Original Research Article

Phenotypic and genotypic characterization of lactic acid bacteria and yeasts involved in spontaneous fermentation of '*ogi'*, a cereal-porridge produced in Nigeria

Abstract

The traditional fermented cereal-porridge 'ogi' is an important dietary staple food popularly consumed in Nigeria. The fermentation is mainly driven by lactic acid bacteria and yeasts during spontaneous fermentation. This study aimed to isolate and ascertain the phenotypic and genotypic characteristics of lactic acid bacteria and yeasts associated with spontaneous fermentation of maize and sorghum. Lactic acid bacteria and yeasts were isolated from fermented ógi' by spread plate method. A total of 9 isolates of lactic acid bacteria and 4 yeast isolates were obtained and were characterized based on general morphology, Gram-staining and biochemical tests. The lactic acid bacteria identified by molecular method were strains of *Lactobacillus fermentum*, *Enterobacter* spp, and *Lactococcus* spp, while the yeasts were strains of *Trichosporon asahii*, *Hypopichia burtoni* and *Pichia kudriavzevii*.

Keywords: Lactic acid bacteria, yeast, spontaneous fermentation, ogi

1. Introduction

Spontaneous fermentation is an uncontrolled fermentation dating way back in time, as a traditional method of preparation and the form of fermentation in most small scale fermentations in the developing countries [1]. The traditional cereal-porridge 'ogi' is produced by spontaneous fermentation. Several genera of microorganisms are known to associated with the fermentation of steeped maize and sorghum for *ogi* production: *Staphylococci, Escherichia, Pseudomonas, Enterococcus, Klebsiella, Bacillus, Lactobacilli, Leuconostoc, Clostridium, Corynebacterium, Streptococcus, Micrococcus* and *Citrobacter* (bacteria), *Aspergillus, Saccharomyces, Penicillin, Candida, Rhizopus, Fusarium, Mucor, Geotrichum*, *Pichia* and *Rhodotorula* (fungi) [1,2-5].

Lactic acid bacteria mainly *Lactobacilli Plantarum* and *Lactobacilli fermentum*, and the yeasts *Saccharomyces cerevisiae* and *Pichia kudriavzevii* are the predominant microbial species in fermentation of cereals for the production of ogi [3,5,6]. Majority of the microbes in fermented steeped maize for *ogi* production have been variously reported to participate in fermentation of some other local food [7,8]. These microorganisms are present in the environment and can inoculate suitable substrates at will.

The major function of LAB is to produce lactic acid that is the acidification of the food while its main application is as starter cultures in the food industry with a variety of fermented diary products, meat, fish, vegetable and cereal products, where it contributes to the flavour, texture and nutritional value of the fermented foods through production of aroma components and use [9-11]. As probiotics, they confer health benefits upon consumption and inhibit proliferation of spoilage and pathogenic microorganisms [11-13]. Together with yeast, LAB are usually the most commonly used microorganisms for the fermentation of foods [3].

Ogi has been produced for a long time by the traditional fermentation technique, without knowledge of the knowledge of the phenotypic and genotypic characteristics of the microorganisms associated with the fermentation. Production ogi on a large scale would require the isolation and characterization of the microorganism involved in the fermentative process.

2. Materials and Methods

2.1. Processing of Maize and Sorghum into “Ogi”

Maize and sorghum grains were separately sorted, cleaned and processed using a modified wet-milling laboratory method developed and reported by Ojokoh [14]. The paste was sieved and allowed to sediment for 48 h.

2.2. Microbiological Analysis

2.2.1 Isolation of Lactic Acid Bacteria and Yeast

Five grams (5g) of “Ogi” paste was mixed by swirling in 45ml sterile 0.1% (w/v) peptone water (Oxiod, England) in a conical flask. Tenfold serial dilutions of the mixture of “Ogi” paste in peptone water were made using 0.1% (w/v) peptone water in test tubes and 0.1ml of appropriate dilutions were inoculated in duplicates by spread plate technique on de man, Rogosa and Sharpe’s (MRS: OXOID, England) agar, for the growth of lactic acid bacteria. The plates were incubated anaerobically in Gallenkamp anaerobic jars for 48 hours. The anaerobic environment was created by the addition of anaerobic gas pack (Thermo SCIENTIFIC, Oxoid Ltd, Basingstoke) to the jars, according to manufacturer’s instruction.

Potato Dextrose agar (PDA: OXOID, England) was used for growth of the yeast. The medium was acidified by the addition of lactic acid (0.1%, v/v) when it was coded after autoclaving and before pouring to eliminate bacteria. The plates were incubated at 30^{O}C for 72 hours.

2.2.2 Characterization of Latic Acid Bacteria

The method of Onwuakor *et al.* [15] was adopted. The parameters for characterization included cultural characteristics, cell morphology, Gram reaction, tests for catalase, indole, oxidase and motility. Other tests included growths in different concentrations of NaCL, different pH environments and temperatures and also the fermentation of carbohydrates (D-glucose, ribose, sucrose, lactose, maltose, mannitol, galactose, salicin, sorbitol, mannose, starch and trehalose) (MERCK, Genermany).

2.2.3 Growth of LAB in different concentrations of sodium chloride (NaCl)

De Man, Rogosa and sharpe’s agar (MRS: OXOID, England) was prepared according to the manufacturer’s information and sterilized by autoclaving at 121^{O}C for 15 min and cooled. It was then inoculated with the test bacteria by pour method after adding 1ml amounts of different concentrations of sterile NaCl to different plates in duplicates. The different concentrations used were 2%, 3%, 4%, 6.5%, 10%. The plates were then incubated anaerobically at 37% for 48 hour. Growth on the plates indicated a positive result.

2.2.4 Growth LAB in different pH environment

The MRS agar medium was prepared in 5 different conical flasks. Then the pH of the medium in each of 4 conical flasks was differently adjusted to 4.4, 7.2, 9.3, 9.6 respectively, using the pH meter, with acetate and phosphate buffers. The 5th flask contained the medium with the original pH (6.5), and was used as control. The media were then sterilized and inoculated with the test organism by pour-plate method and incubated anaerobically for 48h at 37°C. Growth on plate indicated a positive test result.

2.2.5 Growth of LAB at different temperatures

The test bacteria were inoculated by spread-plate method and incubated anaerobically for 48h at different temperatures of 30°C, 40°C, and 45%. Growth on the plates indicated a positive result.

2.2.6 Fermentation of Carbohydrates by LAB

The test medium (MRS broth, Oxoid England) was prepared by mixing 95ml peptone water, and 5ml of 0.2% phenol red (indicator) in a beaker and dispensing 4.75ml volumes into bijou bottles and sterilizing by autoclaving at 121^{o}c for 15 minutes. After cooling, 0.25ml volumes of the filtered test sugar solution was aseptically added to each bottle and mixed to form the test medium. Then the sugar test medium was inoculated with a speck of the test bacterium from an isolated colony. The bottles were then incubated anaerobically at 34^{o}c for 48 hours. A colour change from pale yellow to pink indicated a positive result. The carbohydrates tested for viable aerobic yeast were lactose, sucrose, mannitol, mattose, galactose, fructose, D-Glucose, Ribose, raffinose, arabinose, rhaminose, xylose, mannitol, salicin, sorbitol, mannose, starch and trehalose (MERCK, Germany). A colour change from pale yellow to pink indicated a positive result.

2.2.7 Determination of lactic acid (%)

The method of Aneja [16]was adopted. The LAB were first sub-cultured on MRS Agar and incubated at 37°C under anaerobic conditions for 48 hours.

2.2.8 Fermentation of Carbohydrates

The test medium (MRS broth, Oxoid England) was prepared by mixing 95ml peptone water, and 5ml of 0.2% phenol red (indicator) in a beaker and dispensing 4.75ml volumes into bijou bottles and sterilizing by autoclaving at 121^{o}c for 15 minutes. After cooling, 0.25ml volumes of the filtered test sugar solution was aseptically added to each bottle and mixed to form the test medium. Then the sugar test medium was inoculated with a speck of the test yeast from an isolated colony. The bottles were then incubated anaerobically at 34^{o}c for 48 hours. The carbohydrates tested were glucose, lactose, sucrose, mannitol, mattose, galactose and fructose (MERCK, Germany).

2.2.9 Ethanol Tolerance Test for Yeasts

Potato Dextrose Agar (PDA) (OXOID, England) was prepared according to the manufacturer's instruction, by dissolving it in distilled water in a conical flask. Then 5ml amounts were dispensed into bijou bottles and sterilized by autoclaving at 121^{0}C for 15 minutes. After cooling to 50% (cool enough to touch), 1ml of 2%, 4% and 6% ethanol were added in duplicates to different bijou bottles and poured into sterile Petri dishes and left to solidify. Each plate was then inoculated with the test organism by streaking a loopfull of 24th broth culture grown on Yeast Extract peptone Dextrose broth (MERCK, Germany), per plate in duplicates. A positive result was the growth of the test organism on the medium.

2.3 Molecular Identification Of Lactic Acid Bacteria And Yeasts

2.3.1 Bacterial Genomic DNA Extraction

The physical or boiling method of Okolie et al. [17] was adopted. One isolated colony of each test bacterium was inoculated in Luria Bertani broth (LB: Miller, Amresco) in bijou bottles and incubated anaerobically for 12 hours. Then 1ml of the broth culture was transferred to Eppendorf tube (microcentrifuge tube) followed by the addition of 0.5ml normal saline and centrifuged at 12,000 rpm for 5minutes, using Eppendorf centrifuge (5424), to wash the cells. The supernatant was decanted and 1ml of normal saline was added to the sediment and homogenized for 5 minutes using the votex mixer (Celtech XH-B) and centrifuged again. The washing process was repeated 2 more times with the sediment. Then 0.5ml DNA elution buffer (Zymo Research, USA) was added to the resultant sediment and homogenized using the votex and heated with the heating block (Wealtec HB-2 USA) at 95^{O}C for 20min. The heated bacterial suspension was cooled in the deep freezer at -20^{O}C for 10min and spun for 3min at 14,000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{O}C for other downstream reactions.

2.3.2 Bacterial DNA Quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer (Thermos scientific).

2.3.3 Bacterial 16S rRNA Amplification

The 16S RRNA of the rRNA genes of the isolates were amplified using the 27F: 5’-AGAGTTTGATCMTGGCTCAG-3’ and 1492R:5’-CGGTTACCTTGTTACGACTT-3’ primers on a ABI 9700 Applied Biosystems thermal cycler (Dyad) at a final volume of 50 micoliters for 35 cycles. The PCR mix included: the x2 ream tag Master mix supplied by inqaba, South Africa (tag polymerase. DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95^{o}C for 5 minutes; denaturation, 95^{o}C for 30 seconds; annealing, 52^{o}C for 30 seconds; extension, 72^{o}C for 30 seconds for 35 cycle and final extension, 72^{o}c for 5 minutes. The product was resolved on a 1% agarose gel (Inqaba, South Africa) at 120V for 15 minutes and visualized on a UV transilluminator (prio-surplus).

2.3.4 Fungal genomic DNA Extraction

Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa following the manufacturer's protocol. A heavy growth of the pure culture of the fungal isolates was suspended in 200 microlitres of isotonic buffer in a ZR BashingBead Lysis tube, then 750 microlitres of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashingbead lysis tubes were centrifuged at 10,000 × g for 1 minute.

Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin filter (Orange top) in a collection tube and centrifuged at 7000 × g for 1minute. One thousand two hundred (1,200) microlitres of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes, bringing the final volume to 1600 microlitres. 800 microlitres was then transferred to a Zymo-spin IIC column in a collection tube and centrifuged at 10,000 × g for 1minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliters of the DNA pre-wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000 × g for 1minute followed by the addition of 500 microlitres of fungal/bacterial DNA wash buffer and centrifuged at 10,000xg for 1 minute.

The Zymo-spin IIC Colum was transferred to a clean 1.5 microliters centrifuge tube, 100 microlitres of DNA elution buffer was added to the Colum matrix and centrifuged at 10,000 × g microlitres for 30 seconds to elute the DNA. The ultra pure DNA was then stored at -20 degree for other downstream reactions.

2.3.5 Internal transcribed Spacer (ITS) Amplification of Yeast DNA

The ITS region of the rRNA genes of the isolate were amplified using the ITSIF: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3', primers on an ABI 9700 Applied Biosystems thermal cycler at a volume of 50 microlitres for 35 cycles. The PCR mix included: the x2 Dream tag Master mix supplied by Inqaba, SouthAfrica (tag polymerase, DNATPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95^{o}c for 5 minutes denaturatoin, and 95^{o}C for 30 seconds; annealing, 53^{o}C for 30 seconds; extension, 72^{o}C for 30 seconds for 35 cycles and final extension, 72^{o}C for 5 minutes.

2.3.6 Sequencing

Sequencing was done using the BigDye terminator kit on a 3510 ABI sequencer by Inqaba, South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25µl BigDye$^{(R)}$terminator vl.1/v.3.1, 2.25µl of 5 x BigDye sequencing buffer, 10µl PCR Primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96^{o}C for 10s, 55^{o}C for 5s and 60^{o}C for 4minutes.

2.3.8Phylogentic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit. Similar sequences were downloaded from the National Centre for Biotechnology information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-joining method in MECA 6.0 [18]. The bootstrap consensus tree inferred from 500 replicates [19] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [20].

3. Results

3.1 Phenotypic Characteristics of Isolates

Table 1 shows the phenotypic characteristics of lactic acid bacteria isolated from ogi. Table 1 shows the characteristics of the yeast isolates.

Table 1: Characteristics of lactic acid bacterial isolates

Isolate Code	Gram reaction	Cell Morphology	oxidase	catalsase	Motility	D-Glucose	Ribose	Sucrose	Lactose	Maltose	Fructose	Raffinose	Arabinose	Rhaminose	Xylose	Mannitol	Galactose	Salicin	Sorbitol	Mannose	Starch	Trehalose	Indole	Nitrate red.	Nacl (%)					pH				Temp.			Lactic acid (%)
																									2	3	4	6.5	10	4.4	7.2	.9.3	9.6	30°C	40°C	45°C	
B1	+ve	Cocci	-	-	-	A/G	A/G	A/G	A/G	A	A	A/G	A	A	-	A	A/G	A/G	A	A	-	A/G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1.22
B2	+ve	Cocci	-	-	-	A	A	A	A	A	A	-	-	A	A	-	+	-	-	A	+	+	-	-	+	+	+	+	-	+	+	-	-	+	-	-	0.58
B3	+ve	Cocci	-	-	-	A	A	A	-	A	A	-	A	-	-	-	A	A	-	A	-	A	-	+	+	+	+	+	-	+	+	+	+	+	+	+	0.27
B4	+ ve	Cocci	+	+	+	A	A	A	A	A	A	A	A	-	A	-	A	A	-	-	-	A	-	-	+	+	+	+	+	-	+	+	+	+	+	-	0.60
B5	+ ve	Cocci	-	-	-	A	A	A	-	A	A	-	A	-	-	-	A	A	-	A	-	A	-	+	+	+	+	+	-	+	+	+	+	+	+	+	1.54
B6	+ ve	Cocci	-	-	-	A	A	A	A	A	A	A	A	A	-	A	-	-	A	A	A	A	-	-	+	+	+	+	-	+	+	-	-	+	+	+	0.95
B7	+ ve	Cocci	-	+	-	A	A	A	A	A	A	-	-	-	-	A	A	A	A	A	-	A	-	-	+	+	+	+	-	+	+	-	-	+	+	-	1.24
B8	+ ve	Cocci	-	-	+	A	A	A	A	A	A	A	A	A	A	A	A	-	-	A	-	A	A	+	+	+	+	+	+	+	+	+	+	+	+	+	0.45
B9	+ ve	Cocci	-	-	+	A	A	A	-	A	A	A	A	-	A	A	A	A	A	A	+	A	-	-	+	+	+	+	-	+	+	+	-	+	+	-	0.28

Table 2: Characteristics of yeast isolates

Isolate Code	Cultural Characteristics	Gram Stain	Shape	Sugar Fermentation						Ethanol tolerance
				Glucose	Maltose	Galactose	Sucrose	Lactose	Fructose	
Y1	2mm, mucoid, milky, rough edges	+	Oval	+	+	-	+	-	+	+
Y2	1mm, yellow round edge	+	Oval	+	+	-	-	-	+	+
Y3	3mm, dry surface, pointed centre, smooth edges	+	Oval	+	+	-	+	-	-	-
Y4	1mm pale milky round, smooth edges	+	Oval	+	+	-	+	-	+	+

3.2 Molecular identification of lactic acid bacteria

The obtained 16S rRNA sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate B1-B9 within Lactobacillus and *Enterobacter*sp and revealed a closely relatedness to *Lactobacillus fermentum, Lactobacillus lactis* and *Enterobacter* sp strain LAB respectively (Figure 1).

3.3 Molecular identification of yeast

The obtained ITS sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The ITS of the isolates showed a percentage similarity to other species at 99-100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of ITS of the isolates Y1-Y4 within the *Hypopichia*, *Pichia* and *Trichosporon* spp and revealed a closely relatedness to *Hypopichia burtonii, Pichia kudriavzevii* and *Trichosporon asahii* respectively (Fig. 1). In summary, there were 2 strains of *Hypopichia burtonii*, 1 *Pichia kudriavzevii* and 1 *Trichosporon asahii*.

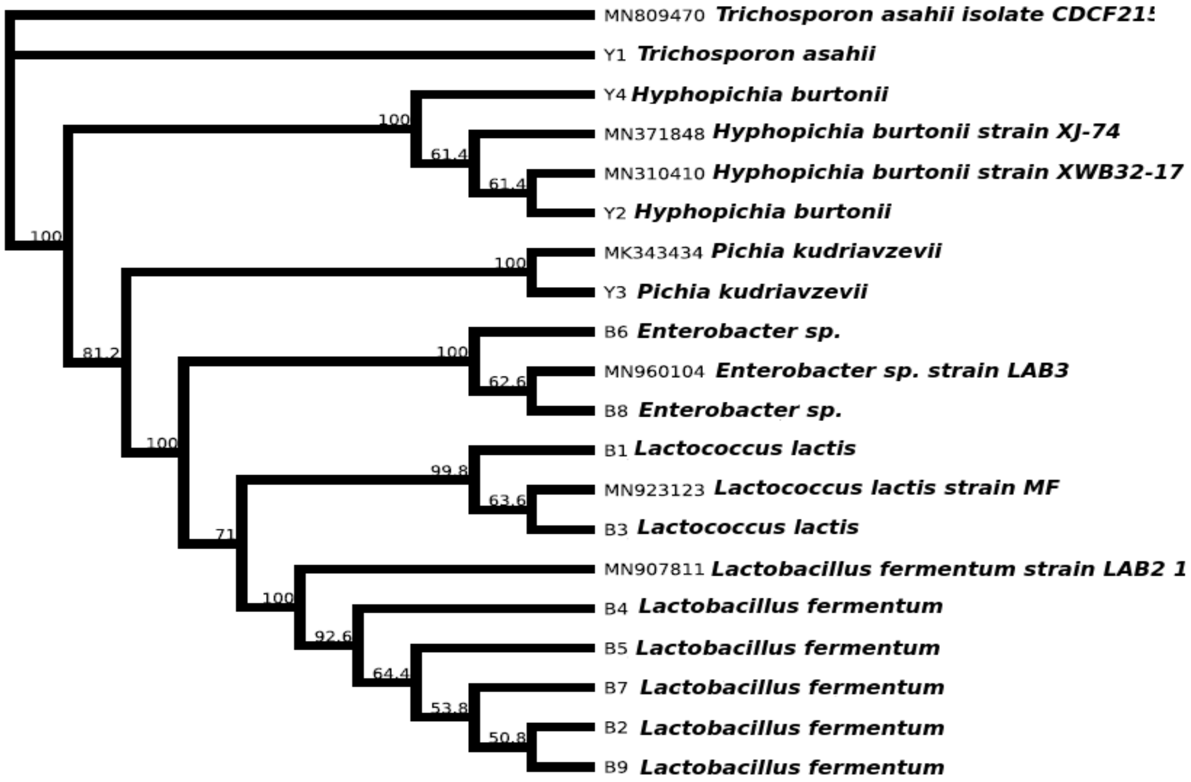

Figure 1: Phylogenetic tree showing the evolutionary distances between the bacterial and yeast isolates

4. Discussion

In the main, lactic acid bacteria (LAB) and yeasts are involved in the spontaneous fermentations of cereals in synergy, where the yeasts help to produce high levels of ethanol and confer desirable flavour, while the LAB principally produce the desirable acids [21,22].

In this study, the LAB isolated from ogi (fermented maize and sorghum blend) were characterized with phenotypic and genotypic methods. It was established that *Lactobacillus fermentum* is the dominant flora as five strains were identified. They were also dominant in the production of lactic acid (1.54%) and tolerated a low pH of 4.4. Houngbedji et al. [22] likewise identified *Lactobacillus fermentum* as the dominant flora in the fermented cereal dough mawe consumed in Benin. In the study conducted by Olatunde et al. [23] 88 LAB were isolated from effluents generated during ogi production of which *Lactobacillus* spp.

predominated in lactic acid production. Agati et al. [5] isolated 12 LAB with amylolytic abilities from fermented cereal dough, which also include *Lactobacillus fermentum* as reported in the present study. Thus in agreement with these and other work [3-5,24], this study also confirmed that some strains of *Lactobacillus fermentum* are amongst key players in the fermentation of ogi.

The other lactic acids isolated were two strains of *Lactococcus lactis* and *Enterobacter sp.* The isolates were able to grow at 45^{o}C, a low pH of 4.4 and produced lactic acids ranging from 0.27-1.22%. They also contribute to the acidification of the medium.

The yeast isolates were strains of *Hypopichia burtonii*, *Pichia kudriavzevii* and *Trichosporon asahii*. The yeast isolates were tolerant to ethanol except *Pichia kudriavzevii.* The yeast, *Pichia kudriavzevii* has been reported for its qualities as a probiotics, flavour enhancer, preservative ability, able to withstand a low pH and can remain metabolically active at 45^{O}C [25]. Houngbedji et al. [22] identified *Pichia kudriavzevii* as the predominant yeast involved in the spontaneous fermentation of cereal-based dough. *Trichosporon asahii* is among dominant yeast in the production of *Fura*, a spontaneously fermented pearl millet product consumed in West Africa [26] and *Nono*, a traditional fermented milk [27]. *Hypopichia burtonii* is commonly associated with the fermentation of rice and coffee [28,29].

The LAB are dominant players in fermenting food matrix like cereal-porridge, supported by the yeast as confirmed in this study. Since the isolated LAB and yeast were characterized with phenotypic and genotypic methods, the correct identities of the isolates were ascertained, and informed that the phenotypic methods should be supported with the genotypic ones as suggested in other studies [24,30].

5. Conclusion

The strains of LAB isolated in this study have been reported strains of probiotic bacteria. The yeasts have also been reported as good candidates as starter culture in cereal fermentation. In combination, the LAB and the yeast isolates can be used as starter culture to produce ogi of desirable nutritional, microbiological and organoleptic qualities.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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