Production of Fermented Weaning Food from *Digitaria exilis* (Acha) using Lactic Acid Bacteria

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Abstract: Several different methods have been used to formulate weaning food through blending of different cereal crops that are locally available. Considering the good prospects of the ready availability of *Acha* cereals, fermentation of malted *Digitaria exilis* (*Acha*) flour using single and combination of lactic acid bacteria to produce weaning food was carried out. One hundred grams of acha grains were steeped in 300ml distilled water in the ratio of (1:3 w/v) for 48 h at 30±2 ^{o}C. Malting was done for 48 h which was followed by oven-drying of malted grains to terminate germination. The dried acha grains were dry milled. Reconstituted malted acha flour was at 30% (w/v) and was allowed to ferment spontaneously for 72 h and sampled every 24 h for the analysis of microbial load and the physiochemical parameters. The highest lactic acid bacteria count ($6.45x10^{14}$cfu/ml) on MRS agar was recorded at 48 h and the least count ($3.72x10^{14}$cfu/ml) at 24 h of fermentation time respectively. The highest aerobic bacteria count ($1.0x10^{14}$cfu/ml) was recorded at 24 h and the least count ($1.0x10^{10}$ cfu/ml) at 72 h of fermentation time. Highest yeast count ($2.15x10^{12}$cfu/ml) was recorded at 48 h and mould by 24 h of fermentation time. The pH of the fermented malted *Acha* slurry decreased drastically while the percentage titratable acidity increased with increase in fermentation time. A total of 11 lactic acid bacteria were isolated and identified based on their morphological, physiological and biochemical characteristics as *Lactobacillus* sp., *Leuconostoc* sp. and *Pediococcus* sp. Isolated lactic acid bacteria (LAB) were screened for probiotic potentials and best two strains *Lactobacillus sp.* (JLAB10) and *Leuconostoc sp.* (KLAB11) were used as starter culture both singly and in combination for the controlled submerged anaerobic fermentation of acha flour. Results of the safety assessment tests revealed that the product is safe for consumption. The study revealed that the production of weaning food from malted *Digitaria exilis* (Acha) as raw material using Lactic Acid Bacteria is possible.
Keywords: Fermentation, Weaning, Acha, Lactic acid bacteria.

INTRODUCTION

Cereals are annual grass members of the family Gramineae. They possess long and thin stalks and include crops such as wheat, rice, maize, sorghum, millet, barley and rye, whose starchy grains are used as food (Sarwar *et al*., 2013). Cereals are highly nutritious and provide food for humans and livestock. They also find application in industries for the production of a wide range of substances such as glucose, adhesives, and alcohols (Mohammed *et al*., 2017; Sarwar *et al*., 2013).

Acha (Digitaria exilis), commonly referred to as hungary rice is a cereal which is cultivated in Nigeria and some West African countries like Sierra Leone, Ghana, Togo and Mali (Gyang and Wuyep, 2005). The crop grows well in a wide variety of soils and mature very quickly compared to other grains. Acha is very nutritious as it is rich in methionine and cysteine. Acha can be ground and mixed with other flours to make bread, pastries and can be used as weaning food (Philip and Itodo, 2006). Fermented foods are those foods which have been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification to the food (Halder *et al*., 2017). This definition means that processes involving ethanol production by yeasts or organic acids by lactic acid bacteria are considered as fermentations. Hence foods submitted to the influence of lactic acid producing microorganisms are considered as fermented foods (Wedajo, 2015).

Fermentation causes changes in the nutritional content of foods: vitamin and amino acid levels may increase, decrease or remain static, depending on the type of microorganism used and the product fermented.

Microorganisms also impart desirable flavours, improve texture and enhance digestibility of foods (Mohammed *et al*., 2017). Fermentation destroys food spoilage organisms and permits preservation of food (Kohajdova and Karovicova, 2007). Lactobacilli in cultured milks are used to supplement the normal intestinal flora in individuals suffering from digestive ailments or enteric diseases (Wedajo, 2015).

Fermentation products in food substrates are based on the microorganisms involved in the fermentation. The indigenous natural fermentation takes place in a mixed colony of microorganisms such as moulds, bacteria and yeasts (Kohajdova and Karovicova, 2007). The bacteria are not harmful to the consumer and have enzymes such as proteases, amylases and lipases that hydrolyze food complexes into simple nontoxic products with desirable textures and aroma that makes them palatable for consumption (Chelule *et al.,* 2010). The fermenting organisms include LAB such as, *Leuconostoc, Streptococcus, Lactobacillus, Enterococcus, Aerococcus* and *Pediococcus* sp. The yeasts isolated are mainly of the species, *Saccharomyces, Candida, Kluyeromyces and Debaryomyces* (Hasan *et al*., 2014). Moulds have been used mainly in milk and cheese fermentation and include *Penicillium, Mucor, Geotrichium,* and *Rhizopus* sp. (Kohajdova and Karovicova, 2007). Some of the microorganisms isolated from fermented food are, however, yet to be identified. In all the foods and beverages examined, LAB is the dominant microorganisms, and therefore, lactic acid fermentation is considered as the major contributor to the beneficial characteristics observed in fermented foods (Holzapfel, 2002). Lactic acid bacteria fermentation is a common way of preparing food traditionally in Africa, as it confers preservative and detoxifying effects on food (Gemechu, 2015). When used regularly, lactic acid bacteria fermented foods boost the immune system and strengthens the body in the fight against pathogenic bacterial infections. Thus, lactic acid bacteria fermentation is not only of a major economic importance, but it also promotes human health in Africa (Chelule, 2010).

In Nigeria and other developing countries, the problem of malnutrition has created the need for urgent research into the development of high nutrient density complementary foods fortified with probiotics from locally available crops as a means of reducing the level of child mortality. Thus, this study focused on the use of Lactic acid bacteria in fermenting malted acha to produce nutritious wholesome weaning food.

MATERIALS AND METHODS

Samples Collection and Preparation

The grains of acha (*Digitaria exilis*) were obtained from Jos Market, Plateau State, Nigeria. The acha cereal grains were stored in a sterile polythene bags and then transported for 14 hr to the Microbiology Unit, International Institute for Tropical Agriculture (IITA), Ibadan for analysis. Acha grains were manually sorted and winnowed so that flawed seeds and stones were removed. One hundred grams (100g) of acha grains were steeped in 300ml distilled water in the ratio of (1:3 w/v) for 48 h at 30±2 ^{o}C on the laboratory bench and sieved with wire mesh filter. Malting/germination of steeped grains were done for 48 h on a sterile stainless steel tray covered with a sterile muslin cloth. The grains were moistened twice daily (mornings and evenings) for 48 h and oven-drying of malted grains to terminate germination was done at 60^{o}C for 20 h using a standard automated oven. Dry milling of malted samples was done using sterile blender and reconstituted with distilled water at a concentration of 1:3 (w/v) and allowed to ferment spontaneously at 30 ± 2 ^{o}C for 72 h. At regular intervals during fermentation (0, 24, 48, 72 h), samples were taken to determine the microbiological load/quality and the physiochemical parameters.

MICROBIOLOGICAL ANALYSIS OF SAMPLES

Nine mililitres (9 ml) of distilled water was dispensed into a 25 ml McCartney bottle and autoclaved at 121^{o}C for 15 min and allowed to cool. One millilitre of the fermenting slurry was introduced into the sterile distilled water, mixed together and serially diluted. An aliquot of 0.1 ml of higher dilution (10^{-6}) and 1 ml of lower dilution (10^{-3}) was pour-plated out onto the appropriate media. The various media such as DeMann Rogosa Sharpe (MRS) agar, Nutrient agar (NA), MacConkey agar, Malt extract agar and Plate count agar were prepared according to manufacturer's instruction and sterilized for 15 min at 121^{o}C. The plates were incubated and observed for growth of distinct colonies which were counted. Lactic Acid Bacteria were randomly selected and sub-cultured on solidified agar plate. Streaking of single colony was performed until pure cultures of LAB isolates were obtained.

Chemical Analysis of Samples

The pH of the fermenting gruel was determined using the glass electrode of the pH meter (Jenway 3520) and the percentage of total titratable acidity was calculated using titration method as described by (AOAC, 2016).

Characterization and Identification of Isolates

The cultural features of each of the LAB isolates such as appearance, shape, elevation and colour were noted after proper examination of the isolates.

Gram staining and biochemical tests were performed using standard methods as described by (Islam et al., 2016) and include catalase test, oxidase test, indole test, starch hydrolysis, production of ammonia from arginine test and sugar fermentation tests.

The physiological characterization tests were carried out as described by (Islam et al., 2016). The tests include Growth of isolates at different temperatures, growth at different pH and growth of isolates at different NaCl concentration.

Probiotic Screening Tests on Isolated Lactic Acid Bacteria

Each of the Lactic acid bacteria isolates was initially examined for colonial, cell morphologies and cell arrangement. Only the Gram positive and catalase negative isolates were then screened for probiotic properties.

Antagonistic activity of LAB strains against enteric pathogens

Antibacterial properties are one of the most important selection criteria for probiotics. The antagonistic activityof LAB cultures against enteric pathogens (*Escherichia coli,Klebsiella* sp., *Salmonella* sp.,*Bacillus* sp., *Staphylococcus aureus, Vibrio cholera* and *Pseudomonas* sp. and *Listeria* sp.) was evaluated using the double layer method of Halder *et al*., (2017).

Antiobiotic susceptibility testing of LAB isolates

Antiobiotic susceptibility profile disc (Oxoid^{TM} Cefaclor-Thermo Fischer, USA) was used according to the manufacturer's instruction. The susceptibility to eight (8) antibiotics namely ofloxacin (5 µg), ceftriazone (30 µg), gentamicin (10 µg), erythromycin (5µg), augmentin (30 µg), cloxacillin (5 µg), cefuroxime (30 µg) and ceftazidime (30 µg) was determined using the agar overlay diffusion method as described by Halder *et al*. (2017). Briefly, a bacterial suspension was made by picking colonies from MRS agar plate using a sterile loop making a suspension in a sterile MRS broth and left for 24 h. With the use of a sterile swab, the suspension was applied on the surface of Muller-Hinton agar in sterile Petri dishes, and the strips were placed on the surface using a sterile forceps. The plates were then incubated at 30 ^{0}C for 48 h after which the readings were taken.

Growth at Different acidic pH

Twenty milliliters (20 ml) of MRS broth that have been previously inoculated with LAB and incubated at 30°C for 48 h. and three separate portions of 20 ml MRS broth containing LAB like the former but whose pH have been adjusted to pH of 2, 3 and 4 respectively were dispensed into screw capped tubes, sterilized and inoculated with the isolates. The tubes were incubated at 30°C for 48-72 hr. Turbidity of the broth was compared with uninoculated control in determining the presence or absence of growth (Islam *et al.,* 2016).

Bile salt Tolerance

The ability of the strains to grow in the presence of bile was determined according to the method of Vinderola and Reinheinmer (2003).

Gastric transit tolerance

A modification of the method of Huang and Adams (2004) was used to determine the tolerance of LAB strains to simulated Gastric intestinal tract conditions. Simulated small intestinal juices was prepared by adding pepsin (1:250, Sigma, USA) to sterile saline (0.5 % w/v, adjusted to pH 8.0 with 0.1 mol/L NaOH) at a final concentration of 1 g/L with 0.3% bile (Sigma, USA). Simulated intestinal juices were sterilized. Culture of the cells of the LAB strains was transferred to 9 ml of simulated intestinal juices and incubated at 37°C for 48 h. The viable counts of LAB were determined using pour plate method with MRS agar after serial dilution. The plates were incubated at 37°C for 48 h before colony counting. The Survival rates were calculated as follows:

Survival rate % $= \frac{\log cfu\, N1}{logcfuN0} \times 100\%$

Where:

N_1 = total viable count of each strains after treatment with simulated GI juices

N_0 = total viable count of each strain before treatment with simulated GI juices.

Cell Surface Hydrophobicity

The method of Dowarah *et al* (2018) was used to determine the cell surface hydrophobicity of the isolates to the hydrocarbons, chloroform and xylene. One millilitre of each of the hydrocarbons was added to test tubes containing 3ml of cell suspensions. The cells were vortexed for 2min. The suspensions were then kept undisturbed at 37°C for 5 min to allow phase separate, and the hydrocarbon layer was allowed to rise completely. After 5 min, the aqueous phase was removed carefully and the absorbance (OD) was measured using a spectrophotometer at 600 nm. The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (hydrophobicity (%). It was calculated using the equation given below:

Hydrophobicity (%) = $(OD_{initial} - OD_{final}) / OD_{initial} \times 100$

Where, $OD_{initial}$ and OD_{final} are the absorbances (at 600 nm) before and after extraction with the hydrocarbons.

Safety Assessment Tests

Safety tests of the screened isolates on the gut were performed using standard procedures. The tests performed include Gelatinase activity test, DNase production test and Haemolytic activity test.

Gelatinase activity and DNase production test were investigated as described by Pooja (2015).

Haemolytic activity was investigated as described by Halder *et al*., (2017). A 16 h old culture was streaked into sterile blood agar. The blood agar was prepared by adding 7% sheep blood, that had been preserved in ethylene diamine tetra acetic acid (EDTA), into sterile blood agar base at 45°C. Plates were incubated anaerobically at 37°C for 48 h after which they were observed for β and α haemolysis.

Product and Sample Formulation

The best two screened lactic acid bacteria with probiotic potential in all the probiotic screening tests carried out include *Lactobacillus* sp.(JLAB10) and *Leuconostoc* sp.(KLAB11). They were further used as starter cultures for the fortification of the malted acha blend. The Malted acha flour was reconstituted at 1:3 (w/v). One hundred grams (100g) each of the oven-dried malted acha flour were poured into three sterile conical flasks for the three different treatments (sample J, K and JK) separately and each mixed with 300ml of sterile distilled water to produce slurry. Measured 1:10 (w/v) inoculum size (MacFarland standard) of isolate J was inoculated into treatment flask labeled J. Similarly, the same procedure was performed for isolate K and inoculated into treatment flask labeled K. However, the treatment flask labeled JK was inoculated with a combination of isolate J and K. All the treatment flasks were corked with sterile cotton wool. This was allowed to ferment at 30±2^{o}C for 48 hr anaerobically.

Where:
Sample K = Blend fermented with single starter (*Leuconostoc sp.)*
Sample J = Blend fermented with single starter (*Lactobacillus sp.)*
Sample JK = Blend fermented with combined starter(*Lactobacillus sp.* and *Leuconostoc sp.*)

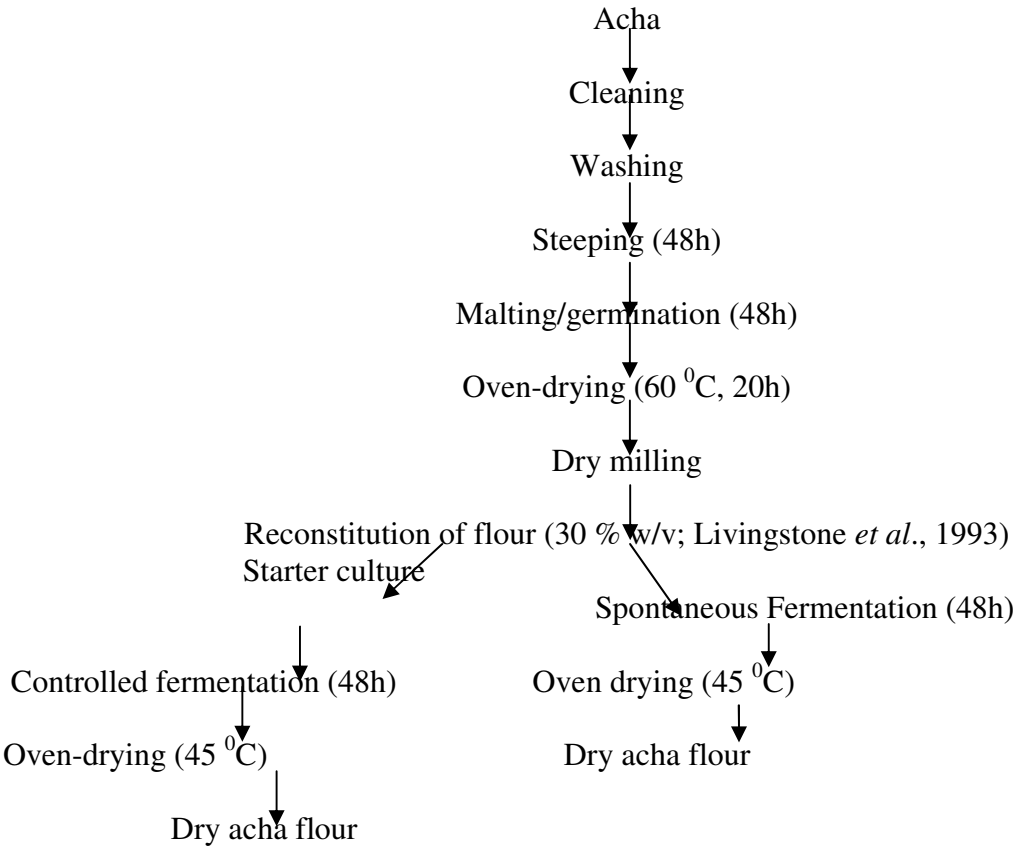

Figure 1: Flow chart of the unit operations involved in the production of starter and non starter fermented malted acha flour.

RESULTS AND DISCUSSION

Table 1 shows the total lactic acid bacteria count on MRS (De Mann Rogosa Sharpe) agar. The values decreased from $3.72x10^{14}$ to $3.5x10^{12}$ cfu/ml. The highest was recorded at 48h, after which there was a decrease in the count at 72h. Colony count on nutrient agar for aerobic bacteria showed that there was decrease in aerobic bacteria count from $1.0x10^{14}$ to $1.0x10^{10}$ cfu/ml. Yeast count increases from 0 to 8.6×10^{11} cfu/ml at 72h. The highest count was recorded at 48 h with no growth recorded on the Maconkey agar, while the total elimination of mould was observed after 24h. This increase in lactic acid bacteria, aerobic bacteria and increase in yeast/mould count, as fermentation time increased was similar to the observation by Mohammed *et al*. (2017) who reported that microbiological studies carried out during fermentation and malting of *wheat* enriched with African locust bean showed a decrease in total aerobic mesophilic and lactic acid bacteria count after 48 h fermentation time. The reduction or total elimination of enteric bacteria and fungi could be as a result of the decline in pH of the medium as a result of lactic acid bacteria growth, which was also reported by Dowarah *et al*. (2018).

The result of chemical analysis is shown in Table 2. The pH of the fermented malted acha slurry decreased drastically while the titratable acidity increased with increase in fermentation time. The pH values ranged from 6.4 to 3.5 while that of the TTA values ranged from 0.3mg/ml to 1.2mg/ml. The decrease in pH as fermentation time increased was possibly because of the accelerated growth rate of lactic acid bacteria which is in line with the submission of Inyang and Idoko (2006). Fermentation caused consistent increase in titratable acidity which could be due to the production of acid by lactic acid bacteria produced during the fermentation. Increase in acidity of several cereal-based foods during fermentation has been widely reported (Sefa-Dedeh *et al*., 2001; Afoakwa *et al*., 2004, 2007).

Table 3 shows the biochemical and physiological characteristics, of all the isolated lactic acid bacteria from the fermenting malted acha slurry. A total of 11 LAB were isolated. Some of the isolates were gram positive rods and a majority were cocci, catalase and oxidase negative respectively. All the isolates were negative to starch while isolate FLAB6, GLAB7 and JLAB10 are negative to ammonia from arginine. All the isolates grew at 30^{o}C, 37^{0}C, and 45^{0}C and also at pH 2, pH 3 and pH 4. All the isolates grew at 3%, 4%, and 6.5% NaCl concentration. Sugar fermentation of lactic acid Bacteria (LAB) showed that all the isolates utilize glucose, galactose, maltose, fructose and sucrose except for isolate ALAB1 and FLAB6 that did not utilize sorbitol and mannitol. All the lactic acid bacteria isolates did not utilize lactose sugar except for isolates ALAB1, BLAB2 and HLAB8. All the LAB isolates utilized mannitol except for isolates ALAB1, CLAB3, DLAB4 andGLAB7. All the selected LAB isolates were able to survive at temperatures of 30 °C, 37 °C and 45°C. However, growth was observed to decrease at 45°C. This agrees with the findings of Prescott *et al*. (2002) who reported that most lactic acid bacteria work best at temperatures of 18 to 37°C. The reason for choosing this temperature range was to detect whether the isolated cultures were able to grow within the range of normal body temperature or not. If the isolates were not able to survive within the selected temperature range then they would not have been able to survive in the human gut. This is an essential factor of probiotics to show their effectiveness. The results obtained were positive for growth at chosen temperature range. Ability of the isolates to tolerate 3.0-6.5% NaCl concentration is in agreement with the report of Hoque *et al*. (2010) who observed the NaCl (1-9%) tolerance of *Lactobacillus* species (*Lactobacillus spp*.) isolated from yoghurts.

The probable identity of the organisms are *Lactobacillus* spp., *Pediococcus* spp. and *Leuconostoc* spp. with *Lactobacillus* spp. having a percentage of 36%, *Leuconostoc* spp. having a percentage of occurrence of 18% and *Pediococcus* spp. having the highest percentage of occurrence of 46% each respectively (Table 4).

Antagonistic activity of the LAB strains against selected clinical isolates is as shown in Table 5. Eight clinical isolates were used; which were *Escherichia coli, Klebsiella* spp., *Salmonella* spp., *Bacillus* spp., *Staphylococcus aureus, Vibrio cholera, Pseudomonas* spp. and *Listeria* spp. The highest antimicrobial activity was produced against *Escherichia coli* (17mm) by *Lactobacillus* spp. (JLAB10). Moderate inhibition was observed against *Staphylococcus aureus, Pseudomonas* spp., *Salmonella* spp. and, *Vibrio cholerae*. However, the LAB isolates produced weak inhibition against *Bacillus* spp., *Klebsiella* spp. and *Listeria* spp. Observed growth inhibition on agar-spot plates indicated that the LAB strains produced antimicrobial products that were able to inhibit growth of *Escherichia coli, Klebsiella* spp., *Salmonella* spp., *Bacillus* spp., *Staphylococcus aureus, Vibrio cholerae* and *Pseudomonas* spp. and *Listeria s*pp. all of which are food contaminants and pathogens. This agrees with different reports which show that lactic acid bacteria produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms. Ability to inhibit Gram positive bacteria was consistent with the findings of Todorov *et al*. (2007) who reported that thirty colonies of *L. plantarum* inhibited the growth of *E. faecium* HKLHS imbedded in MRS agar; Kivinac (2011) isolated *Leuconostoc citreum* KB2, from Boza (a drink from Turkey) which demonstrate antimicrobial activity against *B. cereus*, while another strain *Lactobacillus brevis* KB12 showed antimicrobial activity against *S. aureus* and *B. subtilis* respectively. This further proves the potentials of probiotics to serve as viable alternatives to the use of therapeutic drugs which are often expensive and can have side effects (Brunken *et al.*, 2006).

Table 6 shows the Antibiotic susceptibility patterns of the strains to antibiotics. Eight (8) antibiotics were used in this study; each possessing a unique mechanism of action. All tested LAB strains were resistant to cloxacillin, ceftazidime and cefuroxime. However, they were susceptible to erythromycin, gentamicin, ofloxacin and augmentin respectively. Antibiotic resistance of microorganisms used as probiotic agent is an area of growing concern. This is due to the fact that antibiotic resistant strains can be detrimental as they are capable of transferring the resistant gene to pathogenic bacteria (some of which are part of the human intestinal microflora) when consumed. It is therefore necessary to study the susceptibility patterns of a probiotic before applying it as a functional agent.

Tables 7 and 8 show the viability to acid sensitivity of the isolates after 24hr and the results for growth in 0.3-1% concentration of mixed bile salt method respectively. All organisms were viable. Viability ranged from (9.0×10^3Cfu/ml) – TNTC (Too numerous to count). The important characteristic of a probiotic is its survival at low pH and high bile salts (Brunken et al., 2006). For acid sensitivity test, pH values of 2.0, 3.0, and 4.0 were chosen to determine acid resistance and survival was determined at 6h and 24h as this simulates residence time in the stomach according to Grajek *et al*. (2005). The LAB strains showed variable tolerance at different pH. This is consistent with the findings of Huang and Adams (2004) and Noor Nawaz *et al.* (2017). Generally, all LAB strains were able to grow at a range of pH 3.0 and 4.0. This suggests that pH values lower than 4 could be considered critical for the selection of potential probiotic functional foods.

The presence of bile salts in the environment of bacteria cultures is much more detrimental than the effect of low pH. The choice of the bile concentration selected for our screening (0.3-1% mixed bile-salt solution) was based on its being equivalent to the physiological concentration in the duodenum or the human bile juice (Brashears *et al*., 2003). The LAB strains were able to tolerate 0.3%, 0.5% and 1% bile salt. There was decrease in viability with increasing bile salt concentration. Factors such as membrane characteristics and variation in surface properties may have influenced the bile tolerance of strains as reported by Song *et al* (2015). This may explain the variation recorded among our tested strains.

Table 9 shows the gastric transit tolerance of the 11 isolates. *Lactobacillus* sp. (JLAB10) and *Leuconostoc* sp. (KLAB11) gave the best survival rate to gastric sensitivity test (simulated gastric juice conditions).

Table 10 showed the ability of the isolates to partition into hydrocarbons. Hydrocarbons used in this study were xylene and chloroform. The values obtained ranged from 32.4-45.8%. Partition ability varied amongst the strains and with hydrocarbon. It was observed that the LAB isolates showed higher partition to chloroform than to xylene. Isolates CLAB3, DLAB4, JLAB10 and KLAB11 produced the highest partition to chloroform while CLAB3, JLAB10 and KLAB11 produced the highest partition to xylene. Hydrophobicity to different hydrocarbons has been established as an *in vitro* biochemical marker to assess the colonization potential of the organism (Dowarah *et al*., 2018). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue. This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Wedajo, 2015). The LAB isolates showed moderate to positive hydrophobicity ranging between 32.4-43.6% (xylene) and 32.4-45.8% (chloroform). However, differences in affinity to individual hydrocarbon were observed. The LAB strains showed stronger affinity to chloroform than xylene. Isolate *Lactobacillus* sp. (JLAB10) showed highest affinity to chloroform while *Leuconostoc* sp. (KLAB11) showed highest binding potential to xylene. This suggests that ability to adhere is strain dependent. This is in agreement to the findings of Rinkinen (2004). Factors such as the non-specific reaction by charge, non-specific reaction by hydrophobicity or the presence of the proteinaceous components in the surface layered proteins of the strains that are involved in the adhesion process through their binding to carbohydrate portions as reported by Polak-Berecka *et al* (2014) could have influenced the variability in adhesion observed The results obtained for hemolytic and gelatinase activity showed that all the LAB strains were negative to the tests. The results were consistent with the finding of Kalui *et al*. (2009) and Mami *et al*. (2008). Safety is one of the recommended attributes in the FAO/ WHO (2002) guidelines on evaluation for probiotics. Absence of hemolytic and gelatinase activity is a selection criteria for probiotic strains, indicating that these bacteria are none virulent.

TABLE 1: Microbial load (CFU/ML) of Spontaneous Fermenting Malted Acha Slurry

Microbial Type	Fermentation Time (h)			
	0	**24**	**48**	**72**
LAB	NG	3.72×10^{14}	6.45×10^{14}	3.54×10^{12}
Aerobic bacteria	1.0×10^{14}	2.20×10^{10}	1.02×10^{12}	1.0×10^{10}
Yeast	NG	3.61×10^{10}	2.16×10^{12}	8.6×10^{11}
Moulds	2.0×10^{6}	1.30×10^{7}	NG	NG
Enteric	NG	NG	NG	NG

Values are expressed as mean of triplicates.
KEY: NG = No Growth

TABLE 2: Effect of Fermentation time on the pH and Total Titratable Acidity (TTA) of the Fermented Malted Acha Slurry

Parameter	Fermentation	Time (h)		
	0	24	48	72
pH	6.4	4.8	3.7	3.5
TTA (mg/ml)	0.3	0.5	0.9	1.2

Values are expressed as mean of triplicates

Table 4: Percentage of Occurrence of Lactic Acid Bacteria Isolated from Malted Acha Slurry during Fermentation.

S/N	PROBABLE IDENTITY	NUMBER (n)	OCCURRENCE (%)
1	*Lactobacillus* spp.	4	36
2	*Leuoconostoc* spp.	2	18
3	*Pediococcous* spp.	5	46
	TOTAL	11	100

Table 3: Biochemical and Physiological Characterisation of LAB Isolated from Malted Acha

Isolate Code	Gram Stain	Shape	Colour	Edge	Surface	Catalase	Oxidase	Arginine	Indole	Glucose	Galactose	Maltose	Fructose	Sorbitol	Mannitol	3.0% NaCl	4.5% NaCl	6.5% Nacl	pH 2.0	pH 3.0	pH 4.0	Most Probable Identity
ALAB1	+	C	Milky White	Entire	Smooth	-	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	*Leuconostoc* spp.
BLAB2	+	C	Milky White	Entire	Smooth	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	*Pediococcus*spp.
CLAB3	+	C	Milky White	Entire	Smooth	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	*Leuconostoc*spp.
DLAB4	+	C	Creamy	Entire	Smooth	-	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	*Pediococcous* spp.
ELAB5	+	R	Creamy	Entire	Smooth	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	*Lactobacillus sp*p.
FLAB6	+	R	Creamy	Entire	Smooth	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	*Lactobacillus* spp.
GLAB7	+	R	Creamy	Entire	Smooth	-	-	+	-	+	+	+	+	-	-	+	+	+	+	+	+	*Lactobacillus* spp.
HLAB8	+	C	Creamy	Entire	Smooth	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	*Leuconostoc* spp.
ILAB9	+	C	Milky white	Entire	Smooth	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	*Pediococcus* spp.
JLAB10	+	R	Milky White	Entire	Smooth	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	*Lactobacillus* spp
KLAB11	+	C	Creamy	Entire	Smooth	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	*Leuconostoc*spp.

KEY: C- Curved, R- Round

Table 5: Antagonistic Activity of LAB Isolates against Pathogenic Microorganisms

Isolates	Diameter of zones of inhibition (mm)							
	Lis	Bac	Sal	Esch	Kleb	Stap	Pseudo	Vib
*Leuconostoc*spp. **(ALAB1)**	7.0	9.0	8.0	14.0	10.0	10.0	8.0	8.0
Pediococcus spp. **(BLAB2)**	NA	8.0	10.0	15.0	12.0	13.0	7.0	9.0
Pediococcus spp. **(CLAB3)**	10.0	11.0	13.0	16.0	10.0	10.0	9.0	7.0
Pediococcus spp. **(DLAB4)**	8.0	7.0	9.0	10.0	NA	8.0	9.0	7.0
*Lactobacillus*spp. **(ELAB5)**	9.0	NA	7.0	10.0	6.0	7.0	5.0	5.0
Lactobacillus spp.**(FLAB6)**	NA	7.0	NA	9.0	5.0	7.0	NA	8.0
Lactobacillus spp**(GLAB7)**	8.0	NA	7.0	8.0	8.0	7.0	8.0	9.0
Pediococcus spp. **(HLAB8)**	9.0	9.0	7.0	8.0	8.0	9.0	NA	9.0
Pediococcus spp. **(ILAB9)**	7.0	NA	7.0	9.0	8.0	8.0	9.0	7.0
Lactobacillus spp.**(JLAB10)**	8.0	10.0	9.0	17.0	9.0	13.0	8.0	13.0
Leuconostoc spp. **(KLAB11)**	8.0	9.0	10.0	15.0	10.0	14.0	9.0	10.0

Values are expressed as mean of triplicates

Key: NA = No activity; Lis = *Listeria* sp; Bac = *Bacillus* sp; Sal = *Samonella* sp.; Esch = *Escherichia coli*; *Klebsiella* sp.= *Staphylococcus aureus*; Pseu = *Pseudomonas* sp.; Vib = *Vibrio cholera*

Table 6: Antibiotic Susceptibility Test

	Antibiotics (µg)							
Isolate	Caz	Crx	Gen	Ctr	Ery	Cxc	Ofl	Aug
*Leuconostoc*sp. (ALAB1)	R	R	S	S	S	R	S	I
Pediococcus sp. (BLAB2)	R	R	S	R	S	R	S	I
Pediococcus sp. (CLAB3)	R	R	S	R	S	R	S	I
Pediococcus sp. (DLAB4)	R	R	S	R	S	R	S	S
Lactobacillus sp. (ELAB5)	R	R	I	S	S	R	R	R
Lactobacillus sp. (FLAB6)	R	R	S	R	S	R	I	S
Lactobacillus sp(GLAB7)	R	R	S	I	S	R	S	S
Pediococcus sp.(HLAB8)	R	R	I	S	S	R	R	S
Pediococcus sp.(ILAB9)	R	R	S	R	S	R	S	R
Lactobacillus sp.(JLAB10)	R	S	R	S	S	R	R	S
*Leuconostoc*sp. (KLAB11)	R	R	I	R	S	R	R	R

Key
Where: R= Resistance, S = Susceptible and I= Intermediate
R ≤ 5mm
S ≥ 10mm
I = 6-9mm
Caz = Ceftazidime (30µg), Crx = Cefuroxime (30µg), Gen = Gentamicin (10µg), Ctr = Ceftriaxone (30µg), Ery = Erythromycin (5µg), Cxc = Cloxacillin (5µg), Ofl = Ofloxacin (5µg) and Aug = Augmentin (30µg).

Table 7: Viable Count for Acid Sensitivity

Isolates	Growth at Different Acidic pH (Total Viable Count (cfu/ml)		
	2	3	4
*Leuconostoc*sp. (ALAB1)	$5.0x10^4$	$9.9x10^4$	$2.12x10^5$
Pediococcus sp. (BLAB2)	$3.5x10^4$	$7.0x10^4$	TNTC
Pediococcus sp. (CLAB3)	$4.0x10^4$	$6.5x10^4$	TNTC
Pediococcus sp. (DLAB4	$2.2x10^4$	$6.0x10^3$	TNTC
Lactobacillus sp.(ELAB5)	$1.0x10^4$	$2.7x10^4$	TNTC
Lactobacillus sp. (FLAB6)	$9.0x10^3$	$2.0x10^4$	$7.8x10^4$
Lactobacillus sp(GLAB7)	$2.4x10^4$	$3.9x10^4$	TNTC
Pediococcus sp. (HLAB8)	$3.0x10^4$	$6.8x10^4$	TNTC
Pediococcus sp. (ILAB9)	$2.8x10^4$	$4.5x10^4$	$1.70x10^5$
*Lactobacillus*sp.(JLAB10)	$1.44x10^5$	$2.93x10^5$	TNTC
Leuconostoc sp.(KLAB11)	$1.28x10^5$	$2.28x10^5$	TNTC

Values are expressed as mean of triplicates
Key: TNTC = Too Numerous To Count

Table 8: Tolerance to Bile Salt of Isolated LAB (plate Count for Bile Salt)

Isolates	Growth at (A_{560}) Bile Salt concentration/Growth at 24 h (cfu/ml)		
	0.3	0.5	1.0
*Leuconostoc*sp. (ALAB1)	$9.0x10^4$	$4.0x10^4$	$2.5x10^4$
Pediococcus sp. (BLAB2)	TNTC	$3.5x10^4$	$2.4x10^4$
Pediococcus sp. (CLAB3)	$5.0x10^4$	$2.0x10^4$	$1.0x10^4$
Pediococcus sp. (DLAB4)	$2.3x10^4$	$1.5x10^4$	$9.0x10^3$
Lactobacillus sp. (ELAB5)	$7.0x10^3$	$5.0x10^3$	$3.0x10^3$
Lactobacillus sp. (FLAB6)	$7.0x10^3$	$4.0x10^3$	$2.0x10^3$
Lactobacillus sp(GLAB7)	$4.5x10^4$	$3.3x10^4$	$2.0x10^4$
Pediococcus sp. (HLAB8)	$1.5x10^4$	$1.1x10^4$	$9.0x10^3$
Pediococcus sp. (ILAB9)	TNTC	$7.5x10^4$	$5.5x10^4$
Lactobacillus sp(JLAB10)	TNTC	$1.25x10^5$	$8.0x10^4$
Leuconostoc sp. (KLAB11)	TNTC	$9.8x10^4$	$7.8x10^4$

Values are expressed as mean of triplicates
Key: TNTC= Too numerous to count

Table 9: Gastric Transit Tolerance of Isolates of Isolated LAB

Isolates	Mean Growth (Ab_{560})	Gastric Sensitivity Viable plate count at 24 h (cfu/ml)
*Leuconostoc*sp. (ALAB1)	0.947	$1.40x10^5$
Pediococcus sp. (BLAB2)	0.911	$8.8x10^4$
Leuconostoc sp. (CLAB3)	0.886	$6.5x10^4$
Pediococcus sp. (DLAB4)	0.885	$6.4x10^4$
Lactobacillus sp. (ELAB5)	0.948	$1.25x10^5$
Lactobacillus sp. (FLAB6)	0.967	$1.38x10^5$
Lactobacillus sp(GLAB7)	0.960	$1.34x10^5$
Leuconostoc sp. (HLAB8)	0.921	$1.30x10^5$
Pediococcus sp. (ILAB9)	0.906	$9.2x10^4$
Lactobacillus sp.(JLAB10)	1.064	$2.05x10^4$
Leuconostoc sp. (KLAB11)	0.997	$1.65x10^5$

Values are expressed as mean of triplicates

Table 10: Cell Surface Hydrophobicity test of Isolated LAB

Isolates	Adherence (%) Xylene	Chloroform
Leuconostoc sp. (ALAB1)	32.4	35.4
*Pediococcus*sp. (BLAB2)	33.6	32.5
Leuconostoc sp. (CLAB3)	43.5	40.2
*Pediococcus*sp. (DLAB4)	39.3	42.1
Lactobacillus sp. (ELAB5)	39.5	37.8
Lactobacillus sp. (FLAB6)	38.7	36.8
Lactobacillus sp. (GLAB7)	39.9	38.6
Leuconostoc sp. (HLAB8)	36.5	34.8
Pediococcus sp. (ILAB9)	38.7	37.9
Lactobacillus sp. (JLAB10)	42.0	45.8
Leuconostoc sp. (KLAB11)	43.6	41.7

CONCLUSION

The results of this research have shown that the broad spectrums and arrays of lactic acid bacteria can be isolated from the spontaneous fermentation of acha flour. This can be used for the starter fermentation of acha flour in production of weaning food for infants. This weaning food could be effective for addressing childhood malnutrition and confer other health benefits such as child growth, maintenance of gastrointestinal microflora balance and mental development. Therefore, from this research, it has been established that a formulation blend of weaning food from malted acha flour can be produced with the fortification of the blends with combined starters (*Lactobacillus sp.* and *Leuconostoc sp.*) whose levels in the final product where well below the safe limit.

Further studies should investigate the areas of *in-vivo* study on animals to evaluate the biological impact of the selected probiotic strains used in the fermentation of the malted acha blend.

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