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INHIBITION OF AFLATOXIGENIC FUNGI BY LACTIC ACID BACTERIA ISOLATED FROM FERMENTED MAIZE SLURRY.

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ABSTRACT

Toxigenic fungi are persistent contaminants of food and food products. This study aims at the biocontrol of toxigenic fungi. The inhibitory activity of Lactic acid bacteria (LAB) isolated from fermented cereal slurry against toxigenic *Aspergillus* isolated from maize was investigated. The antagonistic compounds produced by LAB were identified using gas chromatography-mass spectrometry (GCMS). Toxigenic *Aspergillus flavus* and *A. parasiticus* were isolated from maize after screening with an ammonium hydroxide test, and their aflatoxin levels were quantified to be 877 and 797 ng g^{-1} respectively. *Lactobacillus fermentum* and *Lactobacillus paraplantarum* were isolated and their culture-free supernatants (CFS) had a mean inhibitory zone of 15 ± 0.4 to 18 ± 0.6 mm. They were sensitive to pH but slight loss of inhibitory activity when treated with proteolytic enzymes. The inhibitory CFS was subjected to GC-MS. Among the compounds detected, lactic acid, benzenepropanoic acid, 4-hydroxy, pyrrolo [1, 2a] pyrazine 1,4 dione, and fatty acids especially hexadecanoic acid, were the main compounds with potential antifungal activity identified. Lactic acid bacteria inhibited the growth of aflatoxigenic *Aspergilli*, hereby reducing food intoxication.

Keywords: *Antimicrobial, Aspergillus, Biopreservation, Lactic acid bacteria, Toxigenic, GC-MS.*

INTRODUCTION

Fungi are responsible for great losses in food and economic value. The etiological agent mycotic infections in both animals and humans. They produce secondary metabolites known as mycotoxins which are mostly synthesised by molds of *Fusarium, Aspergillus, Alternaria,* and *Penicillium.* The major mycotoxin food contaminants are aflatoxins, fumonisins, ochratoxin A, patulin, citrinin, zearalenone, and deoxynivalenol (Dhakal *et al*., 2023). However, emphasis is placed on the aflatoxins of the *Aspergillus* spp. mainly produced by *Aspergillus flavus, Aspergillus parasiticus*, and *Aspergillus nomius.* Their toxins are associated with important illnesses in humans because they are mutagenic, immunosuppressant, teratogenic, and carcinogenic (Alameri *et al.*, 2023). The prevalence and level of exposure to aflatoxin on a global scale is pegged at about 4.5 billion among dwellers in third-world countries, while the health status of the consumer accounts for over 40% of diseases as aflatoxin also affects the nutritional value of food (Dhakal *et al*., 2023).

In Africa, milk and cereal-based foods are vital in nutrition from infancy to adolescence as well as being a complementary part of adult meals. The effect of spoilage fungi and mycotoxins does not just cut down food supply but poses a great risk due to the food handling culture and medical system. The most effective means to eliminate fungi and inactivate toxins are mainly physical, chemical, or biological means (Alameri *et al.*, 2023), but these methods are characterised by inefficiency, exorbitant prices**,** safety issues and the degradation of nutritional quality of edible agricultural produce. The population of aflatoxigenic *Aspergillus* is a major hindrance in aflatoxin control due to diversity, ability to undergo hetrokaryotic reproduction, and survival for prolonged periods (Nair *et al.*, 2014). The health concern over the use of chemical preservatives necessitates the exploration of food-grade bioactive antifungal compounds to inhibit fungi growth, reduce toxin production, improve food quality and extend the shelf-life of food. Biological agents may outcompete toxigenic fungi or prevent mycotoxin production.

Lactic acid bacteria have been proven to eliminate several food-pathogenic organisms (Hadi *et al.,*2021). They occur as a natural microbial population of spontaneously fermented foods and contribute to safety, shelf-life stability, sensory/textural properties, and the nutritive components of food (Onilude *et al*., 2005; Shehata *et al.*, 2019). The protection of food products by LAB has been attributed to their production of antimicrobial compounds, which include organic acids, hydrogen peroxides, fatty acids, and peptides (Muhialdin *et al.*, 2018). With a Generally Regarded as Safe (GRAS) status and prevalence in nature, Lactic acid bacteria are an unprecedented category of organisms that potentially control the growth of aflatoxigenic fungi.

Aflatoxin is a food safety challenge that poses a tremendous risk to both animal and human health. Elimination of aflatoxigenic strains in food via biological means will limit aflatoxin production, thereby improving the safety of food. In this investigation, Lactic acid bacteria that inhibit aflatoxigenic fungi will be isolated and identified, and the CFS will be characterised.

MATERIALS AND METHODS

ISOLATION AND IDENTIFICATION OF FUNGI

Maize samples were collected from the *Bodija market* in Ibadan, Nigeria (7.42951^{o} N, 3.9204 ^{o} E) and treated using Alsohaili *et al.* (2018) modified technique. The samples were surface sterilised for 2 minutes using 70% ethanol, after which they were rinsed three times with distilled water and dried with paper towels, and milled. The milled maize was vigorously spun before being serially diluted with sterile distilled water. Then, a mL of each dilution from 10^{-5} upwards, were plated on potato dextrose agar (PDA) containing streptomycin (1%) and incubated at 27°C for 7 days. Pure cultures were obtained by the subculturing of distinct colonies on PDA-streptomycin agar for 72 hours respectively.

The fungal isolates were identified using the classical method. Microscopic identification was carried out by mounting fungal mycelium on a slide then stained with lactophenol cotton blue and observed under x 40 objective lens of the microscope. The conidia and conidiophores were observed and compared with the standards described by Barnett and Hunter in 1972.

SCREENING OF AFLATOXIGENIC FUNGI

Ammonium hydroxide test on coconut milk PDA agar

Fungi isolates were subjected to an ammonium hydroxide vapour test (Nair *et al.*, 2014). The media, coconut milk (100 mL)-PDA media (30g/L), was sterilised for 15 minutes at 121^{0}C. Fungi isolates pre-grown in PDA for 72 hours were stabbed on the coconut milk-PDA plates; they were cultured for 5 days at 27^{0}C. On the cover plate, ammonium hydroxide was dropped and the plate was covered again for 10 minutes. The underside of the aflatoxin-producing isolate plate turns pink/red colour, while the non-toxigenic isolates do not change colour. Ammonia vapour assay operates on the basis that aflatoxin synthesis will produce anthraquinone pigments which are usually yellow and turn to red when exposed to ammonium hydroxide

Aflatoxin extraction and quantification

The selected toxigenic *Aspergillus* sp. (10^{6} spores / mL) was inoculated on sterile maize and allowed to incubate for 14 days. The method of Espindola *et al.* (2018) was employed to extract and quantify aflatoxin. Powdered maize (20 g) was suspended in methanol (100 mL; 70%), then, mixed vigorously for approximately three minutes. The mixture was set to rest, after which the toxin-containing supernatant was filtered using filter paper. By the Rapid Aflatoxin B1 ELISA kit instructions, 100 µL of the extract was mixed with 100 µL of enzyme conjugate (Sigma-Aldrich) into a microtiter well. As a control, solutions with known aflatoxin concentrations were used and measurements were taken at OD 450nm using an ELISA-well plate reader.

ISOLATION AND IDENTIFICATION OF LAB FROM FERMENTED MAIZE SLURRY

Lactic acid bacteria were isolated from fermented white maize slurry. Serial dilutions of the sample were prepared and 1 mL of dilution 10^{-6} was inoculated into MRS-$CaCO_3$ (0.1%) agar by pour plate method, then cultured at 30°C for 48 h. Distinct colonies with clear zones were purified by streaking on MRS agar and maintained in MRS broth containing glycerol (30% v/v) and stored at 4˚C (Pessoa *et al.*, 2017).

Preparation of the LAB CFS and spore suspension

The culture-free supernatant (CFS) of the selected LAB was prepared according to the method of Onilude *et al*. (2005). A colony of each selected LAB isolate was grown for 48 hours at 30ºC in MRS broth, then spun at 4000 rpm for 15 minutes. Using a 0.22 µm syringe filter, the CFS was sterilised.

Fungi spores were grown on PDA slants for 7 days. Collection of spore suspension was done by vigorously shaking the fungal slant with sterile water and Tween-80 (1% w/v). It was sieved with sterile muslin cloth, after which, the inoculum was serially diluted and the inoculum size was determined using a pour plate method.

SCREENING OF LAB FOR FUNGAL INHIBITION

Dual agar overlay inhibition test

The antifungal inhibitory capability of the LAB isolates was tested using the dual agar overlay technique (Onilude *et al.,* 2005). LAB isolates were seeded as a straight-line streak on de Man Rogosa and Sharpe agar and cultured at 30°C for 48 hours in an anaerobic jar. The incubated LAB culture was layered with soft agar (0.8 %, agar) PDA containing Aspergillus spp. spores. The plates were incubated for 7 days at 27°C under aerobic conditions, and the inhibition zones were assigned +, + +, + + + and – based on the increasing clearance exhibited on the agar plate. Uninoculated MRS agar overlaid with inoculated PDA served as the control.

Inhibition of *Aspergillus* species by culture-free supernatants (CFS) of LAB

Inhibition of *A. flavus* AG4 and *A. parasiticus* AG3 by the isolated LAB were assayed using agar well diffusion and the modified method of Safdarianghomsheh *et al*. (2022). The LAB isolates were grown for 18 hours in MRS broth and 1 mL of the broth culture was inoculated into 9 mL of MRS broth and incubated for 48 hours in an anaerobic jar. Culture-free supernatants were obtained by the centrifugation of the incubated broth for 10 minutes at 4000 rpm and membrane filtered (0.22 µm-pore-size filter, Millipore). The spore suspensions of *Aspergillus* spp. (10^6 spores mL^{-1}) were mixed separately with 14 mL of PDA and poured into Petri dishes to set. An 8 mm cork borer was used to create an agar well and the CFS (100 µL) was pipetted into the agar wells and allowed to stand for 30 minutes before incubating for 5 days at 28ºC.

MOLECULAR IDENTIFICATION OF SELECTED ISOLATES

DNA Extraction

The Bacteria DNA Preparation Kit designed by JENA Bioscience (Germany) was used to isolate the genomic DNA according to the instruction manual in FOWN Biotechnology Laboratory, Lagos. Centrifugation was used to separate the bacterial cells for a minute at 10000 g, the supernatant was discarded, and the pellets (500 µL) were then re-suspended in 300 µL of re-suspension buffer. Lysozyme solution (2 µL) was added and mixed thoroughly by inverting several times before incubating at 37 °C for 1 hour, then spun at 10000g using a centrifuge, and the supernatant was disposed of. To the pellet, 2 µL of RNase-A solution was added and vortexed vigorously for 30 sec. Proteinase K (8 µL) solution was mixed in by pipetting, then incubated at 60ºC for 10 minutes before cooling on ice for 5 min. The binding buffer (300 µL) was added to the mixture and vortex briefly, cooled on ice for 5mins and centrifuged at 10,000

g for 5 min. Pipetted supernatant into the spin column and centrifuged for 60 seconds at 10000g before the filtrate was decanted. The cell pellet was washed twice by pipetting five hundred microlitre washing buffer into the column and spun for 30 sec at 10000 g, discarding the filtrate. It was again centrifuged at 10000 g for a minute to remove residual washing buffer. The spin column was transferred to an elution tube, and elution buffer (50 µL) was added to the centre of the column, allowed to sit at room temperature for a minute before spinning at 10000 g for 60 seconds.

Polymerase chain reaction (PCR) amplification using Lactic acid bacteria-specific primers

Primer pairs BSF-8 (AGAGTTTGATCCTGGCTCAG) and BSR-534 (ATTACCGCGGCTGCTGGC) were used to amplify the isolated LAB DNA. The PCR reaction was conducted with 25 µl of a reaction mixture using the Solis Biodyne 5X HOT FIREPol Blend Master mix. Thermal cycling was conducted in a Master-cycler (Eppendorf, Germany). Initial denaturation was done for 15 mins at 95°C, then 35 amplification cycles were done for 30 seconds at 95°C, 60 seconds at 58°C and 90 seconds at 72°C. The last extension phase was completed after 10 minutes at 72°C. The amplification product was separated in an agarose gel (1.5%) containing ethidium bromide. A molecular ladder marker (Solis Biodyne, 100bp) was run simultaneously to determine the size of the amplicons. After confirmation of the amplified fragment with electrophoresis, amplicons were sequenced using an ABI 3730XL sequencer (Inqaba Biotec). Sequence similarity was determined using NCBI BLAST (http://www..ncbi.nlm..nih.gov). Utilizing Molecular Evolutionary Genetics Analysis (Mega 11), the phylogenetic tree was built.

CHARACTERIZATION OF THE ANTIFUNGAL METABOLITE OF LAB

The stability of the selected *Lactobacillus* (CFS) to pH, heat, and proteases was assessed as described by Muhialdin *et al*. (2018). The influence of pH on the inhibitory ability of LAB against aflatoxigenic *Aspergillus* spp. (*A. flavus and A. parasiticus*) was investigated. The pH of the metabolite was adjusted to (3.5 4.5, 5.5, 6.5 and 7.5) using appropriate buffers (0.1 M sodium-acetate buffer and potassium phosphate buffer). An agar well diffusion assay was performed, and clear zones of fungal inhibition were measured in millimetres.

The impact of heat on the efficacy of the LAB CFS as an antifungal agent was assessed by heat treatment with a heat block at 45ºC, 70ºC 100ºC for an hour, and autoclave at 121°C for 15 minutes. An agar well diffusion assay was carried out and clear zones of fungal inhibition were measured in millimetres.

The enzymes: pepsin, proteinase K, and trypsin were used to verify the presence of peptides in the CFS. The enzymes were prepared in a buffer and the pH was adjusted to 2 for pepsin and 7.6 for proteinase K and trypsin using 1M HCl and 2 M NaOH. An agar well diffusion assay was performed, and clear zones of fungal inhibition were measured in millimetres.

GC-MS profile of selected inhibitory Lactic acid bacteria

The metabolites were extracted using ethyl acetate (1:3, v/v). The chemical composition of the extracted metabolites was investigated as described by **Chaudhary *et al.* (2020)**. Gas chromatographic-mass spectrometre (GCMS-QP2010SE, Shimadzu Japan) equipped with a capillary column (30 m by 0.25 mm inner diameter; film thickness,

0.25 mm), was used for the analysis. The mass spectra were compared to those in the National Institute of Standards and Technology (NIST) database.

STATISTICAL ANALYSIS

Experiments were performed in triplicate. Statistical analyses were done using Minitab 17.

RESULTS

Fungi isolates from the maize samples varied from 9.3×10^5 to 3.9×10^6 spores / mL. The white maize samples from Bodija: white maize A, white maize B, and white maize C had a total count of 9.3×10^5 to 3.9×10^6, 9.3×10^5, and 3.9×10^6 spores / mL respectively. A total of 21 isolates were obtained on PDA medium and the cultures were identified based on macroscopic and microscopic assessment of morphological features. Four fungi genera were identified, namely; Rhizopus spp., Fusarium sp., Penicillium spp., and Aspergillus spp. The percentage frequency of occurrence (Figure 1), shows that Penicillium spp. was the dominating isolate (38%) followed by Aspergillus spp. (33%), Fusarium sp. (19%) and Rhizopus sp. (10%). The characteristics of the selected Aspergillus spp. are shown on Table 1. The colonies had yellow-green or black colour with velvet appearance and white margin at the edge. Five of the selected isolates were yellowish /green, biserated, globose and smooth; they were identified as *Aspergillus flavus.* While only one was green, uniserated, globose and rough and identified as *Aspergillus parasiticus. The isolates identified as Aspergillus spp.,* were tested for aflatoxin production using the ammonium hydroxide vapour test. The ammonium hydroxide vapour test showed that six isolates AG1 - AG6 were aflatoxin producers (Plate 1).

The aflatoxin produced by the *Aspergillu*s spp. was quantified using ELISA as a confirmatory test and shown in Table 2. All six isolates were aflatoxigenic and produced aflatoxins ranging from 271 ± 05 - 877 ng g^{-1}. *Aspergillus flavus* AG4 produced the highest aflatoxin while the lowest aflatoxin was produced by *Aspergillus flavus* AG1. Based on the aflatoxin produced, *Aspergillus flavus* AG4 (877 ng g^{-1}) and *Aspergillus parasiticus* AG3 (797 ng g^{-1}) were selected for further studies.

Isolation of LAB from fermented cereal slurry

Sixty-two isolates presumed to be Lactic acid bacteria were isolated from fermented white maize slurries. The screened isolates showed an inhibitory activity range of 5 – 18 mm against the selected fungi, *Aspergillus flavus* AG4 and *Aspergillus parasiticus* AG3 using both agar overlay assay and agar well diffusion assay. About 11% (7) of the LAB isolates exerted strong inhibition. The best two LAB with the higher inhibition zones against *A. flavus* and *A. parasiticus* (Table 2) were selected for additional analysis. Based on morphological, biochemical and molecular analysis isolates were identified and reported in Table 4. The selected microbial strains were off-white Gram-positive, rod-shaped, non-motile, and catalase and oxidase-negative. All the isolates showed negative results on MR-VP test and neither could they hydrolyse arginine. They had varied responses to sugar utilization and gas production from glucose. The LAB having the highest antifungal activity was identified using the 16S rRNA gene sequence and the phylogenetic analysis reported in Figure 2. The NCBI BLAST software, using the sequence alignment revealed a high

homology of 98% similarity to *Lactobacillus fermentum* strain Sj8256 (*Accession number: KC759437.1*) and 97% similarity to *Lactobacillus paraplantarum* (*Accession number FR873979.1*).

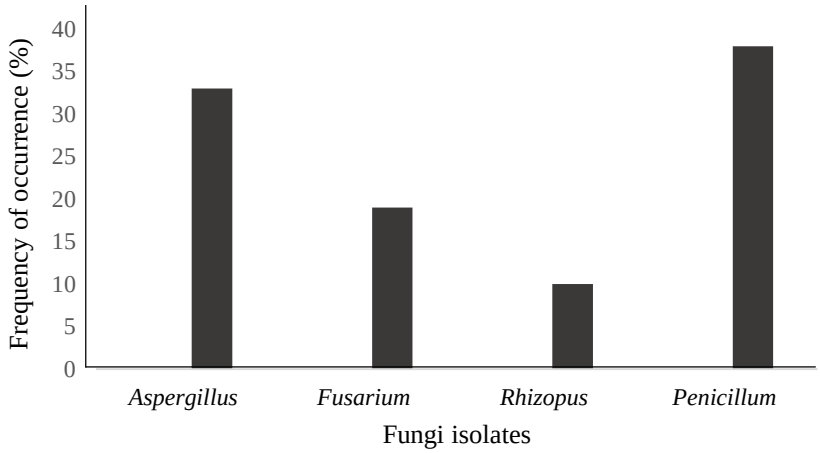

Figure 1: Frequency of occurrence of fungi isolated from white maize

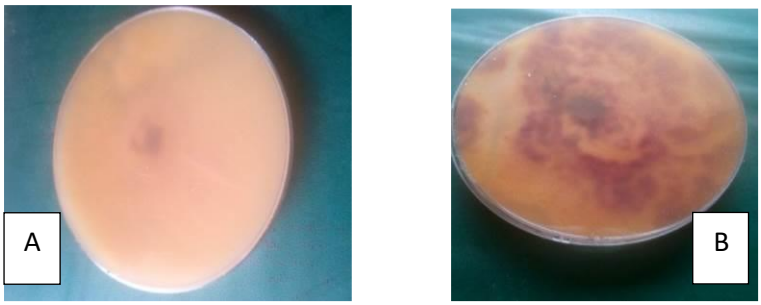

Plate 1: Ammonium hydroxide test for aflatoxin production in coconut milk-potatoes dextrose agar medium.
Key: A - Non aflatoxin producing *Aspergillus. flavus* and B - aflatoxigenic *Aspergillus flavus*

Table 1: Aflatoxin produced by *Aspergillus* spp. using ELISA method

Isolate code	Fungi	Toxin level (ng g^{-1})
AG1	*Aspergillus flavus*	271.5
AG2	*Aspergillus flavus*	581.9
AG3	*Aspergillus parasiticus*	797.0
AG4	*Aspergillus flavus*	877.0
AG5	*Aspergillus flavus*	771.0
AG6	*Aspergillus flavus*	778.2

Characterisation of the antifungal Culture free supernatant (CFS) of *Lactobacillus* spp.

The influence of pH, heat, and proteolytic enzyme on the antifungal activity of LAB metabolite was assayed and shown in Table 5. At pH 3.5 to 7, the antifungal activity of LAB CFS was assessed to ascertain their applicability in food systems. The CFS from the chosen isolates exerted strong activity at pH 3.5 and a decline at pH 7.

The heat stability of the inhibitory *Lactobacillus* spp. CFS was assessed (Table 5.) and varied reactions were recorded. At lower temperatures, the metabolites maintained their heat stability and inhibitory action. After heat treatment at 121 °C for 15 minutes, there was a slight loss of inhibitory activity. Additionally, the CFS treated with proteolytic enzymes revealed that the isolates' antifungal activity had partially declined to the least value of 14.4 mm. The antifungal protein hydrolysis was more pronounced in the *L. fermentum* activity compared to *L. paraplantarum.*

Antifungal compounds of *Lactobacillus* determined with GC-MS

The metabolites of the LAB CFS give an insight into the nature of antimicrobial components present. A total of 42 metabolites were detected from two isolates CFS and selected antimicrobial compound are listed in Table 6. while the GC-MS chromatogram is shown in Figure 2. The prominent antimicrobial compounds of *L. fermentum* and *L. paraplantarum* were lactic acid, n-Hexadecanoic acid, Benzenepropanoic acid, 4-hydroxy, Pyrrolo [1, 2a] pyrazine 1,4 dione, Benzeneacetic acid, 2-propanol, and acids as Tetradecanoic acid, Octadecanoic acid, 3-Hydroxypropyl ester, Pentadecanoic, cis-9,12-Octadecadienoic acid etc, were identified.

Table 2: Inhibition of toxigenic *Aspergillus* spp. by *Lactobacillus* spp.

Fungi strains	Treatment	Fungal Inhibition	
		Dual agar overlay assay	Agar well diffusion assay (mm)
A. flavus	Control	-	-
	L. fermentum	+ + +	17± 0.4
	L. paraplantarum	+ + +	16 ± 0.6
A. parasiticus	Control	-	-
	L. fermentum	+ + +	15 ± 0.4
	L. paraplantarum	+ + +	18 ± 0.3

Result are mean values ± standard deviation; Inhibition zones: - no inhibition, + ≥ 5mm, ++ ≥ 10mm, +++≥ 20

Table 4: Influence of pH, heat, and proteolytic enzymes on the antifungal activity of the *Lactobacillus* spp.

Treatments	Inhibitory zone of activity (mm)	
	L. fermentum	***L. paraplantarum***
pH		
3.5	18.1 ± 0.7^{a}	17.4 ± 0.7^{a}
4.5	15.6 ± 0.7^{b}	16.2 ± 0.5^{b}
5.5	14.8 ± 0.9^{b}	15.0 ± 0.8^{b}
6.5	12.0 ± 0.8^{c}	12.3 ± 0.5^{c}
7.0	6.1 ± 0.4^{d}	5.5 ± 0.5^{d}
Temperature (ºC)		
45	16.9 ± 0.4^{a}	17.0 ± 0.8^{a}
70	16.3 ± 0.4^{b}	16.0 ± 0.2^{ab}
100	15.8 ± 0.5^{b}	15.8 ± 0.6^{ab}
121	14.6 ± 0.5^{c}	15.6 ± 0.8^{b}
Enzymes		
Pepsin	14.7 ± 0.4^{b}	15.6 ± 0.2^{b}
Trypsin	14.4 ± 0.2^{b}	16.3 ± 0.1^{a}
Proteinase K	15.2 ± 1.2^{a}	16.4 ± 0.8^{a}

Means in the same column with different letters are significantly different ($p \leq 0.05$).

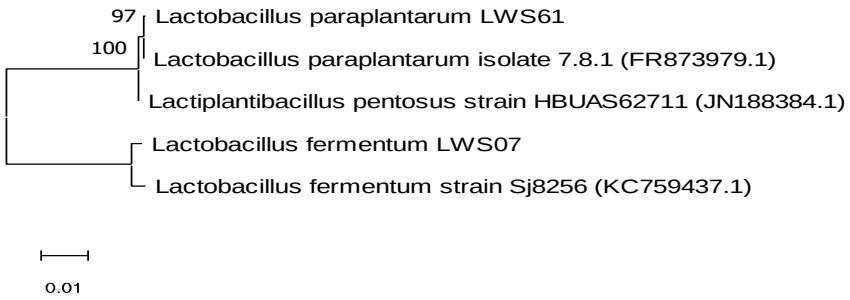

Figure 2: Phylogenetic tree of selected LAB (LWS07 and LWS61) using the Neighbor-joining method.

Table 5: Antimicrobial metabolites of *Lactobacillus* species CFS detected with GC–MS

Molecular formula	**Compound**	***L. fermentum***		***L. paraplanatrum***		**Activity**
		RT	**Area (%)**	**RT**	**Area (%)**	
C_3H_8O	2-Propanol	3.763	2.28	3.828	0.99	*Antimicrobial*
$C_3H_6O_3$	Lactic acid	4.134	46.64	13.773	6.16	*Antimicrobial*
$C_4H_8O_3$	Propanoic acid, 2-hydroxy-, methyl ester	4.613	4.13	3.197	0.28	*Antimicrobial*
$C_8H_8O_2$	Benzene acetic acid	5.083	16.95	4.136	0.53	*Antimicrobial*
$C_{11}H_{18}N_2O_2$	Pyrrolo [1,2 a] pyrazine 1,4 dione,	12.764	2.36	12.407	3.39	Antifungal
$C_9H_{10}O_3$	Benzene propanoic acid, 4-hydroxy	Nd	Nd	9.917	12.22	Antimicrobial
$C_{18}H_{32}O_2$	cis-9,12-Octadecadienoic acid	11.073	3.52	4.779	11.73	Antimicrobial
$C_{16}H_{32}O_2$	n-Hexadecanoic acid	13.868	1.68	13.849	16.96	Antimicrobial
$C_{14}H_{28}O_2$	Tetradecanoic acid	13.658	0.15	14.718	1.98	Antimicrobial
$C_{14}H_{22}O$	Phenol, 2,4-bis-1,1-dimethylethyl	14.726	1.37	10.502	0.43	Antibacterial
$C_{18}H_{36}O$	2,4-di-tert-butylphenol	Nd	Nd	18.219	0.79	Antifungal
$C_{21}H_{42}O_3$	Octadecanoic acid, 3-hydroxypropyl ester	15.442	0.31	16.425	3.11	Antimicrobial
$C_{18}H_{35}NO$	Pentadecanoic	16.940	0.19	14.966	2.87	Antifungal

key: RT -Retention time; Nd – Not Determined

DISCUSSION

Aflatoxicosis caused by aflatoxins which are metabolites of several fungi belonging to the genus *Aspergillus*. Exposure to aflatoxins is mainly via ingestion of contaminated food or animal by-product or inhalation. They are of economic

importance as huge percentages of crops and livestock are lost. Besides, their ability to induce stunted growth and hepatocellular carcinoma (liver cancer) is a health concern. Aflatoxicosis is difficult to treat but its prevention is the only alternative. The main objective of our study was to explore the inhibitory potentials of lactic acid bacteria on aflatoxin-producing fungi. Lactic acid bacteria are naturally habitants of fermented foods hence their predominance in the maize slurry from which sixty-two isolates were obtained. Their dominance may also be due to the nutritional composition of the fermenting substrate. The predominance of LAB in fermented cereals has been reported by several authors (Onilude *et al*., 2005; Pessoa *et al*., 2017; Gizachew *et al*.,2020). Wang *et al*., (2012) suggested that the dominance of *Lactobacillus* spp. may be attributed to it being an indigenous flora of cereals and can tolerate acidic conditions. Among the isolates, 11% could strongly inhibit the growth of *Aspergillus* spp in vitro. The decrease in growth of the toxigenic *Aspergillus* spp. will concomitantly cause a reduction in mycotoxin production. Inhibition of fungi by LAB has been attributed to mycelium alteration due to the damage of the fungal hyphae on interaction with the metabolic products of LAB and its adhesion qualities (Onilude *et al*., 2005). Different species of *Lactobacillus* in fermented Nigerian foods demonstrated inhibitory action against several fungi (Pessoa *et al*., 2017) and Damayanti *et al.* (2021), noted that over 70 % of the isolated LAB from Nigerian fermented foods, had inhibitory activity against *Aspergillus* spp. and *Penicillium* spp. The 16S rRNA gene sequencing of the two best *Aspergillus* inhibiting LAB, showed that *Lactobacillus fermentum* LWS07 and *Lactobacillus paraplantarum* LWS61 are most related to *Lactobacillus fermentum* strain SJ8256 and *Lactobacillus paraplantarum FR873979.1*. at a pairwise sequence similarity percentage of 98% and 97% respectively.

This study showed the stability of the antifungal metabolite over a range of pH, heat, and in the presence of proteolytic enzymes. The antifungal activity of the LAB CFS decreased as the pH increased. This is probably due to the creation of an acidic environment by the presence of organic acids which in an undissociated hydrophobic state diffuse via the membrane to lower the cytoplasmic pH causing cell death. Some antifungal compounds are dependent on pH to exert antimicrobial effect (Wang *et al.,* 2012; Shehata *et al.,* 2019). The fungal inhibitory activity of the LAB CFS was not affected by heat. This is an advantage as they may be adaptable in food thermal processing systems. The inhibitory activity of the CFS-treated with proteases was slightly loss. The partial loss in activity is probably due to inactivation of antifungal protein which suggests that although protein derivatives may have inhibitory effects, they are however not the major inhibitory component of the CFS. According to Shehata *et al*. (2019), inactivation of antifungal compounds by proteolytic enzymes indicates the protein nature of the compound. Muhialdin *et al.* (2018) opined that when treated with proteolytic enzymes, organic acids are stable, therefore a partial loss of activity reveals the presence of a protein molecule.

In this study, the major compounds expressed were propanoic acid, lactic acid benzene propanoic acid, Pyrrolo [1,2a] pyrazine 1,4 dione, benzene acetic acid, 2-propanol and several fatty acids. Organic acids cause membrane disruption, interfere with metabolic activity and enhance toxic anion accumulation in the cell. While fatty acids are cytotoxic to fungal cells, they limit mycelial formation and spore germination. Lactic acid is the major product of lactic acid bacteria and had been noted by several authors to be antimicrobial (Shehata *et al.,* 2019; Damayanti *et al.,* 2021). Lactic acid bacteria produce several compounds such as formic acid, acetic acid propionic acid, butyric acid, n-valeric

acid, and hexanoic acid have been reported to hinder the growth of fungi (Wang *et al.*, 2012). Long-chain fatty acids, particularly C16 and various saturated and unsaturated C18 are known for their antifungal activities. The identification of the metabolites of *L. plantarTm* LPcfr showed that compounds such as hexadecane, hexadeconic acid, dodecanal have antifungal and antioxidant properties (Chaudhary ***et al***., 2020). Vanaja *et al.* (2011) also reported the antimicrobial activity of fatty acids such as palmitic acid, stearic acid and phenol, 2, 4-bis-1,1-dimethylethyl produced by *L. kefiranofaciens, Candida kefir,* and *Saccharomyces boulardii*,

CONCLUSION

Summarily, our study demonstrated that both isolates, *Lactobacillus fermentum* LWS07 and *Lactobacillus paraplantarum* LWS61 could be elicited in biocontrol as they exhibited potent antifungal properties against two prominent species of *Aspergillus*. Antifungal compounds of the selected LAB were non-proteaceous, heat-stable but pH dependent compounds. These *Lactobacillus* species and their metabolites can be utilized as antifungal bioactive agents in pharmaceutical preparation, cosmetics, and food products which will enhance potential health benefits.

CONFLICT OF INTEREST

The authors have no conflict of interest

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