Isolation of Antifungal Lactic Acid Bacteria (LAB) from "Kunu" against Toxigenic *Aspergillus flavus*

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ABSTRACT: The antifungal activity of isolated lactic acid bacteria (LAB) from a locally fermented cereal, "Kunu", was tested against toxigenic *Aspergillus flavus*. The liquid refreshment, "Kunu", was prepared under hygienic condition using millet, sorghum, and the combination of the two grains. The antifungal potential of isolated LAB against toxigenic *A. flavus* was carried out using both *in vitro* and *in vivo* antifungal assays. The LAB count from prepared "Kunu" ranged from 2.80×10^4 CFU/mL to 4.10×10^4 CFU/mL and *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Pediococcus acidilactici*, and *Leuconostoc mesenteroides* were the isolated bacteria. Inhibitory zones exhibited by LAB against toxigenic *A. flavus* ranged from 5.0 mm to 20.0 mm. The albino mice infected with toxigenic *A. flavus* showed sluggishness, decrease in body weight, distortion of hair, and presence of blood in their stool, while those treated with LAB after infection were recovered and active like those in control groups. Except for the white blood cell that was increased in the infected mice as 6.73 mm^3, the packed cell volume, hemoglobin, and red blood cell in infected animals were significantly reduced ($P < 0.05$) to 29.28%, 10.06%, and 4.28%, respectively, when compared to the treated mice with LAB and control groups. The antifungal activity of LAB against toxigenic *A. flavus* can be attributed to the antimicrobial metabolites. These metabolites can be extracted and used as biopreservatives in food products to substitute the use of chemical preservatives that is not appealing to consumers due to several side effects.

Keywords: cereals, aflatoxin, lactic acids, preservatives, Nigeria

INTRODUCTION

"Kunu" is a non-alcoholic beverage produced from selected grains such as millet (*Pennisetum typhoideum*), sorghum (*Sorghum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), and acha (*Dijitap exilis*). Each grain can be used singly or combined in different ratios (1). "Kunu", a staple beverage serves as an alternative drink to quench thirst and is more nutritious compared to carbonated drinks (2). The fermented drink has been appreciated for their aroma and flavour due to addition of medicinal spices such as ginger, black pepper, red pepper, cloves, and sugar coupled with the essential role of lactic acid bacteria (LAB) during fermentation (3,4).

LAB are extensively used as a starter culture in food fermentation due to their ability to utilize sugars and produce different metabolites, which serve as antagonistic compounds and inhibit the growth of pathogenic bacteria and spoilage microorganisms, and thus, maintain the nutritive quality of fermented foods (5). The presence of LAB in foods has been an interesting strategy and alternative means of food preservation over the use of synthetic chemical preservatives. LAB serve as a biotechnological tool for food preservation to improve the quality assurance of food products and to retain the sensory qualities of foods with nutritional benefits (6). Therefore, fermented foods containing LAB can effectively enhance the integrity of gastric mucosa with protective effects against mucosal injury by improving the function of the microbiome in the gastrointestinal tract, exert metabolic activities on indigestible polysaccharides and dietary fibres to provide energy, and short chain fatty acids and essential amino acids that cannot be produced by humans (7,8).

Mycotoxigenic fungi such as *Aspergillus* spp., *Alternaria* spp., *Fusarium* spp., and *Penicillium* spp. cause numerous diseases in cereal crops (9). Although, fungicides are commonly used to reduce the occurrence and destruc-

tive effects of toxigenic fungi, this has not sufficiently exterminated the presence of toxin producing fungi in foods due to their resistant genes, and thus, creating a greater threat to food safety.

These phytopathogenic fungi may also withstand food processing methods and find their way into the finished products, which could cause food borne illnesses due to the production of mycotoxin. Therefore, there is a need for adequate measures to prevent fungal growth in foods. The use of probiotic microorganisms as an emerging bio-control tool against fungal growth is now a better approach to eliminate fungal toxins in foods (10). The presence of LAB in traditionally fermented foods has improved their nutritive values with extended preservation due to inhibitory compounds against spoilage microorganisms. The present study was conducted to isolate LAB from "Kunu" and to assess their antifungal activity against a toxigenic *Aspergillus flavus*.

MATERIALS AND METHODS

Source of toxigenic *A. flavus*

The studied toxigenic *A. flavus* contained aflatoxins B_1, B_2, G_1, and G_2 (11). The fungus was cultivated on potato dextrose agar (PDA, Oxoid, Hampshire, UK) and incubated at $28\pm1^\circ$C for 7 days. After full sporulation, the fungal spores were harvested into sterile peptone water (0.2%) and filtered using sterile cotton filter to avoid the presence of conidia and mycelial debris (12). The spore suspension in peptone water was adjusted to the final concentrations of 10^6 *A. flavus* spores/mL.

Source of grains

Millet and sorghum were purchased from the King's market, Akure in Ondo State, Nigeria. The grains were transferred to laboratory in the Department of Microbiology, The Federal University of Technology Akure, Nigeria.

Preparation of "Kunu"

The traditionally fermented beverage was produced from 500 g of millet, sorghum (500 g), and mixture of millet and sorghum (50% w/w) under control conditions using the methods of Gaffa et al. (1) and Obadina et al. (13) with slight modifications. Each grain was sorted, labeled, washed with clean water, and separately steeped in 1,000 mL of water at $28\pm2^\circ$C for 48 h. Thereafter, the water was removed and the grains were blended separately with 3.25 g of ginger (*Zingiber officinale*), 0.5 g of alligator pepper (*Afromomum melegueta*), 0.25 g of cloves (*Syzygium aromaticum*), 1.25 g of red pepper (*Capsicum* sp.), and 0.25 g of black pepper (*Piper guineense*) to form a smooth slurry. Each of the slurries was sieved to extract the starch, while the shafts were discarded. The filtrates

were allowed to settle for $2\sim3$ h, the supernatant was decanted and the sediment was divided into two portions (2:3). The largest portion was placed in a vessel and boiled to obtain a thick paste, while the other part of the slurry (uncooked) was added and stirred.

Isolation and identification of LAB

A volume (2.50 mL) of "Kunu" was measured into 7.50 mL of sterile water as a stock solution. A 1.0 mL aliquot from the stock solution was serially diluted into 9.0 mL of sterile distilled water. The pour plate method described by Olutiola et al. (14) was adopted. Briefly, 100 μL from the appropriate dilution was transferred into a petri dish and sterile De Man, Rogosa, and Sharpe (MRS, Oxoid) was added. The plates were incubated at 37°C for 48 h. Thereafter, the bacterial growth were counted as colony forming unit per volume (CFU/mL). The bacterial isolates were sub-cultured to obtain pure isolate, which was transferred to slant agar of MRS and kept at -4°C for further use. Gram's staining, spore staining, and biochemical tests such as catalase, nitrate reduction, oxidase, urease production, and sugar fermentation were carried out according to the methods of Cheesbrough (15). Staining reactions and the result of biochemical tests were interpreted for the identification of bacterial isolates to species level according to Cowan and Steel (16).

In vitro antifungal activity of LAB against toxigenic *A. flavus*

The LAB isolates obtained from "Kunu" were initially screened for antifungal activity against toxigenic fungus using the agar overlay method described by Lind et al. (17) with some modifications. Briefly, LAB were cultured in sterile MRS broth and incubated at $35\pm2^\circ$C for 48 h under anaerobic conditions. An aliquot suspension (100 μL) containing each of LAB cells of 10^8 CFU/mL was added into a well of 6.0 mm diameter at the center of the plate containing 10 mL of MRS agar. The inoculated plates were incubated at $35\pm2^\circ$C and observed for growth within $1\sim2$ days. After incubation, the plates were then overlaid with 10 mL of PDA, inoculated with 100 μL of mold spore (10^6 spores/mL) and incubated at $28\pm2^\circ$C for $1\sim5$ days. Thereafter, the zones of inhibition around the bacterial colonies against the fungus were recorded. The inhibitory zones obtained were used to select the best two LAB isolates for the *in vivo* antifungal assay.

In vivo antifungal assay

Albino rats were obtained from the Department of Animal Production and Health, The Federal University of Technology, Akure. The rats were weighed and assigned five to each group in cages at $25\pm2^\circ$C with 12-h light/darkness cycle. The animals had adequate access to food and water *ad libitum* and kept in the cage for 7 days to

acclimate them to the environmental conditions. The use of laboratory animals was approved by the research committee, Department of Microbiology (Ref. no.: 2015-022) in accordance with the institution ethics and international standard of animal welfare described by the National Research Council (18). The mice were grouped and labelled as follow: those fed basal diet as BD, basal diet and toxigenic *A. flavus* as BD+AF, basal diet and *Lactobacillus plantarum* as BD+LP, basal diet and *Lactobacillus delbrueckii* as BD+LD, basal diet, toxigenic *A. flavus,* and *L. plantarum* as BD+AF+LP, basal diet, toxigenic *A. flavus,* and *L. delbrueckii* as BD+AF+LD, and basal diet, toxigenic *A. flavus, L. plantarum,* and *L. delbrueckii* as BD+AF+LPD.

The infectious dose (500 µL) of the test toxigenic *A. flavus* (10^6 spores/mL) was orogastrically administered into the mice. After signs of infection, 500 µL of the suspension containing LAB (10^8 CFU/mL) was administered to the animals. The average weight of the animals was monitored using a digital scale (KERRO BL 200001, MxRady Lab Solutions Pvt. Ltd., Delhi, India).

Collection and examination of blood samples

The animals were disinfected with 70% v/v of ethanol, anesthetized, and their blood samples were collected into ethylenediaminetetraacetic acid bottles using the cardiac puncture method described by Parasuraman et al. (19). The hematological tests packed cell volume (PCV), hemoglobin (HB), red blood cell (RBC), and white blood cell (WBC) were carried out according to the standard methods described by Cheesbrough (15).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS software version 17.0, SPSS Inc., Chicago, IL, USA). The experiment was conducted using a completely randomized design, and data obtained were analyzed by one-way analysis of variance (ANOVA). Means were compared by Duncan's new multiple range test and considered statistically significant when $P \leq 0.05$.
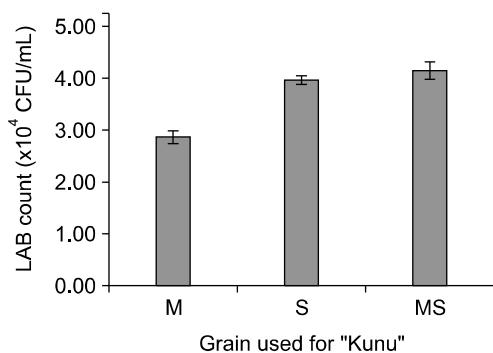

Fig. 1. Lactic acid bacteria (LAB) count in "Kunu" produced from millet (M), sorghum (S), and mixture of millet and sorghum (MS).

RESULTS

The LAB count from "Kunu" produced from millet, sorghum, and the combination of the two substrates ranged from 2.80×10^4 to 4.10×10^4 CFU/mL (Fig. 1). "Kunu" from millet had the lowest LAB count of 2.50×10^4 CFU/mL ($P<0.05$) compared to "Kunu" from sorghum (3.90×10^4 CFU/mL) and the combination of the two grains (4.10×10^4 CFU/mL). The isolated bacteria from "Kunu" were *L. plantarum, L. delbrueckii, Lactobacillus fermentum, Pediococcus acidilactici,* and *Leuconostoc mesenteroides.* These bacteria concurrently occurred in the "Kunu" produced from millet, sorghum, and the combined grains with the highest occurrence (33.4%) of *L. plantarum* (Fig. 2). The inhibitory zones displayed by LAB against toxigenic *A. flavus* are presented in Table 1. *L. delbrueckii* and *L. plantarum* displayed higher inhibitory zones of 15.0 mm and 20.0 mm against toxigenic *A. flavus,* respectively. The two *Lactobacillus* spp. were further investigated for *in vivo* antifungal assay.

The infective dose was able to cause infection, and all the rats survived throughout the experimental period until they were sacrificed. Signs and symptoms observed in the infected animals included unformed stool stained with blood, falling of hair, weakness characterized by
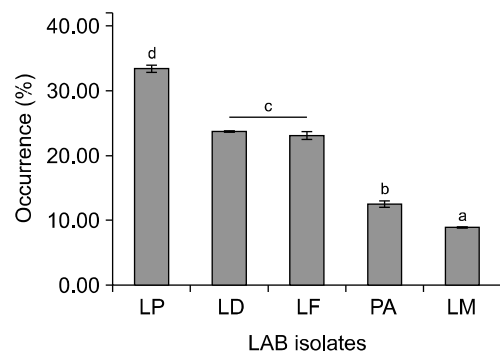

Fig. 2. Occurrence of lactic acid bacteria (LAB) in "Kunu" produced from millet, sorghum, and combined millet and sorghum. Different letters (a-d) are significantly different from each other (*P*<0.05) by Duncan's new multiple range test. LP, *Lactobacillus plantarum*; LD, *Lactobacillus delbrueckii*; LF, *Lactobacillus fermentum*; PA, *Pediococcus acidilactici*; LM, *Leuconostoc mesenteroides.*

Table 1. Inhibitory zones displayed by lactic acid bacteria (LAB) against toxigenic *Aspergillus flavus*

LAB isolates	Inhibited zones (mm)
Lactobacillus plantarum	20.0±0.0[e]
Lactobacillus delbrueckii	15.0±0.0[d]
Lactobacillus fermentum	8.0±0.0[c]
Pedicoccus acidilactici	5.0±0.0[b]
Leuconostoc mesenteroides	0.0[a]

Values are mean±SD of triplicates (n=3).
Different letters (a-e) are significantly different by Duncan's new multiple range test (*P*<0.05).

slow movement, and loss of appetite (Table 2). There was loss of body weight in the infected animals after 21 days, but the treated mice with LAB recovered from the infection and gained body weight (Fig. 3). The control group of mice fed BD, BD+LP, and BD+LD had body weights of 171.30 g, 165.30 g, and 164.80 g, respectively. The final weight (120.30 g) of albino mice infected with toxigenic *A. flavus* was significantly reduced ($P<0.05$) compared to the treated mice with one or two strain(s) of LAB; BD+AF+LP, BD+AF+LD, and BD+AF+LPD, which regained their body weight after treatment as 150.43 g, 148.60 g, and 155.84 g, respectively.

The hematological study on mice challenged with toxigenic *A. flavus* revealed decrease in PCV, HB, and RBC except for WBC as presented in Table 3. The PCV of mice in the control groups: BD, BD+LP, and BD+LD were 34.06%, 33.88%, and 33.77%, respectively. The PCV of the infected group (29.28%) was reduced and significantly different ($P<0.05$) compared to the treated

groups: BD+AF+LP, BD+AF+LD, and BD+AF+LPD with values of 31.50%, 31.70%, and 31.83%, respectively. The HB in infected mice was reduced ($P<0.05$) to 10.06%, while the treated mice with LAB: BD+AF+LP, BD+AF+LD, and BD+AF+LPD had HB values of 10.94%, 10.91%, and 10.95%, respectively, which are similar ($P<0.05$) to the mice in control groups: BD+LP (11.60%) and BD+LD (11.53%). The RBC of mice in the control groups: BD, BD+LP, and BD+LD were 6.75%, 6.47%, and 6.42%, respectively. The treated group of mice from BD+AF+LP, BD+AF+LD, and BD+AF+LPD regained their RBC count after treatment as 6.24%, 5.58%, and 6.59%, respectively, but the RBC of infected mice remained very low ($P<0.05$) with a value of 4.28%. The WBC (6.73 mm^3) in the infected mice with toxigenic *A. flavus* increased and was significantly

Table 2. Sign and symptoms observed in each group of mice

Groups	Clinical signs
BD	Active
BD+LP	Active
BD+LD	Active
BD+AF	Weak, stool stained with blood, loss of hair, appetite and body weight
BD+AF+LP	Recovered with growing hair, no blood in the stool, regain of appetite and body weight
BD+AF+LD	Recovered with growing hair, no blood in the stool, regain of appetite and body weight
BD+AF+LPD	Recovered with growing hair, no blood in the stool, regain of appetite and body weight

BD, mice fed basal diet; BD+AF, mice fed basal diet and toxigenic *A. flavus*; BD+LP, mice fed basal diet and *Lactobacillus plantarum*; BD+LD, mice fed basal diet and *Lactobacillus delbrueckii*; BD+AF+LP, mice fed basal diet, toxigenic *A. flavus*, and *L. plantarum*; BD+AF+LD, mice fed basal diet, toxigenic *A. flavus*, and *L. delbrueckii* BD+AF+LPD, mice fed basal diet, toxigenic *A. flavus*, *L. plantarum*, and *L. delbrueckii*.
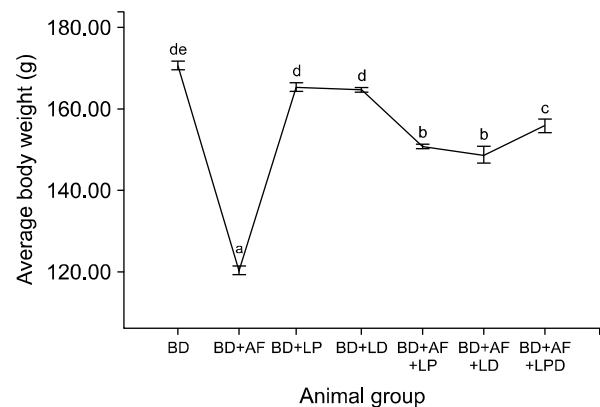
Fig. 3. Body weight of albino rats fed with basal diet, administered with toxigenic fungus and treated with lactic acid bacteria. Different letters (a-e) are significantly different from each other ($P<0.05$) by Duncan's new multiple range test (n=5). BD, mice fed basal diet; BD+AF, mice fed basal diet and toxigenic *A. flavus*; BD+LP, mice fed basal diet and *Lactobacillus plantarum*; BD+LD, mice fed basal diet and *Lactobacillus delbrueckii*; BD+AF+LP, mice fed basal diet, toxigenic *A. flavus*, and *L. plantarum*; BD+AF+LD, mice fed basal diet, toxigenic *A. flavus*, and *L. delbrueckii*; BD+AF+LPD, mice fed basal diet, toxigenic *A. flavus*, *L. plantarum*, and *L. delbrueckii*.

Table 3. Hematological parameters of the abino mice after infection and treatment with lactic acid bacteria

Groups	PCV (%)	HB (%)	RBC (%)	WBC (mm^3)
BD	34.06±0.03[c]	11.75±0.03[cd]	6.75±0.03[cd]	5.40±0.03[a]
BD+AF	29.28±0.08[a]	10.06±0.06[a]	4.28±0.08[a]	6.73±0.08[b]
BD+LP	33.88±0.14[c]	11.60±0.14[bc]	6.47±0.14[c]	5.30±0.14[a]
BD+LD	33.77±0.21[c]	11.53±0.21[bc]	6.42±0.20[c]	5.19±0.20[a]
BD+AF+LP	31.50±0.51[b]	10.94±0.53[b]	6.24±0.52[c]	5.79±0.53[a]
BD+AF+LD	31.70±0.50[b]	10.91±0.50[b]	5.58±0.50[b]	5.46±0.50[a]
BD+AF+LPD	31.83±0.53[b]	10.95±0.51[b]	6.59±0.51[c]	5.45±0.51[a]

Values are mean±SD of triplicates (n=3).
Different letters (a-d) within each column are significantly different from each other at ($P<0.05$) by Duncan's new multiple range test.
PCV, packed cell volume; HB, hemoglobin; RBC, red blood cell; WBC, white blood cell. BD, mice fed basal diet; BD+AF, mice fed basal diet and toxigenic *A. flavus*; BD+LP, mice fed basal diet and *Lactobacillus plantarum*; BD+LD, mice fed basal diet and *Lactobacillus delbrueckii*; BD+AF+LP, mice fed basal diet, toxigenic *A. flavus*, and *L. plantarum*; BD+AF+LD, mice fed basal diet, toxigenic *A. flavus*, and *L. delbrueckii*; BD+AF+LPD, mice fed basal diet, toxigenic *A. flavus*, *L. plantarum*, and *L. delbrueckii*.

different (*P*<0.05) compared to the control groups: BD, BD+LP, and BD+LD and treated groups of BD+AF+LP, BD+AF+LD, and BD+AF+LPD.

DISCUSSION

This study focused on the antifungal activity of isolated LAB from "Kunu". The presence of Lactic Acid Bacteria in prepared "Kunu" corroborates with the findings of Nwachukwu et al. (20). The researchers identified similar bacteria in fermented cereal products indigenous to Nigeria. Carr et al. (21) revealed that members of LAB could be detected in a variety of foods including dairy foods, meat products, vegetables and other fermented foods.

L. plantarum had higher occurrence with pronounced inhibitory property. The predominance and probiotic features of *L. plantarum* has been recognized as a useful species for appreciable fermentative and metabolic processes (5). The isolated bacteria, notably, *L. plantarum, L. delbrueckii, L. fermentum, P. acidilactici,* and *L. mesenteroides* are mostly present in fermented drinks or foods. Species of lactobacilli in fermented food serve as biopreservatives, improving the organoleptic and nutritive properties of the fermented foods (22). Therefore, the presence of LAB in fermented foods has been attributed to health-promoting properties including anti-mutagenic, anti-carcinogenic, and antagonistic actions (7).

This study revealed that LAB inhibited the growth of toxigenic *A. flavus* and reduced the symptoms of infection after treatment. These bacteria have pronounced antimicrobial property against pathogenic bacteria and fungi (12,23). The antimicrobial effect of LAB can be ascribed to the biosynthesis of organic acids, diacetyl, hydrogen peroxide, and bacteriocins during lactic fermentation (24). The antimicrobial compounds produced by LAB in fermented foods would create a competitive advantage and limit the growth of food spoilage microorganisms. The inhibitory mechanisms of LAB against pathogenic microorganisms include creation of a hostile microecology, removal of bacterial receptor sites, secretion of antimicrobial substances, and selective metabolites leading to depletion of essential nutrients, lowering of intracellular pH and accumulation of the ionized form of the organic acid leading to the death of the pathogenic microorganisms (25). Therefore, the use of non-pathogenic bacteria such as LAB and their secondary metabolites could be of interest to food industries to improve the safety and extend the shelf life of foods.

The clinical signs and symptoms recorded in albino rats infected with aflatoxingenic fungus are in accordance with the findings of Zain (26) who reported acute and chronic effects of mycotoxins in human and animals.

Filamentous fungi possess extracellular enzymes, which are used to degrade the structural barriers and complex macromolecules in the host to cause infections (27). The reduced weight in rats exposed to toxigenic fungus containing diet could be the result of watery stool, loss of hair, and blood in the faeces. Lakkawar et al. (28) reported the manifestation of diarrhea in animals as a result of the acute toxic effect of aflatoxin B1. The body weight of treated mice with LAB recuperated, and this shows that LAB neutralize the pathogenic activity of toxigenic *A. flavus* in the tested animals. This is in agreement with Hathout et al. (29) who revealed that rats fed diets containing aflatoxins showed a significant reduction in body weight compared to the treated mice with LAB. Species of *Lactobacillus, Lactococcus, Bifidobacterium, Propionibacterium,* and *Saccharomyces cerevisiae* have a mechanism of detoxifying aflatoxins (30).

The reduction of PCV, HB, and RBC in rats induced with toxigenic *A. flavus* indicates unstable homeostasis and damage to the blood system. The decrease in RBC indicates anemia, which occurs as a result of inflammation, infection and toxemia caused by aflatoxins producing fungi (31). Findings of Abdel-Wahhab et al. (32) also reported decreasing in PCV, HB concentration, and total RBC counts in induced mice with aflatoxins, which resulted to normocytic normochromic anemia due to inhibition of protein synthesis, decrease in total iron binding capacity, and defect of hemopoietic cellular. The exposure of rats to the toxigenic fungus corresponded to a marked increase in the WBC count, which could be a sensitivity to the presence of toxigenic *A. flavus* and its metabolic product as a foreign substance. Wannemacher et al. (33) reported an increase in number of white blood cells and occurrence of lymphocytes when mice were injected with trichothecenes, a T-2 Mycotoxin. However, mice treated with LAB after injection of toxigenic *A. flavus* recovered and their hematological parameters improved. The potential of probiotic bacteria to inhibit the fungal growth and reduce the bioavailability of aflatoxins B1 in infected animals have been attributed to the ability of LAB to bind aflatoxins and decrease its absorption in the intestinal tract of induced rats (34). The use of LAB as an antagonistic agent against toxigenic fungi and their binding affinity to detoxify aflatoxins reveal their therapeutic effect and economic importance. Hence, biocontrol activity of LAB can be adopted to safeguard food losses by eliminating microbial contaminants and preventing spoilage of foods.

In conclusion, this study showed that LAB isolated from "Kunu" displayed protective measures against the pathogenic symptoms caused by toxingenic *A. flavus* in induced mice. Therefore, the presence of LAB in locally fermented "Kunu" ensures its safety and denotes it as a source of probiotic microorganisms that can be used to

control fungal spoilage of foods.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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