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ENHANCEMENT OF SOURDOUGH BREAD USING PROBIOTIC LACTIC ACID BACTERIA ISOLATED FROM CORN STEEP LIQUOR

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Abstract: *Fermented Zea mays (OGI), an edible paste made from fermented grains specific to the region, is an integral part of the human diet. Corn steeping liquor (CSL) is an important by-product obtained from the wet milling of Zea mays. The present study was conducted to investigate the probiotic characteristics and in vitro antibacterial activity of lactic acid bacteria (LAB) obtained from CSL, as well as their impact on the quality of sourdough bread. A total of 46 LAB were isolated and evaluated for their probiotic potential. The probiotic potential of LAB was determined by evaluating their hemolytic capacity, bile salt resistance, phenol tolerance, antimicrobial and antioxidant activities. The lactic acid bacteria with the most promising probiotic potential were molecularly characterized and used for the production of sourdough bread (SDB). Conventional microbiological methods identified Lactobacillus plantarum (21%), Lactobacillus fermenti, and Leuconostoc mesenteroides (17%) as the predominant LAB species. Twelve LAB showed signs of gamma hemolysis. It is noteworthy that isolate CSL23 showed the highest bile tolerance, while isolates CSL15, CSL23, and CSL6 demonstrated the highest phenol tolerance. Isolates CSL15, CSL23, and CSL29 showed significant inhibition zones of 15.00 ± 0.18 mm, 20.5 ± 0.26 mm, and 22.60 ± 0.31 mm against four tested pathogens, in addition to effective antioxidant scavenging activity. Molecular identification revealed that LAB isolates CSL15, CSL23, and CSL29 were Lactiplantibacillus plantarum ROF4, Lactiplantibacillus plantarum ROF5, and Lactiplantibacillus plantarum ROF6, respectively. This finding indicates that all LAB strains belong to the same genus. Therefore, it is evident that the current study demonstrated that Lactiplantibacillus plantarum strains isolated from CSL have the potential to serve as promising probiotic starter cultures that could be used in the production of sourdough bread with improved functional properties.*

Keywords: a*ntimicrobial potential, antioxidant, corn steep liquor, fermented foods, probiotics*

1. Introduction

In recent years, consumers not only consider foods based on their nutritional requirements and perception but also in terms of the precise health benefits (such as antioxidants, antimicrobials, lowering of cholesterol, etc.). Fermented foods, such as *OGI*, can be produced locally at low cost using easily applicable traditional technologies, making them accessible to vulnerable populations [1,2,3]. The fermentation process improves the nutritional value of cereals by increasing the bioavailability of essential nutrients, reducing anti-nutritional factors, and promoting a healthy gut flora. Thus, the inclusion of fermented foods in the daily diet represents a sustainable and effective strategy for improving food security and overall health in underserved communities [4]. The primary raw material used to make *OGI* is maize (*Zea mays*), which is inexpensive, accessible, and frequently used to eliminate hunger [3]. *OGI* is a fermented maize gruel consisting of smooth cereal sediments with a layer of fermented

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water on top called corn steep liquor, which is typically thrown away as wastewater [5, 6]. The microorganisms identified from cereal-based fermented foods are lactic acid bacteria, a greatly significant microbes in food technology [7]. The LAB are rod- or cocci-shaped, Gram-positive, catalase-negative, acid-tolerant, and produce lactic acid as a significant byproduct of fermentative metabolism [8]. The LAB provides foods with a long shelf life and a diversity of flavors, and textures are produced through lactic acid fermentation. Probiotic LAB, including *Lactobacillus* sp., *Bifidobacterium* sp., *Enterococcus* sp., *Streptococcus thermophilus*, and *Pediococcus acidilactici*, have been linked to fermentation and been reported to have health benefits in humans including improved intestinal lactose digestion, shorter diarrhea incidence [9, 1], increased nutritional value due to a high rate of vitamin and mineral digestion and absorption, protection against colon cancer, a decrease in certain allergies, and lower serum cholesterol levels [11, 12]. Even though the efficiency of LAB strains employed as probiotics depends on the species and/or strain, they must nevertheless satisfy all the requirements set for acceptance as probiotics [13]. Probiotic bacteria should be able to survive in the stomach, where the pH can drop as low as 1.5, as well as in the acidic environment of the product, and should also be able to endure the bile concentrations found in the intestines [14]. Low pH in the probiotic strains' development conditions and the generation of organic acids and bacteriocins are mostly responsible for their antibacterial action [15, 16]. Probiotics are said to offer antioxidant and anti-aging properties in addition to their antibacterial properties [17, 18]. The deficiency of adequate antioxidants in the presence of extreme Reactive Oxygen Species (ROS) such as hydroxyl radicals (OH), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2), released during cellular metabolism, can result in *in vivo* oxidative impairment to biological molecules such as proteins, lipids, and chromosomes [19,20]. A plethora of human ailments, encompassing cancer, atherosclerosis, cirrhosis, inflammatory diseases, and damage to cellular macromolecules such as DNA, have been documented in the extant literature to be associated with oxidative damage. Chemical antioxidants, including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and n-propyl gallate (PG), have been shown to possess significant antioxidant properties in the context of oxidation systems. However, these substances have been subject to restrictions in certain nations due to their potential adverse effects [21]. Nevertheless, it is widely acknowledged that antioxidants from natural sources, such as LAB, are likely to be preferable to those produced chemically [22]. Rather than discarding the CSL as a by-product, it could be employed in producing functional foods due to the presence of active probiotic LAB. Sourdough bread is characterised by the presence of metabolically active microorganisms, a consequence of the fermentation process involved in its production. As posited by Fashogbon et al. [23], sourdough bread is constituted of flour, water, and LAB or exopolysaccharide (EPS), which endows the food with an acidic or sharp flavour. This sourdough bread is a traditional loaf that possesses a soft, elastic texture. The acidification process employed during its production has been shown to enhance the quality, taste, and overall desirability of the bread. It also confers a prolonged shelf life, improved flavour, and a gradual decrease in quality over time [18, 19, 20]. The vital activities during SDB fermentation include lactic fermentation, acetic fermentation, proteolysis, synthesis of volatile

compounds, anti-mold activity, and anti-ropiness [24]. Literature reports show that probiotic culture supernatants contain organic acid extracts which have been documented to have antibacterial effects [25, 26, 27]. Consequently, the pursuit of natural, reliable, risk-free, antibacterial, and antioxidant agents has gained attention as an alternative to synthetic substances. CSL could play a leading role, not only as a fermented by-product but as a source of probiotics for the production of functional foods. With implications for improving the quality of sourdough bread.

The present study thus seeks to enhance the production of sourdough bread by means of probiotic LAB isolated from CSL.

2. Materials and Methods

2.1 Sample Collection

The maize grains were obtained from Ajegunle Market in Oyo, Nigeria, in a plastic bag. The conventional method of processing described by Okoroafor et al. [28] was used to produce the *OGI*. The conventional processing technique for *OGI* was conducted within the Microbiology laboratory at the Department of Microbiology and Biotechnology, Ajayi Crowther University, Oyo, Nigeria.

It should be noted that all experiments were performed in triplicate, unless otherwise stated. The maize grains were then sorted and cleaned by subjecting them to three cycles of washing in distilled water. Following a 48-hour soaking period, the grains were subjected to a wet milling process using a two-fold grinding mill (Kenwood Heavy Duty Grinder, UK).

The wet-milled grains, produced in the local area, were subjected to a wet-sieving process using a muslin cloth. These grains were then left to ferment for a period of 72 hours at ambient temperature. The gruel produced was then permitted to settle for a period of 24 hours into *OGI* and CSL.

2.2 Determination of pH and Titratable Acidity (TTA) of the Corn Steep Liquor

The pH and TTA of the CSL were calculated according to the approved methods of the American Association of Cereal Chemists (AACC) [29]. For the pH measurement, 10 mL of the CSL was aseptically obtained in sterile tubes. A standardized pH meter (Oakton pH 550 Benchtop, Japan) electrode was inserted, and the results were recorded. Ten (10) ml of CSL was titrated against 0.1 M Sodium hydroxide (NaOH) using phenolphthalein as an indicator [30], and the TTA was calculated using the equation below.

$$\text{TTA of Lactic Acid Bacteria (mg/mL)} = \frac{(mL\ NaOH\ x\ M\ NaOH\ x\ M.E.)}{\text{Volume of sample used}} \quad (1)$$

Where mL NaOH is the volume of NaOH used, M NaOH is the Molarity of NaOH used, ME = Milliequivalent factor = 90.08 mg

2.3 Isolation and Identification of Lactic Acid Bacteria

The LAB was isolated from freshly prepared CSL using the pour plate method [31]. In brief, CSL samples were serially diluted 1:10 in sterile water, and 1 mL of each dilution was added to De Man, Rogosa, and Sharpe (MRS) agar (LabM, Lancashire, United Kingdom). Plates were allowed to be set at room temperature and then incubated at 37 ℃ for 24 h. Distinct colonies were selected and sub-cultured to obtain pure cultures [32,33]. The pure cultured isolates were characterized, and preliminary identification of isolates was also done with the use of Bergey's manual of identification as described by Morante-Carriel et al. [31] and Rozman et al. [33].

2.4 Screening of LAB for probiotic characteristics

2.4.1 Hemolytic ability

The hemolytic activity of LAB isolates was determined according to the protocol of Mangia et al. [34] and Fečkaninová et al.

[35]. Overnight cultures of LAB isolates in MRS broth were streaked onto blood agar base (Diagnostic Pasteur, France) plates containing 5% (v/v) of sheep blood and incubated overnight at 37 °C. The hemolytic activity of the LAB strains was determined by visual inspection after incubation. Alpha (α) hemolytic colonies produce a small greenish to brownish media discoloration. Beta (β) hemolytic colonies produce a lightened yellow zone surrounding the colonies. Gamma (Υ) hemolytic colonies produce no visible change to the media.

2.4.2 Resistance of the LAB to bile salts and phenol

One mL of an overnight LAB culture was inoculated into 9 mL MRS broth containing bile salt (2 or 4% w/v) or phenol (0.2 or 0.4 v/v) and incubated at 37 ℃ for 24 h. The turbidity of suspensions was determined using a spectrophotometer (BIOCHROM S21 Libra visible 325–100 nm, New York) at 620 nm compared to a control. The medium under consideration contains MRS broth, together with bile salt or phenol, yet does not contain any LAB cultures. The experiment was done in triplicate.

2.4.3 The antimicrobial potential of the LAB isolates

The antibacterial capability of the LAB isolates was determined using the agar well diffusion method [38]. The following experiment was conducted for a period of 24 hours in order to cultivate the foodborne pathogens under investigation. *Klebsiella* pneumoniae was isolated from a fresh catfish organ. The following samples were cultivated in brain heart broth: *Shigella dysenteriae* (isolated from meat pie), Staphylococcus aureus (isolated from meat pie), Escherichia coli (isolated from fresh catfish organ), and Pseudomonas aeruginosa (isolated from meat pie). The cell suspension of the test pathogens was applied to a pre-prepared Muller Hinton agar plate surface using a sterile cotton swab, thereby creating a lawn of the indicator strain. The plates were then permitted to dry at ambient temperature, after which a sterile corn borer with a diameter of 5 mm was utilized to create uniform wells in the agar. The LAB isolates in MRS broth were subjected to a centrifugation process (4 °C at 5,000 rpm, 10 min), and 60 µL of culture-free filtrate was added to each well and incubated for 24 h at 37 °C. Following the incubation period, the plates were examined for a zone of inhibition (ZOI) surrounding the well [39].

2.5. Antioxidant potential of LAB isolates

2.5.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity

Free Radical Scavenging Activity was determined as described by Kariyawasam et al. [39]. In brief, a stock solution (48 mg/100 mL) of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany) in 95% v/v ethanol was prepared, and centrifuged using a cold centrifuge at 10 ℃ at 5,000 rpm for 10 min. 1 mL of supernatant was added to 0.1 mL of the broth culture, gently shaken, and incubated at room temperature for 30 min in the dark. After the incubation, absorbance at 517 nm was determined, and DPPH scavenging activity was calculated using the equation below

$$\text{DPPH (\%)} = \frac{\text{Absorbance of standard} - \text{Absorbance of control}}{\text{ABsorbance of control}} \text{X } 100 \quad (2)$$

2.6. Identification of LAB using 16S rRNA

2.6.1. DNA Extraction

The best probiotic LAB isolates with the highest antioxidant and antimicrobial potential were molecularly characterized. Microbial DNA was extracted using the Promega USA KIT. A single colony from MRS broth containing the LAB isolate was transferred into an Eppendorf tube and centrifuged at 14,000 rpm for 2 min. EDTA

(480 µL of 50 mM) and 120 µL of lysozyme were added to the pellets obtained. The mixture was incubated in a 37 ℃ water bath for 1 h and re-centrifuged for 2 min at 14,000 rpm. The pellets obtained were taken, and 600 µL of nuclei lysis solution was added, homogenized, and incubated at 80 º C for 5 min. Three microliters of RNase Solution were added, homogenized, and incubated in 37 °C water for 1 h. After this process, Protein precipitation solution (200 µL) was added to the mixture, vortexed, incubated on ice for 5 min, and then centrifuged at 14,000 rpm for 15 min. The supernatant was transferred into a new Eppendorf tube, and 600 µL of isopropanol was added, homogenized, and centrifuged for 2 min at 14,000 rpm to obtain the pellet. The pellet was then removed, dried for 15 min at room temperature, and rehydrated with 50 µL of rehydration solution for 30 min at 65 °C [40].

2.6.2. Preparation of PCR and 16S rRNA gene amplification

Ten microliters of the primer concentrates [R primer (16S-1525R, Tm 54.3 °C, 5' AAGGAGGTGWTCCARCGCA -3) and the F primer (16S-27F, Tm 47 °C, 5' AGAGTTTGATCCTGGCTCAG -3)] were mixed in dH2O (90 µL). The 100 µL concentrates of the two primer mixtures were subsequently dissolved in a solution of Tris-EDTA (TE) buffer. The 16S rRNA primers (Master Mix PCR 12.5 L, Primer F 1.0 L, Primer R 1.0 L, dH2O 9.5 L, 24.0 L, DNA 1.0 L) were utilised to prepare the 16S rRNA gene for amplification. The Applied Biosystems 2720 Thermo cycler was utilised to execute the PCR amplification process. This process encompassed an initial denaturation step at 94 °C for a duration of 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s. The subsequent annealing phase occurred at 55 °C for 60 s, followed by an extension step at 72 °C for 60 s. The final extension step was performed at 72 °C for a duration of 10 min. The amplification process was then extended to a total duration of 10 min at 72 °C. Subsequently, the samples were chilled at 4 °C on an agarose gel.

Preparation of 1.5% agarose and running of gel electrophoresis

Tris-Acetate-EDTA (TAE) buffer (40 mL) was used to dissolve agarose (obtained from 1.5% x 40 mL = 0.6 gram), which was then heated for 30 sec and then cooled at 60 °C. 5 µL DNA stain dye (Ethidium bromide) was added to the agarose gel and mixed properly. The agarose gel was slowly poured into the gel plate to get until about 0.5 mm thickness was achieved. The gel comb, having 8 wells, was placed into the plate before pouring the gel and removed carefully after solidification. The gel, along with the gel plate, was placed into the electrophoresis tank. A 4 µL 100-bp DNA Marker was placed in the first well, and a negative control was maintained in the second well. A 5 µL PCR products were loaded from the third well onwards. The gel tank was covered, and the gel was run at 100 volts for 30 min. The gel run was stopped when the DNA Marker had run half the length of the gel. The gel was taken very carefully from the gel tank after unplugging. Then the gel was observed under a UV transilluminator [40].

2.6.3. Construction of Phylogenetic Tree

The Maximum Likelihood approach, which is based on the Tamura-Nei model, was used to infer the evolutionary history. The tree displayed the highest log probability (-2647.56) by employing the Neighbor-Join and BioNJ algorithms on a matrix of pairwise distances calculated using the Maximum Composite Likelihood (MCL) technique.

The initial tree for the heuristic search was automatically generated, and the topology with the highest log likelihood value was chosen; ten nucleotide sequences were examined. The final dataset contained 795

locations in total. In MEGA 7, evolutionary analyses were performed.

2.7. Production of sourdough bread using LAB isolated from CSL

In Sourdough bread preparation, a mixture containing 200 g of wheat flour, 120 mL of water, 2.5 g of salt, and 100 mL of sourdough starter (a mixture of equal volume of wheat flour and water, allowed to ferment for 5 days) was made according to the method of Fashogbon et al. [22].

The mixture was divided into four parts, and 10 mL of each LAB strain was added in the following proportion to the four different groups:

A: Mixture alone (Control).
B: Mixture with isolate CSL 15
C: Mixture with isolate CSL 23
D: Mixture with isolate CSL 29.

All ingredients were thoroughly mixed and kneaded in an electric mixer (Binatone electric mixer, Model: HM-350S, United Kingdom) for 15 min. The mixture was left for 40 min and kneaded again until a small piece of the dough could be stretched between four fingers without breaking.

The prepared dough was cut into smaller pieces (600 g), placed in a baking pan, covered with foil paper, allowed to proof (38 ℃ RH = 85%) for 45 min, and baked in an oven (ROBHOT HLY-309E, India) at 180 ℃ for 40 min. Once cooled, the bread was sliced, packed in plastic bags, and stored at 25 ℃ for further analysis.

2.8. Determination of acidification properties of the LAB sourdough bread (pH and TTA)

Ten grams of each sourdough was homogenized in 90 mL of distilled water, and the pH and TTA were determined as described above.

2.9. The Physical Properties of the LAB sourdough bread

2.9.1. Determination of Weight, Height, and Specific Volume of Sourdough Bread

According to the method of Cakir et al. [41], the height of the sourdough bread samples was measured in cm using a ruler, while the weight was determined using a weighing balance (Iscale Weighing Machine, India). The volume of the loaf was measured using the rapeseed displacement method with the American Association of Cereal Chemists (AACC) number 10-05.01 [42]. Briefly, a graduated container was filled with 1000 mL of rapeseeds, a loaf of bread was gently placed into the same container until fully submerged in the seed, and more seed was added until the loaf was completely submerged. Air gaps were removed by tapping the container, and the final volume of the seeds was measured. Loaf volume is calculated using the formula:

Loaf volume (mL) = Final volume – Initial volume.

The specific volume was calculated by dividing the volume by the weight. All measurements were carried out in triplicate.

2.9.2. Determination of Crumb Hardness and relative humidity of the LAB sourdough

Crust hardness was measured on days 1, 4, and 7 using a Brookfield CT3 texture analyzer (Weitech Scientifics, India) in accordance with AACC method number 74-10A. The AACC method for moisture determination using an air-dried oven was adapted to determine the moisture content of bread [43]. Bread samples (2 g) were weighed in pre-dried moisture dishes. The samples were heated at 100 ±2 °C for 3 h, removed, and placed in a desiccator to cool down before being reweighed.

2.10. Proximate analysis of sourdough bread

The crude fiber content was determined by the Acid/Alkali digestion method, the protein content by the micro Kjeldahl method, and the oil content using a Soxhlet extractor with hexane. The moisture and ash content were determined by drying to constant weight at 105 for 4 h in an oven and lighting the ash in a muffle furnace at 55 °C for 4 h, respectively [22].

2.11. Sensory evaluation/ organoleptic analysis on the bread sample

Ten untrained panelists evaluated the bread's sensory qualities using a modified version of the AACC technique 74-34 [43]. The panelists were requested to rate the samples for appearance, taste, aroma, crust, color, crack, and overall acceptability in 1, 4, and 7 days after the bread production using a 9-point hedonic scale ranging from dislike extremely (1) to like extremely (9) for each sensorial characteristic.

2.12. Statistical Analysis of Data

Data were statistically analyzed with SPSS 22.0 version (IBM, New York, USA) and subjected to analysis of variance. Tukey's test was used to separate the differences between the means ($p \leq 0.05$).

3. Results

3.1. Physicochemical parameters of the LAB isolates

The pH and TTA before fermentation (BF) of the CSL were 6.9 and 0.12% respectively. However, after 3 days of fermentation (AF), the pH decreased to 3.8 while the TTA increased to 0.26%. The total viable count of the CSL colony-forming unit ranged from 6.0 x 10^6 to 2.9 × 10^7 CFU/mL at dilutions 10^5 and $10^{7,}$ respectively, as shown in Table 1. One of the properties of probiotics is the presence of viable cells in adequate amounts to confer health benefits. The result from this research revealed that the pH of the CSL dropped from 6.9 to 3.8 in just 72 h. These findings are in line with those of Akin-Osanaiye and Kamalu [44], who found that during maize's spontaneous fermentation, the pH of the fermented grain dropped from 6.6 to 3.4 after 48 h. The drop in pH and high acidity level of the CSLs result from the presence of the fermenting microbes and the production of organic acids such as lactic acid. An increase in the TTA after fermentation is an indication that the LAB metabolizes sugar present in the grains [21].

3.2. Preliminary Characterization of LAB isolates

A total of 46 LAB were isolated after being subjected to physiological and biochemical tests. LAB isolates were cream in color, circular to irregular shape, undulated to the entire margin, raised and flat elevation, dull and shiny appearances, and were either opaque or translucent.

The examination of the cell shows rod and cocci, Gram-positive, catalase-negative, non-spore-forming, and non-motile. The isolated organisms were negative for oxidase, indole, methyl red, Voges-Proskauer test, and could not synthesize H_2S gas.

3.3. Screening for probiotic characters

3.3.1. The hemolytic potential of the LAB isolates

Out of the 46 LAB isolates, 12 (26%) had gamma hemolysis, while the remaining 34 (74%) of the LAB isolates had beta hemolysis. Notably, 12 out of the 46 LAB strains examined were non-hemolytic, and so they were selected for subsequent evaluations. The first in vitro safety parameter to be assessed was bacterial hemolytic activity. Similar findings showing that most LAB strains were non-hemolytic have already been published [50, 51]. Alpha hemolysis is the oxidation of hemoglobin to green methemoglobin by the bacteria, which gives the bacterial colonies a green appearance (false hemolysis). Red blood cells around the colony and in the media are completely lysed during beta-hemolysis, leaving the area clear and pale (yellow). According to Pradhan et al. [52], gamma-hemolysis is the absence of hemolytic activity and does not affect the colony.

3.3.2. Bile and Phenol Tolerance of the LAB Isolates

The 12 LABS with gamma hemolysis were further used to determine the tolerance to bile salts and phenol, as shown in Figure 2. The tolerance to bile salts ranged from 56.6

Table 1

Physicochemical parameters and total viable count of LAB isolated from corn steep liquor

Samples	pH		TTA (%)		Total Viable Count (CFU/mL)	
	BF	AF	BF	AF	10^5	10^7
CsI	6.9	3.8	0.12	0.28	6.0 x 10^6	2.9 x 10^8

Key: BF – Before fermentation, AF – After Fermentation

-91.5% and 45.5-94.2% at 2 and 4% concentrations, respectively. However, at 2% bile salt, isolates CSL15 and CSL 29 had the highest tolerance to bile salt, while the least was observed in isolate CSL11. At 4%, isolates CSL15, CSL 23, and CSL29 had the highest tolerance to bile salt, while the least was observed in isolate CSL44. The tolerance to phenol at 0.2% concentration ranged from 27.43 - 51.34% and 53.9 - 97.91% at 0.4%. Isolate CSL15 has the highest tolerance to phenol at 0.2% while isolate CSL 26 has the lowest tolerance. At 0.4%, isolates CSL15, CSL23, and CSL29 had the highest tolerance, and isolate CSL6 had the lowest tolerance in 0.4% phenol (Figure 1). Phenol tolerance is important for isolates to survive gastrointestinal conditions, where the gut bacteria have the ability to determine aromatic amino acids that are derived from dietary proteins and may lead to the formation of phenols [53-55]. The result from this study revealed that isolates CSL15, CSL23, and CSL29 had high bile salt and phenol tolerance. Numerous phenol tolerance cases in LAB that were isolated from naturally fermented food sources have been reported earlier [56]. The host's small intestine requires bile salt tolerance for bacterial colonization and metabolic activity [57,58]. The outcome of this research demonstrates the ability of the LAB isolates to endure gastrointestinal conditions in humans.

3.3.3. The antimicrobial potential of the LAB Isolates

The antimicrobial activity results showed that the spectrum of inhibition was different for the isolates tested against five food-borne pathogens (Table 2).

Among the 12 LABs, isolates CSL15, CSL23, and CSL29 showed strong antibacterial activity against *K. pneumoniae, S. dysenteries, S. aureus,* and *E. coli* at a range of 10.00±0.11-22.60±0.31mm. CSL29 had the highest ZOI of 22.00±0.31mm against *K. pneumoniae*, 16.00±0.170 mm against *S. dysenteriae*, 20.00±0.41 mm against *S. aureus,* and 10.00±0.06 mm against *E. coli*. The least and highest ZOI were recorded as 3.00±0.00 and 5.00±0.00 mm, respectively. The degree of antimicrobial property among the isolates was recorded in this order of CSL29 > CSL23 > CSL15. However, not all LAB isolates showed any ZOI against *P. aeruginosa*. Antibacterial activity against infections is another essential feature of probiotic properties.

Three of the LAB strains (CSL15, CSL23, and CSL29) exhibited antagonistic effects on the test pathogens. Probiotics have a complicated and multifaceted antibacterial action, primarily via competing with binding sites and nutrients, activating the immune system of the host to selectively reject infections, and generating inhibitory metabolites against unfavorable microbes [59]. The antagonistic action of LAB is maintained by the release of several antimicrobial substances, including alcohols, hydrogen peroxide, bacteriocins, organic acids (lactic, acetic, and so forth), and antimicrobial peptides [60–61]. Nallala et al. [61] isolated three LAB from maize

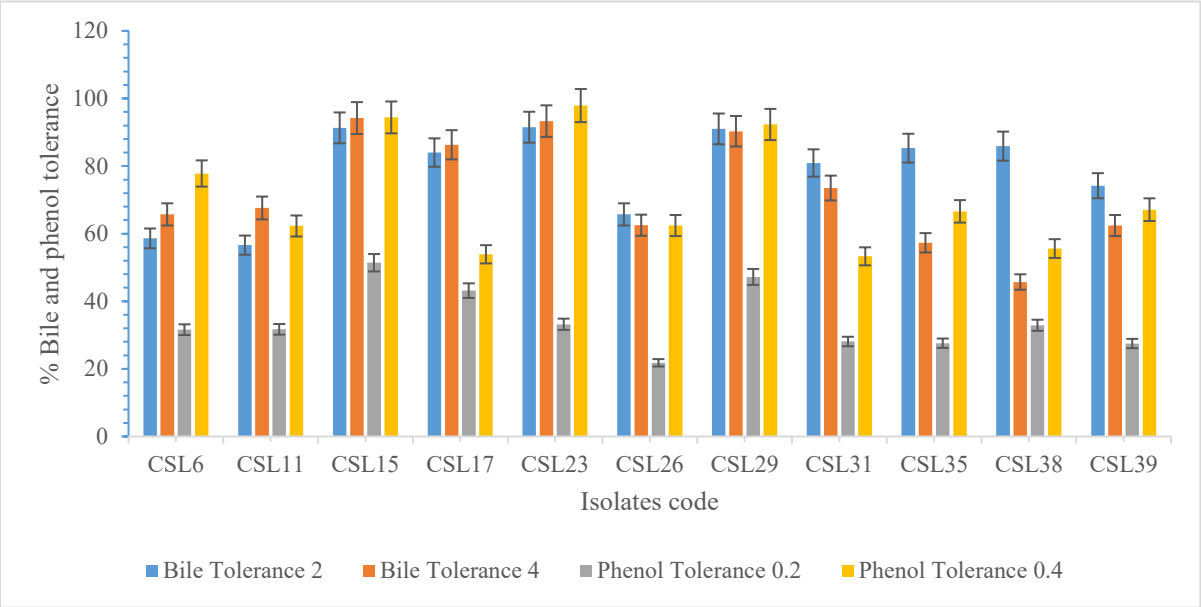

Fig. 1. % Bile salt and phenol tolerance of LAB isolated from CSL

waste liquor and demonstrated their antibacterial activity against several indicator microbes.

3.4. Antioxidant potential of the LAB isolates

The DPPH radical scavenging assay result is presented in Figure 2.

The DPPH value ranged from 51.2-79.2%. CSL23 demonstrated the highest DPPH scavenging activity (79.2%), followed by isolate CSL15 (77.8%) and isolate CSL26 (76.4%), while the least was recorded in isolated CSL38 (51.2%).

In this current study, CSL was used to isolate putative probiotic lactic acid bacteria with good antioxidant potential.

The DPPH free radical scavenging activities indicated that isolates CSL23, CSL15, and CSL26 showed the highest antioxidant activity. Strong antioxidant activity was also demonstrated by *P. acidilactici* ATCC 8042 *in vitro*, and *L. plantarum* C88 as reported by Mohammed et al. [62]. Oxidative stress evolves when the body's antioxidant capacity is insufficient to handle or eliminate free radicals [63]. Free radicals are extremely unstable chemicals that interact with other molecules in the body to damage DNA, enzymes, and cellular membranes.

3.5. Identification of LAB using 16S rRNA

The three eminent probiotic properties with the highest probiotic activity were identified by 16S rDNA gene sequence and phylogenetic analysis as reported in Table 3, and Figure 3. The isolates were grouped with certain reference probiotic strains (RPS). The molecular analysis revealed that isolate CSL 15 had 96.27% similarity to *L. plantarum* ROF4, Isolate CSL 23 had 92.92% similarity to *L. plantarum* ROF5, while isolate CSL 29 had 92.58% similarity to *L. plantarum* ROF6 with accession numbers OR405527, OR405526, and OR405528, respectively. In the study conducted by Ajayeoba and Ijabadeniyi [6], the isolation and characteristics analysis of LAB from *OGI*. liquor showed *L. plantarum* stain RPS with a similarity value of 81% using BLAST analysis.

According to Amelia et al. [64], the molecular analysis of LAB from dadiah also obtained *L. plantarum* strain DAD-13 with a similarity value of 97 – 100% using BLAST analysis. Ohaegbu et al. [65] also reported the isolation of *L. plantarum* strain ci-4w in Nigerian traditional fermented foods.

Table 2

Antibacterial potential of LAB isolated from CSL

Isolates code	Test Pathogens/Zone of Inhibition (mm)				
	K. pneumoniae	***Shig. dysenteries***	***S. aureus***	***E. coli***	***P. aeruginosa***
CSL6	6.00±0.15	3.00±0.00	5.00±0.18	5.00±0.07	3.00±0.13
CSL11	5.00±0.32	3.00±0.19	6.00±0.35	3.00±0.00	3.00±0.02
CSL15	15.00±0.18	10.00±0.11	15.07±0.32	8.00±0.21	3.00±0.50
CSL17	3.00±0.10	3.00±0.01	4.00±0.20	4.00±0.09	3.00±0.31
CSL23	20.00±0.26	12.00±0.12	12.70±0.10	15.00±0.09	3.00±0.22
CSL26	00.00±0.12	6.00±0.20	8.00±0.04	3.00±0.00	3.00±0.32
CSL29	22.60±0.31	16.00±0.17	20.30±0.41	10.00±0.06	3.00±0.31
CSL31	7.00±0.12	3.00±0.11	3.00±0.00	0.00±0.00	3.00±0.21
CSL35	3.00±0.01	5.00±0.28	3.00±0.00	4.00±0.07	3.00±0.02
CSL38	5.00±0.09	30.00±0.20	3.00±0.00	30.00±0.03	3.00±0.03
CSL39	10.00±0.61	8.00±0.20	3.00±0.00	7.00±0.15	3.00±0.31
CSL44	5.00±0.22	5.00±0.12	4.00±0.30	3.00±0.02	3.00±0.20

Values are presented as means ± standard deviation and mean values.

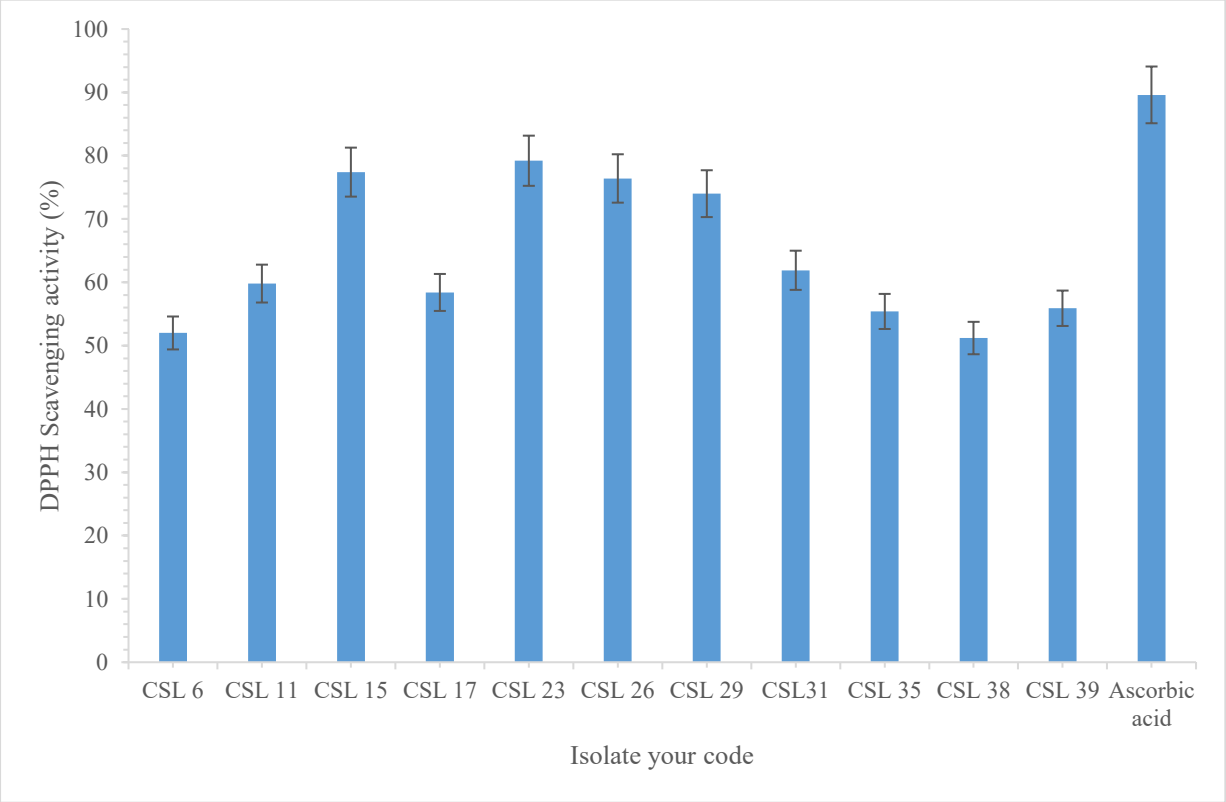

Fig. 2. % DPPH scavenging activity of the LAB isolated from CSL

Table 3

16S-rRNA sequencing of isolated LAB from CSL

Isolate Code	Species identity	Foreign	Accession number	Similarity Percentage (%)	Molecular Identification
CSL 15	*Lactobacillus plantarum*	OR405526	*Lactobacillus plantarum* strain 4619	96.27	*Lactiplantibacillus plantarum* foreign ROF4
CSL 23	*Lactobacillus plantarum*	OR405527	*Lactobacillus plantarum* strain HT-W91-B2	92.92	*Lactiplantibacillus plantarum* foreign ROF5
CSL 29	*Lactobacillus plantarum*	OR405528.	*Lactobacillus plantarum* strain PS1-3	92.58	*Lactiplantibacillus plantarum* foreign ROF6

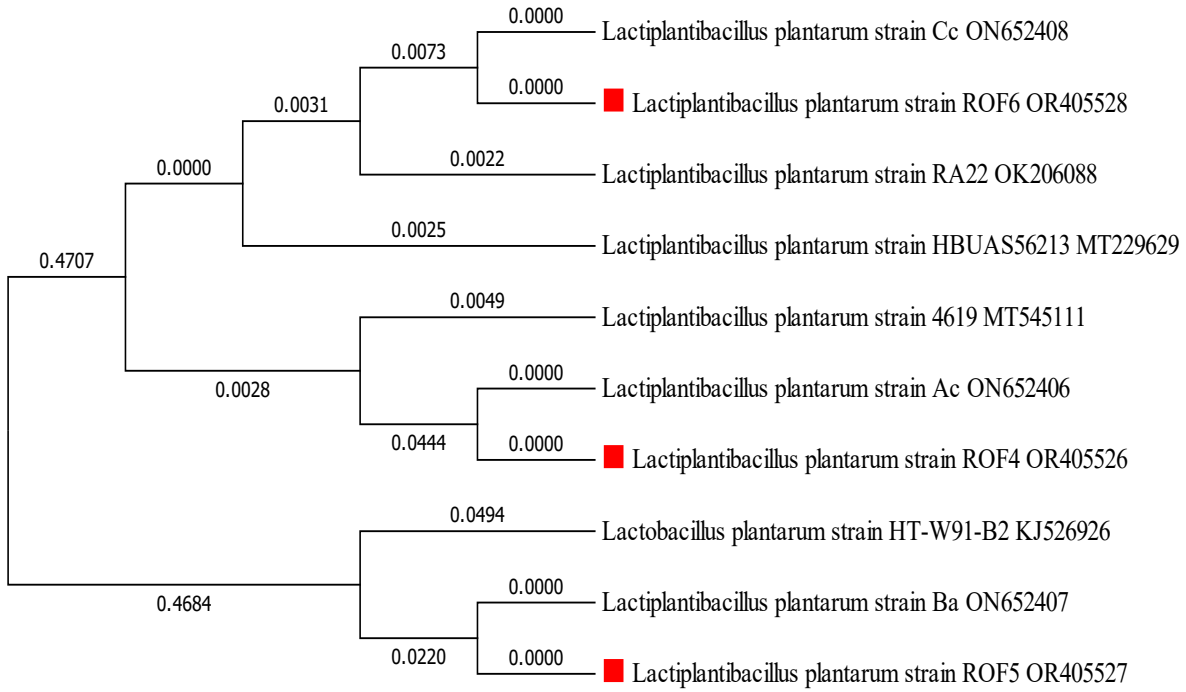

Fig. 3. Molecular phylogenetic analysis of LAB amplicons using the Maximum Likelihood method

3.6. Acidification and physical properties of the probiotic starter fermented sourdough

Various LAB used in this study showed different ranges of pH and TTA in the sourdough bread, as shown in Table 4. The pH value ranged from 3.9±0.15 – 5.9±0.58 while the TTA ranged from 0.21±0.32 - 0.39±0.21%. The lowest pH and the highest TTA were recorded in sample D (Sourdough bread inoculated with *L. plantarum* ROF6). The control sample had the highest pH and the lowest TTA. The height, weight, and specific volume of the bread samples ranged from 1.3±0.03 – 2.8±0.10 cm, 173.8±0.24 – 311.1±0.19 g, and 4.34±0.62 – 7.99±0.41 cm^3/g, respectively. Sample D (Sourdough bread inoculated with *L. plantarum* ROF6) had the highest value for weight (311.7±13.7 g) and height (2.8±0.24 cm), followed by samples C (Sourdough bread inoculated

with *L. plantarum* ROF5) and B (Sourdough bread inoculated with *L. plantarum* ROF4). In contrast, sample A (control) had the lowest value. All the bread samples containing LAB starter in the sourdough were significantly higher in volume compared to the control without the LAB ($p \leq 0.01$). Similar researchers reported that sourdough samples seeded with LAB had the lowest pH and the highest TTA compared to the control (without LAB cultures) [66-68]. Low pH can benefit the dough by boosting the activity of amylases and proteases in the flour and enhancing the digestion of bread Limbad et al. [69, 70, 71].

Table 4

Acidification and physical properties of the sourdough

Sample Code	pH	TTA (%)	Weight (g)	Height (cm)	volume (mL)	Specific volume (cm^3/g)
A (Control)	5.9±0.58	0.21±0.32	173.8±7.91	1.3±0.23	1050±9.33	6.04±0.35
B	4.3±0.51	0.25±0.43	251.1±28.74	2.2±0.26	1090±7.25	4.34±0.62
C	4.3±0.04	0.33.0±0.41	250.3±24.5	2.5±0.24	2000±8.78	7.99±0.41
D	3.9±0.15	0.39±0.21	311.7±13.7	2.8±0.24	2100±24.88	6.73±0.27

∗ Data are the mean of three replication analyses and standard error of the means (±SEM); Sample A: Control; Sample B: Sourdough bread inoculated with *L. plantarum* ROF4; Sample C: Sourdough bread inoculated with *L. plantarum* ROF5; and Sample D: Sourdough bread inoculated with *L. plantarum* ROF6.

3.7. Effect of LAB on the Crumb Hardness of Sourdough

The effect of LAB on the texture of produced sourdough bread revealed a significant increase ($p \leq 0.05$) in the crumb hardness after day 1, 4, and 7 of production. The results showing the mean values for the crumb hardness as shown in Figure 4 revealed that sample A (control) had the highest level of crumb hardness on the first day of production (39.91±0.05 N) to the last day of production (49.20±0.13 N) while the lowest hardness was recorded in sample D (26.1±0.21 N) to the last day of production (32.24±0.27 N). The humidity of the sourdough bread ranged from 40.43±1.75 – 45.25±0.52%, 36.39±0.52 – 42.67±0.00% and 30.19±0.33 – 35.01±0.22 % at day 1, 4 and 7 respectively. It was observed that the humidity dropped as the days increased. Sample D had the lowest humidity at day 7. Contrary to the findings of Torrieri et al. [72], bread containing sourdoughs inoculated with LAB starters was less stiff than control samples after six days of storage. This research supports the findings of Hadaegh et al. [67], who found that control sourdough had the highest level of crumb hardness among the sourdough samples. The findings of Palla et al. [73] and Saeed et al. [74] also indicated that the concentration and sourdough starter strains had an impact on the samples' crumb hardness.

3.8. The Proximate analysis and organoleptic properties of Sourdough bread produced with *Lactiplantibacillus plantarum* ROF4, 5, and 6.

The physical properties of the sourdough bread samples are shown in Table 5. The result revealed a significant difference in the crude protein, crude fat, % ash, % carbohydrate, and moisture of the different sourdough samples. Sample D has the best proximate analysis. The average values for sensory evaluation of the different bread samples are shown in Table 6. The appearance and color of the samples containing LAB starters were significantly preferred by the panelists. Based on the results achieved by instrumental analysis, this study's results showed that Sample D had the best ratings for color, appearance, taste, texture, and general acceptance. The three sourdough breads used in this study had protein ranging from 16.20.73% –

11.70.21%, which is very close to the results of Gobbetti et al. [75] and Limbad et al. [68]. According to reports, the ash level of flour has an impact on the sensory quality of sourdough bread; the lower the ash content of the flour, the better the bread's sensory quality. All sourdough bread produced in this study has a low ash concentration of less than 1% [75, 76]. Additionally, this is consistent with the findings of Hadaegh et al. [66]. According to Păcularu-Burada et al. [77], the organoleptic properties of sourdough are regulated by the activity of LAB, resulting in various flavors. Therefore, in line with other studies, the appearance and color of the samples containing LAB starters were significantly more preferred by the panelists [77].

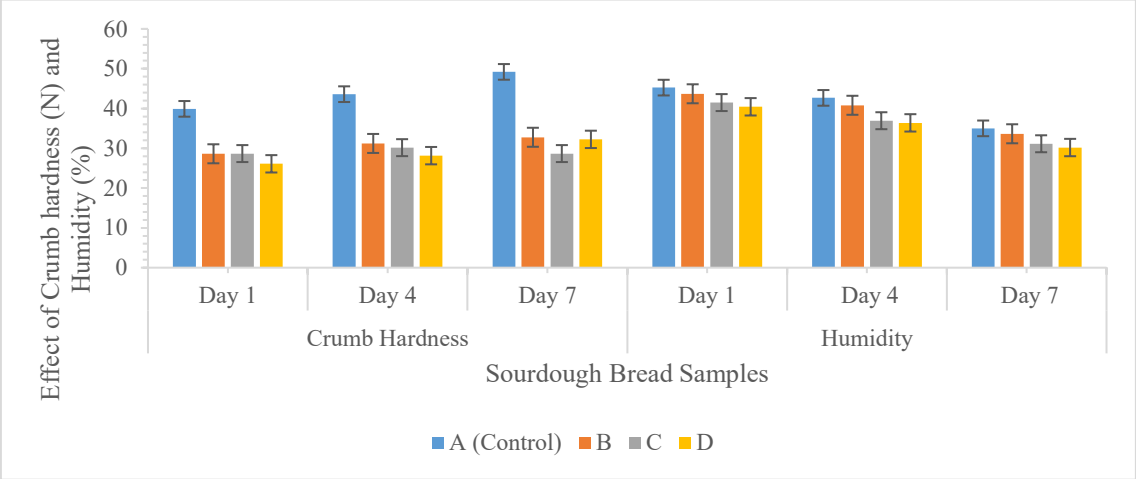

Fig. 4. LAB on crumb hardness of sourdough

Data are the mean of two replication analyses and standard error of the means (±SEM); Sample A: Control sample (Sourdough bread without LAB); B: Sourdough bread inoculated with *L. plantarum* ROF4; Sample C: Sourdough bread inoculated with *L. plantarum* ROF5; and Sample D: Sourdough bread inoculated with *L. plantarum* ROF6.

Table 5

Proximate analysis of the probiotic starter produced sourdough bread

Samples	% Crude protein	% Crude fat	% Ash	% Carbohydrates	% Moisture
A	16.2±0.73	1.61±0.32	0.43±0.47	54.7±0.51	38.4±0.17
B	11.2±0.01	1.3±0.11	0.38±0.36	43.1±0.38	30±0.06
C	11.7±0.21	1.29±0.49	0.34±0.29	42.4±0.62	31.3±0.1
D	11.4±0.28	1.35±0.13	0.4±0.81	44.4±0.20	29.1±0.29

∗ Data are the mean of two replication analyzes and standard error of the means (±SEM); different letters in each column indicate statistically significant differences; Sample A: Control; Sample B: Sourdough bread inoculated with *L. plantarum* ROF4; Sample C: Sourdough bread inoculated with *L. plantarum* ROF5; and Sample D: Sourdough bread inoculated with *L. plantarum* ROF6.

Table 6

Organoleptic properties of probiotic starter produced sourdough bread

Samples	Dye	Appearance	Keys	Textures	Overall Acceptability
A	5.79±0.06	5.38±0.61	5.32±0.21	5.67±0.21	5.11±0.02
B	8.20±0.18	6.93±0.49	5.89±0.44	7.35±0.71	6.62±0.10
C	7.940±0.52	7.03±0.37	6.00±0.19	7.12±0.39	7.10±0.71
D	8.530±0.68	7.88±0.13	6.49±0.43	6.91±0.83	7.47±0.20

∗ Data are the mean of three replication analyses and standard error of the means (±SEM); Sample A: Control; Sample B: Sourdough bread inoculated with *L. plantarum* ROF4; Sample C: Sourdough bread inoculated with *L. plantarum* ROF5; and Sample D: Sourdough bread inoculated with *L. plantarum* ROF6.

4. Conclusion

The effect of different probiotic LAB strains on qualities such as: humidity, weight, height, volume) of the produced sourdough bread was studied. The best three probiotic LAB were molecularly identified as *L. plantarum* ROF4, *L. plantarum* ROF5 and *L. plantarum* ROF6. These LAB isolates were able to tolerate bile salt and phenol, exhibiting good antimicrobial and antioxidant potential. Sourdough fermentation with the use of LAB starter cultures apparently increased the weight, height, and volume of the bread. To a greater extent, the presence of *L. plantarum* ROF4, *L. plantarum* ROF5, and *L. plantarum* ROF6 in the sourdough bread greatly enhanced the proximate and organoleptic properties of the sourdough bread produced in this study.

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Conflict of Interest

The authors declare that they have no conflict of interest

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Supplementary list

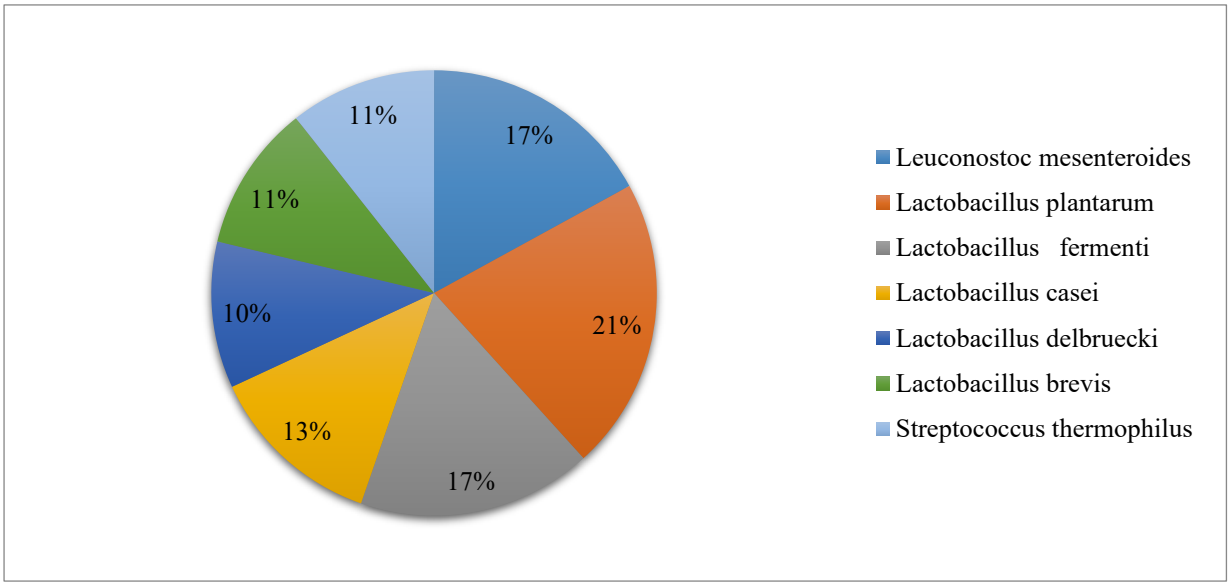

Fig.1 Percentage frequency of the LAB isolates

Table 2

Hemolysis ability of isolated LAB from CSL

Isolates wavering	Hemolysis Test	Isolates wavering	Hemolysis Test	Isolates wavering	Hemolysis Test
CSL 1	γ	CSL 16	γ	CSL 31	B
CSL 2	γ	CSL 17	β	CSL 32	γ
CSL 3	γ	CSL 18	γ	CSL 33	γ
CSL 4	γ	CSL 19	γ	CSL 34	γ
CSL 5	γ	CSL 20	γ	CSL 35	β
CSL 6	β	CSL 21	γ	CSL 36	γ
CSL 7	γ	CSL 22	γ	CSL 37	γ
CSL 8	γ	CSL 23	β	CSL 38	β
CSL 9	γ	CSL 24	γ	CSL 39	B
CSL 10	γ	CSL 25	γ	CSL 40	γ
CSL 11	B	CSL 26	B	CSL 41	γ
CSL 12	γ	CSL 27	γ	CSL 42	γ
CSL 13	γ	CSL 28	γ	CSL 43	γ
CSL 14	γ	CSL 29	γ	CSL 44	B
CSL 15	γ	CSL 30	γ	CSL 45	γ
				CSL 46	γ

Key: *γ* – Gamma hemolysis; *β* – Beta hemolysis