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Evaluation of the antagonistic effect of some species of lactic acid bacteria isolated from fermented food against toxigenic *Aspergillus flavus*

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ABSTRACT

The presence of mycotoxigenic molds in food potentially poses hazards to healthy living and needs to be controlled. There is a greater risk that the resistance phenomenon will increase in the future due to the frequent use of antibiotics and preservatives, unlike biopreservation. Lactic acid bacteria are capable of producing secondary metabolites with antimicrobial properties. This study was conducted to evaluate the antagonistic effect of lactic acid bacteria isolates against toxigenic mold, *Aspergillus flavus*. Lactic acid bacteria were isolated from palm wine, ogi, and yogurt samples, and their antagonistic activity against toxigenic mold was investigated using the agar overlay method. *Streptomyces* spp. had the highest counts of 15.1×10^4 cfu/ml, while the least count of 2.0×10^4 cfu/ml from *Lactobacillus plantarum* was obtained. *Pediococcus damnosus* and *Lactobacillus plantarum* were present in both yogurt and palm wine samples. Similarly, *Streptococcus* spp. was identified in both yogurt and pap (ogi) samples. *Aspergillus flavus* was susceptible to half of the lactic acid bacterial isolates with varying degrees of inhibition. Total inhibition of toxigenic mold was observed on the control plate and plates inoculated with *L. lactis, S. thermophilus, Leuconostoc mesenteriodes, P. damnosus,* and *L. bulgaricus*. The antifungal activity of lactic acid bacteria suggests their biocontrol efficacy against *A. flavus*. Also, the antimicrobial properties of lactic acid bacteria can make them a suitable candidate in food preservation. Furthermore, this study can stand as a preliminary step in multistep research to investigate the anti-fungal and anti-aflatoxigenic potential of lactic acid bacteria from fermented foods against toxigenic *A. flavus*.

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1. Introduction

The inefficient quality control during food processing has resulted in food contamination by mycotoxigenic fungi (1). Mold contamination does not only cause food and feed deterioration but also adversely affects. The health status of man and animals; since it is capable of producing toxic metabolites known as mycotoxins causing food poisoning and liver cancer (2).

Filamentous molds are the main spoilage organisms of various food products, such as fermented dairy (cheese, yogurt), bread, stored crops, feed hay, and silage (3).

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Food/feed contamination with various types of toxigenic molds is a serious problem nowadays. The growth of mold in foods causing alterations in their texture and harms the external aspect of the products has led to significant economic losses (4).

The loss of 5 to 10% of the world's food production has been linked to fungal contamination, and 27% of the foods produced in the United States are annually destroyed by toxigenic fungi (5). Additionally, the toxigenic and spoilage fungi responsible for numerous diseases and health risks occur due to their mycotoxin production potential, which includes aflatoxins or allergenic conidia, spores, and mycelia (3).

Mycotoxins are fungal metabolites, usually found in cereal crops and animal forage (6). For example, aflatoxins (AFs), one of the dangerous secondary metabolites (mycotoxins) are produced mainly by *A. flavus*, which produces both aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2), and are commonly found in grain and feed under storage (4). Similarly, *A. parasiticus* produces aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) (7). AFB1 and ochratoxin A (OTA) type of mycotoxins are the most common and dangerous mycotoxins with a high degree of hepatotoxicity, immunotoxicity, and nephrotoxicity (8). Nevertheless, among these mycotoxins, AFB1 is the most toxic and is well known for its toxic, carcinogenic, and teratogenic mutation effects (9).

Because of their diverse toxic nature i.e., neurotoxic, hepatotoxic, immunotoxic, carcinogenic, teratogenic, mutagenic, and nephrotoxic; the persistence of aflatoxigenic molds in foods poses threat to the health of animals and humans upon consumption of mold-contaminated foods (8). The major classes of mycotoxins that are of the greatest agro-economic importance are aflatoxins, ochratoxins, fumonisins, trichothecenes, emerging *Fusarium* mycotoxins, enniatins, ergot alkaloids, *Alternaria* toxins, zearalenone and patulin (5).

To remove these aflatoxins from foods is necessary; however, how to remove AF with a less adverse effect on humans or drastically reduce nutritional components of food products has become a major issue. Biological methods are non-polluting, protect the environment, specific-based, and work at high temperatures. The prevalence of mycotoxins in animal feeds, raw and processed foods are known to be more profound due to their stability under various processing conditions (10). Mycotoxins can be eliminated either after they are produced or by inhibiting the growth of the fungus that produces them. Heating, ammonia treatment, screening, and radiation are now used to remove mycotoxins in food; however, they are costly, impractical for industrial use, or damage key nutrients in the grain (7). As a result, controlling mold contamination in animal feeds and food crops at harvesting, pre-processing, processing, post-processing and those under storage is critical. Despite the control measures in the decontamination of fungal spores in feedstuffs, the resistance of fungal strains to some chemical and biopreservatives, and their persistence in the cereal grains in the storage

Environment has been of major concern to human health (11). Hence, there is a need to devise possible techniques in the control of food contamination with pathogenic fungi by exploring the natural biological component of LAB, i.e., bacteriocins in food preservation (12,13).

The long shelf-ability, safety concern, and removal of toxic contaminants from food has been linked to diverse LAB, yeasts, and their products in food fermentation (15). *Saccharomyces cerevisiae* and LAB are two distinct genera of potential decontaminating microorganisms that are commonly employed in food fermentation and preservation (11). For instance, LAB has been employed as starter cultures in the food industry for millennia and are capable of producing various bioactive metabolites, which include hydroxyl fatty acids, organic acids, fragrance compounds, hydrogen peroxide, bacteriocins, bacitracin, reuterin, proteinaceous compounds, and phenolic compounds (16). Certain factors, such as temperature, incubation time, growth medium, pH, and nutritional factors influence the antifungal activity of LAB in reducing the problem of toxicogenic mold contamination in foods (17). The probiotic activity has been found in many LAB strains and some *S. cerevisiae* strains. Food fermented with LAB tends to enhance their nutritional profiling and probiotic potential, which makes them regarded as Generally Recognized As Safe (GRAS) (18). The vast majority of LAB's antimicrobial activity publications have focused on antibacterial effects, with only a few findings on antifungal effects.

The antifungal properties of LAB on some mycotoxigenic fungi have been reported by a few authors (2,13,19,20), but the number of published studies on antifungal activity of LAB is still less studied, which necessitated this study. Furthermore, a limited number of reports have shown that a good selection of LAB can allow the control of fungal growth, thus reducing health risks due to exposure to mycotoxins (21). There is an open area for research possibilities for preventing fungal growth and elimination of mycotoxins from food or their transformation into less dangerous compounds, using the strains of LAB. Hence, this research was performed to assess the effect of different LAB found in palm wine, *ogi,* and yoghurt samples against the toxigenic *A. flavus* with promising industrial and health properties.

2. Materials and Methods

2.1. Sample preparation and microbial analysis

Fermented food substrates, such as palm wine, *ogi,* and yoghurt were purchased from Ondo Central Market and kept in sterile bottles, while toxigenic fungus, *A. flavus* was collected from the Food Microbiology Laboratory, Department of Biological Sciences, University of Medical Sciences, both in Ondo City, Nigeria. The microbial analysis was performed by employing serial dilution, pour and streak plating procedures. First, a serial dilution up to the sixth dilution was accomplished using test tubes containing 9 ml sterile distilled water. For each sample, 1 ml was pipetted and aseptically dispensed into separate test tubes to form a stock solution.

Consequently, 1 ml was pipetted from each stock solution and serially diluted until 10^{-5} dilutions were attained.

From the dilutions, 10^{-2} and 10^{-4}, 0.1 ml was pipetted and carefully dispensed into different sterile Petri dishes, before pour-plating with sterilized molten MRSA (de-Man Rogosa and Sharpe agar). After solidification, the plates were incubated aerobically at 37°C for 24 h. The colony formation observed on the plate's revealed LAB growth. Pure cultures were obtained by repeatedly streaking on freshly prepared MRSA. Bacterial isolates were identified on the basis of cultural, cellular morphology, Gram's reaction, and advanced bacterial identification software. The pure isolates were preserved in 3% glycerol and stored at 4°C for further use (13).

2.2. Antagonistic properties of LAB against toxigenic *A. flavus*

The antagonistic effect of LAB isolates against toxigenic mold (*A. flavus)* was determined using agar overlay, according to the method of Olonisakin *et al.* (22) with slight modifications. LAB was streaked on each MRSA plate. The spore suspensions of *A. flavus* were prepared by introducing a loopful of the culture into 10-milliliter sterile water with vigorous shaking. This spore-containing medium was poured onto the MRSA plates containing LAB isolate and incubated at room temperature for 48 h. The observed zones of inhibition showed antagonistic activity and inhibitory zones were measured in millimeters (mm) and recorded. All experiments were performed in replicate.

2.3. Statistical analysis of experimental data

The data obtained were analyzed using analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS), version 25.0. Results were presented as mean±standard deviation.

Statistical significance was set at $p \leq 0.05$ at 95% confidence interval using the Duncan Multiple Range Test (DMRT) to evaluate the level of differences among means of the different samples.

3. Results

3.1. Total LAB counts from different samples

The total LAB counts from different samples are shown in Figure 1. *Streptomyces* spp. had the highest counts of 15.1×10^4 cfu/ml, followed by *Pediococcus damnosus* with a count of 10.1×10^4 cfu/ml, while the least count of 2.0×10^4 cfu/ml from *Lactobacillus plantarum* was obtained.

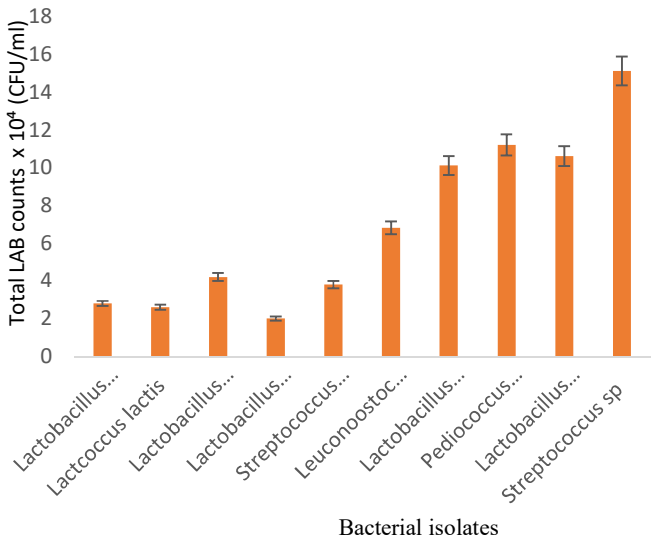

Figure 1. Total LAB counts (cfu/ml) from different samples

3.2. Morphological characterization of LAB isolates

Based on the morphological characterization of LAB isolated as shown in Table 1, all the isolated were creamy, except for *Pediococcus damnosus* and *Streptococcus* spp. from pap with whitish coloration. Most isolates have flat elevation, except *P. damnosus,* with a convex elevation, while *L. delbrueckii* displayed pulvinate elevation. All the isolates are opaque, circular, smooth texture, and entire margin. *P. damnosus, L. bulgaricus, Lactococcus lactis,* and *L. delbrueckii* were dull in appearance, while other isolates showed glister appearance.

3.3. Biochemical characteristics of LAB isolates

The biochemical characterization of LAB isolates is shown in Table 2. The isolates were Gram-positive (+) and catalase-negative (-), respectively. *Lactobacillus* genera were the most predominant in the samples. *Lactobacillus fermentum, L. lactis, L. delbrueckii, L. plantarum,* and *Streptococcus thermophilus* were isolated from yogurt samples, *Leuconostoc mesenteriodes* and *L. plantarum* were isolated from palm wine samples, while *Pediococcus damnosus, L. bulgaricus* and *Streptococcus* spp. were isolated from pap (*ogi*) samples. Furthermore, *L. plantarum* was found in both yogurt and palm wine samples; likewise, *Streptococcus* spp. was found in both yogurt and pap (*ogi*) samples.

3.4. Antifungal activity of LAB against toxigenic *A. flavus*

The antifungal activity of LAB against toxigenic *A. flavus* is presented in Table 3. The results obtained showed that *A. flavus* was susceptible to half of the LAB isolated from the samples.

No fungal growth was observed on the Petri plates inoculated with *L. lactis, S. thermophilus, L. mesenteriodes, P. damnosus,* and *L. bulgaricus,* and the control plate containing sodium hypochlorite. *L. plantarum* isolated from palm wine exhibited a high zone of inhibition of 25.0 mm, followed by *L. plantarum* from yogurt with the value of 24.0 mm, and while the least zone of inhibition of 16.0 mm was recorded from Petri plated inoculated with *L. delbrueckii* isolated from yogurt (Fig. 2).

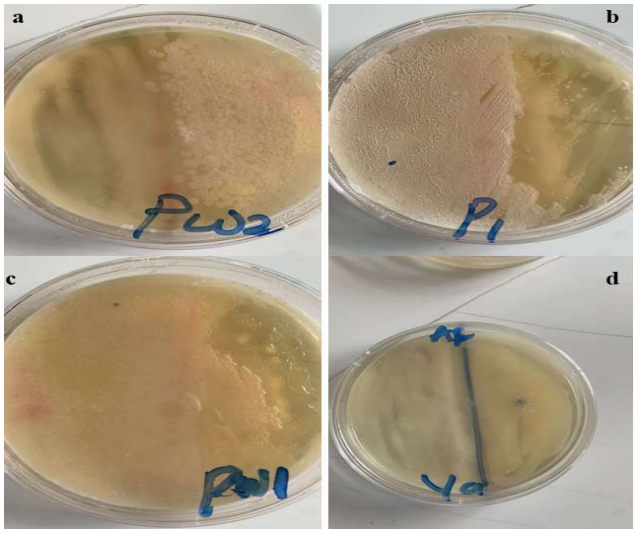

Figure 2. Zone of inhibition of LAB from palm wine (a, and c), pap (*ogi*) (b), and yogurt (d) samples against toxigenic *A. flavus*

Table 1. Morphological characteristics of LAB isolates

	Shape	Elevation	Colour	texture	Margin	Opacity	Pigmentation	Appearance	Size	Tentative organism
Y1	CC	Flat	Cream	ST	ET	OP	Non-p	glistering	Small	*Lactobacillus fermentum*
Y2	Ir	Flat	Cream			OP	Non-p	dull	MD	*Lactococcus lactis*
Yo	Ir	Pulvinate	Cream	ST	ET	OP	Non-p	dull	MD	*Lactobacillus delbrueckii*
Yc	CC	Flat	Cream	ST	ET	OP	Non-p	glistering	Small	*Lactobacillus plantarum*
Ya	CC	Flat	Cream	ST	ET	OP	Non-p	glistering	Small	*Streptococcus thermophilus*
Pw1	CC	Flat	Cream	ST	ET	OP	Non-p	glistering	Small	*Leuconostoc mesenteriodes*
Pw2	CC	Flat	Cream	ST	ET	OP	Non-p	glistering	MD	*Lactobacillus plantarum*
P1	CC	Convex	White	ST	ET	OP	Non-p	Dull	Small	*Pediococcus damnosus*
P2	CC	Flat	Cream	ST	ET	OP	Non-p	Dul	Big	*Lactobacillus bulgaricus*
P4	CC	Flat	White	ST	ET	OP	Non-p	glistering	small	*Streptococcus* spp

Key: Y1, Y2, Yo, Yc, Ya: Yogurt samples, Pw1, Pw2: Palm wine samples, P1, P2, P4: Pap samples, Non-p: non-pigmented, CC – circular, Ir – irregular, ST – smooth, ET – entire, OP – opaque, MD – moderate.

Table 2. Biochemical characteristics of lactic acid bacteria isolates

	Gram reaction	**Shape**	**Catalase**	**Citrate**	**Glucose**	**Maltose**	**Mannitol**	**Indole**	**Raffinose**	**Rhamnose**	**Ribose**	**Sucrose**	**Fructose**	**Lactose**	**H2S**	**Gas**	**Tentative organism**
Y1	+	R	-	+	+	+	-	-	+	-	+	+	+	+	+	+	*Lactobacillus fermentum*
Y2	+	R	-	-	+	+	+	-		-		+	+		-	+	*Lactococcus lactis*
Yo	+	R	-	-	+	+	-	-	-	-	+	+	+	+	-	+	*Lactobacillus delbrueckii*
Yc	+	R	-	-	+	+		-	+				+	+	-	+	*Lactobacillus plantarum*
Ya	+	C	-	+	+	+	-	+	+	+		+	-	+		+	*Streptococcus thermophilus*
Pw1	+	C	-	-	+	+			-	-		+	+	+	_		*Leuconostoc mesenteriodes*
Pw2	+	R	-	-	+	+		-	+				+	+	-	+	*Lactobacillus plantarum*
P1	+	C	-	-	+	+	-	-	-	-	-	+	+		_	+	*Pediococcus damnosus*
P2	+	R	-	Nd	+	+	-		+	-	-	-	-	-		+	*Lactobacillus bulgaricus*
P4	+	C	-	+	+	+	-	+	+	+		+	-	+		+	*Streptococcus* spp.

Key: Y1, Y2, Yo, Yc, Ya: Yogurt samples, Pw1, Pw2: Palm wine samples, P1, P2, P4: Pap samples, R – rod, C – cocci, nd – not determined

Table 3. Antifungal activity of LAB against *Aspergillus flavus*

Lactic acid bacteria	Zone of inhibition (mm)
Sodium hypochlorite (Control)	No growth
Lactobacillus fermentum	20.0±0.20[b]
Lactococcus lactis	No growth
Lactobacillus delbrueckii	16.0±0.12[a]
Lactobacillus plantarum	24.0±0.30[c]
Streptococcus thermophilus	No growth
Leuconostoc mesenteriodes	No growth
Lactobacillus plantarum	25.0±0.30[d]
Pediococcus damnosus	No growth
Lactobacillus bulgaricus	No growth
Streptococcus spp.	20.0±0.06[b]

Key: 0-10 = resistant, 11-13 = intermediate, ≥14 = susceptible. Values are expressed in mean±standard deviation. $p<0.05$ at 95% confidence interval was considered to be statistically significant

4. Discussion

The study revealed a total LAB count from notable traditional fermented foods. *Lactobacillus delbrueckii* from yogurt sample had the highest counts with the least count from *L. plantarum*. Besides LAB from the palm wine sample, *L. plantarum* had the highest counts, while *Leuconostoc mesenteriodes* had the lowest counts.

Total bacterial counts of pap (*ogi*) vary with no difference; however, *Streptococcus* spp. had the highest counts with the least counts recorded from *Pediococcus damnosus*.Among all the samples collected, *Streptococcus* spp.from pap (*ogi*) had the highest counts with a value of 15.1 cfu/ml. The variation in total bacterial loads recorded for the samples collected may be due to the differences in sugar and nutrient composition of one sample from the other and the ability of the bacterial isolates to metabolize the substrates. Abegaz (23) reported differences in LAB counts from fermented foods due to the acidification of the fermentation medium.

Lactic acid bacteria regarded as the best probiotics were granted the status of Generally Regarded As Safe (GRAS) by the Food and Drug Administration (FDA) (24), and their benefits for the gastrointestinal tract and immune system are known (25). These groups of microorganisms exist broadly in vast habitats, such as plants, dairy and dairy products, and fermented food products (17). According to the results obtained from this study, *L. mesenteroides* and *L. plantarum* isolated from palm wine samples agree with the findings of Djeni *et al.* (26), who reported 60% *Lactobacillus* and *Leuconostoc* genera as the most abundant bacteria in palm wine samples in Côte d'Ivoire. Similarly, Kouamé *et al*. (27) reported the abundance of LAB species during traditional production of *Elaeis guineensis* wine, *Raphia hookeri* wine, and *Borassus aethiopum* wine in Côte

d'Ivoire. The presence of *Leuconostoc* genus in milk corroborates the findings of Fatma and Benmechernene (28). Similarly, identifiable LAB from this study has been implicated in different palm wines across many countries. For instance, isolation of *L. paracasei, L. fermentum, L. nagelii, Leuconostoc mesenteroides, L. plantarum, S. mitis,* and *F. durionis* from *Borassus akeassii* palm wine in Burkina Faso has been documented by Ouoba *et al*. (29).

Other LAB species, such as *F. fructosus, L. delbrueckii, L. sucicola,* and *L. nagelii* isolated from Tunisian date palm sap have been reported in other studies (30,31). Furthermore, the results obtained corroborate the findings of Adamu-Governor *et al*. (32) who reported *L. plantarum* and *Leuconostoc mesenteroides* from oil palm (*Elaeis guineensis*) and raphia palm (*Raphia regalis*) sap from the South-West of Nigeria.

Previous reports also reveal that palm wine can harbor heavy microbial loads because it is rich in simple sugars, which serve as metabolic substrates for microorganisms to grow efficiently in a growth medium. Lactic acid bacteria, especially *Lactobacillus* spp. also prefer such basic sugars and predominate in fermented palm wine during fermentation. They also produce organic acids and antimicrobial substances, which inhibit the growth of most other bacteria (28).

The higher prevalence of rod-shaped LAB ascertain in this study corroborate the study by Ali *et al.* (33) who reported that the family Lactobacillaceae commonly predominates during food fermentation because they are the most aciduric all LAB.

Lactic acid bacteria were isolated from *ogi*, a natural cereal food. Traditional *ogi* fermentation has shown that besides other viable microorganisms, LAB is the most predominant microorganism inherent in most cereal-based products (34). From this study, *P. damnosus, L. bulgaricus,* and *Streptococcus* spp. were isolated from pap (*ogi*) samples. Previous authors have reported LAB species, such as *Lactobacillus* spp. and *Streptococcus* spp. from fermented *ogi* samples (34,35). Other findings have also established a diverse population of LAB and yeasts in fermented *ogi-baba* - a West African fermented sorghum gruel and *ogi* from maize (36,37).

The findings in this research indicate diverse species of LAB in yoghurt. *Lactobacillus fermentum, Lactococcus lactis, L. delbrueckii, L. plantarum,* and *Streptococcus thermophilus* were isolated from yogurt samples. Research has shown that the LAB *Lactobacillus* genera are by and large one of the most predominant species of LAB with a high occurrence rate from plant sources through fermentation (38). These bacterial isolates have been tested as probiotic candidates for their probiotic potential and applied in most food fermentation (39). The selection of desirable LAB, such as *L. helveticus, L. casei, Leuconostoc mesenteroides, L. brevis, L. delbruecki, Lactococcus lactis,* and *L. plantarum*, as starter For commercial use in traditional fermentation technology protects typical organoleptic properties of traditional drinking yoghurt (40).

Lactic acid bacteria and their metabolites play an important role in improving the microbiological quality of foods (35). Some identifiable strains of LAB involved in the fermentation process; potentially, have been explored in food preservation and the control of food-borne pathogens (25). Lactic acid bacteria produce antimicrobial substances, which are mainly in the form of organic acids, bacteriocins, and other compounds

(20). Bacteriocins have antimicrobial properties, which contribute to the antagonist potential of LAB to inhibit the growth of similar or closely related bacterial strains. Bacteriocins are typically considered narrow-spectrum antibiotics and with lesser effects on the growth of molds and yeasts (22). The results of antifungal activity of LAB isolates were similar to the findings those of Kanak and Yilmaz (41), who reported lactobacilli strains with antifungal activity with an average zone diameter of 40.4 mm for *A. flavus* (42). Additionally, Taheur *et al.* (13) confirmed the inhibitory effect of *L. plantarum*, *L. graminis,* and *P. pentosaceus* against *Aspergillus* spp. due to the secretion of antifungal substances by *L. plantarum.* Sevgi and Tsveteslava (43) reported inhibition of several toxigenic fungi by LAB. Similar results have also been observed in *Lactococcus lactis, Streptococcus thermophilus,* and *L. delbrueckii* isolated from various sources (25). A previous study by Azeem *et al.* (44) reported 20% AFB1 binding capacity of *L. casei*, which is less than that of *L. paracasei* 120 (28%), whereas *L. delbrueckii* had the maximum

Antifungal (67.43% reduction) and anti-aflatoxigenic (94.33% reduction) activity against *A. flavus*. Our findings suggested that the selected LAB with antifungal activity can be a new biocontrol agent against pathogenic fungi; specifically, the toxigenic type assayed in this study. Hence, this study can act as a preliminary step in a multistep study to investigate the anti-fungal and anti-aflatoxigenic against toxigenic *A. flavus*.

5. Conclusion

According to the results obtained from this study, different species of LAB were isolated from the three fermented samples; namely, yogurt, palm wine, and pap (*ogi*) collected from different sources. *Lactobacillus* species were reported dominant. The study also confirmed that five LAB isolates suppressed the growth of *A. flavus.* Also, it was revealed that the LAB isolates (*Lactococcus lactis, Streptococcus thermophillus, Leuconostoc mesenteriodes, Pediococcus damnosus,* and *Lactobacillus bulgaricus*) completely suppressed the growth of *A. flavus* capable of producing mycotoxins. This study suggests that isolated/selected LAB can be a new potential biocontrol agent against this toxigenic mold (*A. flavus).*

The indiscriminate use of some chemicals to preserve foods and their side effects in food products have necessitated further research in exploring LABs as biocontrol agents to inhibit the growth of pathogenic bacteria and other microorganisms in foods. The nutritional composition of foods provides excellent and rich sources of essential micronutrients for various

LABs to carry out the fermentation process for a desirable product, which enhances consumers' choice and acceptability of fermented foods. Results showed that the foods sample used in this study contain various LABs and their metabolites. The bacteriocin-producing potential of LABs with inhibitory characteristics on some pathogenic bacterial growth suggests their use as the best alternative to chemical preservatives with shelf-life longevity of most fermented foods.

Conflict of interest

The authors declared no conflict of interest.

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