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ANTIFUNGAL ACTIVITY OF *Pediococcus pentosaceus* ISOLATED FROM BAMBARA GROUNDNUT (*Vigna subterranea* (L.) Verdc.) SEEDS AGAINST *Aspergillus flavus*

Amidou S. OUILI[1], Camelia Filofteia DIGUȚĂ[2], Ynoussa MAIGA[1], Cheik Omar Tidiane COMPAORE[1], Aboubakar Sidiki OUATTARA[1], Florentina ISRAEL-ROMING[2], Florentina MATEI[2, 3]

[1]Université Joseph KI-ZERBO 03 BP 7021 Ouagadougou 03, Laboratoire de Microbiologie et de Biotechnologie Microbienne, Burkina Faso
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Marasti Blvd, District 1, Bucharest, Romania
[3]Transilvania University of Brașov, Faculty of Food Industry and Tourism, 148 Castelului Street, 500014, Brașov, Romania

Corresponding author email: camelia.diguta@biotehnologii.usamv.ro

Abstract

In this study, 22 lactic acid bacterial (LAB) strains were isolated from Bambara groundnut seeds and assessed for their ability to suppress the development of two strains of aflatoxigenic Aspergillus flavus. The antifungal activity of LAB strains was assessed via the overlay assay. Also, HPLC methodology was used to quantify the production of lactic and acetic acid by the LAB strains with high antifungal activity. After incubation at 30°C for 72 hours, five of these LAB strains (52B1, 52C1, 74B3, 67B1, 67A9) showed high inhibitory activity against Aspergillus flavus strains. These LAB strains exhibiting antifungal potential were identified as Pediococcus pentosaceus through 16S rDNA sequencing. Furthermore, the lactic acid bacteria (LAB) strains reported lactic acid production ranging from 12.08 mg/ml to 15.38 mg/ml and acetic acid production ranging from 0.06 mg/ml to 1.68 mg/ml. Notably, antifungal experiments are conducted in a controlled setting. Further, it would be worthwhile to explore the direct application of Pediococcus pentosaceus strains or their antimicrobial agents to food products to prevent fungal growth during storage. By doing so, the use of fungicides can be avoided and lead to safer food products.

Key words*: Bambara groundnut, Pediococcus pentosaceus, antifungal activity, Aspergillus flavus.*

INTRODUCTION

Bambara groundnut (*Vigna subterranea* (L.) Verdc.), an African crop (Bamshaiye et al., 2011; Oyeyinka et al., 2017), is the third most produced and consumed legume in Africa after groundnut (*Arachis hypogaea* L.) and cowpeas (*Vigna unguiculata* L. Walp.) (Aremu et al., 2006). Bambara groundnut has been assessed for its potential to aid food security in sub-Saharan Africa due to its valuable nutritional value and ability to thrive in poor soils (Dan-Jimo et al., 2007; Arise et al., 2015; Oyeyinka et al., 2017; Feldman et al., 2019; Lin Tan et al., 2020). Bambara groundnut seeds are high in carbohydrates (58.3-64.4%), protein (23.7%), and fats (4.3-5.5%) as well as essential micronutrients such as calcium, iron, potassium, and sodium, according to Oyeyinka et al. (2018) and Lin Tan et al. (2020). It has higher methionine content than other frequently consumed legumes (Halimi et al. 2019; Oyeyinka et al., 2019). Additionally, certain studies have acknowledged its capacity to serve as a source of income for small-scale farmers (Bamshaiye et al., 2011; Oyeyinka et al., 2017). During lean times, this crop can supplement the family's diet improving its nutritional value, particularly as a protein source (Bonny & Djè, 2011). Once ripe, the fresh seeds can be boiled or roasted. Additionally, they can be mixed with maize flour to enhance the flavor of traditional foods. Ground seeds often make patties, fritters, and pancakes, fried in oil (Baudoin & Mergeai, 2001).

Despite the advantages and the interest that African populations have in Bambara groundnuts, the main challenge is the storage of harvested seeds. Traditional storage methods are primarily used by farmers for storing Bambara

groundnut seeds after shelling such as in the hermetically sealed cans, jars, plastic bags, canaries, or granaries. (Ouoba et al., 2016; Komi et al., 2018; Ouili et al., 2022a). Nevertheless, such methods do not always guarantee the safety of seeds during storage. Stored products are subject to deterioration of all kinds due to the action of pests, including fungi (De Groot, 2004; Komi et al., 2018). Fungal contamination of Bambara groundnut seeds can reduce their nutritional value as well as their organoleptic properties (Olagunju et al., 2018). Furthermore, the growth of fungi in seeds holds the potential to produce mycotoxins, which pose a threat to the health and safety of consumers (Pereira et al., 2013; Olagunju et al., 2018; Adebiyi et al., 2020; Okayo et al., 2020).
To control the fungal growth in Bambara groundnut seeds, farmers use several chemicals during storage. These include bextoxin and phostoxin (Ouoba et al., 2016; Ouili et al., 2022a). Unfortunately, they are not always used wisely or by the manufacturer's recommendations. The arbitrary application of these substances puts users at risk of intoxication and environmental contamination (Ouoba et al., 2016). For these reasons, crop protection research could concentrate on the use of biocontrol agents such as lactic acid bacteria (LAB). LAB species have long been used as food preservatives for both human and animal consumption to prevent spoilage and extend shelf life (Ananthi et al., 2016). Lactic acid bacteria, including the genera *Lactobacillus* and *Pediococcus* genera, (Zamfir et al., 2014; Matei et al., 2018; Ștefan et al., 2018; Utoiu et al., 2018; Constantin et al., 2023; Coulibaly et al., 2023), as well as yeasts such as *Saccharomyces boulardii* and *S. cerevisiae* (Diguță et al., 2023; Mogmenga et al., 2023), have undergone extensive research for their antimicrobial properties and potential health benefits. LAB species provide a sustainable alternative to chemicals as they have the capacity to a broad variety of antifungal compounds that efficiently suppress fungi growth and also minimize mycotoxin synthesis (Belkacem-Hanfi et al., 2014; Le Lay et al., 2016). Indeed, Belkacem-Hanfi et al. (2014) reported that *Lactobacillus graminis, Lactobacillus plantarum, Pediococcus pentosaceus*, and *Weissella cibaria* can inhibit by up to 53% the radial growth of *Aspergillus* isolated from wheat. Asurmendi et al. (2013) isolated LAB from brewing grains that had the ability to suppress the growth of at least two strains of *Aspergillus*. Similar findings were reported by Ogunremi et al. (2022), who isolated LAB strains from kunu-zaki, a Nigerian fermented beverage, and observed *in vitro* inhibition of *Aspergillus flavus* and *Penicillium crustosum* strains. Moreover, Le Lay et al. (2016) demonstrated the ability of LAB and their supernatants to inhibit the growth of *Aspergillus niger* and *Penicillium corylophilum*. The antifungal properties of lactic acid bacteria (LAB) are mainly attributed to acid or protein metabolites, which include organic acids such as acetic acid, phenyllactic acid, cyclic dipeptides, and fatty acids, as well as ethanol, fatty acids, reuterine, and hydrogen peroxide (Le Lay et al., 2016; Ouiddir et al., 2019; Sadeghi et al., 2016; Garnier et al., 2020). The most effective antifungal compounds in LAB, as stated by Le Lay et al. (2016) and Quattrini et al. (2018), are lactic and acetic acids. The antifungal compounds can neutralize the electrochemical potential of target cells' plasma membranes, thereby enhancing their permeability, and leading to the death of susceptible fungi (Dalié et al., 2010; Ogunremi et al., 2022).
The aims of this investigation were: (i) the isolation of LAB strains from Bambara groundnut seeds; (ii) the assessment of their antifungal activity against two aflatoxigenic *Aspergillus flavus* strains; (iii) the identification of LAB strains with antifungal potential by 16S RNAr sequencing, and (iv) the quantification of lactic and acetic acids in cell-free supernatants of LAB strains with high antifungal potential using HPLC.

MATERIALS AND METHODS

Isolation and purification of LAB

LABs were isolated from Bambara groundnut seeds collected in Burkina Faso during the 2020 harvest. Ten grams of Bambara groundnut seeds were finely ground and suspended in 100 ml of MRS (Man, Rogosa and Sharp) broth (Oxoid, UK). The mixture was then incubated at a temperature of 30°C for 48 hours. After conducting several decimal dilutions, an inoculum of 0.1 ml from the 10^{-8} dilution was introduced onto MRS agar plates (Oxoid, UK).

After incubation in anaerobic jars (Schuett-biotec GmbH, D-37079 Göttingen, Germany) at a temperature of 30°C for 48 hours, the bacterial cultures were transferred and purified by successive subculturing onto MRS agar. Pure strains were identified using Gram staining, catalase tests, and mobility. LAB was used to treat Gram-positive, non-motile, and catalase-negative bacteria, which were stored at a temperature of -20°C in MRS broth containing 50% glycerol.

Antifungal activity of LAB isolates

Two *Aspergillus* section *Flavi* strains (AVBF26, AVBF66) previously isolated from Bambara groundnut seeds and able to produce aflatoxins B_1 and B_2 were selected for the antifungal tests (Ouili et al., 2022b). These tests were carried out using the overlay assay described by Cabo et al. (2002). Briefly, 10 µl of each overnight LAB culture were spotted onto MRS agar medium in Petri plates and incubated at 30°C for 48 hours. The plates were then plated with 15 ml of Potato Dextrose Agar (Merck™, Darmstadt, Germany) containing 10^8 spores/ml of each *Aspergillus flavus* strain. As control plates, pure fungal cultures were prepared. The plates were then incubated at a temperature of 30°C. Antifungal activity of each fungal growth was observed every 24 hours, for five days. LAB strain was evaluated by the appearance of clearly visible halos around colonies, which were graded as follows: no inhibition, + (6-10 mm zone of inhibition), + + (10-20 mm zone of inhibition), and + + + (>20 mm zone of inhibition). All experiments were conducted in duplicate.

Molecular identification of LAB and fungal strains

The 16S rDNA region of LAB strains was amplified using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The 5.8S ITS region of fungal strains was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), as described by White et al. (1990). The PCR reaction was performed in 50 µl volume containing 0.5 µM of each primer, 0.2 mM dNTP, 10X DreamTaq green buffer supplemented with MgCl2, 0.125 U DreamTaq polymerase (ThermoFisher Scientific, Baltics, UAB, Vilnius, Lithuania), and 10 ng/µl DNA. PCR amplification was performed utilizing a MultiGene PCR System (MyCycler thermal cycler, BIO-RAD, Hercules, USA) and following protocols specified by Coulibaly et al. (2023) for 27F/1492R primers and by Esteve-Zarzoso et al. (1999) for ITS1/ITS4 primers. PCR products were detected by 2% (w/v) agarose gel electrophoresis supplemented with 0.7 µg/ml ethidium bromide and visualized using a GelDoc-It imaging system (Analytik Jena, USA).

Sequencing was performed using the Sanger method by CEMIA (Cellular and Molecular Immunological Application, Greece), with the same primers as those for PCR. The sequences obtained were compared with the sequences available in the NCBI database (The National Center for Biotechnology Information) using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/BLAST) for the purpose of species identification. The phylogenetic tree of LAB was constructed using the neighbour-joining method (Saitou & Nei, 1987).

Quantification of lactic and acetic acids

The organic acids (lactic acid and acetic acid) were identified through high-performance liquid chromatography (HPLC). The samples underwent centrifugation, followed by a tenfold dilution with ultrapure water and filtration through a 0.20 µM Millex PTFE membrane. Then, 10 µL of the resulting solution was injected into an HPLC system (Waters; Millipore, Milford, MA, USA). Analytical separation was carried out using a SUPELCOGEL H Column and 0.1% H3PO4 as the mobile phase. UV detection was carried out at 210 nm. Quantification of organic acids was carried out by measuring the peak area, using a calibration curve derived from injecting diverse volumes of organic acid standard solutions (Utoiu et al., 2018).

RESULTS AND DISCUSSIONS

Morphological identification of LAB strains and fungal strains

A total of 22 LABs were identified based on their morphological characteristics (catalase-negative, Gram-positive, and non-motile) and stored at -20 C in glycerol.

Initially, the two fungal strains were identified as belonging to *Aspergillus* section *Flavi* by macroscopic (e.g. mycelium and colony reverse colours, exudate production) and microscopic observations (e.g. conidia, conidiophore). Furthermore, these strains produced aflatoxins B1 and B2 (Ouili et al., 2022b).

Antifungal activity of LAB strains against *Aspergillus flavus*

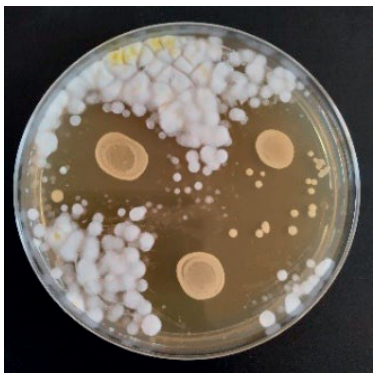
The lactic acid bacteria (LAB) strains were classified into two groups: 17 strains showed no antifungal activity towards the tested fungal strains, while five strains (52B1, 52C1, 74B3, 67B1, 67A9) exhibited antifungal activity (refer to figure 1). All five of these LABs showed an inhibition diameter greater than 20 mm against the tested *Aspergillus flavus* strains (AVBF26, AVBF66) (Table 1). Differences in inhibition halos existed across fungal strains, attributable to variations in LAB sensitivity towards fungi. Some strains exhibited inhibition halos for 48 hours, while others displayed them for 72 hours of incubation at 30°C. On the third day of incubation, mycelial growth was observed throughout the clear zone of certain Petri dishes, indicating the fungistatic effects of the bacteria used on the studied fungi. By contrast, the bacteria in the remaining Petri dishes sustained inhibition for more than five days. Notably, the number of fungal spores used (10^8 ml spores) in the experiment is probably greater than expected in practical food preservation scenarios, highlighting the potent inhibition capacity of the lactic acid bacteria that we examined in our investigation.

Table 1. Antifungal activity of LAB strains

Fungal strains	**LAB strains**				
	52B1	52C1	74B3	67A9	67B1
AVBF26 (*A. flavus*)	+++	+++	+++	+++	+++
AVBF66 (*A. flavus*)	+++	+++	+++	+++	+++

+++, >20 mm zone of inhibition.

a: no inhibition of 70B7 against AVBF26



b: presence of halos of 67A9 inhibition against AVBF26

c: total inhibition of 67B1 against AVBF66

Figure 1. Examples of antifungal activity displayed by the LAB strains against *A. flavus* (after 72 hours)

One of the main lactic acid bacteria that inhibit the growth of fungi is *P. pentosaceus* (Magnusson et al., 2003; Riolo et al., 2023). *P. pentosaceus* has been shown to possess inhibitory effects against numerous common fungi, such as *Aspergillus, Fusarium, Penicillium,* and *Candida albicans* (Magnusson et al., 2003; Sellamani et al., 2016; Riolo et al., 2023). Strains of *P. pentosaceus* isolated by Sadeghi et al. (2016) demonstrated the ability to suppress the growth of *Aspergillus flavus* and *Aspergillus niger*, whereas *P. pentosaceus* species that were isolated by Belkacem-Hanfi et al. (2014) led to a reduction in the growth of multiple fungi, including *Aspergillus flavus*.

Molecular identification of LAB strains and fungal strains

DNA sequencing has proven to be an efficient methodology to identify microorganisms because of their reliability, simplicity, and swiftness (Unban et al., 2020). Following the sequencing, the fungal isolates were determined as *Aspergillus flavus* species, exhibiting a 100% similarity rate with diverse sequences present in the NCBI database.

The identification of the five LABs with antifungal potential was accomplished through sequencing of the 16S rDNA gene, and the obtained sequences were compared with those accessible in the NCBI database. The sequence comparison indicates that all LABs are affiliated with the *Pediococcus pentosaceus* species as their sequence similarity is >99% (Table 2). The phylogenetic tree analysis also reveals that the LAB strains are closely linked to *P. pentosaceus* (Figure 2). Other techniques that can be used to identify microorganisms include combining 16S rDNA gene sequencing with MALDI-TOF-MS and chemometrics or using whole genome sequencing. Regardless, due to its simplicity and cost-effectiveness relative to alternative techniques, 16S rDNA gene sequencing is still the most commonly used. Previous studies have reported the sequencing of 16S rDNA genes from *P. pentosaceus* isolated from various foods (Djossou et al., 2011; Asurmendi et al., 2014; Belkacem-Hanfi et al., 2014, Utoiu et al., 2018). *P. pentosaceus* strains have been found in food, plants, and animals, where they act as preservatives or growth promoters (Ghadban, 2002; Matei et al., 2018; Utoiu et al., 2018; Diguță et al., 2020; Coulibaly et al., 2023).

Table 2. BLAST analysis of sequenced sequences of the LAB strains using the NCBI database

LAB strains	Similarity (%)	Identified species
52B1	99.55%	*Pediococcus pentosaceus* (JX141321.1)
52C1	99.40%	*Pediococcus pentosaceus* (OM760896.1)
74B3	99.77%	*Pediococcus pentosaceus* (MK818761.1)
67B1	99.27%	*Pediococcus pentosaceus* (JX141316.1)
67A9	99.33%	*Pediococcus pentosaceus* (OK513531.1)

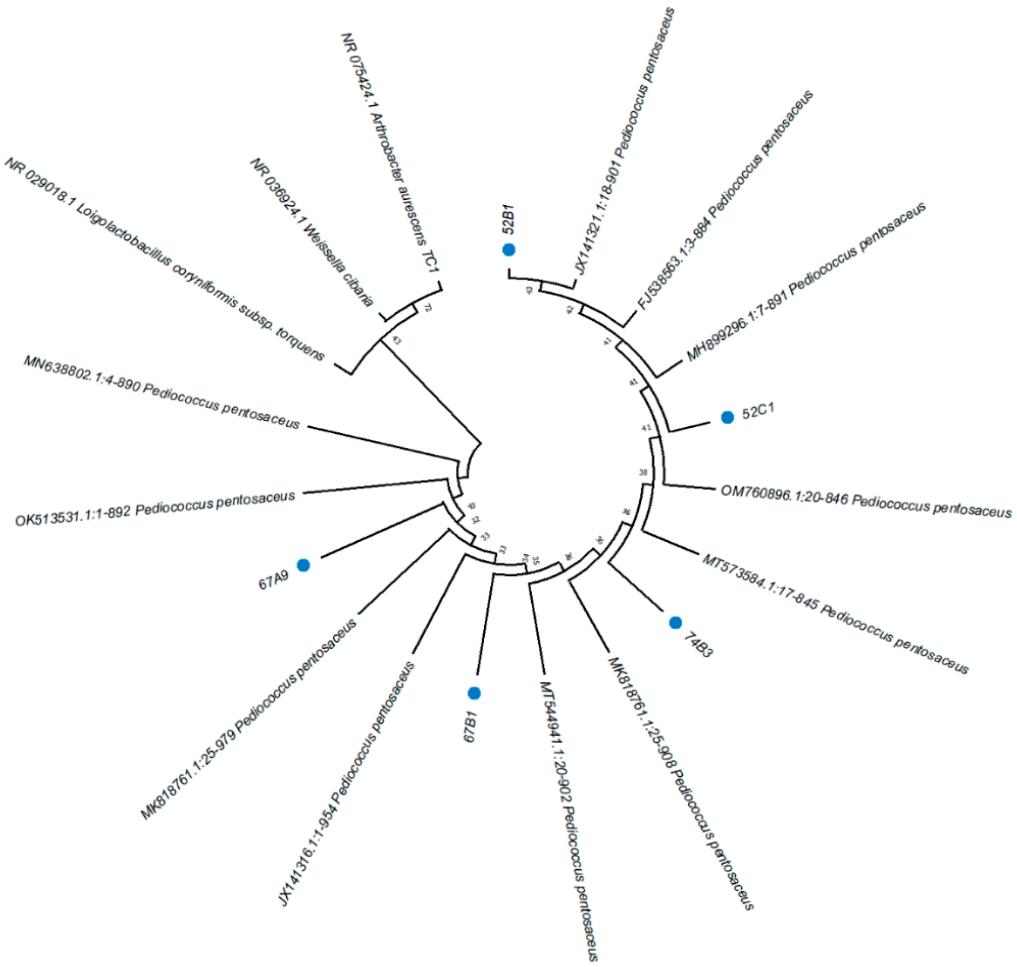


Figure 2. Phylogenetic trees for LAB strains were constructed using the neighbour joining method using 16S rDNA. Percentages next to branches indicate the frequency of duplicates where related taxa clustered in the 1000 replicate bootstrap test

Quantification of antifungal compounds (lactic and acetic acids)

Lactic and acetic acids are widely recognized as primary metabolites produced by LABs. The acids are not only involved in antifungal activity, but they also play a significant role in many other processes (Dal Bello et al., 2007; Huh & Hwang, 2016). All five tested LAB supernatants recorded lactic acid ranging from 12.08 mg/ml to 15.38 mg/ml (Table 3). Strain 74B3 produced the highest concentration, while strain 67B1 produced the lowest (Table 3). Acetic acid was produced by 80% of the active bacterial strains, with average quantities ranging from 0.06 mg/ml (strain 67A9) to 1.68 mg/ml (strain 74B3) (Table 3).

Table 3. Lactic and acetic acid content (mg/ml) in LAB supernatants

LAB strains	Lactic acid (mg/ml)	Acetic acid (mg/ml)
67B1	12.02	<L
74B3	15.38	1.68
52B1	12.78	0.34
52C1	15.04	0.68
67A9	12.58	0.06

<L: Limite de quantification

Ogunremi et al. (2022) isolated LAB from kunu-zaki, a Nigerian cereal-based fermented drink, that produced lactic acid (1.80-6.28 mg/ml) and acetic acid (0.96-1.53 mg/ml). Le Lay et al. (2016) founded that LAB isolated from milk produced concentrations of lactic acid (2.47-5.7 mg/ml) and acetic acid (0.53-2.35 mg/ml) in the supernatant. Furthermore, Guimarães et al. (2018) recorded lactic acid ranging from 3.4 to 4.3 mg/ml in the supernatants of LAB isolated from Kefir grains. Acetic acid concentrations obtained by the authors are comparable to those found in our investigation. However, the reported concentrations of lactic acid are lower than those found in our study. The variance in concentrations might be associated with the species of LAB, their distinct physiology, and their divergent origins.

This organic acid synthesis can partly explain why the lactic acid bacteria analyzed in our study blocked fungal strains. Lactic and acetic acids are substances that inhibit fungal growth. Organic acid synthesis decreases the pH of the environment, creating an unfavorable setting for the development of disease-causing microorganisms in food products (Peyer et al., 2016). Organic acids exert their antifungal activity by penetrating cell membranes in their undissociated form, which lowers intercellular pH and disrupts metabolic activities, ultimately inhibiting growth or causing fungal cell death (Guimarães et al., 2018). Lactic acid bacteria (LAB) produce several antifungal compounds, including lactic and acetic acids, which in combination create unfavourable conditions for fungal development (according to Cabo et al., 2002; Crowley et al., 2013; Le Lay et al., 2016: Özcelik et al., 2016; Guimarães et al., 2018; Ogunremi et al., 2022).

CONCLUSIONS

Out of the 22 strains of lactic acid bacteria isolated from Bambara groundnut, only 5 strains exhibited high antifungal properties against two *Aspergillus* strains. These fungal strains were identified through analysis of 5.8 rDNA-ITS gene sequence homology as belonging to *Aspergillus flavus.* BLAST analysis of the 16S rRNA indicated that the LAB strains with high antifungal activity showed that they all belong to the *Pediococcus pentosaceus* species. Except for strain 67B1, the quantification of organic acids indicated that all strains are able to synthesize both lactic and acetic acids. These LAB strains show potential as candidates for developing methods to prevent fungal growth during food storage, including Bambara groundnuts, intending to replace or minimize the use of chemical preservatives and reduce consumer exposure to the hazards associated with aflatoxins and chemical products.

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