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ANTIBACTERIAL SENSITIVITY PROFILE OF LACTOBACILLUS SPECIES AND CHARACTERIZATION OF AFLATOXIN PRODUCING FUNGI ISOLATED FROM YAM AND PLANTAIN FLOUR

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ABSTRACT

This study investigated the beneficial effects of fermenting microorganisms as well as the presence of aflatoxins in milled flour samples. Thermotolerance, pH tolerance, phenol tolerance, temperature sensitivity, *in-vitro* antimicrobial potentials of lactic acid bacteria (LAB) isolates and aflatoxin detection in the milled flour sample were analyzed. LAB isolates were identified and characterized as *Lactobacillus* species using standard procedures. Probiotic properties and antimicrobial activity of LAB isolates were investigated using standard methods. In addition, quantitative and qualitative detection of aflatoxins in the milled flour samples were also studied. The results showed that all the LAB isolates were *Lactobacillus* species which survived in high temperature range of 50 ^{0}C-60 ^{0}C, acidic pH (pH 2, 3, 4 and 5), phenol tolerance (0.1%, 0.2%, 0.3% and 0.4%). *In -vitro* antimicrobial properties of the LAB species (PSEO1 and PSEO4) showed that they were able to inhibit the growth of *Escherichia coli*(ATCC 43816) and *Klebsiella pneumoniae*(25922) with the corresponding zones of inhibition: 25mm and 20 mm. Due to the effect of storage time (6 months), yam and plantain flour samples moisture content yielded 9.50% and 9.20% with the corresponding total number of aflatoxins: 859.3 and 899.0. In conclusion, LAB species isolated from yam and plantain flour samples have probiotic potentials. Moreover, the longer the storage time, the higher the number of aflatoxins. Thus, they should be consumed within the shortest time possible to avoid caking and moisture accumulation which tends to decrease the number of beneficial microbes present in them.

Contribution/Originality: This study documents the inherent probiotic bacteria isolated from yam and plantain flour. These microorganisms enhance the improvement of the gastrointestinal tract. Therefore, storing these food products in a humid-free environment will prevent moisture accumulation and aflatoxin contamination. This in turn reduces the risk of food-borne intoxications.

1. INTRODUCTION

White yam (*Dioscorea rotundata*) and plantain (*Musa parasidiaca*) are good sources of carbohydrate, protein and dietary fibre (Akissoe, Joseph, Christian, & Nago, 2003; Jonathan & Olowolafe, 2001). These food products are

undoubtedly important staple foods in that they contribute substantially to the calories consumed by humans. But, due to lack of adequate storage facilities, yams tubers and plantain fruits are prone to degradation and spoilage by gradual microbial and physiological deterioration shortly after harvesting (Okigbo & Nwakammah, 2005). The need to however reduce the risk of spoilage has prompted the people in West Africa most especially Nigerians to devise a means of processing these food commodities into less perishable products such as fermented dried yam and plantain chips which are locally known in Yoruba as "elubo gbodo" and "elubo ogede" respectively (Olorunda & Adelusola, 1997). These fermented dried chips are being milled and processed into flour that can be reconstituted with hot water to make paste or dough known as 'amala' and 'kokonte' popularly consumed among the Yoruba and Ashante people of Nigeria and Ghana respectively. It has been documented from previous studies that 'elubo' consists of 12.3% moisture and 80.6% carbohydrate which makes the food a very good source of energy (Adebayo-Tayo, Onilude, Ogunjobi, Gbolagade, & Oladapo, 2006; Jonathan., Ajayi, & Omitade, 2011; Okigbo & Nwakammah, 2005). The low sodium content of plantain flour makes it suitable for consumption by hypertensive patients while its relatively high energy content makes it suitable for consumption by diabetic patients (Federal Institute of Industrial Research Oshodi Nigeria (FIIRO), 2009; Folayan & Bifarin, 2011). In addition, these food products can also be sources of probiotics that confers health benefits on humans. Most probiotics are lactic acid bacteria such as *Lactobacillis casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus infantis* (Yu, Yuan, Deng, & Yang, 2015; Zavala et al., 2016). The health benefits conferred by the presence of these probiotics include: inhibition of the proliferation of pathogenic microorganisms in the digestive tract, reduction of blood cholesterol, improvement of the immune system and reduction of the risks of colon cancer (Behnsen, Deriu, Sassone-Corsi, & Raffatellu, 2013; Gorbach, 2000; Huang, Haug, Gesemann, & Neuhauss, 2012). However, the challenge of food contamination with aflatoxigenic fungi still remains a great issue of public health concern. The presence of aflatoxins in these food commodities creates a negative impact because the use of adequate and appropriate techniques required to prevent mould growth during harvest and post-harvest practices is seldom in place, coupled with inadequate storage facilities (Adebayo-Tayo et al., 2006; Jonathan & Olowolafe, 2001). In view of this, yam and plantain flour may get contaminated with aflatoxigenic fungi such as : *Aspergillus* species, *Mucor* sp, *Rhizopus* sp and *Penicillium* sp. These organism have the ability to reduce the nutrient contents and also produce aflatoxins that may pose a serious health hazard to the consumers (Adebayo-Tayo et al., 2006; Peraica, Radić, Lucić, & Pavlović, 1999). The intake of aflatoxin contaminated food products above levels considered to be safe is harmful to humans and this is a public health challenge. Aflatoxins are potent carcinogens, mutagens and teratogens. Literature have documented that there are eighteen (18) different types of aflatoxins and the major ones have been identified as aflatoxin B1, B2, G1 and G2 (Jonathan. et al., 2011). However, the occurrence of aflatoxin B1 have been identified in yam and plantain flour, cassava and maize flour as well as garri flakes sold in local African markets with concentrations sometimes above the tolerance level (Okigbo & Nwakammah, 2005). Therefore, the aim this study is to characterize and determine the lactic acid bacteria *(Lactobacillus* species) and aflatoxin producing fungi isolated from yam and plantain flour.

2. MATERIALS AND METHODS

2.1. Sample Collection

Six months old 'elubo gbodo' (*D. rotundata*) and 'elubo ogede' (*M. parasidiaca*) were purchased in local markets situated at Osogbo, Ife, Moro and Sekona, Osun State, Nigeria. The samples were collected inside sterile polypropylene bags and transported immediately to the Microbiology laboratory for further analyses.

2.2. Preparation of Samples

The collected samples were checked by visual inspection for the presence of lumps, foreign matters such as stone and other impurities.1 g of each samples (*D. rotundata* and *M. parasidiaca*) was weighed and added into as test

tube containing 9 ml of distilled water to make a stock which was then dispensed into 9 test tubes. 1 ml of the stock was added to the first test tube containing 9 ml of distilled water and the process was followed for subsequent test tubes. 1 ml of 10^{-3} and 10^{-5} dilution of each sample were aseptically inoculated into sterile Petri dishes and 15 ml of sterilized MRS agar was poured into the plate and incubated at 42 ^{0}C for 48 hr anaerobically. Fungal isolates were grown on yeast extract sucrose agar (YES) and incubated at 28 ^{0}C for 7 days .

2.3. Characterization and Identification of Bacterial Isolates

The cultural characteristics of the isolates on the MRS agar plates were observed by checking the appearance, Gram staining and cell morphology were also examined. Pure cultures of lactic acid bacteria were sub-cultured and preserved in 1.5% (v/v) glycerol agar and kept at -80 ^{0}C.

2.4. Biochemical Characterization

The morphological and biochemical characteristics of the LAB isolates were carried out using catalase, indole, phenol tolerance and sugar fermentation tests (glucose and sucrose) while the fungal isolates were identified using the Aplexopoulos (fungi compendium) to study properties such as the elevation, surface and colour (Kurtzman, Fell, Boekhout, & Robert, 2011).

2.5. Thermotolerance Test

A colony of each catalase negative and Gram-positive isolate was suspended in 10 ml of sterile MRS broth in test tubes and heated at temperatures between the range of 50-60 ^{0}C for 1 hr. After cooling, they were incubated at 37 ^{0}C for 48 hr. MRS broth only without microorganisms serves as control tests (Guimarães, Moriel, Machado, Picheth, & Bonfim, 2006).

2.6. PH Tolerance Test

MRS broth at pH 2,3,4 and 5 were prepared by adjusting with 10N HCl and 1N NaOH. Fresh bacteria cultures were inoculated into respective MRS broth inside test tubes and incubated at 37^{0}C for 48 hr. MRS broth without the inoculation of microorganisms serves as control tests (Daniyan & Nwokwu, 2011).

2.7. Temperature Sensitivity Test

Selected bacteria cultures were grown at varying temperatures (42, 43, 44 and 45^{0}C) for 48-72 hr. 0.1 ml of inoculum was transferred to MRS plates using the pour plate method and incubated at 37^{0}C for 48 hr (Tambekar & Bhutada, 2010).

2.8 Antibacterial Activity of Isolated Lactic Acid Bacteria (LAB) Species

Antibacterial activity of isolated LAB species was determined against selected pathogens using the dual agar overlay. LAB isolates were inoculated on MRS agar plates and incubated at 42 ^{0}C for 24 hr anaerobically. The plates were then overlaid with 15 ml of nutrient agar containing the selected pathogens (10^6 cfu/ml) and incubated at 37 ^{0}C. After incubation, zones of inhibition were measured and recorded appropriately (Adeniyi, Adetoye, & Ayeni, 2015).

2.9. Isolation and Characterization of Aspergillus Species

Yam and plantain flour samples were grown on yeast extract sucrose agar (YES) and incubated at 28 °C for 7 days. The isolates were examined for aflatoxin production using the thin layer chromatography (TLC) technique . Agar plugs were prepared by cutting the fungal colony to a diameter of 5 mm. The plugs were immersed in 2 mL chloroform for extraction of toxin from fungi. The extracts (10 µL) were spotted on TLC plates for quantification.

Subsequently, 10 µL of aflatoxins solution B1and B2 which shows light and deep blue colour and G1 and G2 which shows light and deep green colour were used as reference standards and spotted along with the fungal samples extract (Jonathan. et al., 2011).

Developing Tank: The plates were developed in a solvent tank containing chloroform : toluene : acetone (75:15:10) for 1h and observe under long wave UV-visible light. Sample extracts were compared with reference standards spots.

3. RESULTS

Figure 1 is a map of Osun State showing the various collection sites of the yam and plantain flour samples. The result of biochemical characterization and sugar fermentation tests of LAB species is presented in Table 1. It was observed that the isolated lactic acid bacteria were all Gram- positive, catalase negative, indole negative, thermotolerance positive, phenol positive and can as well ferment glucose and sucrose.

Table 2 shows the result of the pH tolerance and temperature sensitivity of the isolated LAB (*Lactobacillus* species). All the LAB species were able to grow at temperature range of 42 ^{0}C, 43^{0}C, 44^{0}C and 45^{0}C, with a dense growth at pH 2, moderate growth at pH 3 and slight growth at pH 4.

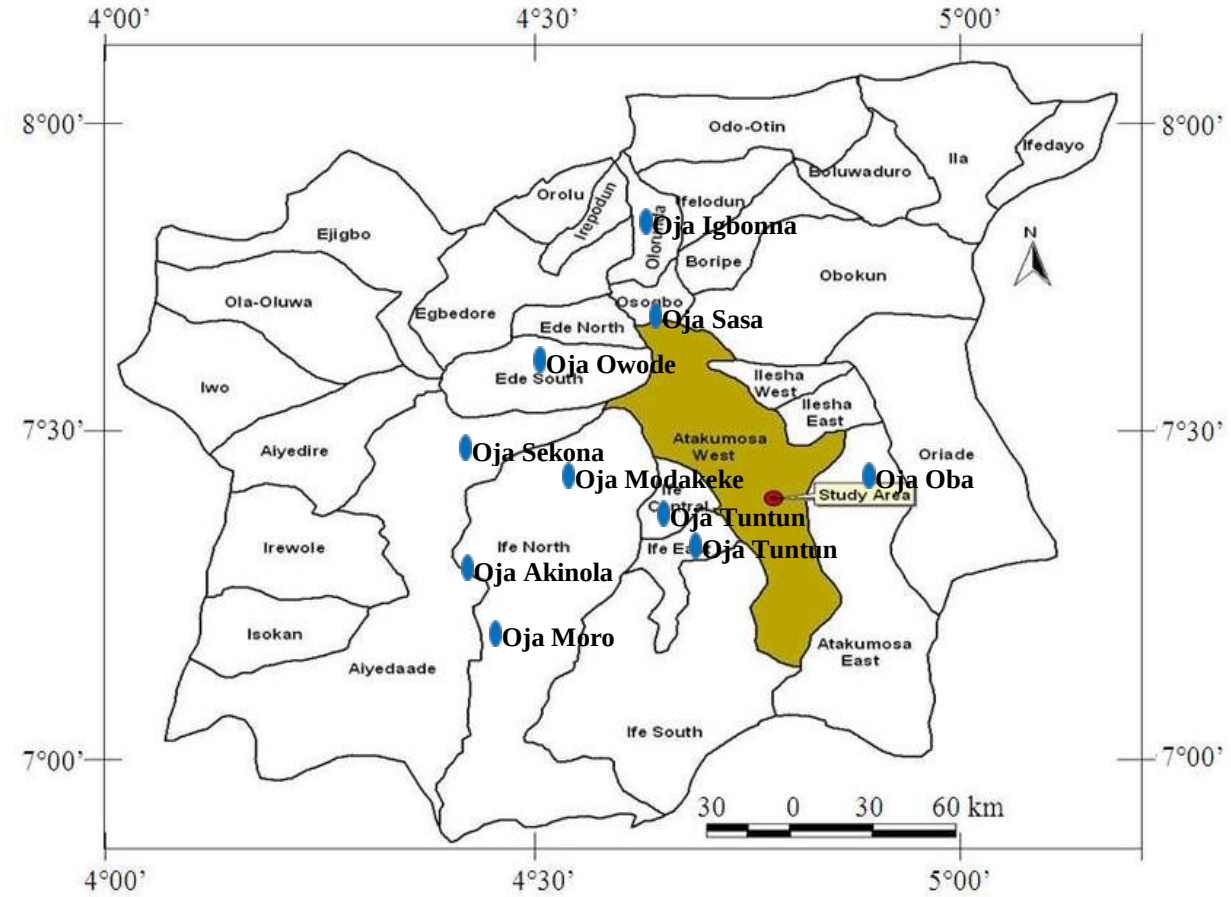

Figure-1. Osun State map showing the different yam and plantain sample collection sites marked with blue color.

Legend
- Markets locations
- Express way
- Major Road
- Minor Road
- Boundary

Table-1. Gram's reaction and biochemical characterization of LAB species isolated from six (6) months old yam and plantain flour samples.

Isolate code	Gram's reaction	Catalase	Indole	Thermotolerance	Phenol tolerance	Glucose	Sucrose	Control	Probable organism
YSG1	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG2	GPCB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG3	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG4	GPCB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG5	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG6	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG7	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG8	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG9	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
YSG10	GPCB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO1	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO2	GPCB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO3	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO4	GPCB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO5	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO6	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO7	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp
PSEO8	GPB	-	-	+	++	A^+G^+	A^+G^+	A^-G^-	*Lactobacillus* sp

Note: YSG= Yam flour sample; PSEO= Plantain flour sample; GPCB= Gram positive coccobacilli; GPB= Gram positive bacilli; ++= (Turbid, positive), A+= Positive with gas production; A-= Negative without gas production; G= Growth; G - = No growth.

The zones of inhibition produced by the isolated LAB species against test organisms using the dual agar overlay method is presented in Table 3. It was observed that PSEO2 showed the highest zone of inhibition (25 mm) against *Escherichia coli* (ATCC 43816) while PSEO1 had the least zone of inhibition (2 mm) against *Klebsiella pneumoniae* (ATCC 25922).

Graphical representation of the zones of inhibition produced by the isolated LAB species against selected test organisms is presented in Figure 2.

Table-2. pH tolerance and temperature sensitivity of isolated bacteria.

Isolate code	pH 2	pH 3	pH 4	pH 5	42 °C	43 °C	44 °C	45 °C	Control
YSG1	+++	++	+	++	+	+	+	+	-
YSG2	+++	++	+	++	+	+	+	+	-
YSG3	+++	++	+	++	+	+	+	+	-
YSG4	+++	++	+	++	+	+	+	+	-
YSG5	+++	++	+	++	+	+	+	+	-
PSEO1	+++	++	+	++	+	+	+	+	-
PSEO2	+++	++	+	++	+	+	+	+	-
PSEO3	+++	++	+	++	+	+	+	+	-
PSEO4	+++	++	+	++	+	+	+	+	-
PSEO5	+++	++	+	++	+	+	+	+	-

Note: +++= Dense growth; ++= Moderate growth; += Slight growth - = No growth ; += Growth; YSG= Yam flour samples; PSEO= Plantain flour samples.

Table-3. Zones of inhibition (mm) produced by isolated LAB species against selected test organisms.

Isolate code	*E.coli* (ATCC 43816)	*K. Pneumoniae* (ATCC 25922)
YSG1	23	17
YSG2	18	13
YSG3	12	18
YSG4	17	12
YSG5	22	19
YSG6	14	12
YSG7	15	11
YSG8	19	8
YSG9	13	9
YSG10	11	6
PSEO1	7	2
PSEO2	25	10
PSEO3	16	11
PSEO4	19	20
PSEO5	15	8

Note: YSG= Yam flour samples; PSEO= Plantain flour samples.

Table 4 shows the aflatoxin producing fungi present in yam and plantain flour samples. The moisture content of yam and plantain flour samples was recorded to be 9.50% and 9.20% respectively. *Aspergillus flavus* was the most predominant aflatoxin producing fungi isolated from yam and plantain flour samples with the corresponding values: 8.5×10^2 cfu/g and 8.9×10^2 cfu/g respectively while A. tamari was the least aflatoxin.

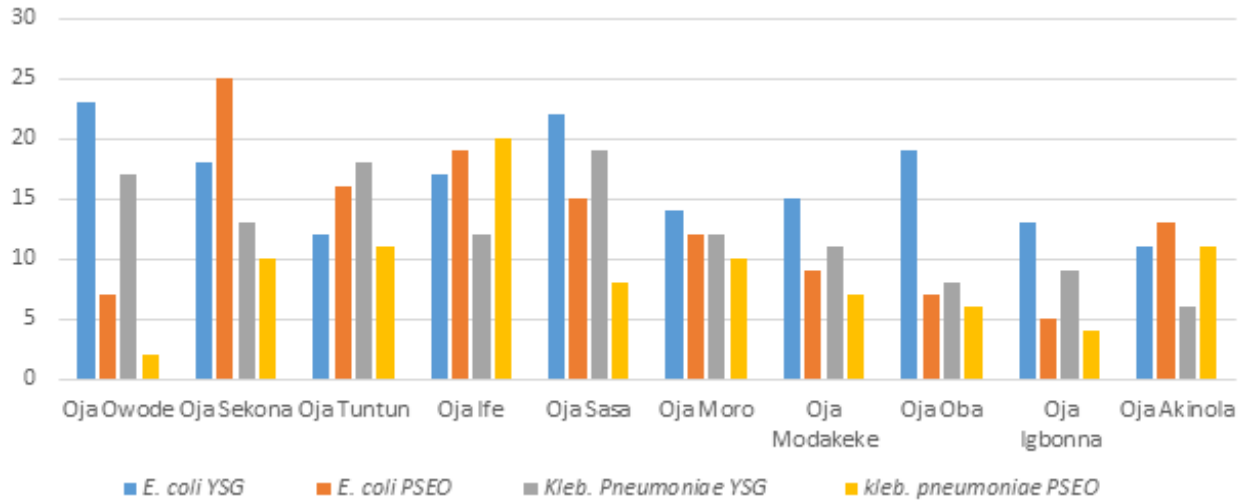

Figure-2. Zones of inhibition shown by lactobacillus species isolated from yam and plantain flour samples against test organisms (*Escherichia coli* ATCC 42816 and *Klebsiella pneumoniae* ATCC 2592).
Note: *E.coli* YSG= *E.coli* isolated from yam flour samples; *E.coli* PSEO= *E. coli* isolated from plantain flour samples; *K. pneumoniae* YSG= *K. pneumoniae* isolated from yam flour sample; *K. pneumoniae* PSEO= *K. pneumoniae* isolated from plantain flour samples

Table-4. Micro-fungi count of aflatoxins present in yam and plantain flour samples using Thin layer chromatography technique.

	Moisture Content	*A. flavus*	*A.niger*	*A. tamari*	*A. clavatus*	*A. japonicum*	*A. fumigatus*	*A. ochraceus*	*A. parasiticus*	*A. tereus*
Yam	9.50	$8.5\text{x}10^2$ **B1**	1.2 **B2**	1.0 **G1**	1.5 **G2**	1.2 **B1**	1.0 **B2**	1.0 **G1**	1.2 **G2**	1.2 **B2**
Plantain	9.20	$8.9\text{x}10^2$ **B1**	1.0 **B2**	1.0 **G1**	1.4 **G2**	1.2 **B1**	1.1 **B2**	1.0 **G1**	1.1 **G2**	1.2 **B2**

Note: A= *Aspergillus*; AFB1= Aflatoxin B1; AFB2= Aflatoxin B2; AFG1= Aflatoxin G1; AFG2= Aflatoxin G2).
Total number of AFs present in yam flour = 859.3
Total number of AFs present in plantain flour = 899.0

4. DISCUSSION

This study was designed to investigate the physicochemical parameters (pH, thermotolerance, phenol tolerance and temperature sensitivity), inherent microorganisms and aflatoxin detection present in yam and plantain flour samples. Lactic acid bacteria (*Lactobacillus* species) were isolated from the two flour samples. They were all Gram positive, catalase and indole negative, and were also able to ferment glucose and sucrose sugars. Earlier reports have documented the isolation of lactic acid bacteria from non-dairy food products like cassava flasks, yam and plantain flour and from dairy foods like yogurt, cheese and milk (Adolfsson, Meydani, & Russell, 2004; Fossi & Ndjouenkeu, 2017). *Lactobacillus* species from fermented products are important sources of probiotic microorganisms that helps to improve the microbiota of the gastrointestinal gut of humans and animals. The potential probiotic bacteria (LAB) species isolated from the two samples used in this study were able to tolerate different heating temperatures within the range of 42^{0}C to 60^{0}C which corresponds to the temperatures for processing different industrial dairy and non dairy products such as cassava flasks, cheese and yogurt. This observation support the claims of Fossi and Ndjouenkeu (2017). Thermotolerance is a vital industrial property as most of the probiotic products such as cheese and milk are processed at temperatures above 40 ^{0}C. Therefore, Yang et al. (2017) documented that the viability of probiotic bacteria at an elevated temperature is essential to make the probiotic product effective after consumption. Shokryazdan et al. (2014) and Azat et al. (2016) have reported that the optimal pH for probiotic organism survival is pH 2.0. All strains isolated from the samples were tested in acidic conditions at pH 2.0-5.0 which confirms the LAB species to be acid-tolerant. Hoque et al. (2010) and Pundir, Kashyap, and Kaur (2013) have also reported that *Lactobacillus* species isolated from fresh vegetables, fruits and curds survived in pH 2.0 to5.0 pH tolerance is a vital criterion to be considered for the growth and beneficial effects of probiotic microorganisms in the gastrointestinal tract. From this study, the LAB isolates only survived at low phenol concentrations (0.1% and 0.2%) while 5% of the isolates survived in 0.3% to 0.4% phenol concentrations. This is in conformity with the previous reports of Hoque et al. (2010) and Sultana, Refaya, R., and Kohinur (2017) that all the LAB isolates were able to ferment glucose and sucrose sugars with the production of gas. Klaenhammer and De Vos (2011) have previously documented that probitoic bacteria must be capable of fermenting different sugars, yielding lactic acid as their end product.

To further investigate the functional characteristics of the LAB isolates, in-vitro antimicrobial potential of the isolates were investigated using the dual agar overlay method. LAB produces antimicrobial compounds such as lactic acid, acetic acid, diacetyl, fatty acids, aldehyde bacteriocins and carbon dioxide among others. Ponce, Moreira, Del Valle, and Roura (2008) and Maragkoudakis et al. (2009) have documented that organic acids synthesized by LAB leads to a reduction in pH and an increase in hydrogen peroxide production which can serve as antagonistic substances. Furthermore, one of Food and Agriculture Organization/ World Health Organization (2002) criteria for selecting a probiotic organism is its ability to show antimicrobial activity against pathogenic test organisms. PSEO2 and PSEO4 showed significant antagonistic activity against *Escherichia coli* (ATCC 43816) and *Klebsiella pneumoniae* (ATCC 25922) with the corresponding zones of inhibition 25 mm and 20 mm respectively. Mohammed-lawal and Balogun (2010) documented that *L. acidophilus* isolated from a yogurt stock culture showed significant antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus* and *S. epidermidis* that causes wound infections. In addition, Turchi et al. (2013); Shokryazdan et al. (2014) and Azat et al. (2016) had earlier reported that LAB isolates from human intestine showed antimicrobial activity against a wide range of Gram-positive and Gram-negative pathogens both *in-vivo* and *in-vitro*.

A total number of 9 aflatoxigenic fungal species were isolated from the six months stored yam and plantain flour samples. The isolated fungi were *Aspergillus flavus*, *A. niger*, *A.tamari*, *A. clavatus*, *A, clavatus*, *A. fumigatus*, *A. ochraceus*, *A. parasiticus* and *A. tereus*. The presence of aflatoxigenic fungi and aflatoxins in the yam and plantain flour samples were investigated. *Aspergillus flavus* was the most predominant fungal species isolated from the yam and plantain flour sample giving a yield of 8.5×10^2 and 8.9×10^2 cfu/g respectively. Therefore, the predominance of

Aspergillus species in these samples could be responsible for the high level of aflatoxins detected in them. This is in support of the reports of Jonathan. et al. (2011). Aflatoxins B1, B2, G1 and G2 were detected in the two flour samples. Bankole, Ogunsanwo, and Mabekoje (2004) had earlier documented that The Federal Institute of Industrial Research (FIIRO) acclaimed that the levels of aflatoxins (AFB_1) residues permitted in Nigerian food products by should not exceed 20 µg/kg. The maximum level of aflatoxin B1 concentration in human food consumption should range between 5 to 50 ppb. The moisture content of the two samples were 9.50% and 9.20% respectively which is similar to the report of Nageh, Ahmed, Usama, Abdel-Rahim, and Abdallah (2016) that recorded a moisture content of 9.31% to 12.4% in wheat samples. Further more, Adebayo-Tayo et al. (2006) reported that moisture content is required for the growth of aflatoxins which supports the results obtained from this work. Therefore, flour quality evaluation based on qualitative and quantitative detection of aflatoxin contamination is very necessary for the consumption of safe food products.

5. CONCLUSION

From the results and observations obtained from this study, it can be concluded that yam and plantain flour are good source of energy giving diets with inherent probiotic bacteria that enhances the improvement of the gastrointestinal tract. However, these food products can also become vehicles of food -borne infections because the longer the storage period, the higher the level of moisture accumulation and aflatoxin concentrations. Proper storage in a humid-free environment is therefore recommended for the safe keeping of these food products.

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