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Molecular Identification and Antifungal Potentials of Lactic Acid Bacteria Isolated from Soil

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Abstract

Lactic acid bacteria (LAB) are a large group of bacteria that produce lactic acid as a major end product of fermentation and are found in various environments, including plant materials, gastrointestinal tract of humans and animals. The increasing prevalence of fungal infections and growing resistance of fungi to conventional antifungal therapies necessitate the urgent exploration of new and effective antifungal agents. In this study, LAB were isolated from soil samples from the Botanical Garden of Ahmadu Bello University in Zaria, Nigeria. The fungicidal potency of isolates were tested against some fungi, namely *Aspergillus flavus, A. fumigatus A. Brasiliensis, Talaromyces purpureogenus, Penicillium notatum, and Fusarium oxysporum* employing the overlay technique. All isolates exhibited potent antifungal activity, with inhibition diameters of 30.2±0.1 to 45.3±0.3 mm. The identity of isolates was confirmed by 16S rRNA sequencing and comparing the sequences with the biological sequences in the National Centre for Biotechnology Information (NCBI) database. The similarity of isolates obtained were 99.74% for *Lactiplantibacillus plantarum* (KR816154.1), 99.80% for *Lactobacillus pentosus* (AB362757.1), 99.10% *Levilactobacillus brevis* (CP031208.1), *Lactiplantibacillus plantarum* (OQ224994.1) 99.26%, *Lactiplantibacillus plantarum* (KY203913.1) 99.26% and *Lantiplantibacillus plantarum* OQ224994.1 99.85%. The phylogenetic relationship established close similarities between *Lactobacillus* species isolated and other species from NCIB GenBank.

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INTRODUCTION

Lactic acid bacteria (LAB) have garnered significant attention in recent years for their potential as antifungal agents (Muhialdin *et al*., 2020). The increasing prevalence of fungal infections and the growing resistance to conventional antifungal drugs have necessitated the exploration of novel alternatives (3). The family lactobacillaceae encompasses the following genera: Weissella, Vagococcus, Tetragenococcus, Symbiobacterium, Streptococcus, Pediococcus, Oenococcus, Leuconostoc, Lactococcus, Lactobacillus, Enterococcus, Carnobacterium, Alloiococcus, and Aerococcus (Aspri *et al*., 2017). LAB, are commonly found in fermented foods and the human microbiome, have been discovered to produce antifungal compounds that inhibit fungal growth (Ema *et al*., 2020). Botanical gardens, with their diverse range of plant species, offer a unique environment for the isolation of LAB with potential antifungal properties (Luciana *et al*., 2020). Recent studies have investigated the molecular identification and antifungal potential of LAB isolated from botanical gardens, yielding promising results. For instance, a study by Yu et al. (2020), isolated LAB from a botanical garden in China and identified several species with antifungal activity against *Candida albicans*. Lactic acid bacteria are acknowledged as a sustainable and ecologically beneficial approach to food and

feed preservation and potential sources for new antimicrobials. The main element accountable for the preservation effect as well as their antimicrobial properties is the decrease in pH caused by the production of lactic acid (Tulini *et al.*, 2016; Alemayehu *et al.*, 2014). Lindgren and dobrogosz (1990) indicated that LAB generate supplementary antimicrobial substances in addition to lactic acid during fermentation with lactic and acetic acids as the predominant and most abundant. Consequently, the pH decreases. The chemicals that are generated include acetoin, formic acid, hydrogen peroxide, diacetyl acetic acid, and propionic acid (Bangar *et al.*, 2021). This study aims to determine molecular identification, antifungal potential of lab isolated from botanical garden of Ahmadu Bello University Zaria.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria

One (1) g each of 10 (ten) soil samples from the North, South, East and West cardinal points of the garden, were taken and placed into 9 ml of sterile distilled water. Ten- fold serial dilutions were made from the mix. Pour plate technique was used for isolation by dispensing 1 ml from the last 3 dilutions (10^{-7}, $10^{-8}$and 10^{-9}) into sterile petri-dishes followed addition of twenty (20) ml of molten De Man Rogosa and Sharpe (MRS) agar supplemented with 0.7% precipitated calcium carbonate. The plates were incubated anaerobically at 37 °C for 48 hours. Colonies with clear distinct zones around them were picked and subcultured onto fresh MRS agar plates to obtain pure cultures (Awan and Rahman, 2005).

Antifungal Activity of Lactobacillus Isolates

Antifungal activity of LAB was done using an overlay assay was employed as described by Lind *et al.*, (2005). Bacteria were inoculated in 2 cm lines on MRS agar plates and allowed to grow at 30 °C under anaerobic condition for 48 hrs. Ten milliliter of molten potato dextrose agar containing 1 ml of (120 x 10^4 spores/ml) inoculum of mould spores were then poured onto the agar plates and incubated at room temperature for 3-5 days. Ten (10) µg of standard antifungal drug ketoconazole was used as control. The zones of inhibition around the colonies were measured using a metric ruler

Molecular Identification of Lactic Acid Bacteria Isolates

Genomic DNA Extraction Genomic DNA of LAB isolates were extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005)**.** About 100 mg of the bacterial cells were added to a ZR BashingBead™ Lysis Tube. 750 µl of BashingBead™ buffer was added into the tube and was processed for 20 minutes. The ZR BashingBead™ Lysis Tubes were thereafter centrifuged at 10,000 rpm for 1 minute. Subsequently, 400 µl of the resulting supernatant was transferred into a Zymo-Spin™ III-F Filter in a collection tube and centrifuged at 8000 rpm for 1 minute. Zymo-Spin™ III-F Filter was discarded, 1200 µl of Genomic Lysis Buffer was added to the filtrate obtained and mixed well. 800 µl of the mixture was transferred to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuged at 10,000 rpm for 1 minute. The Zymo-Spin™ IICR Column was transferred to a new collection tube and 200ul of DNA Pre-Wash buffer was added to it and was centrifuged at 10,000 rpm for 1 min. 500 µl of g-DNA Wash buffer was added to the ZymoSpin™ IICR Column in a collection tube and centrifuged at 10,000 rpm for 1 min. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube and 50µl of DNA Elution Buffer was added directly to the column matrix. The assembly was thereafter centrifuged at 10,000 rpm for 30 seconds to elute the DNA.

PCR amplification

The isolated genomic DNA of the LAB isolates was used as template to amplify conserved region of the 16S rRNA gene using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) as below:

Table 1: Components of NEB Catalog N0. M0486

Components	Volumes for a 25µL reaction
Template DNA	2-3µl
10µM Forward Primer	0.25µl (10nM)

10µM Reverse Primer	0.25µl (10nM)
One Taq Quick Load 2X Master Mix with Standard Buffer	6.25µl
Nuclease free water	Varies (25-total of volumes above) µl

Primers
For the PCR reaction, the following primers were used 27F – AGAGTTTGATCMTGGCTCAG and 1492R – CGGTTACCTTGTTACGACTT as forward and reverse primers respectively as adopted by Iqaba Biotec West Africa Ibadan, Nigeria.

Thermal Cycling
The extracted DNA were then subjected to the following thermal cycling conditions using the Eppendorf Master cycler nexus gradient 230.

Table 2: Thermal Cycling conditions

Stage of PCR	Temperature (°C)	Time (min:secs)
Initial Denaturation	94	5:00
Denaturation1	94	0:30
Annealing1	50	0:30
Extension1	68	1:30
Denaturation2	94	1:30
Annealing2	53	0:30
Extension2	68	1:30
Denaturation3	94	0:30
Annealing3	55	0:30
Extension3	68	1:30
Final Extension	68	10:00
Hold	4	Hold

Gel electrophoresis After PCR amplification, 2 µl of each of the PCR product was ran on 1% agarose gel stained with 5 µl of SafeView Red for 30 minute

Post-PCR Purification The PCR products were cleaned using an enzymatic technique (ExoSAP). The ExoSAP master mix contained 0.6 ml micro-centrifuge tube filled with 50µl

Exonuclease I (20U/µl) (NEB M0293L) and 200µl Shrimp Alkaline Phosphatase (1U/µl), (NEB M0371). The reaction mixture was prepared by mixing 10 ul of the amplified PCR product, 2.5 ul Exo SAP Mix (step 2) and were incubated at 37C for 15m and further at 80C for 15minutes.

Sequencing

DNA fragments were sequenced using the Nimagen Brilliant DyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000, analyzed by Genetic Analyzer and sequencing data were used for analysis as adopted by Iqaba Biotec West Africa Ibadan, Nigeria.

Phylogenetic Relationship of LAB isolates

The DNA sequences obtained were edited and consensus sequences were obtained using the Bioedit 7.7.1 software package. Final sequences were aligned using multiple sequence alignment software CLUSTAL 2 for each of the sequences. Phylogenetic tree construction was carried out using the Maximum Likelihood method based on the Tamura-Nei model with Molecular Evolution Genetics Aanlysis (MEGA) version 11 (Belay *et al*., 2018).

RESULTS AND DISCUSSION

Isolation of Lactobacillus species from Soil

Twenty-three (23) *Lactobacillus* species were isolated from the soil samples obtained (Table 3)

Table 3. *Lactobacillus* species isolated from soil samples

Location	Soil samples collected	*Lactobacillus* species
North	2	5
South	2	4
East	2	4
West	2	3
Central	2	5
Total	10	21

Lactic acid bacteria mostly exist as autochthonous microorganisms in raw milk and are employed as inoculants in the manufacturing of fermented dairy goods. (Aspri *et al*., 2017). LAB are found in several environments, including dairy products, meat, vegetables, the digestive and urinary systems of people and animals, as well as soil and water, this illustrates the widespread and nomadic character of LAB (Liu *et al*., 2014). Several factors have been identified to affect the distribution and abundance of LAB in the soil these include pH, organic matter, nutrient availability, bio-fertilizers, plant-microbe interaction as well as climate and environmental conditions (Haitam *et al*., 2024).

Antifungal Activities of Lactobacillus isolates

A total of six (6) Lactobacillus species exhibited robust antifungal activity against the tested fungi. The N1 isolate demonstrated susceptibility to *Aspergillus flavus* with diameter of inhibition of 31.3±0.5 mm. *A. brasiliensis* by 35.0±0.8 mm, while *Penicillium notatum* had 40.3±0.5 mm. *Talaromyces purpureogenus, P. notatum, and Fusarium oxysporum* were inhibited by isolate N2 with inhibition diameter measurements of 35.1±0.1 mm, 32.7±0.2 mm, and 31.0±0.0 mm, respectively. S3 repressed *A fumigatus* by 40.5±0.2 mm and *Talaromyces purpureogenus* by 31.7±0.5 mm. S2 had a significant susceptibility to *A. flavus* by 32.2±0.4 mm, whereas *A. brasiliensis* is 30.6±0.7 mm. Isolate W4 demonstrated the highest activity, measuring 45.3±0.3 mm against *A. flavus*, *A. brasiliensis* is

36.0±0.8 mm and *P. notatum* is 41.4±0.4 mm. C2 was active against *A. fumigatus* with 30.5±0.3 mm and *F. oxysporum* measures 30.2±0.1 mm (Table 4).

Table 4. Antifungal activity of *Lactobacillus* species against Test Fungi

I	Zone of inhibition (mm)						
	A.f	A.fu	A.b	T. p	P. n	F.o	Kt (10µg)
N1	31.3±0.5	-	35.0±0.8	-	40.3±0.5	-	23.6±0.5
N2	-	-	-	35.1±0.1	32.7±0.2	31.0±0.0	25.2±0.3
S3	-	40.5±0.2	-	31.7±0.5	-	-	22.0±0.1
S2	32.2±0.4	-	30.6±0.7	-	-	-	20.3±0.4
W4	45.3±0.3	-	36.0±0.8	-	41.4±0.4	-	27.2±0.5
C2	-	30.5±0.3	-	-	-	30.2±0.1	20.1±0.8

Key: I: Isolates A.f: *Aspergillus flavus* A.fu: *Aspergillus fumigatus.* A.b: *Aspergillus brasilliencis.* T.p: *Talaromyces purpurogenus*. P.n: *Penicillium notatum*. F.o: *Fusarium oxysporum* Kt: Ketoconazole - : no inhibition

Lactic acid bacteria quickly lower the pH of the growth medium during fermentation and this process generates a range of secondary metabolites that possess antifungal characteristics (Indriana *et al.*, 2020). Studies by Sadeghi *et al*. (2019), indicated that secondary metabolites include fatty acids, organic acids, carbon dioxide, volatile compounds, hydrogen peroxide, peptides and other substances that have the capacity to inhibit the growth of fungi. LAB generates a significant and diverse amount of proteolytic enzymes, including intracellular peptidases, cell-wall proteinases, and several peptide transporters. The primary function of these compounds and enzymes are to break down mycotoxins and other cellular components produced by fungi into less toxic and less harmful chemicals (Bangar *et al.*, 2021). Lactobacillus has demonstrated the ability to efficiently eliminate aflatoxin-producing fungi, specifically those that belong to the Aspergillus, Penicillium, and Fusarium species, by creating phenylacetic acid, citric acid, phenyl lactic acid, and other organic acids. The antifungal properties of *Lactobacillus* species examined in this study align with the results of previous investigations conducted by Tulini et al. (2016), and Ademola et al. (2021), which shown a wide range of effectiveness.

Molecular Confirmation of Identity of Isolates with Antifungal activities

The aligned amplicons of 16S RNA Gene of *Lactobacillus* species revealed DNA fragments of varying sizes ranging from 0.050 - 10.0Kb. Amplified DNA fragments from wells SALA, 22, KUN, SM2, and W4 all aligned to 1 kb size on the ladder, which confirms the presence of the 16S RNA genes in our isolates. However, the DNA fragment from sample KUM2 failed to amplify (Plate 1).

Plate 1: The amplicons of 16S RNA Genes of *Lactobacillus* species with a size of 1kb.

Key: LADDER: DNA ladder. SALA, KMU-2, 22, KUN, SM2 and W4 a**re** wells contain DNA samples. **NTC**: Nuclease free water as negative control.

Bacterial species possess a minimum of one copy of the 16S rRNA gene, which comprises both highly conserved and variable sections. This gene is employed for the aim of distinguishing and defining bacterial species (Mohammed *et al*., 2011). The 16S rRNA gene is advantageous due to its ubiquitous presence in all bacterial species and the existence of species-specific variations, which are commonly utilized for identification purposes (Mohania *et al*., 2008). The results of our study are consistent with the investigations conducted by Adeyemo and Onilude (2014) and Gbemisola et al. (2020), which specifically examined the isolation and identification of Lactobacillus plantarum from fermenting grains. Furthermore, the study performed a molecular examination of the Phytase Gene in Lactic Acid Bacteria that were extracted from Ogi and Kunun-Zaki. Ogi and Kunun-Zaki are traditional African food and drink made from fermented cereal. The analysis was conducted utilising 16S rRNA. However, the process of increasing the amount of isolate C2, specifically known as KMU-2, has not been successfully accomplished. The failure to amplify the PCR fragment can be attributed to various factors, such as the use of inaccurate primer sequences or errors in the PCR primers, inappropriate temperatures for annealing and extension, incorrect primer concentration, impurities in the primers, damage or degradation of the template, presence of inhibitory substances in the template, and inadequate template concentration in the reaction (Todd, 2012).

Table 5: Similarities between Lactobacillus isolates and Biologic sequences within National Center for Biotechnology Information (NCBI) data base.

Isolate	Prediction (%)	E-value	Accession Number	BLAST Predicted Organism
N1	99.74	0.00	KR816164.1	*Lactiplantibacillus plantarum*

N2	99.80	0.00	AB362757.1	*Lactobacillus pentosus*
S2	99.10	0.00	CP031208.1	*Levilactobacillus brevis*
S3	99.26	0.00	OQ224994.1	*Lactiplantibacillus plantarum*
W4	99.85	0.00	KY203913.1	*Lactiplantibacillus plantarum*

The findings derived from the BLAST matched to the level of similarity between the searched sequences and the NCBI database. The genotyping of the Lactobacillus isolates, together with their NCBI accession numbers and the organisms in GenBank that showed significant similarities, were presented in Table 3. The confirmation of the identification of our isolates has been achieved with a similarity rate over 99%. This study agrees with the investigation conducted by Haleh et al. (2021), regarding the phenotypic and phylogenetic characterization of *Lactobacillus* species found in traditional Lighvan cheese which found that the isolates retrieved from the Gebank showed a high level of similarity, with a 99.0-100% identification match to the isolates mentioned in the earlier study. In a study conducted Mohamedkassm et al. (2017), indicated that *Lactiplantibacillus plantarum* demonstrated a reduction of 96%, *Lactobacillus pentosus* revealed an 81% decrease, and *Levilactobacillus brevis* showed a loss of 95%.

Phylogenetic relationship of LAB isolates

Figure 2 illustrates the evolutionary pattern of Lactobacillus species obtained from both isolated samples and the NCBI database. *Levilactobacillus brevis* CP031208.1 had the highest degree of resemblance to *Levilactobacillus brevis* LC654672.1, as shown by a bootstrap support value of 100. Nevertheless, *Lactiplantibacillus plantarum* KR816164.1 displayed a weak connection with *Lactobacillus plantarum* subsp. The plant species Plantarum MF6, specifically identified as KX430840.1, has a bootstrap support score of 26. The bootstrap test indicated that the related taxa formed clusters in 1500 out of the total number of replicates. The findings of our inquiry unveiled significant similarities as well as discernible discrepancies in comparison to the study conducted by (Saeed *et al*., 2020). The analysis uncovered a high probability that the Iraqi samples are intimately associated with samples from China, Japan, and India. The indigenous strains of *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus pentosus* exhibit a distribution pattern that closely mirrors isolates from Brazil, Turkey, China, Italy, Saudi Arabia, India, Romania, Belgrade, and France, indicating a shared genetic ancestry. Chen et al. (2015), stated that lactic acid bacteria, like other bacteria, can acquire genetic material from their environment through horizontal gene transfer. This allows them to adjust and thrive in novel environments. The 16S rRNA sequence analysis methodology is superior in terms of accuracy and reliability when compared to previous identification methods (Ren and Suo, 2017).

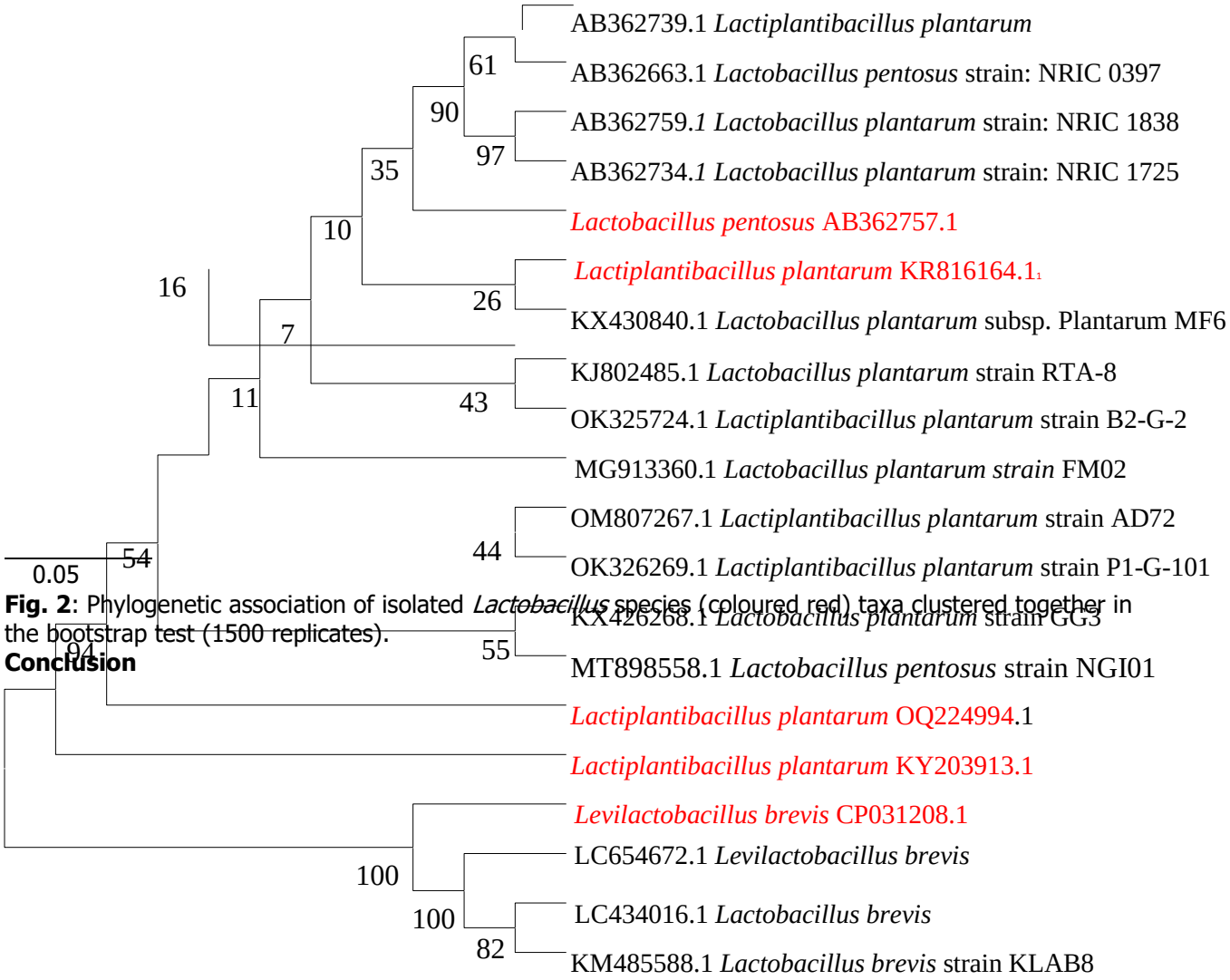

Fig. 2: Phylogenetic association of isolated *Lactobacillus* species (coloured red) taxa clustered together in the bootstrap test (1500 replicates).

Conclusion

This study isolated and identified *Lactobacillus* species namely; *Lactiplantibacillus plantarum*, *Lactobacillus pentosus,* and *Levilactobacillus brevis* from soil samples of various locations in the Botanical Garden of Ahmadu Bello University Zaria, Kaduna State. All isolates selected demonstrated strong activity, the highest activity of 45.3±0.3 mm against *Aspergillus flavus* and minimum activity was recorded at 30.2±0.1mm inhibition diameter against *Fusarium oxysporum*. The phylogenetic relationship established very high similarities of *Lactobacillus* species isolated from this study to other *Lactobacillus* species from the NCBI GenBank database with the highest bootstrap support value

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