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Unveiling *Leifsonia* sp.: A Novel Bacterium from *Casuarina equisetifolia* Root Nodules – Isolation, Identification, and Biochemical Characterization

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Abstract

The survival rate of bio fertilizers is enhanced when they contain nodule bacteria. The primary objective of this research is to identify a superior endophytic bacteria that promotes plant growth in *Casuarina equisetifolia*. Six isolates were collected from the root nodules; with one isolate demonstrating significant plant growth promoting capabilities. Approximately 90% of the isolates from C. *equisetifolia* were Actinobacteria, which are Gram-positive organisms. All isolates were evaluated for their Plant Growth Promoting (PGP) activity, and one isolate was recognized as the predominant plant growth promoting *Actinobacterium*. Among the Nodule Associated Bacteria (NAB) isolates, L1 exhibited the highest PGP characteristics. The phylogeny of the isolate was determined through 16S rRNA sequencing. The biomass characteristics of *Zea mays*, including the number of leaves, shoot length, girth, and root length, demonstrated significant growth and stability when inoculated with a 50% concentration of *Leifsonia* sp. It is recommended that *Leifsonia* sp. be utilized as a successful endophytic bacterial inoculant for the growth of C. *equisetifolia*.

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Introduction

Casuarina equisetifolia L., a member of the *Casuarina*ceae family, native to Australia, New Guinea, Southeast Asia and India. It is a remarkable evergreen tree that exhibits both dioecious and monoecious characteristics. *Casuarina equisetifolia* exhibits rapid growth during its initial seven years, achieving an annual increase of 1.5 to 2.5 meters. The maximum volume yield is generally observed between the ages of 15 and 20 years, reaching approximately 7 to 10 cubic meters per hectare annually. One of the notable features of this tree is its ability to produce high-quality fuel wood and excellent charcoal. Additionally, it possesses termite resistance and exceptional durability, making it a valuable material for construction purposes.

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The rustling of the wind passing through its canopy creates a distinctive whistling sound, earning it the name "Gaalimara" or Wind tree. Moreover, this tree plays a crucial role in safeguarding coastal environments from storms and tsunamis, and it has become a key component in various coastal afforestation programs aimed at creating bioshields.[1] Furthermore, C *equisetifolia* is utilized in agroforestry systems alongside vegetable and pulse crops, further highlighting its versatility.[2] It is also employed in the production of paper and serves as scaffolding in building construction.[3]

C. equestifolia utilizes various Actinobacteria such as *Frankia* sp., *Micromonospora* sp., and others to fix nitrogen. *Casuarina* plants, known as good pioneer plants, can be employed in degraded lands to enhance fertility. This aids in the pedogenetic process and enables nitrogen fixation with the assistance of *Frankia*.[4] Actinobacteria possess the capability to boost plant growth by producing growth-promoting substances like auxins, gibberellins, and more.[5] *Micromonospora* is recognized as a root nodule endophytic bacteria that supports the growth of *Casuarinas* (*C. equestifolia* and C. *junghuhiniana*) and enhances biocontrol potential against *Ralstonia solanacerum*.[6]

Leifsonia sp., an endophytic bacterium found in sugarcane and grasses, has been identified by Young.[7] While some species within this genus are known to be pathogenic, others have been shown to be beneficial for plants. According to Kang, the culture filtrate of *L. soli* has been found to significantly enhance the biomass, hypocotyl, and root lengths of cucumber seeds when compared to controls that were not inoculated or treated with distilled water.[8] Lin observed that *Casuarina* has a rich microbiome in its litter, roots, seeds, and branchlets.[9] The current study focuses on isolating *Leifsonia* sp. from the root nodules of C. *equisetifolia* and investigating its potential as a plant growth promoter in *Zea mays*.

Materials and Methods

Sample Processing

Root nodules obtained from the rhizospheric area of *Casuarina equisetifolia* were gathered. The nodules underwent three washes with tap water to eliminate any debris. Subsequently, they were surface sterilized twice with 1% Mercuric chloride (HgCl3) and rinsed five times with distilled water. The root nodules were then crushed in 1 ml of distilled water using a mortar and pestle.

Isolation of Endophytic Bacteria

The root nodules that had been crushed were subjected to the serial dilution technique and subsequent plating. The Defined Propionate Media (DPM) plates were then placed in an incubator at a temperature of 28°C for a duration of 21 days. Following the incubation period, the plates were carefully examined using a stereo microscope. The morphology of the colonies obtained from the isolates was duly recorded.

In Vitro Screening of Isolates for PGPR activity

Antagonistic activity

The strains were centrally inoculated on Starch Casein agar plates, followed by an incubation period at 30°C for 24 hours. The pathogens for the test was subcultured from the Forest Protection division, ICFRE-IFGTB Coimbatore stock cultures. Subsequently, different test pathogens such as *Sclerotium*, *Alternaria*, *Phytophthora*, *Diploidia, Fusarium* species were streaked along the periphery of the plates after the initial 24-hour incubation. The plates were then incubated once more at 30°C for an additional 24 hours.[10] The presence and characteristics of the inhibition zone were observed and determined to be either beneficial or not. Percent inhibition was calculated as follows,

Percentage of Inhibition(%) = Radius of pathogen away from antagonist – Radius of pathogen / Radius of pathogen away from antagonist. × 100

Organic Acid Production

The test organism was introduced into the Minimal Salt Medium (MM9) broth and allowed to incubate for a period of 2-3 days at a temperature of 30°C. Following the incubation period, methyl red indicator was added. The alteration in color observed in the media serves as an indication of a positive result.[11]

Ammonia Production

After being freshly grown, the cultures were introduced to 10 ml of peptone water and left to incubate for 48 hours at a temperature of 30°C. Subsequently, 0.5ml of Nessler's reagent was introduced to each tube. The presence of a brownish-yellow color signifies a positive result.[12]

Phosphate Solubilization Activity

The selected isolates were introduced as spots in the middle of the Pikovaskayas medium and kept in an incubator at a temperature of 30°C for a duration of 7 days. The presence of a distinct transparent area signifies a positive outcome for phosphate solubilization, as stated by Nautiyal.[13] Phosphate solubilization efficiency was calculated by,

E = Solublization Diameter — Colony Diameter / Colony Diameter × 100

Hydrogen Cyanide Production

The chosen isolate L1 was introduced onto the nutrient media plates which were enriched with 4.4 gm of glycine per liter. Subsequently, a Whatman No.1 Filter paper that had been pre-soaked in a solution of 2% NaCO3 in 0.5% Picric acid was placed on the surface of the agar plate. The plate was then sealed using parafilm and left to incubate for a period of 4 days at a temperature of 30°C. A change in color of the filter paper from a deep yellow hue to reddish brown was considered as a positive result.[14] The quantitative analysis was carried out by employing UV-Visible Spectroscopy at wavelengths of 480, 520, and 640 nm.

IAA Production

The *Actinobacterium* was introduced into Luria Bertani broth supplemented with 100mg/l of tryptophan. The culture was then placed in an incubator set at 250 rpm and 30 °C for a period of 3-5 days to allow for growth. Once fully grown, the cultures were harvested and subjected to centrifugation at 10000 rpm for 10 minutes. Subsequently, 2ml of the supernatant was combined with 4 ml of Salkowski's reagent (composed of 50 ml of 35% HClO4 in 1 ml of 0.5 M FeCl3 Solution) and left to incubate in darkness for 30 minutes. The development of a pink coloration indicated a positive result.[15] The Optical Density was determined through UV-Visible Spectroscopy at a wavelength of 540 nm.

Molecular Characterization

The culture underwent 16SrRNA sequencing to conduct phylogenetic and genomic analysis. The culture was identified through the utilization of BLAST software. The MEGA software was employed for neighbour joining and phylogenetic analysis.

Pot Trail Analysis: (Optimization)

Different concentrations of the selected inoculant (*Leifsonia* sp.) were prepared. The chosen strain was administered at varying concentrations to the experimental plant *Zea mays*. Specifically, the concentrations used were as follows.

Treatment 1 - 25% of the strain, Treatment 2 - 50% of the strain, Treatment 3 - 75% of the strain, Treatment 4 - 100% of the strain, Treatment 5 - Control (no inoculation).

Five replicates (n=5) were maintained for each treatment. The biomass data obtained was then subjected to statistical analysis utilizing SPSS Software. To determine significant differences among the groups, Duncan's test was conducted with a significance level set at P < 0.5%.

Results

Isolation of Endophytic Bacteria

Six isolates were chosen from the spread plates, and the colony morphology of these isolates from the medium was examined. (Table 1)

Table 1: Colony morphology of the isolates, isolated from the root nodules of *Casuarina equisetifolia*

Isolates	Media	Shape	Size (cm)	Elevation	Margin	Color	Opacity
L1	DPM	Circular	1.67	In agar	Entire	White	Transparent
L2	DPM	Circular	0.73	In agar	Entire	White	Transparent
L3	DPM	Circular	1.43	In agar	Entire	White	Transparent
L4	DPM	Circular	1.56	In agar	Entire	White	Transparent
L5	DPM	Circular	0.45	In agar	Entire	White	Transparent
L6	DPM	Circular	0.56	In agar	Entire	White	Transparent

Antagonistic Activity

The growth of the pathogens in the plate inhibited the isolate L4. The metabolism and physiology of L4 were significantly affected by the presence of the pathogens, leading to an inability to uptake nutrients from the media. As a result, the growth of the organism was not visible on the plate. In contrast, isolate L1 exhibited strong inhibitory effects on all pathogens, with more than a 50% reduction in growth. *Diploidia* was the most inhibited pathogen, followed by Phytophtora, *Alternaria*, *Fusarium*, and *Sclerotium*. The diameter of growth for L1 was measured at 2.5 cm. Isolate L2, on the other hand, inhibited the growth of pathogens in the order of *Phytopthora*, *Diploidia*, *Alternaria*, *Fusarium*, and *Sclerotium*. The growth of L2 was minimal, measuring only 0.23±0.5 cm in diameter. Similarly, L3 showed similar inhibitory effects to L2, with very limited growth observed at 0.22±0.1 cm in diameter. Isolates L5 and L6 specifically inhibited the growth of *Diploidia*, *Phytophthora*, and *Alternaria*.(Table 2) Among the isolates, L1, L2, L3, L5, and L6 were specifically picked for further examination, with L4 being omitted from consideration due to its reduced antagonistic activity.

Table 2: Percent Inhibition of radial growth of Pathogen was calculated using Percent inhibition formula.

Isolates	Sclerotium	Alternaria	Phytopthora	Diploidia	Fusarium	Result
L1	73.16%	75.00%	89.00%	93.00%	68.45%	+++
L2	62.47%	89.34%	75.67%	90.12%	73.65%	+++
L3	42.32%	72.45%	66.08%	86.71%	67.89%	++
L4	0.00	11.92%	23.07%	24.10%	13.27%	---
L5	36.28%	84.90%	90.62%	75.16%	47.83%	++
L6	45.23%	76.23%	82.33%	76.10%	32.17%	++

(--- indicates no antagonistic activity, ++ indicates antagonistic activity, +++ high antagonistic activity)

In Vitro PGPR Analysis

The L4 isolate was not accepted during this stage. The chosen isolates underwent processing for the analysis of plant growth-promoting rhizobacteria (PGPR). The presence of a green color in the organic acid production test signifies the production of specific organic acids for nutrient uptake. On the other hand, the brown color observed in isolates L5 and L6 indicates a lower amount of ammonia production. In contrast, isolates L1 and L2 exhibited a yellow color, indicating a significant amount of ammonia production. Notably, L3 produced a golden brown color, suggesting a substantial production of ammonia. Based on the color observations, the order of ammonia production is L3>L1=L2>L5=L6. IAA production was observed only in L1 isolate, which it produced pink to reddish color after 30 minutes of incubation. The isolates L2, L3, L5 and L6 was colorless to light yellow. From the primary screening the L1 isolate was screened positive as effective PGPR. The L1 isolate showed increase in the amount of IAA proportionally in increase in the concentration of tryptophan (Table 3).

Table 3: Quantitative measurement of IAA (L1) in UV-Visible spectroscopy at 540 nm in 24 and 48 hours.

Isolate	24hrs	42hrs
L1	µg/ml	µg/ml
0.1	1.233	1.703
0.5	1.787	1.937
1	1.904	2.369
5	2.033	2.408
10	2.078	2.796
15	2.448	2.902

Table 4: Quantitative analysis of HCN production by L1 isolate by using UV-Visible spectroscopy (µg/ml).

Isolate	480 nm	540nm	620nm
L1	0.015	0.006	0.002

The L1 isolate's HCN production caused the filter paper to exhibit an orange shade, indicating the bio control capability of the L1 isolate. The plate's ACC deaminase activity led to the growth of approximately 30×10-1 colonies of the organism. This activity also resulted in a reduction of ethylene. Additionally, the organism L1 demonstrated phosphate solubilizing activity, with an efficiency of 50% solubilization.

Molecular Characterization of the Isolate

The isolated specimen exhibited a 99.68% match with *Leifsonia* sp. *Actinobacterium* in the BLAST software analysisGene Bank Accession ID = PP542598.1 *Leifsonia* sp. *strain Sathiqanum* 16S ribosomal RNA gene, partial sequen - Nucleotide - NCBI

The FASTA sequence of the organism is presented below,

TTACTAACCGACTCCCGACTTCATGAGGTCGA
GTTGCAGGACCTCAATCCGAACTGAGAGC
GGCTTTTTGGGATTCGCTC

CACCTTACGGTATTGCAGCCCTTTGTACCGG
CCATTGTAGCATGCGTGAAGCCCAAGACA
TAAGGGGCATGATGATTTGA

CGTCATCCCCACCTTCCTCCGAGTTGA
CCCCGGCAGTCTCCTATGAGTTCCCGCC
ATTACGCGCTGGCAACATAGAACGA

GGGTTGCGCTCGTTGCGGGACTTAA
CCCAACATCTCACGACACGAGCTGACGA
CAACCATGCACCACCTGTTCACGAGTG

TCCAAAGAGTTCCCTATTTCTAGGGCG
TTCTCGTGTATGTCAAGCCTTGGTAAGG
TTCTTCGCGTTGCATCGAATTAATC

CGCATGCTCCGCCGCTTGTGCGGGCCCCG
TCAATTCCTTTGAGTTTTAGCCTTGCGGCCG
TACTCCCCAGGCGGGGCGC

TTAATGCGTTAGCTGCGACACGG
AAACCGTGGAATGGTCCCCACATCTAGCGC
CCAACGTTTACGGCGTGGACTACCAGG

GTATCTAATCCTGTTCGCTCCCCACGC
TTTCGCTCCTCAGCGTCAGTTACGGCCCAAA
GAACTGCCTTCGCCATCGGTGT

TCCTCCTGATATCTGCGCATTCCACCGC
TACACCAGGAATTCCATTCTCCCCTACCGC
ACTCTAGTCTGCCCGTACCCAC

TGCAGGCCCGAGGTTGAGCCTCGGGTTTT
CACAGCAGACGCGACAGACCGCCTACGA
GCTCTTTACGCCCAATAATTCCG

GACAACGCTAGCACCCTACGTATTACCG
CGGCTGCTGGCACGTAATTAGCCGGTGCTTT
TTCTGCAGGTACCGTCACTTT

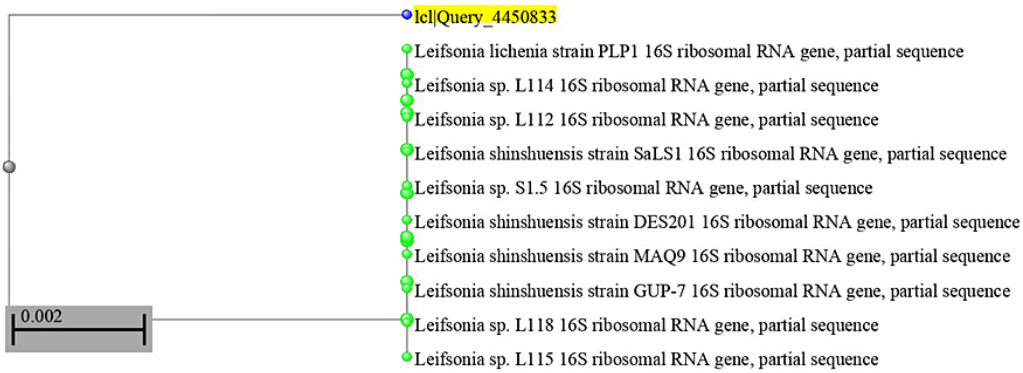

Fig. 1: Phylogenetic tree of the organism Leifsonia sp. from BLAST software.

Pot Trial Analysis: (for Optimization)

The analysis conducted on the pot trial has determined that the presence of a specified amount of *Leifsonia* sp. will result in an enhanced growth of *Zea mays*. Notably, When 50% of *Leifsonia* sp. was introduced, a significant improvement in plant growth was observed, followed by a 25% culture. This information is presented in table 5, sections a and b.

Table 5: (a) The table represents the germination percentage of different treatments in 5th and 10th day of inoculation & number of leaves present in the 7th and 15th day of inoculation. (a, b, c) represents the treatments are significantly different from each other in Zea mays for 5 replicates in each treatment to standardization the quantity of the culture for applying in agricultural fields.

Treatment	Germination percentage (5th day)	Germination percentage (10th day)	Number of leaves (7th day)	Number of leaves (15th day)
T1	3.66±0.57bc	4.66±0.577b	2.00±0.00b	4.33±0.57b
T2	4.00±1.00c	5.00±1.00b	3.00±0.00c	6.00±0.00c
T3	2.66±0.57b	4.00±0.577b	2.00±0.00b	3.00±1.00a
T4	3.66±0.57bc	4.33±0.577b	2.00±0.00b	2.33±0.57a
T5	0.00±0.00a	0.66±0.00a	0.667±0.577a	2.33±1.54a

[Values within a column followed by single letters (a,b,c) show significant varietal difference by Duncan's test]

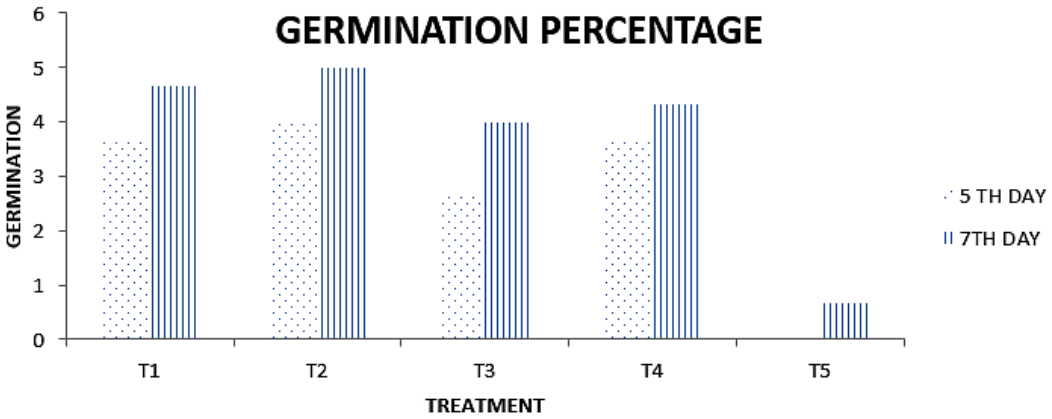

Fig. 2: Germination percentage of the different concentration of *Leifsonia sp. on Zea mays*. T2 exhibits high germination percentage.

Table 5: (b) The table represents the shoot length, girth and root length of different treatments, 15th day of inoculation. a, b, c represents the treatments are significantly different from each other in Zea mays for 5 replicates in each treatment.(n=5)

Treatments	Shoot length (cm)	Girth (cm)	Root length(cm)
T1	10.33±1.60c	0.436±0.046c	9.100±0.041c
T2	8.50±0.00bc	0.436±0.02c	7.23±0.020bc
T3	8.16±0.28bc	0.297±0.32b	7.144±0.032bc
T4	6.83±1.89b	0.440±0.36c	5.30±0.036b
T5	1.00±1.00a	0.103±0.105a	1.23±0.105a

[Values within a column followed by single letters (a, b, c) show significant varietal difference by Duncan's test]

Discussion

Leifsonia strains have different roles, including being pathogenic, commensal, or beneficial. We successfully isolated a beneficial *Actinobacterium* from the root nodules of *Casuarina equisetifolia*. According to Karthikeyan,[2] *Casuarina equisetifolia* can fix atmospheric nitrogen through a symbiotic relationship with *Frankia*. Our current research has identified that the endophytic Actinobacteria *Leifsonia* plays a crucial role in nitrogen fixation and ammonia oxidation. Previous studies by Karthikeyan have shown that the growth and biomass of *Casuarina*'s are enhanced when inoculated with *Frankia*, are strongly increased nitrogen fixation by *Frankia*.[16] Additionally, *Leifsonia* has been found to promote plant growth and biomass at specific culture concentrations. In a recent study, Karthikeyan reported that *Micromonospora maritima* exhibits significant antagonistic activity against Ralstonia solanacerum.[6] Our observations indicate that *Leifsonia* sp. itself demonstrates antagonistic effects against plant pathogens such as *Alternaria*, *Sclerotium*, *Phytophthora*, *Diploidia*, and *Fusarium* species. The antimicrobial activity against *Phytophthora* and *Diploidia* represents a noteworthy development.

Nordsettt[17] conducted research on the advantageous effects of *Leifsonia* sp. in enhancing water stress tolerance in plants, potentially through its involvement in bacterial osmotic stress, production of osmoprotectants, and synthesis of vitamin B9. Their in vitro studies revealed that *Leifsonia* acts as a Plant Growth Promoting bacterium, with L1 exhibiting superior abilities in indole-3-acetic acid (IAA) production, hydrogen cyanide (HCN) production, and phosphate solubilization compared to other isolates. Kang, observed that treatment with *L. soli* led to a significant increase in the growth of Waito rice seedlings compared to the control group.[8] Furthermore, it was found that 50% and 25% concentrations of *Leifsonia* culture resulted in enhanced shoot length, girth, and root length of *Zea Mays*. Kang, also demonstrated that *Leifsonia* sp. is a highly effective plant growth promoting bacterium.[18] Nordsett suggested that *Leifsonia* sp. holds promise as a plant growth-promoting bacterium for future applications.[17] Rastogi documented the synthesis of bacterial cellulose derived from *Leifsonia* species, highlighting its potential applications in food packaging and medical implants.[19] The current investigation emphasizes the significant role of these species in agriculture, particularly in promoting the growth of *Zea mays*. The current study highlights that a 50% concentration of *Leifsonia* culture can serve as an improved bio inoculant for plants.

Conclusion

According to the research, *Leifsonia* sp. has the potential to serve as a superior bioinoculant, capable of colonizing the inner tissues of the plant and functioning as an endophytic bacterium. When applied at a concentration of 50%, *Leifsonia* has been shown to enhance both the growth and physiological processes of the plant. This research indicates encouraging outcomes with *Leifsonia* sp. under controlled settings; however, subsequent investigations should prioritize field trials to evaluate the long-term effectiveness and ecological adaptability of this bacterial inoculant on C. *equisetifolia*. Such studies are essential for understanding the practical applicability and consistency of plant growth-promoting benefits across various soil types and climatic environments. Future investigations should seek to clarify the precise mechanisms by which *Leifsonia* sp. enhances growth in C. *equisetifolia*. This may encompass the synthesis of phytohormones (such as auxins and gibberellins), nitrogen fixation, nutrient solubilization, and the inhibition of plant pathogens. Gaining insights into these mechanisms will facilitate the advancement of efficient bio fertilizers. Evaluating the effectiveness of *Leifsonia* sp. as a bioinoculant for additional tree species would be beneficial, particularly within afforestation and reforestation initiatives. The wider applicability of this endophytic bacterium could yield substantial ecological and economic advantages, especially for rapidly growing species like C. *equisetifolia*. A thorough genomic examination of *Leifsonia* sp. will shed light on its genetic capabilities related to plant growth promotion, stress resilience, and compatibility with host plants. Additionally, metabolic profiling may uncover critical enzymes or metabolites produced by the bacterium that contribute to its advantageous effects. Researching the effects of *Leifsonia* sp. inoculation on soil health and microbial diversity is crucial for understanding its broader ecological implications and potential to enhance soil quality and ecosystem functioning.

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Ethics Statement
The research didi not involve human participants, animal subjects, or any material that requires ethical approval.

Author Contributions
- **Jini Viju Pamboor Chack:** Data collection, Analysis, Methodology and results writing-Orginal draft
- **Arumugam Karthikeyan:** work plan, Guidance, Review and Editing of article.

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