/N	Identified Species	Similarity index (%)	Query cover (%)
1	*Lasidiplodia hormozganensis*	99.82	99
2	*Lasidiplodia theobromae*	99.64	99
3	*Lasidiplodia parva*	99.64	99
4	*Lasidiplodia theobromae*	99.64	99
5	*Lasidiplodia theobromae*	99.64	99
6	*Lasidiplodia sp.*	99.64	99
7	*Lasidiplodia pseudotheobromae*	99.46	99
8	*Lasidiplodia pseudotheobromae*	99.46	99
9	*Lasidiplodia theobromae*	99.46	99
10	*Lasidiplodia theobromae*	99.46	99

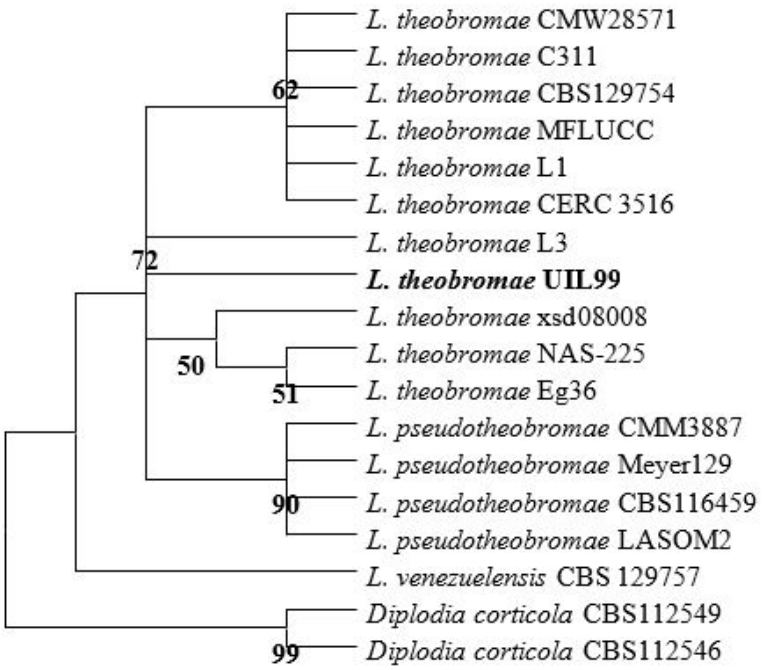


Fig. 2 *Phylogenetic tree of Lasiodiplodia species inferred from alignment of the Internal Transcribed Spacer (ITS) sequences. Bootstrap values by Maximum Likelihood less than 50% are omitted. The tree was rooted to D. corticola*

3.3 Discussion

L. theobromae has a global occurrence with several records in Africa where it causes diseases and harvest losses in Mango, Cashew, Cocoa, Banana, Yam and *Pinus elliottii* seeds [40], [41], [42], [43]; in Asia, it caused diseases on fruit of Melon in Thailand, kenaf seeds in Malaysia, Bast in China, *Psidium guajava* in Malaysia and *Cassia fistula* in China [44, 45], 46, 47, 48] and in Europe [49, 50, 51, 52]. However, this is the first report of *Lasiodiplodia theobromae* as an endophyte in leaves of *Vitellaria paradoxa* using a gene sequence analysis. *Lasiodiplodia theobromae* has been reported as an endophyte in *Acacia mangium* [53], *Viscum coloratum* [54], *Cattleya*, *Phalaenopsis* and *Oncidium* [55], *Cinnamomum zeylanicum* [56], *Boswellia ovalifoliolata* [57], and *Theobroma cacao* [58].

Adedeji and Adeniyi (2015) reported *L. theobromae* as being present in tea leaves [59] while [60] found *L. theobromae* to be responsible for the leaf necrosis on tea plant (*Camellia sinensis*). *L. theobromae* was also reported to be responsible for the root rot and collar rot disease of *Jatropha curcas* in Benin, Nigeria [61], the dieback of cashew in Nigeria [62] and the dieback of mango [63, 64].

There haven't been reports on the role of *L. theobromae* in *Vitellaria paradoxa* (Shea butter tree), however, it has been previously isolated from shea butter fruit in a bid to identify the fungal species responsible for its bio-deterioration in storage [65]. The biology of *V. paradoxa*, particularly its high oil content and prolonged fruit maturation [66], creates a moist, nutrient-rich environment conducive to fungal colonization. This ecological niche supports the growth of *L. theobromae*, which thrives in warm, humid conditions and invades through wounds or natural openings [21]. *Lasiodiplodia theobromae* has also been implicated for postharvest losses in mango fruits [67]. This study thus provides information on the presence of *L. theobromae* in the leaves of *Vitellaria paradoxa* as an endophyte. The transition between its endophytic and pathogenic lifestyles is influenced by environmental factors and host stress conditions [68].

The presence of *L. theobromae* as an endophyte and a potential pathogen causing numerous diseases suggest the possibility of it being pathogenic only under certain conditions. This corresponds to the study by [51] which sought to identify potential pathogenicity genes that were expressed when *L. theobromae* was exposed to heat as a form of abiotic stress. The dominance of a specific endophyte within a plant host as observed in this study often suggests a specialized symbiotic relationship, ranging from mutualism to latent pathogenicity[69]. The isolation of *Lasiodiplodia theobromae* from *Vitellaria paradoxa* highlights its dual role as both endophyte and latent pathogen [68, 70]. Similar patterns were observed in *Theobroma cacao*, where *Colletotrichum gloeosporioides* dominates under humid conditions [71], and in *Mangifera indica* where *Fusarium* species are prevalent, influenced by seasonal shifts [72].

These studies underscore how host species, climate, and geography shape endophytic communities, suggesting that *L. theobromae*'s dominance in *V. paradoxa* may reflect both environmental adaptation and potential pathogenicity. The frequent isolation of *L. theobromae* from *Vitellaria paradoxa* implies a persistent endophytic phase that may shift to pathogenicity under stress conditions, such as environmental stress or mechanical injury. However, it should be noted that factors such as geographic location and seasonality can influence the composition and diversity of endophytic fungal communities in plants [73].

The use of molecular method proved particularly important in this study due to the fact that *Lasiodiplodia theobromae* sporulate poorly in commonly used media like Potato Dextrose Agar (PDA) [74]. This further supports the reports by [75], [76] and [77] on the need to adopt the use of molecular methods due to the increasing number of non-sporulating fungal isolates.

In a study by [78], members of the Botryosphaericaceae were found to frequently act as endophytes in latent phase that can cause diseases in native and non-native plant hosts around the world. In the study, *L. theobromae*, and *L. mahajangana,* were however implicated as the dominant species. This suggests the possibility of *Lasiodiplodia theobromae* being linked to the occurrence of diseases in plants.

L. theobromae has also been implicated in several reports as being responsible for substantial yield loss in economically important plants across the world. It is reported to have a host range of more than 280 plant species [79] and to be present in more than 70% of farms surveyed in Nigeria where it is further linked to the colossal yield loss of around 80% of harvest [80]. This suggests its relative ability to invade plant tissues and adapt to different environmental conditions and also validates this study since the organism was isolated from *Vitellaria paradoxa,* which is an economically important tree in Nigeria and the whole of West Africa [81]. According to [52], its adaptability and ability to cause diseases in plants is largely due to its capacity to produce bioactive compounds directly involved in host fungus interactions. However, despite its threatening disease capacity, *L. theobromae* have been reported to have good antibacterial activity [82] and also contain a rich amount of secondary metabolites [52].

A more detailed morphological characterization of the *Lasiodiplodia* isolate was however not performed for this study due to the fact that identification of Botryosphaeriaceae species using morphology is virtually impossible and is only able to distinguish between genera [83, 84, 85, 86]. Thus the best way to differentiate species within Botryosphaeriaceae is through the use of DNA sequence data, preferably, combining sequences from multiple loci [85, 86].

The outcome of this study implicates the need for further investigations on the roles of the secondary metabolites of *L. theobromae* in the metabolism of *Vitellaria paradoxa* and its pathogenicity potentials, high-throughput studies of the entire endophytic community at different life stages and different parts of *V. paradoxa*.

4. Conclusion

This study brings to light the occurrence and molecular identification of *L. theobromae* on leaves of *V. paradoxa* collected from the university of Ilorin campus, Nigeria. This study also represents the first report of *L. theobromae* as an endophyte in leaves of *V. paradoxa* and is still preliminary, and may be useful for further studies to correlate *Lasiodiplodia theobromae* with the different diseases associated with *Vitellaria paradoxa*. Indeed, it is also evident that performing complementary studies based on sequencing more than one gene region is important in order to support reliable species identification. Additional studies on the impact, biology, fungicide sensitivity, and epidemiology of these fungi may prove vital towards the development of suitable management strategies to reduce the disease impact on the productivity and longevity of *Vitellaria paradoxa.*

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Conflict of Interest

The authors have declared no conflict of interest.

Author Contribution Statement

The authors confirm contribution to the paper as follows: LAA: ***conceptualization, data curation, funding acquisition, methodology, software, writing****, TBB:* ***funding acquisition, writing- reviewing****, AAO:* ***conceptualization, methodology, software****, MFO:* ***conceptualization, methodology, writing****, AKA:* ***supervision, investigation, writing- reviewing****.*

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