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Characterization of potential probiotic and safety properties of *Levilactobacillus brevis* isolated from traditionally fermented milk, *Amabere amaruranu*

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ABSTRACT

The study aimed to determine the probiotic and safety properties of a bacterial strain isolated from Kenyan traditionally fermented milk called Amabere amaruranu. Probiotic characteristics of the isolate were assessed based on its ability to survive artificial simulated conditions of the digestive tract including temperature sensitivity, low pH and phenol tolerance, and antagonistic activity against human bacterial (*Staphylococcus aureus*, *Escherichia coli,* and *Salmonella enterica Typhimurium*) and fungal (*Candida albicans*) pathogens. Safety analysis was based on hemolytic activity and antibiotic susceptibility against most common antibiotics namely: nalidixic acid, ampicillin, azithromycin, ciprofloxacin, tetracycline, gentamicin, and chloramphenicol using zone of diameter of inhibition (ZDI). The isolate survived and grew in low pH (2.0–3.5), tolerated 0.4% phenol, and survived temperature ranges of 20°C, 30°C, and 37°C but showed partially reduced growth at 45°C. It exhibited strong antagonism against all pathogens (ZDI>20 mm). Antagonism was strongest towards *S. enterica* serovar Typhimurium (ZDI = 59 mm) and lowest towards *E. coli* (ZDI = 33 mm). The isolate was sensitive to azithromycin (ZDI = 31± 2.08), chloramphenicol (ZDI = 26 ±2.34 mm), gentamycin (ZDI = 26 ±1.41mm), and tetracycline (ZDI = 32 ±1.73 mm), resistant to nalidixic acid (ZDI = 0.0 ±0.00 mm), while susceptibility towards ampicillin and ciprofloxacin was intermediate. The isolate also exhibited γ -hemolytic activity and was identified as a *Levilactobacillus brevis* strain based on 16S rRNA gene sequencing. The isolate exhibited probiotic potential and was safe, affirming its potential application as a probiotic in the formulation of functional foods.

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1. Introduction

Probiotics are live microbial cultures, which when consumed in adequate amounts exert health benefits to the host (1).

Exertion of health benefits by probiotics is achieved through modification of the inherent microbial community thereby improving the ambient environment of the gastrointestinal tract, and ameliorating the host's response to diseases (2).

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Members of the genera Lactobacillus, Bifidobacterium, Lactococcus, Pediococcus, Enterococcus, Streptococcus, Leuconostoc, and Saccharomyces have been the most studied probiotic strains with potential beneficial effects on human health (3). The ability of these organisms to produce antimicrobial compounds, which inhibit the growth of pathogens makes them highly desirable by consumers (4). The antimicrobial compounds produced include bacteriocins, reuterin, proteinaceous compounds, organic acids, and secondary metabolites that not only lower the pH hence affecting pathogen growth but also exhibit toxicity to the pathogens (5).

The ability to reduce lactose intolerance in milk, alleviate diarrheal diseases by competitive exclusion of pathogens, reduce blood cholesterol, increase immune modulation and prevent cancer, makes probiotics to be considered prudent agents to confer a number of health benefits to the host (6). The Food and Agriculture Organization and World Health Organization (FAO/WHO) have defined guidelines that establish the safety and effectiveness standards for the selection criteria of probiotics (7). The suggested selection criteria include safety assessment of the organism and the ability to resist harsh conditions of the human gastrointestinal tract based on tolerance to body temperature, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonize the gastrointestinal tract, production of antimicrobial substances, ability to stimulate a host's immune response, and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity (3,8).

A food can be regarded as a functional food if it satisfactorily demonstrates to be beneficial to one or more target functions in the body, beyond adequate nutritional benefits, in a way that is relevant to the improved stage of health including well-being and reduced risk of disease to the host (6). Recently, consumers have become aware of their health and nutrition, increasing the demand and consumption of functional foods (9). The current probiotics being used in Kenya to produce functional foods like yogurt and other fermented foods are imported and hence expensive.

The isolation and application of probiotic microorganisms from traditionally fermented food sources can extend the possibility of recovering local strains with improved functional characteristics. In a previous study (10), Lactobacillus strains were isolated from a Kenyan traditionally fermented milk product (locally known as amabere amaruranu or mursik depending on the community making the product). Lactobacillus strains are lactic acid-producing bacteria that are common inhabitants of fermented foods (11). Lactobacillus strains are facultative anaerobes whose strains are known to have significant health effects such as lowering blood glucose levels in type 1 diabetes, high antioxidant activity, good in vitro adherence to human intestinal cells, and immune-modulating ability (12, 13). This indicates that although the role of the isolate in milk fermentation is not known, it could be conferring health benefits to the consumers. In the present study, we investigated the probiotic potential and safety status of the isolate and identified it through 16S rRNA gene sequencing to validate its use in food formulation.

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2. Materials and Methods

2.1. Test organism strain

The isolate that was used in this study was previously isolated from a Kenyan traditionally fermented milk product, *amabere amaruranu* (10).

2.2. Determination of temperature tolerance

The temperature tolerance assay was performed according to Wang *et al*. (14) with modifications. The overnight cultured isolate was inoculated into MRS broth and then incubated aerobically at different temperature ranges of 20°C, 30°C, 37°C and 45°C for 2 h and 4 h. Thereafter, 0.1 mL volume of the inocula from each of the temperature-time-treatment were transferred to MRS agar plates by a surface plating method and incubated aerobically at 37°C for 48 h. The growth of the test organism on the plate was used to designate the number of survivors after exposure to the different temperature-time treatments. The survival cell counts were expressed as log values of colony-forming units per mL. The results were obtained from triplicate cell counts.

2.3. Determination of acid tolerance

An acid tolerance test was performed according to the method described by Chen et al. (15). MRS broth that had been regulated to pH 2.0, 2.5, 3.0, and 3.5 with 0.1 M HCl was used. A volume of 0.1 mL of MRS broth culture of the isolate (grown at 30°C for 12 h) was added to the acidified MRS broth. Acid-free MRS broth was used as a control. This was followed by aerobic incubation for 2 h and 4 h at 30°C. Thereafter, 0.1 mL volume of the inocula from the different treatments were transferred to MRS agar plates by a surface plating method and incubated aerobically at 30°C for 48 h. Growth of the test organism on the plate was used to designate the isolate as acid tolerant. The results that were obtained from triplicate colony counts were used to determine the survival rate (16).

2.5. Determination of phenol tolerance

The experiment on phenol tolerance was done as per the method by Yasmin et al. (17) with modifications. Here, test tubes containing MRS broth were adjusted to contain 0.4% (w/v) phenol. They were then inoculated with 1 mL of fresh overnight culture of 10^9 cfu/ mL inocula of the isolate and incubated at 30°C for 24 h. After 24 h, surface plating was done onto MRS agar plates and thereafter incubated for 48 h at 30°C. The growth was determined by colony counts on the MRS agar plates. This was done in triplicate and the control was done similarly but without phenol treatment.

2.6. Antibiotic susceptibility assay

The antibiotic susceptibility of the isolate was performed against seven antibiotics by the disc diffusion method as described by Chen et al and Yasmin et al. (15,17). The antibiotics used were; ampicillin (10-µg/disc), azithromycin (15-µg/disc), chloramphenicol (30-µg/ disc), ciprofloxacin (30-µg/disc), gentamicin (10-µg/disc), nalidixic acid (30-µg/disc), and tetracycline (30-µg/disc). The test organism was inoculated into MRS broth and then incubated aerobically at 30°C for 12 h. After 12 h, a volume of 0.1 mL of 10^8 cfu/ mL inocula was surface plated on MRS agar. The antibiotic discs were then placed centrally on the surface of the plates containing MRS agar with the test organism and then incubated aerobically at 30°C for 48 h.

The zone of diameter inhibition (ZDI) measurement

values were obtained and interpreted according to the criteria method of Halder et al. (19) whereby ZDI: <15 mm, ZDI: >21 mm, and ZDI: 16–20 mm were grouped as resistant, sensitive, and intermediately susceptible respectively. This was done in triplicates.

2.7. Hemolytic activity assay

The hemolytic activity of the isolate was performed using a method by Meleh et al. and Wang et al. (14,20). The MRS broth culture that was grown overnight was streaked onto 5% defibrinated sheep blood agar plates and incubated aerobically at 30°C for 48 h. Following incubation, the plates were examined for hemolytic activity and classified on the basis of the lysis of red blood cells in the medium around the isolate colonies. The greenish zones around colonies (α-hemolysis), clear zones around colonies (β-hemolysis), and no zones around colonies (γ-hemolysis) on the blood agar plates. Candida albicans was used as a positive control. Strains with γ-hemolysis are considered safe (17).

2.8. Molecular characterization of the Lactic acid bacteria isolates

Genomic DNA extraction from the isolate and handling was previously described by Katiku et al. and Sichangi et al. (10,21). Samples of dissolved DNA were sent to Inqaba biotechnical industries Ltd, Pretoria, South Africa, for 16S rRNA partial gene sequencing using primer pairs; 907R (5'CCGTCAATTCCTTT(AG)AGTTT3') and 1492R (5'GG(CT)TACCTTGTTACGACTT3'). The partial 16S rRNA gene sequence data was aligned and analyzed to find the closest homologous organisms in the nucleotide databases using the BLASTN program that is available from the National Center for Biotechnology Information (22) and retrieved from the Gene Bank database. The phylogenetic tree was made with the neighbor-joining and bootstrap methods in the NCBI database. The consensus sequence was deposited in the gene databank (GenBank) (Accession No: MW843637) (22).

2.9. Statistical analysis

Each of the experiments was run in triplicate and the data was analyzed using SPSS software version 20.0.

3. Results

3.1. Tolerance to temperature

In this study, the isolate that was identified as *Levilactobacillus brevis* (previously *Lactobacillus brevis)* was subjected to different temperatures, and its growth was monitored (Fig. 1). The isolate survived well at temperature ranges of 30°C and 37°C whereas its survival at 20°C was slightly affected but its survival was significantly reduced at 45°C. At 30°C and 37°C, the organism had a significant increase in its cell numbers from two to four hours whereas at 45°C the cell numbers showed a steady decline from zero to two hours and from two hours to four hours incubation time at 61% to 43%, respectively. At 20°C there was a steady increase in the cell numbers from 0 to 2 h after which there was a decline in the cell numbers (viable colony counts) of the organism up to four hours incubation time.

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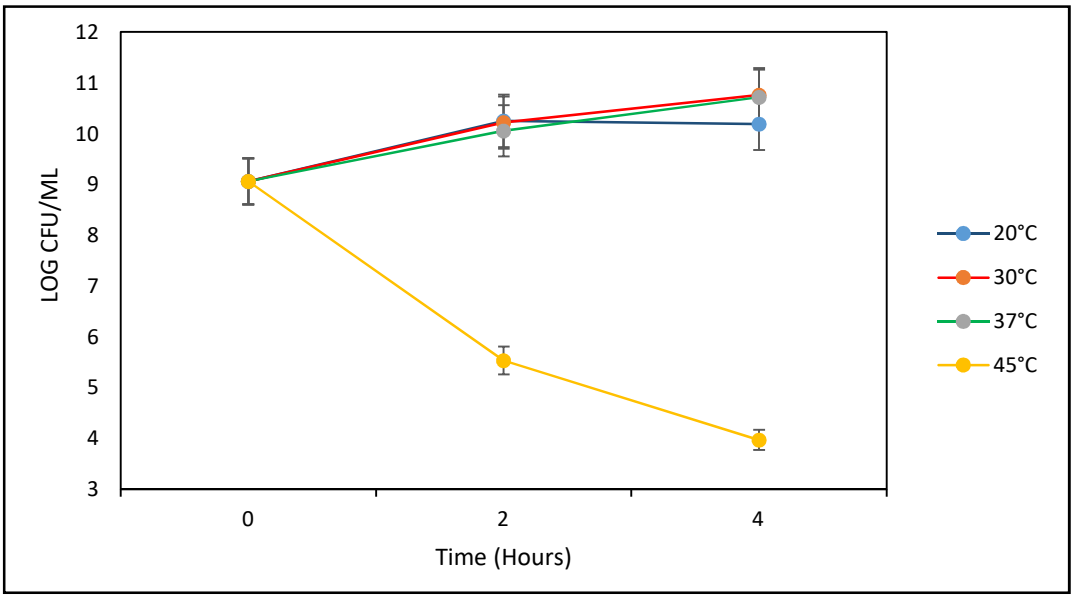

Figure 1. Effect of temperature on the growth rate of *Levilactobacillus brevis* strain

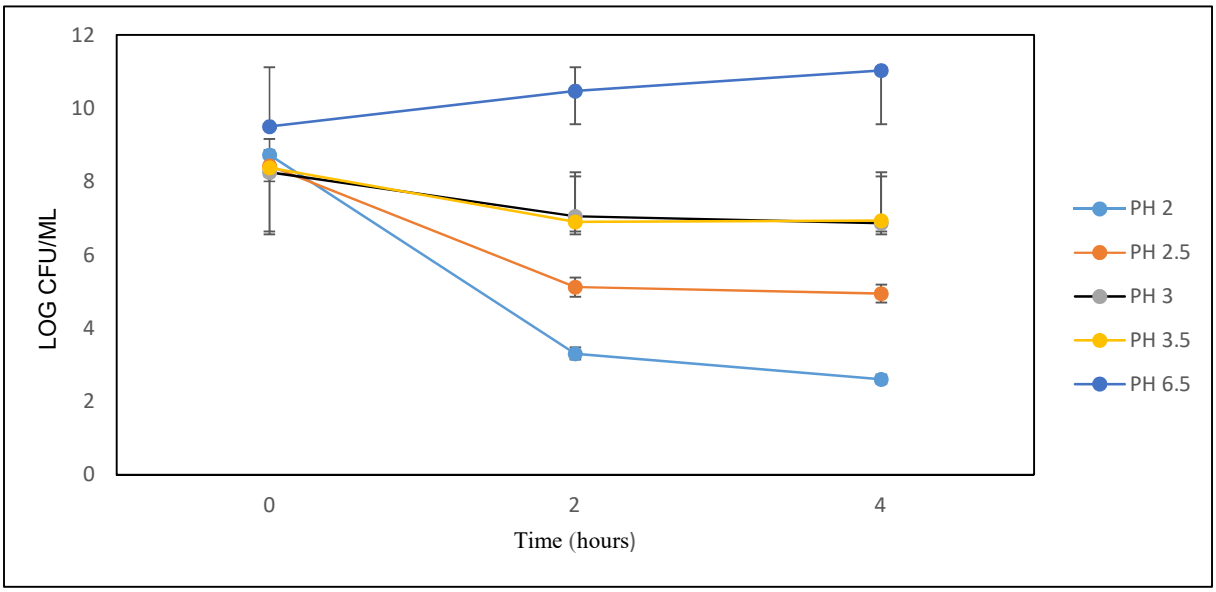

Figure 2. Acidic pH tolerance of *Levilactobacillus brevis* isolate

3.2. Tolerance to low pH

The ability of the *Levilactobacillus brevis* isolate to tolerate acidic pH is shown in Fig. 2. The survival of the microbial isolate on acidic pH was significantly affected by the acidic pH (2.0 – 3.5) compared with the control pH (pH 6.5), whereby microbial cell counts increased after 2 and 4 h of pH exposures. The pH that had a significant effect on cell numbers was pH 2.0 which had the lowest cell numbers after 2 and 4 h pH exposures. The severity of pH increased with a decrease in pH. A significant effect of pH was noted between 0 and 2 h, whereas between 2 and 4 h, the effect in cell numbers was minimal. This trend was not similar to the control which showed a continuous increase in the number of colony counts even after four hours incubation time. At pH 3.0 and 3.5 the survival ability increased at the same rate which means there was no clear difference in the organism's ability to survive low acidity at these two pH values.

3.3. Tolerance to phenol

The isolated *Levilactobacillus brevis* strain was able to tolerate 0.4% (w/v) phenol. This was observed through the viable colony counts on the MRS agar plates after 24 h incubation at 30°C (Fig. 3). At 0 h, the colony counts (Log 10 = 1.83 cfu/ mL) for 0.4 % phenol treatment was significantly lower compared with the colony counts (Log 10 = 2.46 cfu/ mL) of the control treatment (No phenol). However, phenol was resisted by the microbial isolate since colony counts after 24 h culture in 0.4% phenol treatment (Log 10 = 2.46 cfu/ mL) was similar to the control (Log 10 = 2.53 cfu/ mL).

3.4. Antibiotic susceptibility

The antibiotic susceptibility of the *Levilactobacillus brevis* isolate was performed using different commonly used antibiotics and is depicted in Fig. 4 as means ±SD. The results presented in Table 1 reveal that the isolate was sensitive to azithromycin, chloramphenicol, gentamycin, and tetracycline (ZDI: >21 mm), whereas it was resistant to nalidixic acid (ZDI <15 mm) and exhibited intermediate susceptibility to ampicillin and ciprofloxacin (ZDI: 16-20 mm).

3.5. Antagonistic activity

A halo of growth inhibition produced on agar plates by the *Levilactobacillus brevis* isolate against indicator pathogenic bacteria (*Salmonella enterica* serovar *Typhimurium*, *Escherichia coli*, *Staphylococcus aureus,* and *Candida albicans*) showed the results of the antagonistic activity (Table 2). Based on the results, the *Levilactobacillus brevis* isolate had strong antagonistic activity against all the indicator organisms (ZDI>20 mm). Strong inhibition was however highest towards *Salmonella enterica* serovar *Typhimurium* (ZDI = 59 mm) whereas, *Escherichia coli* (ZDI = 33 mm) had the least inhibition among the indicator organisms recruited in the study.

3.6. Hemolytic activity

The hemolytic activity of the *Levilactobacillus brevis* isolate was evaluated on 5% defibrinated sheep blood agar plates. The result in Fig. 5 indicates that the isolated *Levilactobacillus brevis* had no clear transparent or greenish zone on the blood agar plates, surrounding their colonies and thus they were classified as γ-hemolytic or non-hemolytic. The data shown are means ± SD of triplicate values of independent measurements.

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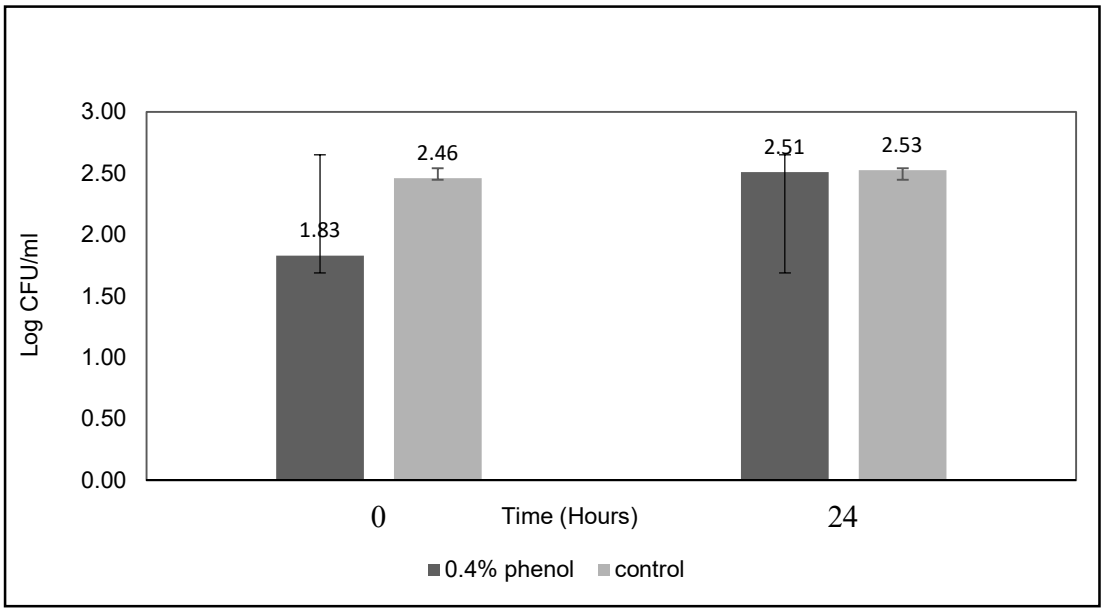

Figure 3. Phenol resistance of *Levilactobacillus brevis* isolate

Table 1. Antibiotic susceptibility test results on the isolated *Levilactobacillus brevis* strain.

Antibiotic Name	Concentration (µg/disc)	Zone of Diameter Inhibition (ZDI) in mm	Susceptibility
Ampicillin	10	16.0 ± 1.0	Intermediate susceptibility
Azithromycin	15	31.0 ± 2.1	Sensitive
Chloramphenicol	30	26.0 ± 2.3	Sensitive
Ciprofloxacin	30	18.0 ± 0.6	Intermediate susceptibility
Gentamicin	10	26.0 ± 1.4	Sensitive
Nalidixic Acid	30	0.0 ± 0.0	Resistant
Tetracycline	30	32.0 ± 1.7	Sensitive

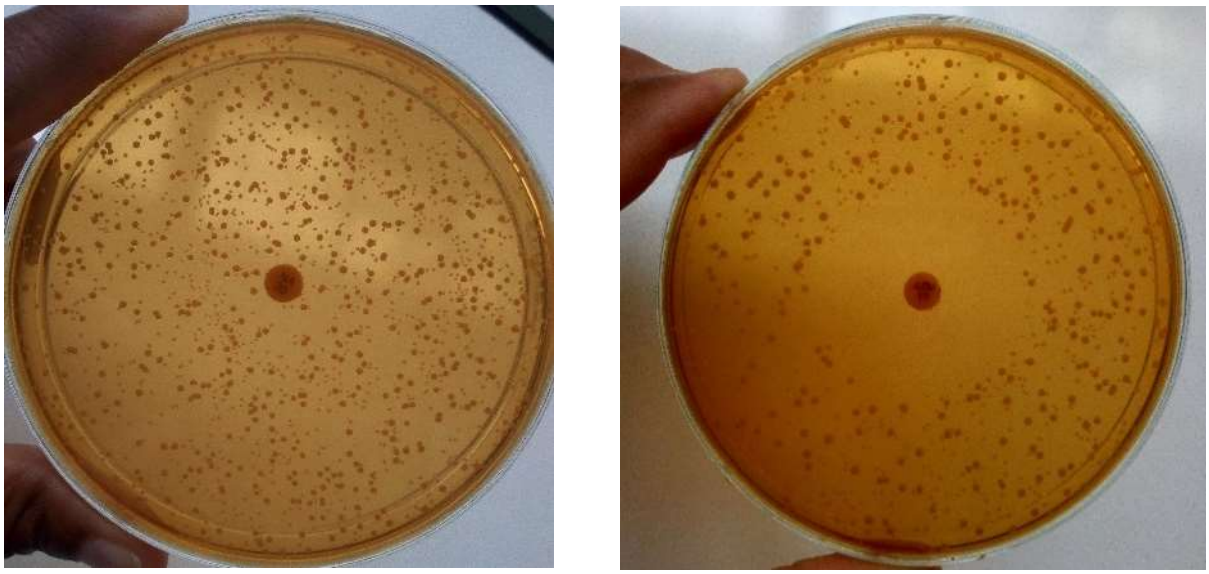

Figure 4. Antibiotic susceptibility test result on isolated *Levilactobacillus brevis;* **A**, resistant; **B**, Sensitive

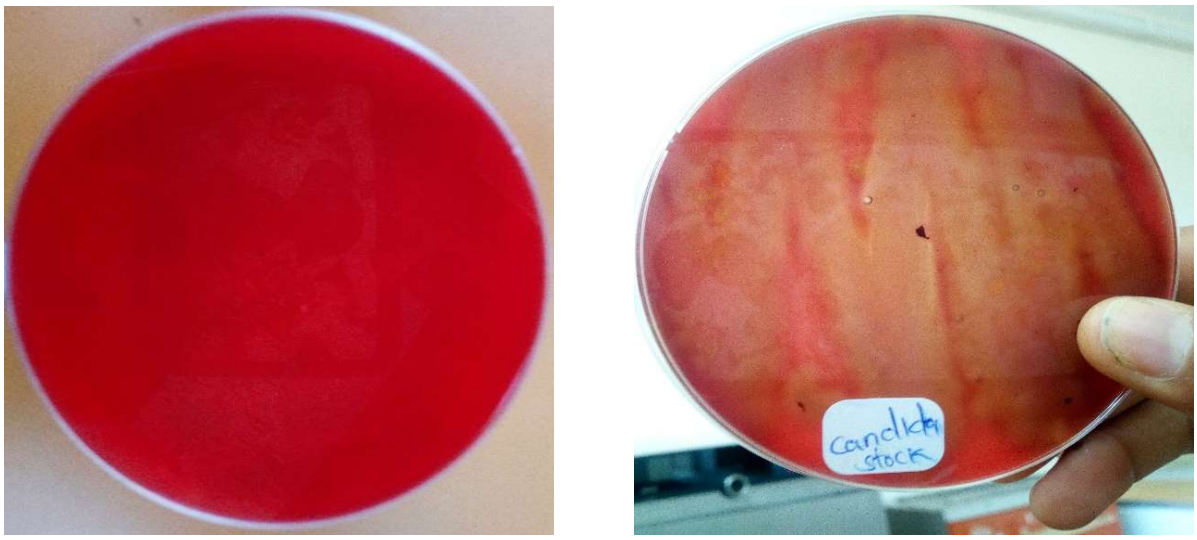

Figure 5. Hemolytic activity test of the isolated *Levilactobacillus brevis* on sheep blood agar. A, *Levilactobacillus brevis* (test organism); B, *Candida albicans* (control)

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Table 2. Antibacterial activity of the isolated *Levilactobacillus brevis*, in terms of ZDI, following agar overlay method.

Indicator Microorganism	Zone of Diameter Inhibition (mm)
Candida albicans	41.0 ± 7.1
Escherichia coli	33.0 ± 8.4
Salmonella enterica serovar Typhimurium	59.0 ± 8.8
Staphylococcus aureus	49.0 ± 4.1

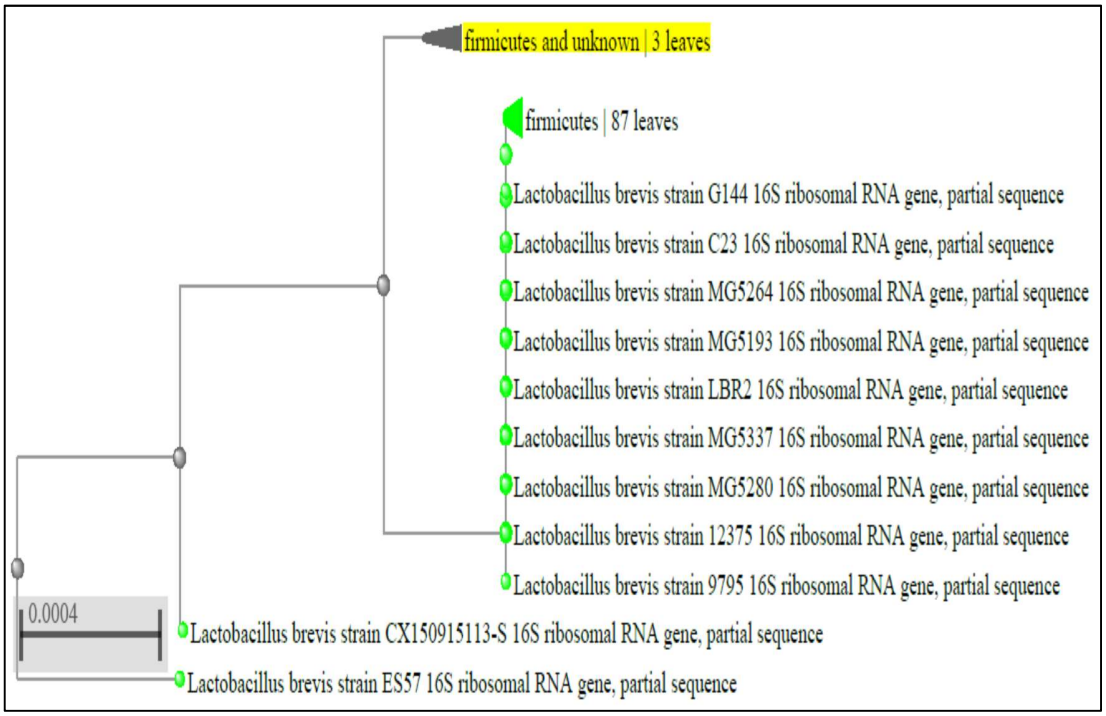

Figure 6. Neighbor-joining tree based on 16S rRNA sequence of the *Levilactobacillus brevis* EGER31 strain (Firmicutes and unknown) obtained from BLAST search showing the position of isolate and related strains

3.7. Genetic identification of the isolate

The BLAST-search for homology of the 16S rDNA sequences of the isolate with known sequences in the NCBI database indicated that the isolate was a strain of *Levilactobacillus brevis*. Phylogenetic analysis demonstrated that the *Levilactobacillus brevis* isolate aligned most closely with *Levilactobacillus brevis* strains in the Gene Bank (Fig. 6). The 16S rRNA sequence similarity with this type strain was over 98.09%, clearly indicating that our isolated strain was *Levilactobacillus brevis.*

4. Discussion

Probiotics, gut microorganisms with beneficial effect on human health have emerged as an important prophylactic or therapeutic strategy for many health conditions (23). Isolation and screening of microorganisms from various naturally occurring processes has become an important process for obtaining and improving probiotic strains with medical relevance for commercial and scientific purposes. In this study, the probiotic capacity of a microbial isolate confirmed to be *Levilactobacillus brevis* by cultural, morphological and biochemical characteristics, and DNA sequencing and obtained from spontaneously fermented milk (amabere amaruranu) (10) was evaluated. This was done to determine if the strain is able to survive and pass through the gastrointestinal tract to colonize and confer associated health benefits to the host and if it had a good safety profile.

Survival and growth in the human gastrointestinal tract temperature is an essential factor in the effectiveness of probiotics. In this study, the *Levilactobacillus brevis* isolate was able to survive and grow at temperature ranges of 30°C and 37°C whereas it survived poorly at temperatures of 45°C, results similar to those reported for other Lactobacillus strains (1). At 20°C, the survival was slightly affected perhaps due to reduced optimal metabolic activity of the organism that slowed down its thriving ability. Temperature is a very important parameter because it affects the survival, growth and viability of probiotics during mammalian gastric transit and industrial production condition (1,24). The purpose of choosing these temperature ranges was to investigate whether the isolated *Levilactobacillus brevis* isolate could survive within the normal body temperature ranges and industrial production conditions or not. If the isolate could not be able to survive and grow within the selected temperature range then it would have proved that it cannot be able to survive in the human gastrointestinal tract and industrial production conditions, therefore fail to qualify as a potential probiotic. However, it was noted that the optimum temperature for the growth of the Levilactobacillus brevis isolate ranged from 30°C to 37°C, which were the same as those recorded for Lactobacillus strains in other studies (1).

Acid tolerance, especially survivability at pH 3.0, has been considered an important criterion for characterization of the probiotic potential of microorganisms (25-27). The *Levilactobacillus brevis* isolate was able to grow at pH 2.5, 3.0, and 3.5 for incubation time of 2 and 4 h, whereas at pH 2.0 the organism showed decreased survival and growth ability (as shown in Fig. 2 in the results section above) compared with the control pH (6.5). Various *Levilactobacillus strains* (a major group of probiotics) have been reported to have tolerance to acid at pH of 2.0–3.0, for 2–6 h (28 - 33), however there was reduction in viability at pH 2.0 (30,31), similar to our results in this study. Secretion of gastric acid is the stomach's primary

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defense mechanism against colonization by pathogens. After ingestion, probiotics should be able to withstand the low gastrointestinal pH in order to exert health benefits to the host. Therefore, low pH (gastric juice) tolerance is one of the criteria in the selection of probiotics (17,24,34). The results of this study suggest that the *Levilactobacillus brevis* isolate can tolerate low acid of between pH 2.0 and 3.5. Therefore it can survive in the gastrointestinal tract, thrive and balance the natural intestinal microflora thereby imparting positive health benefits to the host.

The isolated *Levilactobacillus brevis* strain was able to resist and grow at 0.4% phenol concentration. This was an important test done to check the survival of the isolate under gastrointestinal conditions because, phenol is a toxic metabolite that is usually produced during the deamination of some aromatic amino acids when they get disintegrated in the intestines (35). Phenol has a bacteriostatic effect in the gastrointestinal tract which may compromise the ability of the Levilactobacillus brevis to survive while there and exert probiotic benefits to the host (17).

The antagonism capacity against pathogenic bacteria is an important attribute when selecting potential probiotics (19). The *Levilactobacillus brevis* isolate from spontaneously fermented milk had broad antibacterial spectrum (ZDI: 33–59 mm), against indicator pathogens. The isolate had excellent antibacterial activity against *Salmonella enterica* serovar *Typhimurium* (ZDI: 59 mm) and *Staphylococcus aureus* (ZDI: 49 mm), which were significantly higher than that of the control organism, *Candida albicans* (ZDI; 41 mm). The antibacterial activity against *E. coli* had the lowest inhibition with ZDI of 33 mm. However, the antagonism capacity obtained in this study with the *Levilactobacillus brevis* isolate was higher than those reported for most Lactobacillus isolates (19, 36 - 38). This could be due to the fact that, the antagonism towards a pathogenic bacterium by probiotic strains is strain/ isolate-specific as well as pathogen (target bacteria)-specific (19). Probiotics possess antagonistic activity against human pathogens by production of various antimicrobial compounds that help manage GIT infections (39). Once probiotics are consumed, they adhere to the gut and produce extracellular antimicrobial components by converting carbohydrates, proteins and other compounds into important substrates that kill pathogens (40). The observed strong antagonistic property of the *Levilactobacillus brevis* isolate could be due to possible acid production that increased the acidity of the medium, competition for nutrients between the pathogens and the *Levilactobacillus brevis* isolate, and due to the production of bacteriocins and other inhibitory metabolites that enabled the organism to stay competitive and exclude the pathogens. In addition, the antagonistic activity of the *Levilactobacillus brevis* isolate was probably due to the production of antimicrobial substances such organic acids, hydrogen peroxide, and secondary metabolites that have the capability of inhibiting the growth of fungal pathogens such as *Candida albicans* used in this study (34, 39).

Food and Agricultural Organization (FAO), World Health Organization (WHO) and the European Food Safety Authority (EFSA) have recommended that new probiotic strains intended for use in food product development be examined for their safety by testing their antibiotic resistance, toxin production and

hemolytic potential, and assessing metabolic activities such as D-lactate production to prevent the occurrence of a potential adverse event (17,41). A new probiotic strain should be incompetent to cause hemolysis and gelatin liquefaction in the human body and it should be sensitive to antibiotics to avoid disseminating the resistance to other organisms especially pathogens in the same niche. In this study, the hemolytic activity of the *Levilactobacillus brevis* isolate was evaluated on 5% defribrinated sheep blood agar plates. The isolate did not cause α- or β-hemolysis but rather showed γ-hemolytic or no hemolytic activity. The lack of hemolytic activity is important as this means that the strain is non-virulent and cannot cause infections, therefore it can be used to formulate safe probiotic products for human consumption (42,43). The test isolate *Levilactobacillus brevis* was sensitive to azithromycin, chloramphenicol, gentamicin and tetracycline antibiotics. It also had moderate susceptibility to ampicillin and ciprofloxacin, while it was resistant to nalidixic acid (Table 1). The antibiotic sensitivity and the intrinsic antibiotic resistance property of *Levilactobacillus brevis* have not been reported but the current isolate was sensitive to most of the antibiotics tested (Table 1). The ability to resist commonly used antibiotics makes probiotics be considered safe to consume after antibiotic therapy. This is because they can maintain the balance of the microflora in the gastrointestinal tract (17).

5. Conclusion

Although this Levilactobacillus brevis isolate has not been recorded as a probiotic in human food development, it was found in traditionally spontaneously fermented milk. To demonstrate its applicability as a probiotic, in this study, we sought probiotic validation of the *Levilactobacillus brevis* isolate. This candidate organism exhibited promising probiotic potential due to its high resistance to harsh gastrointestinal tract simulated conditions (pH, temperature, and phenol resistance) as well as antibiotic susceptibility. The organism also showed strong antagonistic activity towards human pathogens and was sensitive to most commonly used antibiotics and had no hemolytic activity meaning it is not pathogenic to the consumer. Cumulatively, the *Levilactobacillus brevis* isolate has emerged as a potential probiotic candidate, which can be safely used to formulate functional foods. However, more probiotic parameters and in vivo activity of this isolate as a probiotic should be investigated to further prove its immunomodulatory, nutritional, and health benefits.

Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

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