



## Research article

## Genomic, biochemical and microbial evaluation of probiotic potentials of bacterial isolates from fermented sorghum products

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## ABSTRACT

Fermented products, including *Ogi-baba* and *Pito*, provide several health benefits, particularly when probiotics are used in the fermentation process. Probiotic microorganisms exert strain-specific health-promoting activities on humans and animals. The objective of this study was to investigate the probiotic potentials of Lactic-acid bacteria (LAB) isolates from indigenous fermented sorghum products (*Ogi-baba* and *Pito*). The LAB isolates were screened for potential probiotic properties by antagonistic activity against eight enteropathogenic clinical bacteria isolates (*Escherichia coli*, *Klebsiella* sp., *Helicobacter pylori*, *Bacillus* sp., *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp. and *Listeria monocytogenes*) as indicator organisms using the agar well diffusion technique. The organisms were also screened for acidity, bile tolerance, antibiotic susceptibility, production of lactic acid, diacetyl and hydrogen peroxide.  $\beta$ -galactosidase assay was also done. Genomic DNA was extracted from the two selected LAB isolates; the 16S rRNA were amplified and sequenced. The sequence data were subjected to Basic Local Alignment Search Tool (BLAST) and molecular phylogenetic analyses to identify the isolates. The isolates were identified as strains of *Lactobacillus plantarum* and *Pediococcus pentosaceus*. The sequence data for these two isolates were submitted to the Genbank with accession numbers KP883298 and KP883297 respectively. The *P. pentosaceus* strain (PB2) exhibited  $\beta$ -galactosidase activity as well as *L. plantarum* strain (OB6). The study revealed exceptional probiotic potentials of two LAB namely *Lactobacillus plantarum* strain (OB6) and *Pediococcus pentosaceus* strain (PB2) isolated from fermented sorghum products, *Ogi-baba* and *Pito* respectively. Hence, the two LAB strains may be potentially used as probiotic to prevent some enteropathogen-induced gastrointestinal disorders; reduce the incidence of respiratory tract infections and for the management of lactose intolerance.

## 1. Introduction

Fermentation is a commonly used food processing technology, and lactic acid fermentation is probably the simplest and safest means of preserving food. Many African staple foods are fermented with lactic acid bacteria (LAB), and lactic acid-fermented foods constitute a significant portion of indigenous diets in many low income countries [1]. *Ogi-baba* and *Pito* are fermented beverages, commonly from sorghum (*Sorghum bicolor*) which is a very common food taken in the Mid-western, West-central, Western, Benue-Plateau and North-central states of Nigeria [2, 3, 4, 5]. Nigeria, being among foremost producers of *Sorghum bicolor* in the world [6], has scanty record of microbial ecology of fermented sorghum products [7]. A characteristic of many sorghum foods and beverages is

their ability to undergo lactic acid fermentation by lactic acid bacteria, generally referred to as probiotic, during their production.

The term probiotic is currently used to name ingested microorganisms associated with beneficial effects to humans and animals [8, 9]. Probiotics may produce various compounds, which are inhibitory to the pathogen's growth, such as organic acids (lactic and acetic acids), bacteriocins, and reuterin [10]. There is increasing evidence that probiotics are beneficial in gastrointestinal (GI) infections, such as diarrhoea, dysentery, typhoid and respiratory tract infections to mention a few [11, 12]. Also, it has been reported to be effective against infectious diseases [13].

Infectious diseases are the biggest problem in human being and every year gastrointestinal infections are responsible for significant morbidity and mortality worldwide [14]. Infectious diseases are predominantly

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caused by bacteria otherwise referred to as enteropathogenic bacteria [15]. Enteropathogenic bacteria comprised of *Salmonella* species, *Shigella* species, *Proteus* species, *Klebsiella* species, *E. coli*, *Pseudomonas* species, *Vibrio cholerae* and *S. aureus* are major etiologic agents of GI infections [16, 17]. The rise in antibiotic resistant bacteria has awakened the scientific community to the prophylactic and therapeutic uses of probiotics, and to reconsider them as alternatives to antibiotics [18, 19].

More importantly, the emergence of antibiotic resistance in enteric pathogenic bacteria has facilitated the prophylactic and therapeutic uses of probiotics [20]. Consequently, probiotics should be considered as alternatives to antibiotics [21]. In the Nigerian traditional medicine, there are have been a reported activity of the sorghum products, *Ogi-baba* and *Pito* in the management of gastro-infectious diseases, however, there has been no scientific study in the open literature that has assessed on a genomic, biochemical and microbial scale the probiotic potential of the bacterial isolated before now. This study is therefore designed to investigate the hypothesis that these indigenous sorghum products possess probiotic potentials. The aim of the present study is to investigate the probiotic potentials of bacterial isolates from traditional fermented sorghum products such as *Ogi-baba* and *Pito*.

## 2. Material and methods

### 2.1. Preparation of samples

The common red variety of *Sorghum bicolor* grains in Nigeria was purchased from local retail market outlet in Mile 12 market, Kosofe, Lagos state. The seeds were authenticated by University of Lagos Herbarium and ascribed a voucher number: LUH 7417. The seeds were carefully freed from foreign materials by hand picking. All experiments were in triplicate.

*Ogi*, a gruel was prepared by soaking sorghum grains in water for 3 days followed by wet milling and sieving to remove bran, hulls and germ [22]. The pomace which was retained on the sieve was discarded while the filtrate was fermented for 2 days to yield *Ogi-baba*, which is sour sediment [23].

*Pito*, traditional beverage drink was prepared by soaking sorghum grains in water for 24 h, followed by malting for 5 days in baskets lined with moistened banana leaves. The malted grains were kilned at 55 °C for 24 h to remove moist and hops. The dry grains were wet milled with water (1:1 v/v) and boiled for 4 h. The resulting mash was allowed to cool and later filtered through a fine mesh basket. The filtrate thus obtained was allowed to stand at ambient temperature and allowed to ferment overnight [24]. *Pito*, the product thus obtained, was a dark brown liquid with characteristic taste.

### 2.2. Isolation of bacteria

Bacterial cells were isolated by serial dilution followed by spread plate technique [25] from the fermented products. Fermented sorghum gruels were labelled as OB (*Ogi-baba*) and beverages as PB (*Pito*) and were stored in broth at temperature 0 °C to -4 °C for future analyses.

### 2.3. Bacteriological identification

One milliliter of samples were taken from the fermented products (gruel and beverage) under aseptic conditions. Serial dilutions of the samples were prepared in 0.1% peptone water. MRS agar supplemented with 0.01% (g/v) sodium azide in order to inhibit the growth of Gram-negative bacteria was used. The diluted samples were spread on the MRS agar plates and then incubated anaerobically at 37 °C for 48 h using a Gas-pak system. Colonies were isolated, sub-cultured and purified by repeated streaking. The morphological, physiological and biochemical examination of the isolates were determined by the standard procedure of Gram staining, catalase test and gas production test.

### 2.4. Morphology and staining reaction

The isolated sample colonies were microscopically characterized.

Purification of the culture was confirmed by Gram staining: Samples were grown in MRS broths at 37 °C for 24 h under anaerobic conditions. After incubation cultures were transferred aseptically into 1.5 ml microfuge tubes and centrifuged for 5 min at 6,000 rpm. Then, supernatant was removed and cells were re-suspended in sterile water. Gram staining procedure was applied. Then, under light microscopy gram positive and purified isolates were determined [26].

### 2.5. Biochemical characterization of isolates

#### 2.5.1. Carbohydrate fermentation

Isolates were characterized according to their fermentation profiles, by the ability to ferment eight (8) different carbohydrates, namely: glucose, xylose, galactose, maltose, lactose, fructose, sucrose and dextrose. Active cells and sugar solutions were prepared separately in 10 ml MRS broths and incubated at 37 °C for 24 h. The overnight cultures were centrifuged for 10 min at 10,000 rpm. Pellets were washed twice with distilled water and re-suspended in MRS containing pH indicator bromocresol purple (0.04 g/l). Sugar solutions (100 µg/ml) were prepared. The solutions were filter sterilized with filters (0.22 µm pore diameter). The sugar filtrates (400 µl) were pipetted into new tubes containing 1.6 ml of suspended cells. Thus 2% (w/v) final sugar concentration was obtained.

Also positive and negative controls were used to indicate any contamination. 1.6 ml of suspended cells and 400 µl of glucose solution (2% w/v) were used as positive control while 2 ml of suspended cells was used as negative one [27]. After overnight incubation at 37 °C, the turbidity and the colour change from purple to yellow was recorded as positive fermentation results compared with the positive and negative controls.

#### 2.5.2. Gas production from glucose

In order to determine the homo-fermentative and hetero-fermentative characterization of isolates, CO<sub>2</sub> production from glucose test was done. MRS broths and inverted Durham tubes were prepared and inoculated with 1% (v/v) overnight fresh cultures. Then the test tubes were incubated at 37 °C for 48 h. Gas occurrence in Durham tubes was observed during 48 h which is the evidence for CO<sub>2</sub> production from glucose [27].

#### 2.5.3. Catalase test

Catalase test was performed on isolates to observe catalase production. For this purpose, overnight cultures of isolates were grown in MRS broth under anaerobic conditions. After 24 h, fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures. The reaction was allowed to go on for about 2 min and examined. The formation of gas bubbles indicates the presence of catalase enzyme [27].

#### 2.5.4. Temperature tolerance

MRS broths containing bromocresol purple (0.04 g/l) indicator was prepared and inoculated with 1% of overnight cultures of isolates. The mixtures were incubated for 5 days at 15 °C (refrigeration) and 48 h at 45 °C (in water-bath). After incubation period at specified temperatures colour change from purple to yellow was observed indicating acid production.

#### 2.5.5. Acidic tolerance

Isolates were grown in MRS broths at pH 2.0 ± 0.2, previously adjusted using 1 M HCl. The MRS broths were inoculated with 1% (v/v) of overnight cultures of isolates and incubated at 37 °C for 48 h. Aliquots of the cultures were taken after incubation period and growth was determined by measuring absorbance (A) at 540 nm. Respective samples were inoculated in MRS (pH 7.0 ± 0.2) as control.

**Table 1.** Morphological and Biochemical Analyses of Bacterial Isolates from fermented sorghum products (*Ogi-baba* and *Pito*).

Code	OB1	OB2	OB3	OB4	OB5	OB6	OB7	PB1	PB2	PB3	PB4
Colour of colony	White	White	White	Pale yellow	White	White	Pale yellow	white	white	White	white
Gram's reaction	+	+	+	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Cocci	Cocci	Rod	Rod	Rod	Cocci	Cocci	Rod	Cocci
Catalase	-	-	-	-	-	-	-	-	-	-	-
Gas from Glucose	-	-	+	-	+	-	-	-	-	+	-
Growth at 15 °C	+	+	+	-	+	+	+	-	+	-	+
Growth at 45 °C	-	-	-	+	+	-	+	+	-	+	-
Growth at pH 2.0 ± 0.2	-	-	+	-	+	+	+	+	+	-	+
Growth in 2% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth in 5% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth in 10% NaCl	-	-	-	-	-	-	-	-	+	-	-
Arginine Hydrolysis	-	-	-	+	+	-	+	+	+	-	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Xylose	-	-	+	+	-	-	+	+	-	-	+
Maltose	+	+	-	+	+	+	+	-	+	-	-
Fructose	+	+	+	+	+	-	+	+	+	+	+
Galactose	+	+	-	+	+	+	+	+	+	+	+
Glucose	-	-	-	-	+	-	-	-	-	+	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	-	-	+	+	+	-	+	+	+
Probable Microorganism	Lb	Lb	Le	St	Lb	Lb	Lb	Pc	Pc	Lb	Pc

+: positive; -: negative; Lb: *Lactobacillus* sp.; Le: *Leuconostoc* sp.; St: *Streptococcus* sp.; Pc: *Pediococcus* sp.

#### 2.5.6. NaCl tolerance

Isolates were tested for their tolerance to different sodium chloride (NaCl) concentrations. A volume of 5 ml of nutrient broths (supplemented with 0.5% dextrose) containing 2%, 5% and 10% NaCl concentrations (w/v) were prepared and 0.04 g/l of bromocresol purple indicator was added. These tubes were inoculated with 1% overnight cultures and then incubated at 37 °C for 48 h. The change of the colour from purple to yellow indicated acid production.

#### 2.5.7. Ammonia production

Arginine containing MRS broths and Nessler's reagent were used in order to detect ammonia production from arginine. MRS containing 0.3% L-arginine hydrochloride was transferred into tubes as 5 ml and inoculated with 50 µl overnight cultures. Tubes were incubated at 37 °C for 24 h. After incubation, 100 µl of cultures transferred onto a white background. The same amount of Nessler's reagent was pipetted on the

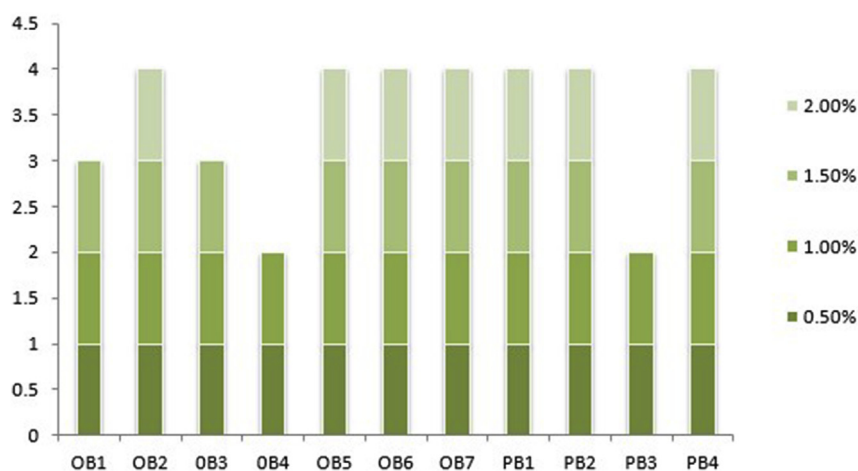
cultures. The change in the colour was observed. Bright orange colour indicated a positive reaction while yellow indicated the negative reaction. A negative control, which did not contain arginine, was also used as negative control [26].

#### 2.5.8. Bile tolerance

Isolates were inoculated into MRS broths containing Oxgall (Sigma) of varied concentrations (0.5, 1.0, 1.5 and 2.0%) and incubated at 37 °C for 24 h. After incubation period 100 µl of cultures were transferred to MRS agar by pour plate method and incubated at 37 °C for 24 h, anaerobically. The growth of isolates on the agar plate was used to confirm isolates as bile salt tolerant.

#### 2.5.9. Determination of organic antibacterial agents

The bacterial isolates were grown of MRS broth for 24 h and samples were taken for Lactic acid, hydrogen peroxide and diacetyl production.



**Figure 1.** Bile tolerance of LAB isolates from fermented sorghum products (OB and PB).

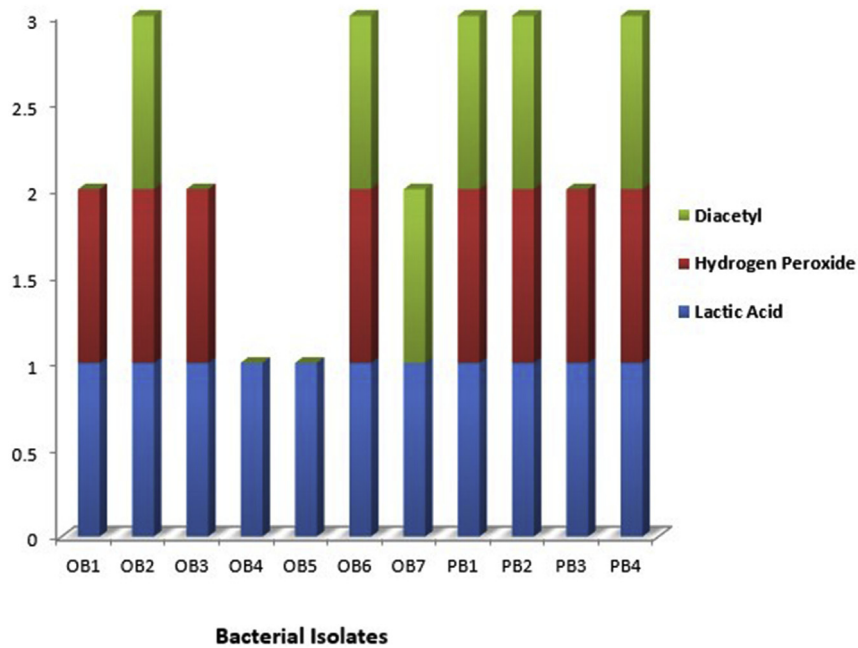


Figure 2. Production of antibacterial gases by bacterial isolates from fermented sorghum products (OB and PB).

Table 2. Antibiotic sensitivity profiles of bacterial isolates from fermented sorghum products (*Ogi-baba* and *Pito*).

Antibiotic	OB1	OB2	OB3	OB4	OB5	OB6	OB7	PB1	PB2	PB3	PB4
Chloramphenicol 25 µg	R	R	R	R	R	R	R	R	R	R	R
Erythromycin 5 µg	S	S	R	S	S	S	S	S	S	R	S
Fusidic acid 10 µg	S	R	S	S	S	S	S	S	S	S	S
Oxacillin 5 µg	R	R	R	R	S	R	R	R	R	S	R
Novobiocin 5 µg	R	R	R	R	R	R	R	R	S	R	S
Penicillin G 1 unit	S	S	S	R	R	S	S	S	S	S	S
Streptomycin 10 µg	R	R	R	S	S	S	S	R	S	R	R
Tetracycline 25 µg	R	R	S	S	S	R	R	R	S	R	S

Keys: S: Sensitive; R: Resistant ( $\geq 18$  mm).

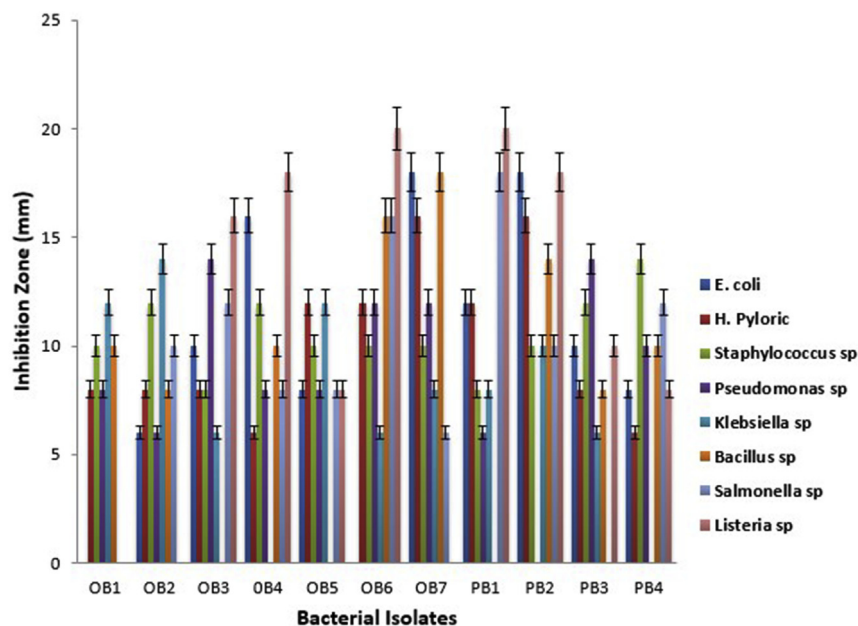


Figure 3. Antibacterial activities of LAB isolates from fermented sorghum products (OB and PB).

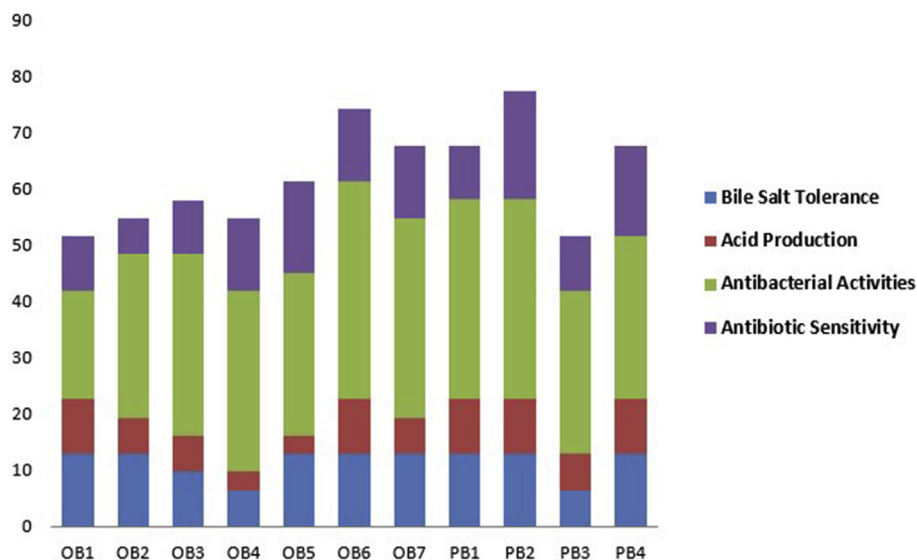


Figure 4. Probiotic potentials of LAB isolates from fermented sorghum products (OB and PB).

>KP883298.1 OB6 16S ribosomal RNA gene, partial sequence

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GTCTGATGGAGCAACGCCGCTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAAC
ATATCTGAGAGTAACTGTTTCAGGTATTGACGGTATTTAACCCAGAAAAGCCACGGCTAACTACGTGCCAGCA
GCCCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTT
TAAGTCTGATGTGAAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAACTGGGAACTTGAGTGCAGAA
GAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACCAGTGGCGAAGGCG
GCTGTCTGGTCTGTAACCTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAG
TCCATGCCGTA AACGATGATTGCTAAGTGTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTA
AGCATTCCGCCTGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGG
TGGAGCATGTGGTTAATTTCGAAGCTACGCGAAGAACCTTACCAGGCTTTGACATACTATGCCAATCTAA
GAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGAGA
TGTTGGGTTAAGTCCCAGCAACGAGCGCAACCCCTTATTATCAGTTGCCAGCATTAAAGTTGGGCACTCTGGT
GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGC
TACACAGTGTCTACAATGGATGGTACAACGAGTTGCGAGCTCGGAGAGTAAGCTAATCTCTTAAAGCCA
TTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTA
    
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Figure 5. 16S rRNA sequence of bacterial isolate OB6 from fermented sorghum product (Ogi-baba).

2.5.10. Hydrogen peroxide

Diluted sulphuric acid (25 ml) was added to 25 ml of the broth culture of the isolates. Titration was carried out with 0.1 N potassium permanganate 1 ml of 0.1 N potassium permanganate is equivalent to 1.070 mg of hydrogen peroxide. A decolourization of the sample was regarded as end point [28].

2.5.11. Lactic acid

NaOH (0.1 N) was titrated against 25 ml broth culture of the isolates using 3 drops of phenolphthalein as indicator. The NaOH was added until the colour changes to pink. Each millilitre of NaOH is equivalent to 90.08 mg of lactic acid [28].

2.5.12. Diacetyl

Into conical flasks were dispensed 25 ml broth culture of isolates and 7.5 ml of hydroxylamine solution were used for the residual titration. Titration was done with 0.1 N HCl to a greenish end point using bromophenol blue as indicator. The equivalent factor of HCl to diacetyl is 21.5 mg [28].

2.6. Antibiotic susceptibility profile

Isolates antibiotic susceptibility was determined by the ring overlay technique on solid MRS agar at pH 7 with the use of antibiotic multi-

discs (MASTRING-S™ M13): chloramphenicol (25 µg), erythromycin (5 µg), Fusidic acid (10 µg), Oxacillin (5 µg), Novobiocin (5 µg), Penicillin G (1U), Streptomycin (10 µg) and Tetracycline (25 µg) [29].

2.7. Determination of antimicrobial activities

Nutrient agar was seeded with the indicator organisms (*E.coli* and some enteropathogens) by streaking the entire surface of the culture plates and incubated at 37 °C for 6 h. Holes (6 mm in diameter) were aseptically punched out of the agar plates, and then, 100 µl overnight MRS broth cultures of the isolates were introduced into the holes and incubated aerobically at ambient temperature for 6 h and anaerobically at 37 °C for the next 18 h. After incubation period, inhibitions observed by clear zones extending laterally from the border of the isolate was noted and recorded in mm diameter [30].

2.8. Determination of probiotic potential of the isolates

Each study of the bacterial isolates was scored. Percentage probiotic potential was calculated as observed score divided by maximum score as done by Tambekar and Bhutada [31].

$$\text{Probiotic Potential} = \text{Observed Score} / \text{Maximum Score} \times 100 \quad (1)$$

**Table 3.** BLAST Lineage report of OB6 isolated from fermented sorghum product (OB: *Ogi-baba*).

Closest Genetic Match	% of Similarity
<i>Pedococcus pentosaceus</i>	100
<i>Pedococcus acidilactici</i>	99
<i>Pedococcus damnosus</i>	96
<i>Pedococcus ethanolidurans</i>	97
<i>Pedococcus pavulus</i>	95
<i>Lactobacillus kisonensis</i>	95

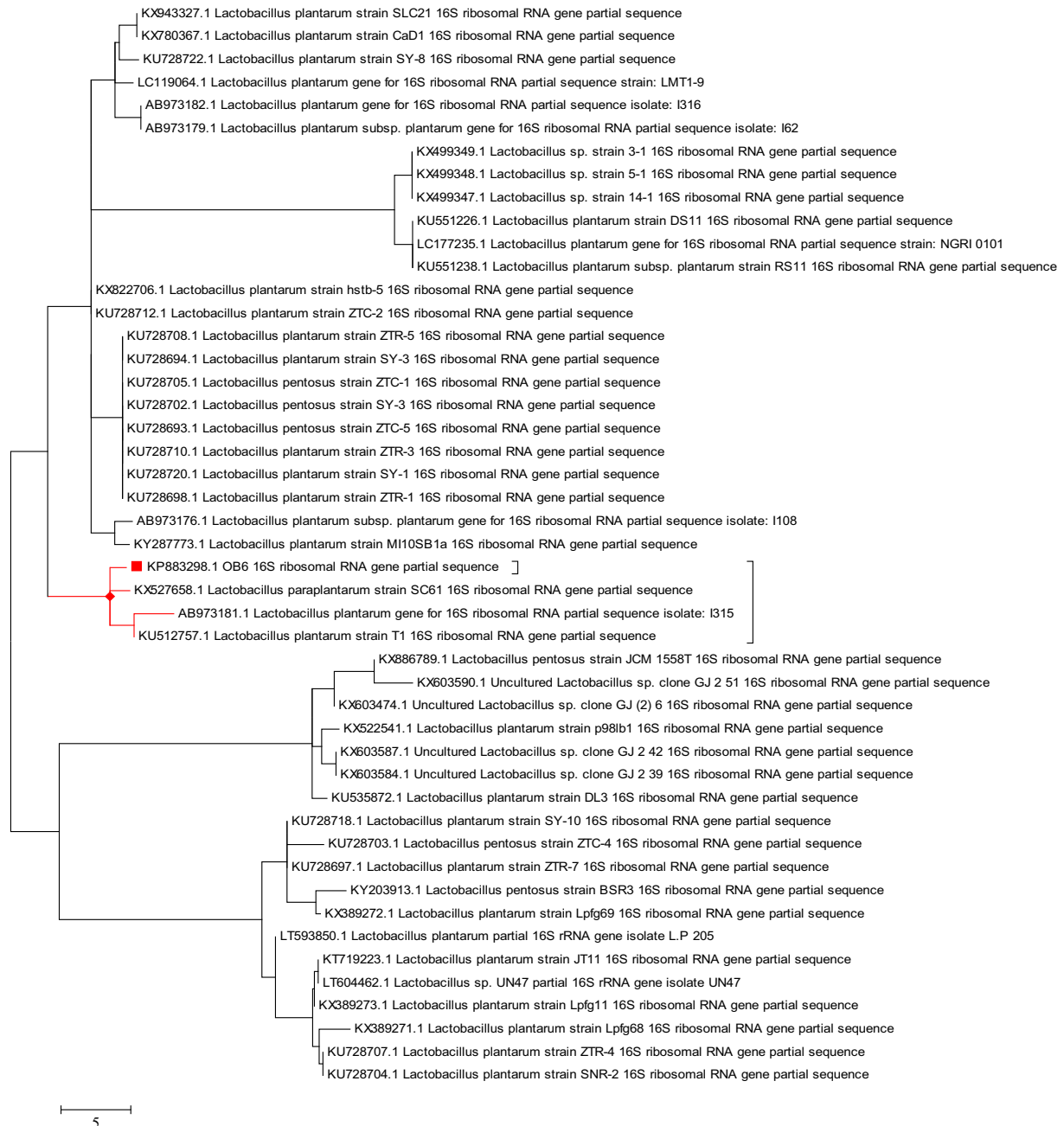
**2.9. Genetic characterization of selected isolates**

**2.9.1. Genomic DNA isolation and PCR amplification of 16S rRNA gene**

DNA was isolated from selected isolates grown in 1.5 ml of MRS broth at 37 °C for 24 h. The cells were harvested by centrifugation at 10,000

rpm for 5 min. After centrifugation, the pellet was collected and washed twice with 1 ml of 0.5 g/l sodium phosphate buffer. 1 ml double distilled water (ddH<sub>2</sub>O) was added to the pellet, vortexed and subjected to heating at temperature of 95 °C for 30 min. The suspension was then cooled immediately on ice for 30 min and centrifuged at 14,000 rpm for 1 min. The supernatant was decontaminated with equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1). DNA was precipitated with absolute ethanol from aqueous phase and washed with 70% ethanol. The washed DNA was retained for further analyses [32]. DNA pellets were air dried and re-suspended in TE buffer. An amplicon was amplified from the small subunit of 16S rRNA gene using primer set 16S1 (5'-GAGTTTGTATCCTGGCTCA-3') and 16S2 (5'-CGGCTACCTTGTTACGACTT-3').

Amplification of 16S rRNA gene was performed in a 25 µl reaction volume containing approximately 50 µg genomic DNA as the template. 5 µl of 0.2 mM deoxynucleoside triphosphates, dNTPs (Promega UI20A - UI23A,



**Figure 6.** Molecular Phylogenetic analysis of OB6 isolated from fermented sorghum product (OB: *Ogi-baba*) by Maximum Likelihood method.

### >KP883297.1 PB2 16S ribosomal RNA gene, partial sequence

CTGATGGAGCACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTG  
GGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  
GCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCCGAGCGCAGGCCGGTCTTTTAA  
GTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAGTGGGAGACTTGAGTGCAGAAGAG  
GACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCCGAAGGCCGGCT  
GTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCC  
ATGCCGTAAACGATGATTACTAAGTGTGGAGGGTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGT  
AATCCGCCTGGGGAGTACGACCCGCAAGGTTGAAACTCAAAGAATTGACGGGGGCCCGCACAAAGCGGTGG  
AGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGTCTTGACATCTTCTGACAGTCTAAGAG  
ATTAGAGTTCCCTTCGGGGACAGATGACAGGTGGTGCATGGTTGTCGTCAGTCTCGTCTGATGATGT  
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTAAAGTTGGGCACCTAGTGTAG  
ACTGCCGGTGACAAACCGGGAAGGTGGGGACGACGTCAAATCATCATGCCCTTATGACCTGGGCTAC  
ACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACC CGGAGGTTAAGCTAATCTCTTAAACCATT  
TCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATC

Figure 7. 16S rRNA sequence of bacterial isolate PB2 from fermented sorghum product (Pito).

Madison, WI, USA), 5 µl of 2.5 mM MgCl<sub>2</sub>, 5 pmol each (0.1 µl volume) of the DNA primer in PCR buffer (Promega, UK), and 5 µl of 1.25 units Taq DNA polymerase (Promega, UK) and 8 µl distilled water. Amplification conditions were as follows: an initial denaturation step of 5 min at 96 °C, 30 amplification cycles, each consisting of 30 s denaturation at 96 °C, 60 s annealing at 50 °C and 60 s elongation at 70 °C. Reactions were terminated with a final extension step for 5 min at 70 °C. PCR amplification was performed in a BIORAD thermal cycler (BIORAD iCycler, USA).

The quality of the extracted DNA was determined by agarose gel electrophoresis 1.2% (w/v), and visualized on UV-transilluminator [33].

#### 2.9.2. Sequencing and phylogenetic analysis of 16S rRNA

The PCR product was sequenced in and submitted to LARAGEN laboratory and 16S rRNA was obtained. The 16S rRNA sequences of the isolates were compared by alignment against 16S rRNA sequences of LAB available in the Genbank Database using the Basic Local Alignment Search Tool (BLAST). The sequence data was aligned and analyzed to identify the bacteria with closest neighbors based on the program at: <http://www.ncbi.nlm.nih.gov/BLAST> [34].

Phylogenetic analysis of LAB isolates 16S ribosomal RNA gene sequence through Maximum likelihood methods were carried out using MEGA 5.2.2 software and determined by tree reconstructed using Neighbor-Joining method using Tamura-Nei model [35, 36].

#### 2.9.3. β-galactosidase assay of the selected isolates

Overnight culture was streaked onto MRS agar plate containing 0.01% X-gal (5-Bromo, 4-Chloro, 3-indolyl-D-Galactopyranoside) and 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Hi Media, India) dissolved in Dimethyl sulphoxide (DMSO) as an inducer. The plate was incubated for 24 h at 37 °C and colour (blue/white) formation was observed for β-galactosidase activity [34].

#### 2.10. Statistical analyses

Statistical analysis was performed using SPSS 20.0 software (International Business Machine 'IBM' Corporation, 2014). Data are expressed

Table 4. BLAST Lineage report of PB2 isolated from fermented sorghum product (PB: Pito).

Closest Genetic Match	% of Similarity
<i>Lactobacillus plantarum</i>	100
<i>Lactobacillus pentosus</i>	99
<i>Lactobacillus vaccinostercus</i>	96
<i>Lactobacillus diolivorans</i>	94
<i>Lactobacillus kisonensis</i>	94

as mean ± SD for triplicate results. Genetic data were analyzed using bioinformatics tools such as Basic Local Alignment Search Tool (BLAST) and Molecular Evolutionary Genetics Analysis (MEGA) Version 5.2.2.

#### 2.11. Submission of strains to public strain database

The strains are made publically available. They were submitted to Laragen Incorporation, 10601 Virginia Ave, Culver City, CA90232, California, USA.

### 3. Results

#### 3.1. Identification of LAB isolates from fermented sorghum products

In the study, a total of 11 bacterial strains were isolated from fermented sorghum products. Seven isolates from gruel are labelled OB1 to OB7 and Four isolates from beverage which a labelled PB1 to PB4.

#### 3.2. Microbiological and biochemical characterization of LAB isolates from fermented sorghum products

The isolates were tentatively identified as six *Lactobacillus* species; three *Pediococcus* species; one *Streptococcus* specie and one *Leuconostoc* specie based on morphological, phenotypic physiological and biochemical characterization (Table 1). The isolates were characterized to be Gram-positive, catalase-negative and tolerant to 5% (w/v) NaCl. The isolates showed varied capabilities to ferment different sugars as confirmed by acid production (Table 1).

#### 3.3. Bile tolerance of LAB isolates from fermented sorghum products

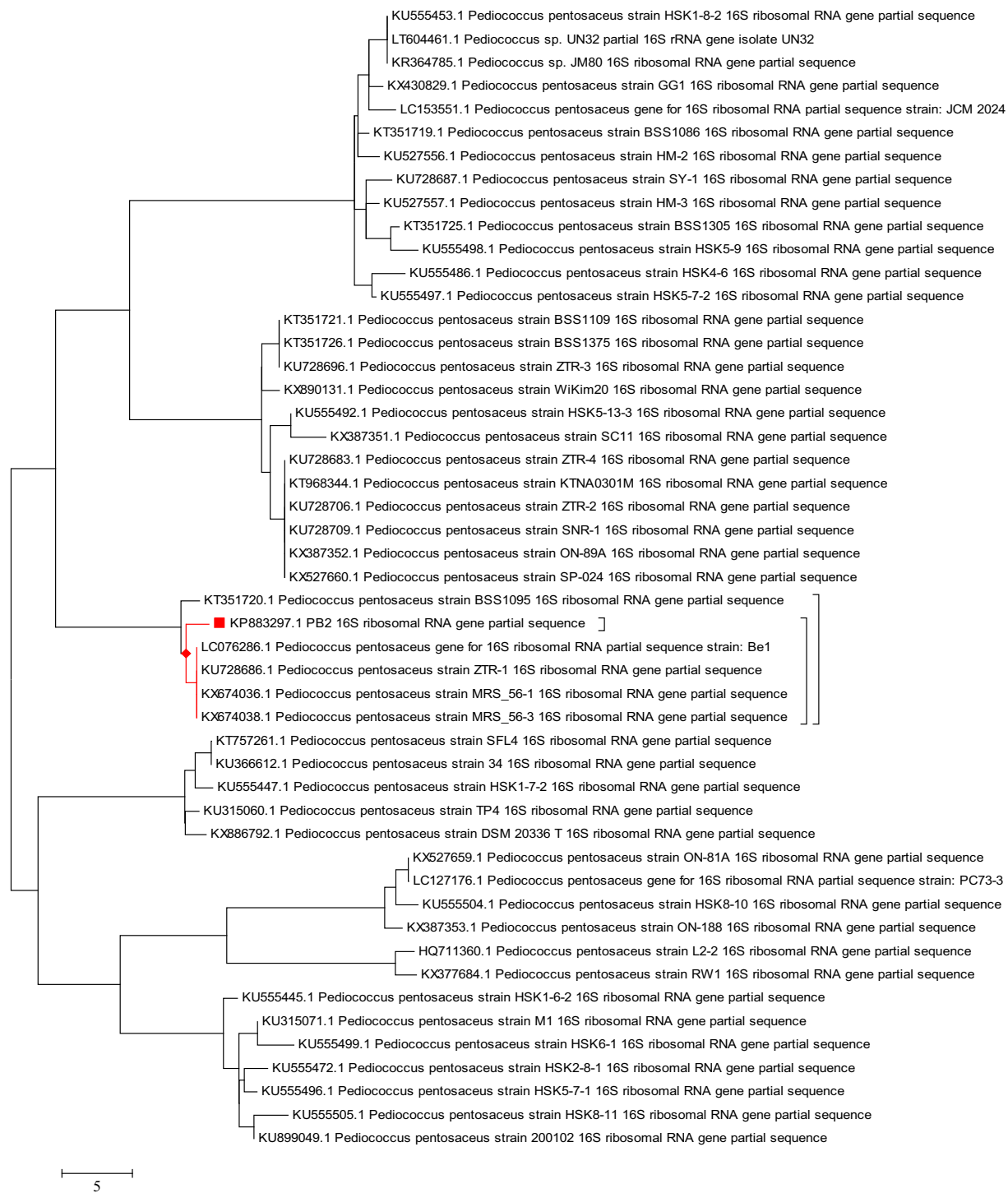
Tolerance to bile salts was observed in six isolates (OB5, OB6, OB7, PB1, PB2 and PB4) at 2% (Figure 1).

#### 3.4. Production of antibacterial agents by lab isolates from fermented sorghum products

All isolated strains produced lactic acid. However, over 75% of the isolates (OB1, OB2, OB3, OB6, PB1, PB2, PB3 and PB4) produced diacetyl whereas about 55% of isolated strains (OB1, OB6, OB7, PB1, PB2 and PB4) produced hydrogen peroxide (Figure 2).

#### 3.4.1. Antibiotic susceptibility of LAB isolates from fermented sorghum products

All isolates demonstrated high susceptibility Erythromycin 5 µg, Fusidic acid 10 µg and Penicillin G 1 unit. Isolates OB5 and PB2 exhibited



**Figure 8.** Molecular Phylogenetic analysis of PB2 isolate from fermented sorghum products (PB: Pito) by Maximum Likelihood method.

sensitivity to additional antibiotics such as Oxacillin 5 µg, Streptomycin 10 µg and Tetracycline 25 µg. However, all the isolates showed resistance to Chloramphenicol 25 µg (Table 2).

### 3.4.2. Antibacterial activities of LAB isolates from fermented sorghum products

The isolated strains exhibited more pronounced antibacterial activities against indicator Gram-positive bacteria, such as *Listeria* sp., *Bacillus* sp., and *Staphylococcus* sp., than the indicator Gram-negative bacteria (*E. coli*, *H. pylori*, *Pseudomonas* sp., *Klebsiella* sp., and *Salmonella* sp.) (Figure 3).

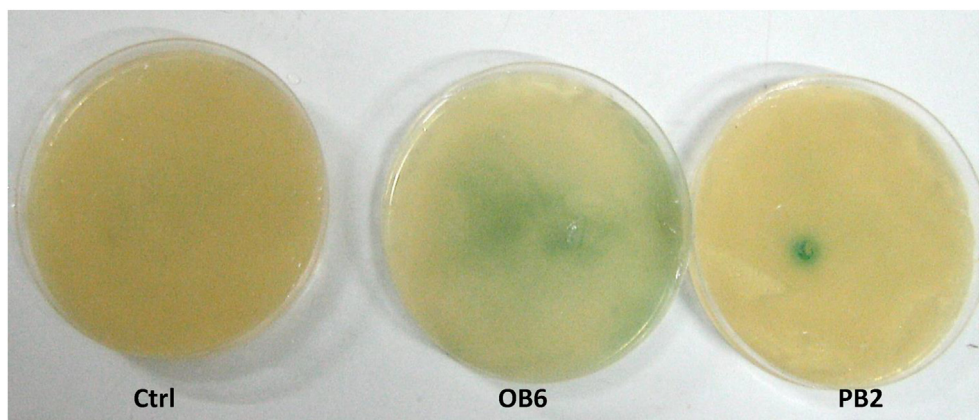
### 3.4.3. Probiotic potentials of LAB isolates from fermented sorghum products

The probiotic potentials of the isolates were calculated and observed to range between 51 and 78%; PB2 having highest of 77.42% (Figure 4).

### 3.4.4. 16S rRNA sequence of OB6 isolate from fermented sorghum gruel 'Ogi-baba'

Figure 5 shows the 16S rRNA gene partial sequence of OB6 after amplification by PCR. The sequence datum of 963 nucleotides was submitted and ascribed ascension number: KP883298.





**Figure 9.**  $\beta$ -galactosidase activity of selected strains OB6 and PB2 isolated from fermented sorghum products (*Ogi-baba* and *Pito*). OB6: *L. plantarum* OB6 grown on nutrient agar supplemented with X-gal; PB2: *P. pentosaceus* PB2 grown on nutrient agar supplemented with X-gal; Ctrl: nutrient agar supplemented with X-gal without bacterial isolate.

#### 3.4.5. Molecular identification of OB6 isolate by BLAST search

The alignment of 16S rRNA gene partial sequence of OB6 in the Genbank by BLAST run showed 100% identity with *Lactobacillus plantarum* strain 16S ribosomal RNA gene (see Tables 3).

#### 3.4.6. Phylogenetic identification of OB6 isolate from Ogi-baba

The tree with the highest similar sequences is shown. The tree is drawn to scale, with query sequence (ascension number: KP883298) belonging to OB6 isolate having 100% similarity with three *Lactobacillus plantarum* strains (Figure 6).

#### 3.4.7. 16S rRNA sequence of PB2 isolate from fermented sorghum beverage 'Pito'

Figure 7 shows the 16S rRNA gene partial sequence of PB2 after amplification by PCR. The sequence datum of 966 nucleotides was submitted and ascribed ascension number: KP883297.

#### 3.4.8. Molecular identification of PB2 isolate by BLAST search

The alignment of 16S rRNA gene partial sequence of PB2 in the Genbank by BLAST run showed 100% identity with *Pediococcus pentosaceus* strain 16S ribosomal RNA gene (see Tables 4).

### 3.5. Phylogenetic identification of PB2 isolate from Pito

The tree with the highest similar sequences is shown. The tree is drawn to scale, with query sequence (ascension number: KP883297) belonging to PB2 isolate having 100% similarity with four *Pediococcus pentosaceus* strains (Figure 8).

#### 3.5.1. $\beta$ -galactosidase assay of *P. pentosaceus* PB2 from Pito

Both strains (*L. plantarum* OB6 and *P. pentosaceus* PB2) showed blue/green colonies on MRS agar supplemented with X-GAL (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) and IPTG (isopropyl  $\beta$ -D-1-thiogalactopyranoside) and thus  $\beta$ -galactosidase activity (Plate 1) (see Figure 9).

## 4. Discussions

Food fermentations contribute substantially to food safety and food security, particularly in the rural areas of many developing countries [37]. Many foods have not been examined thoroughly to establish their health-benefits like probiotic properties. Fermented sorghum products are among such food condiments which have never been evaluated for the microbial ecology and beneficial effects to the consumers. However, this study enabled the isolation of lactic acid bacteria (LAB) and analyses of the ability to exhibit profound probiotic characteristics such as

antibacterial property, acid tolerance, bile acid tolerance as well as antibiotic susceptibility.

Enteropathogenic bacteria are of global public health concern because of the associated significant morbidity and mortality especially in low and middle income countries where they place tremendous burdens on fragile health care systems [38].

Strains of *Lactobacillus* species which are most employed as probiotics were found to predominate amongst the population of bacteria isolated from fermented sorghum which confirms the report of Ruiz *et al.* [39]. The presence of *Pediococcus pentosaceus* strain (PB2) with homo-fermentative nature indicates that it will grow substantially faster than other bacteria present in the same ecological. This may enhance its very rapid domination and establishment in a wide range of environments and will it suitable for development of a probiotic [40, 41].

It was observed that PB2 tolerated temperature as high as 45 °C. This ability to grow at high temperature is a desirable trait because a high fermentation temperature could reduce contamination by other micro-organisms less tolerant to heat [42]. Additionally, strain PB2 was more tolerant to high NaCl concentration (10% w/v) when compared with other isolates of study. Bacteria adapt to hyper-osmolarity by accumulation, synthesis and transport of compatible solutes to restore turgor. It has also been well documented that osmo-protectants could play additional positive roles and have beneficial effects on membrane integrity, thermo-protection, protein folding and stability [43] which could be a contributory factor to the thermo-tolerant ability of PB2. The tolerance of some of the isolated strains (including PB2) to acidity and bile salt is vital for bacterial survival and growth in the gastro-intestinal tract. These attributes are the main requirements for bacteria to be considered probiotics [44, 45]. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host [46, 47]. This will help probiotics to reach the small intestine and colon and contribute in balancing the intestinal micro-flora [31, 48].

This study also found the antibacterial properties of the isolated strains against enteropathogenic bacteria that are of clinical importance. The antimicrobial properties of the isolated LAB were detected to have resulted from production of lactic acid, diacetyl and hydrogen peroxide. In addition to production of organic acids and hydrogen peroxide PB2 produced other anti-enterogenic agent (diacetyl). The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic, acetic, propionic acids) which result in lowered pH [49, 50] and production of other compounds including carbon dioxide, hydrogen peroxide, diacetyl and bacteriocins among others [34]. The production of acids lowers the pH of the digestive tract and inhibits the growth of the pathogenic microorganisms [51].

The isolated LAB strains exhibited potent antimicrobial activity against *E. coli*, *Listeria* sp. and other putative entero-pathogens.

Antimicrobial activities account for restoration of gastrointestinal and respiratory tracts microbiota whereby boosting and stimulating immune activity. At present, good scientific evidence exists to support the ability of probiotics to boost human immunity, thereby preventing colonization by pathogens and reducing the incidence and severity of infections [52].

Antibiotic resistance of enteric LAB is of major concern because they are highly amenable to conjugation [53, 54] and also successful donor organisms for the transfer of antibiotic resistance genes to unrelated enterococci [55, 56], lactobacilli [57], other Gram-positives including *Bacillus subtilis* [58], *Staphylococcus* [59] and *Listeria* spp. [60]; and even Gram negative bacteria [61, 62, 63]. Conversely, sensitivity of OB6 and PB2 isolates to majority of tested antibiotics makes them potentially useful as a probiotic and an alternative to antibiotic therapy.

Phylogenetic trees were constructed by the software showing the ancestral relationship among the sequences following the use of alignment tool 'BLAST' which matched submitted sequences of selected strains of isolates OB6 and PB2, (accession numbers: KP883298 and KP883297 respectively) with deposited ancestral gene sequences in GenBank. The Maximum Likelihood phylogenetic tree produced different clusters showing their evolution relationship within different species of LAB implicated *Lactobacillus* sp. and *Pediococcus* sp. The phylogenetic affiliations of the isolates confirmed OB6 as *Lactobacillus plantarum* and PB2 as *Pediococcus pentosaceus*. These evolutionary acquisitions have made necessary changes in the genetic control of ontogeny, and this, in turn, might have caused adaptive changes in the 16S RNA gene [64].

$\beta$ -Galactosidase activity reduces lactose intolerance and is an important probiotic property [34, 65]. Therefore, the expression  $\beta$ -Galactosidase activity by *Pediococcus pentosaceus* (PB2) and a strain of *Lactobacillus plantarum* (OB6) indicates potential use in the management of lactose intolerance.

## 5. Conclusion

Fermentation is a major food processing technique that enhances the nutritive value of the products and at the same time uses probiotics for healthcare benefits. The beneficial attributes of probiotics involved in local fermentation offer exploitable means for the improvement of local fermented foods thereby helping to maintain the human gastrointestinal and respiratory tracts microbiota. This study has generated useful information on exceptional probiotic potentials of both *Lactobacillus plantarum* OB6 and *Pediococcus pentosaceus* PB2 isolated from fermented sorghum products, *Ogi-baba* and *Pito* respectively, and has opened up a vista of possible approaches to ameliorate infections of gastrointestinal and respiratory tracts in humans.

## Declarations

### Author contribution statement

Ahmed Adebisi Otunba: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Akinniyi Adediran Osuntoki and Daniel Kolawole Olukoya: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Benjamin Ayodipupo Babalola: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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### Data availability statement

Data associated with this study has been deposited at BLAST under the accession number KP883298 and KP883297.

## Declaration of interests statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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