Introduction

Acha (Digitaria exilis & Digitaria iburua), also known as fundi, hungry rice and Asian millet, is an underutilized and nutritious cereal mainly consumed as a whole grain, porridge and couscous, ground or mixed with other flours to make pastries by Africans and Asians (Chukwu and Abdul-Kadir, 2008). Acha is high in protein, leucine, methionine and valine than other cereals (wheat, maize, millet, sorghum, barley, oat, etc.) (Princewill-Ogbonna, 2015).

Fermentation is the oldest known form of food biotechnology which has been practiced for thousands of years by ancient men as an important mechanism for imparting longevity to food and beverages (Borgstrom, 1968). It is a process whereby useful products are synthesized either by a single organism or by the combination of two microorganisms (Wood, 1997). The role of microorganisms in fermentation cannot be overemphasized. More importantly, the most relevant group of microorganisms is the Lactic Acid Bacteria (LAB) (Ritcher and Vadamuthu, 2001), which are capable of producing acid and some other bioactive and antimicrobial compounds.

LAB is gram positive, non-spore forming, catalase-negative, devoid of cytochromes, anaerobic but aero-tolerant, fastidious, acid tolerant and strictly involved in the process of fermentation (Khalid, 2011). They have been used in food fermentation and their proteolytic activity is of great importance for the production of flavor compounds in a product (Moulay et al., 2013). The weight of LAB in the food is usually very small, but their effect on the nature of the food, especially in terms of flavor and other organoleptic properties is profound (Okafor, 2009).

Over the years, it has been discovered that poor handling and preparation of cereals have significantly increased the contamination of these foods by pathogenic microorganisms. This study therefore aimed at isolating LAB with probiotic properties useful in the fermentation of cereals to reduce incidence of pathogenic microorganisms as well as improving the overall quality of fermented foods.
2. Materials and Methods

2.1. Sample Collection

Freshly harvested and unprocessed white Acha (Digitaria exilis) was purchased at Dawaki market, Kanke Local Government Area of Plateau state, Nigeria and was transferred to the Postgraduate laboratory of the Department of Microbiology, University of Ibadan, and was stored in air tight containers at 40°C until required for further use.

2.2. Sample Preparation

Acha grains were sorted manually to remove unwanted materials. They were afterwards washed thoroughly in distilled water, while floating grains were removed via decantation and the cleaned, sorted grains were steeped in distilled water in a 1:3 w/v (Ikujenlola, 2014) for 8-9 hours and subjected to germination using the modified method of Ariahu et al. (1999). Steeping, germination and malting of the grains were carried out using the methods of Genath et al. (2011) and Ikujenlola (2014).

2.3. Isolation of Lactic Acid Bacteria

A ten-fold serial dilution of the fermenting slurry was performed by introducing 1 mL into 9 mL of sterile distilled water. Thereafter, using the standard pour plate technique, 0.5 mL of dilutions 10⁻⁴ and 10⁻⁵ were inoculated into sterile petri dishes prior to the pouring of cooled, molten De Mann Rogosa and Sharpe (MRS) agar. The plates were incubated anaerobically for 48 hours at 35±2°C. Observed LAB isolates were sub-cultured via subsequent streaking unto fresh MRS agar until pure cultures of LAB were obtained, which were then preserved on MRS agar slants, at 30±2°C until growth became visible, and were kept in a refrigerator at 4°C until needed.

3. Selection of Probiotic Lab Isolates

3.1. Growth at Different Temperatures

Overnight grown active cultures were inoculated at 1% in MRS broth tubes and incubated up to 3 days at 15°C, 30°C and 45°C. Extent of growth was visually recorded based on intensity of turbidity (Pooja et al., 2015).

3.2. Growth at Different pH Concentrations

Twenty-four hours old cultures of LAB isolates adjusted to 0.5 MacFarland’s standard were inoculated into MRS broth adjusted to pH values of 1, 2, 3, 4, 5 and 6 using 0.5N Sodium hydroxide and 0.5N hydrochloric acid to create an alkaline and acidic environment. The broths were incubated at 35±2°C for 24 hours. Tolerance of the isolates was determined by spectrometric observation at 560nm optical density (OD) against the uninoculated broth as control (Pooja et al., 2015).

3.3. Sodium Chloride (NaCl) Tolerance

MRS broth was adjusted to different concentration (4, 6, 8 and 10% (w/v)) of NaCl. After sterilization, one colony of fresh overnight culture of LAB was inoculated into sterile MRS/NaCl broth and incubated at 35°C. The growth was evaluated by pour plate method on MRS agar and growth was monitored for 24 hours (Pannapa and Pattra, 2015).

3.4. Tolerance to Bile Salt

One milliliter (1mL) cell suspension of 24 hours old culture of each isolate adjusted to 0.5 Mac Farland’s standard was added into 20 mL of freshly prepared sterile MRS media containing 0.05%, 0.1%, 0.3% 0.5% and 1% of No.3 bile salts (Oxoid, England). The broths were incubated for 6 hours and observed for turbidity spectrometrically at 620nm. Cell viability was determined by serial dilution and plating onto sterile MRS agar after 6 hours of incubation (Neethu et al., 2015). Broth without bile salt served as control.

3.5. Antimicrobial Activity against Selected Pathogens

The agar well diffusion method was used for detecting antimicrobial activity against pathogens such as Escherichia coli, Bacillus cereus, Klebsiella pneumonia, Staphylococcus aureus, Listeria monocytogenes and Salmonella typhi. These pathogens were collected from the Department of Microbiology, University of Ibadan, Nigeria. The selected LAB isolates were grown in sterile broth for 48 hours at 35 ± 2°C while the pathogens were grown on sterilized nutrient agar plates for 24 hours. Cell free supernatant of the LAB isolates was obtained by centrifuging the broth grown isolate in a cold centrifuge (4°C) at 5000rpm for 15 minutes. Inoculum of the pathogens was suspended in normal saline and was adjusted to 0.5 Mac Farland’s Standard. Sterile swab stick was used to transfer the adjusted pathogens to sterile solidified Mueller Hinton agar plates and a sterile cork borer of 5mm in diameter was used to bore wells on the Mueller Hinton agar plates containing the pathogen. The cell free supernatant of the LAB isolates was dispensed into each well and incubated aerobically at 37°C for 24 hours. Zones of inhibition around each well indicated the antimicrobial activity of the lactic acid bacteria against the particular pathogen (Qing et al., 2015).

3.6. Cell Surface Hydrophobicity

Cell surface hydrophobicity was carried out using a modified method of Rosenberg et al. (1980), using three hydrocarbons namely xylene, chloroform and benzene. The isolates were cultured in MRS broth at 37°C for 24 to 48 hours. Isolates were harvested at 4000 rpm for 15 minutes and washed twice in PBS (phosphate buffer saline, pH 7.0) and re-
suspended in PBS and the Optical Density (OD) determined at 600 nm. Thereafter, 3 mL of the bacterial suspension was mixed with 1 mL of the hydrocarbons and vortexed (using “mrc” VORTEX MIXER) at speed 10 for 120 seconds. It was then incubated at 37°C for 30 minutes for the separation of the aqueous and organic phase. 1mL of the lower aqueous phase was removed carefully with a syringe and the optical density (OD at 620 nm) was determined. The hydrophobicity index (HPBI) was calculated as the decrease in optical density of the initial bacterial suspension due to cell partitioning into a hydrocarbon layer as the following equation:

\[
\text{HPBI} = \frac{A1 - A2}{A1} \times 100
\]

Where A1 = Optical density before extraction with hydrocarbons
A2 = Optical density after extraction with hydrocarbons

3.7. Tolerance to Gastric Acidity

The test for tolerance to gastric acidity was carried out using the method described by Lucía (Abadia-Garcia et al., 2013) with some modifications. Simulated gastric juice was prepared by suspending pepsin (3gL⁻¹) in sterile saline 0.5% w/v and adjusted to pH 1.5, 2.5 and 3.5. It was sterilized using a membrane filter (0.22μm). The imitative intestinal juice was prepared by using the method of Chavarri et al. (2010). The isolates grown for 18 to 24 hours were harvested by centrifugation at 4000 rpm for 10 mins. The cells were washed twice in PBS and the total viable count of washed cells was determined by plating 0.1mL of cell suspension before assay for transit tolerance. 0.2 mL of the washed cells was mixed with 1 mL of pepsin and 0.3mL of NaCl. The content was vortexed using mrc Vortex mixer at speed 10 for 10 seconds and incubated at 37°C for 3 hours. During the assay 0.1 mL of the aliquot was withdrawn at 1 minute, 90 minutes and 180 minutes and plated on MRS agar, incubated at 37°C for 24-48 hours for viable count determination.

4. Safety Assessment

4.1. Hemolytic Activity

Hemolysis activity was investigated as described by Gerhardt et al. (1981) with some modification. 3μl of a 6 h old culture broth was spot inoculated into sterile blood agar. The blood agar was prepared by adding 7% sheep-blood (freshly collected and preserved in EDTA), into Trypticase Soy Agar. Plates were incubated anaerobically at 37°C for 48 h after which they were observed for clear zones surrounding colonies (positive reaction for beta hemolysis). A strain of S. aureus was used as positive control (Pooja et al., 2015).

4.2. DNase Test

DNase agar medium (Oxoid) was used to check production of DNase enzyme and was streaked with the LAB isolates, followed by plate incubation at 30°C for 48 hours. When clear pinkish zone was noticed around the colonies after incubation, it was read as a positive result for DNase production (Gupta and Malik, 2007).

4.3. Gelatinase Activity

Gelatinase activity of the isolates was investigated as described by Harrigan and McCance (1990) with slight modification. Twenty-four hours old culture was spot inoculated into sterile nutrient gelatin agar (Oxoid, England). The plates were incubated anaerobically for 48 h at 37°C after which they were flooded with saturated ammonium sulfate solution and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis). A strain of Staphylococcus aureus was used as positive control.

5. Identification of Isolates

LAB isolates with the best desirable probiotic potential were identified using basic morphological, biochemical and sugar fermentation properties (Olutiola et al., 1991).

6. Results and Discussion

Of all the 47 bacteria isolated during the spontaneous fermentation of the Acha samples on MRS agar, 36 (76.59%) were Gram-positive, catalase negative, non-motile, non-sporulating and oxidase negative and therefore considered to belong to the bacterial group Lactic Acid Bacteria (LAB) (Wood and Holzapfel, 1995). Similarly, Wakil and Olorode (2018) isolated 40 LAB isolates from fermenting Acha. This observation may attest to previous reports that LAB isolates are the major group of bacteria responsible for the fermentation of cereals and are isolated throughout the process (Adegbeye et al., 2017).

A vast majority of the LAB isolates were able to grow at the varying temperature ranges (Table 1) and thus used for further probiotic screening. This attribute is essential for probiotic bacteria because humans are warm-blooded and as such when these LAB are ingested; they must be able to survive the high body temperature of humans as well as that of the gastro intestinal tract (GIT).

The ability of a probiotic to colonize and contribute to the balance of the microflora of the GIT is usually hinged on their ability to tolerate some levels of bile salt, a substance usually encountered in the GIT. Eighty two percent (82%) of the isolates from this study tolerated bile concentrations within 0.05-1.0% (Table 2). Meanwhile, the average concentration of bile salt is a healthy human is 0.3% (Dunne et al., 2001). The growth of LAB in high bile concentrations is in corroboration with several reports from different authors (Santos et al., 2016; Freire et al., 2017; Wakil and Olorode, 2018); In addition, Giri et al. (2018) reported that Lactobacillus plantarum L7 used as a starter for preparation of a fermented cereal-based beverage tolerated up to 0.3% of bile.
Growth at low pH is a major attribute when considering a microbe as a probiotic. It was observed that the growth of the LAB isolates was in direct relationship with increasing pH, with all growing profusely at pH 3 and 4 (Figure 1), thereby showing that the isolates could tolerate this pH. Similar observations have been reported by several authors (Hawaz, 2014; Thakkar et al., 2015; Wakil and Olorode, 2018) for LAB isolated from different fermenting gruel. This attribute indicates that should these isolates be consumed, they can withstand the low pH encountered through the digestive tract, that is, from the mouth through the small intestine. This ability to survive at low pH may translate to the probiotics being capable of colonizing the GIT and thus displace the unwanted enteropathogens.

Table 3 shows the antimicrobial activity of the LAB isolates against Bacillus cereus, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi, Listeria monocytogenes and Escherichia coli. It was observed that majority of the isolates significantly inhibited the growth of the pathogens; with S. aureus being the least (37.5%) inhibited while 66.7% of the isolates inhibited the growth of K. pneumoniae. This antagonistic activity can be attributed to the production of several inhibitory substances such as acid, peptides, bacteriocins, alcohol, etc., by the isolates and according to the study by Giri et al. (2018), 55.5% of 9 LAB isolates showed significant antimicrobial activity when tested against same genus of bacteria as used in this study.

According to a report by Pundir et al. (2013), the threshold point of NaCl tolerance for LAB is within 1-6%, this is because at these levels of NaCl, many pathogenic or bacterial cells will plasmolysze due to high solute concentrations in the growing medium. The LAB isolates from this study tolerated NaCl concentrations within 4-6% (Table 4), this is a similar result to what was obtained by Adebayo-Tayo and Onilude (2008). The ability of LAB to tolerate high NaCl concentrations is essential as it is mostly used as an inhibitory substance in fermented foods to enhance preservation, as such LAB must tolerate it.

The isolates showed varying degree of hydrophobicity to the different hydrocarbons with a range 3.4 to 32.4%. Meanwhile, the isolates showed the highest hydrophobicity to xylene with least to benzene (Table 4). Similarly, Giri et al. (2018) reported significant differences in the degree of cell surface hydrophobicity by LAB isolated from a rice-based fermented beverage in Seoul, South Korea. The cell hydrophobicity potential of these isolates will ensure that they adhere efficiently to the epithelial cells of the GIT and as such exert their probiotic effects.

The gastric juice present in the small intestine is very acidic with a pH of 2.5, thus for any isolate to serve its probiotic potential, it must survive this adverse condition and thus establish itself to inhibit unwanted enteropathogens. Just as Agaliya and Jeevaratnam (2012), it was observed that LAB isolates showed reduction in growth during the first 90 minutes after which there was significant increase in the colony counts after 180 minutes (Table 6), thus translating to these isolates capable of surviving the 90 minutes lap to get the food from the mouth to the stomach.

All of the seven LAB isolates subjected to safety assessment tests such as DNase, gelatinase and haemolytic activity, 5 (71.42%) were negative, leaving just LAB 120 and LAB 132 that were positive for gelatinase and haemolytic activity respectively (Table 7). The inability of the isolates to produce DNase and gelatinase has been reported as a desirable attribute for microorganisms to be used as probiotics (Kalui et al., 2010). Similar observations of lack of haemolytic and gelatinase activity were reported in Lactobacillus spp. isolated from ikki (Kalui et al., 2009). Should an isolate produce gelatinase and ingested, there is a risk of the mucoid lining becoming deranged, a condition that could lead to the development of autoimmune diseases and exposure to infections.

All the five isolates belonged to the genus Lactobacillus. Their identities are shown in table 8. Probiotic properties of LAB isolate especially from the genus Lactobacillus has been reported by several authors (Kalui et al., 2009; Ojokoh and Onasanya, 2017; Wakil and Olorode, 2018) and their use in formulation of complementary foods.

### 7. Conclusion

This study has shown that fermentation of cereals, e.g. Acha is a reliable source of LAB. These LAB isolated showed great probiotic potential and thus can be used as starters in the formulation of safe and quality weaning foods.

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