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Probiotic potential of lactic acid bacteria isolated from Ethiopian traditional fermented *Cheka* beverage

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Abstract

Introduction : Lactic acid bacteria (LAB) are a cluster of microbes distributed in a variety of environments and have potential probiotic activity to improve human well-being. This study was aimed at assessing the probiotic potential of LAB isolated from *Cheka*, an Ethiopian traditionally fermented beverage.

Method Pure isolates obtained from 16 *Cheka* samples from Konso ($n=8$) and Derashe ($n=8$) were characterized morphologically, biochemically, and physiologically by considering basic criteria to identify the LAB. The probiotics properties of the LAB were evaluated in vitro at low pH values (2.0 and 3.0), and two bile salt concentrations (0.3 and 0.5%) for 3 and 6 h. The 16 S rRNA gene sequencing was done using an ABI 3730xl sequencer, and the gene sequences were aligned.

Results Of the 27 pure isolates, 11 isolates were proven to be LAB with non-motile, negative for catalase, and non-spore former characteristics. Based on cultural characteristics and sugar fermentation ability, the 11 isolates were assembled into the genera *Lactobacillus* (55%), *Lactococcus* (18%), *Pediococcus* (18%), and *Leuconostoc* (9%). At pH 3.0 and a bile salt concentration of 0.3%, isolate ChK-11 showed a better survival rate (97 and 94%) than other isolates [ChK-7 (93 and 80%) > ChD-5 (84 and 76%) > ChD-8 (46 and 36%) > ChK-4 (41 and 34%)] for 6 and 3 h, respectively. According to 16 S rRNA sequencing results, isolates ChK-11 and ChK-7 were found to be *Weissella paramesenteroides* and *Leuconostoc pseudomesenteroides* with sequence similarity of 99 and 91%, respectively.

Conclusions In the present study, probiotic LAB (*Weissella paramesenteroides* and *Leuconostoc pseudomesenteroides*) was successfully isolated and sequenced from *Cheka* samples. The findings of this in vitro study indicated that fermented beverages like *Cheka* are a source of the LAB with probiotic functional properties. Overall, *Weissella paramesenteroides* and *Leuconostoc pseudomesenteroides* isolates, which showed promising probiotic properties under in vitro conditions, can be used for starter culture development for the *Cheka* fermentation process.

Keywords *Cheka*, Derashe, Konso, Lactic acid bacteria, Probiotic, Starter culture

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Introduction

One of the oldest forms of biotechnology called fermentation has been used to preserve and enhance the food flavor, texture, shelf life, and functional qualities (Angelescu et al. 2019; Wang et al. 2018). Fermented foods and beverages contain microorganisms (bacteria and yeasts) that utilize the supplements in the food as growth nutrients and energy sources (Mokoena et al. 2016). Among the varieties of microbes responsible for food and beverage fermentation, lactic acid bacteria (LAB) are the most dominant ones and can serve as probiotics (Angmo et al. 2015; de Júnior et al., 2015). Probiotics can be defined as food supplements that contain a single or mixed viable microbial culture purposefully consumed to enhance consumer health (Zhou et al. 2022). When LAB are consumed as probiotics, they play a positive role in maintaining a desirable microbial stability in the gut, and leading to enhance the health status of the consumer (Amenu and Bacha 2023).

Traditional fermented foods and beverages contain probiotic LAB that may be used as a starting culture for large-scale manufacturing of the product and have a required functional feature that makes them useful as probiotics against food-borne infections (Pabari et al. 2020). *Cheka* is a fermented traditional beverage in Ethiopia consumed as food and drink in different communities, especially Konso zone and Derashe special woredas located in the southern part of the country (Worku 2016). People of all ages, including babies, adult, expectant mothers, and nursing women, can consume *Cheka*. On average, an adult male can consume up to 8 L of *Cheka* per day, which is much greater than females ability to consume the same product. It gives customers with low incomes access to an inexpensive beverage source and significantly improves their nutritional security (Worku 2016). The most common ingredients used during *Cheka* preparation are maize, sorghum, malt, and water, but in the case of Derashe people, vegetables like cabbage and moringa leaf are also used. During the preparation, the ingredients pass through spontaneous fermentation using conventional utensils in a non-hygienic environment (Worku 2016). This kind of traditionally fermented food generally depends on naturally occurring microbes, which may result in unpredictable quality and stability of the final products (Birmeta et al. 2019). To enhance flavors and purported health benefits, it may be helpful to have a detailed understanding of the microbiological and functional characteristics of such fermented foods and beverages (Angelescu et al. 2019). Most Ethiopian traditionally fermented foods are consumed without further heating or any other frame of processing and handling. Hence, they are ideal for isolating LAB which may have probiotic properties. So far, the isolation of LAB with probiotic properties targeting *Cheka* is lacking. Thus, evaluating the probiotic potential of LAB isolated from *Cheka* was the goal of this study. The specific objectives of this study were (i) isolating bacteria from fermented *Cheka* samples collected from Konso and Derashe, (ii) characterizing isolates using morphological, biochemical, and physiological techniques, and (iii) molecular characterization of selected isolates involved in *Cheka* fermentation. This research provides insights and promising technology for the fermentation of *Cheka*.

Materials and methods

Descriptions of the study area

Cheka samples were obtained from Derashe and Konso, which are found within the Southwestern part of the Southern Nations, Nationalities, and Peoples of Ethiopia. Konso zone and Derashe special woreda are found at a distance of almost 595 and 550 kilometers from Addis Ababa, respectively. The towns are found at an elevation of 2,561 and 1,650 meters above sea level, respectively. The latitude and longitude coordination of the town is also 5°30′N, 37"30′E and 5°15′N and 37°29′E as shown in (Fig. 1). Derashe's administrative center is Gidole, with an estimated total population of 204,041, while Konso Zone's administrative center is Karat, with an estimated total population of 309,998. In Konso, spring (Belg) is the primary rainy season and the season when most crops are produced. Konso has a 23°C average annual temperature and 802 mm of yearly precipitation. Sorghum and maize are the principal crops, with a small amount of cotton, yam, cassava, sweet potato, and taro as root and tuber crops (Gashure et al. 2022). The Derashe district's climate is defined by mean annual temperatures that fall between 15 and 28°C and mean annual rainfalls that fall between 600 and 1600 mm. In the study region, maize, sorghum, teff, and wheat are the main crops that are grown.

Experimental design and collection of *Cheka* samples

The random sampling design was applied to collect samples from household brewers in Konso and Derashe. The areas were selected based on their potential to produce and consume *Cheka*. Sixteen *Cheka* samples ($n=8$) from Konso and ($n=8$) from Derashe brewers each with 200 mL using sterilized bottles placed in the icebox. The samples were transported to Arba Minch University Microbial Biotechnology Laboratory and kept under refrigeration at 4 °C until analysis was done.

Local Cheka preparation practices

In order to get information how the *Cheka* is prepared, thirty ($n=30$) respondents were selected to participate in a questionnaire-based survey to get information regarding the ingredients use, duration of fermentation and preparation methods. Prior to delivering questionnaires for data collection, the repondents were given a brief

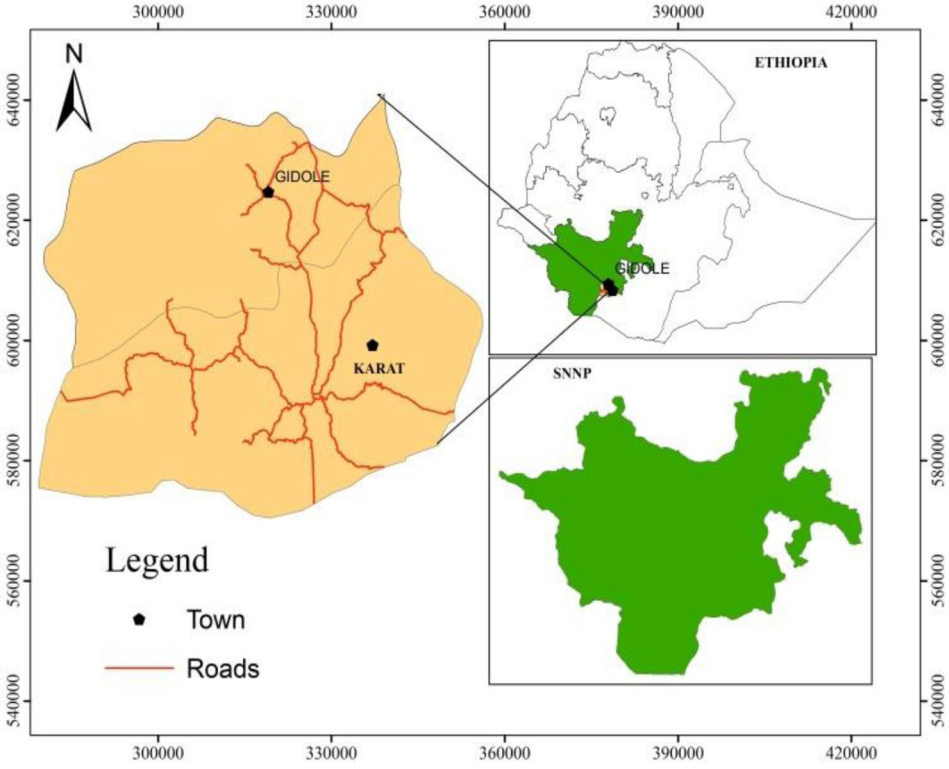

Fig. 1 Location map of the study area

explanation of the purpose. When necessary, the questions were translated into Amharic. This was done to thank the respondents for their assistance in maintaining the traditional knowledge of the study area and to increase their confidence in their ability to provide accurate information.

The process of *Cheka* preparation, duration of fermentation, and ingredients (maize, sorghum and/or wheat) being used may be the same or different types from one household brewer to another, but the process has three major phases (Fig. 2). Brewers can go for grinding of grains (wheat, sorghum, and maize), preparation of malt and porridge is the general activity done in *Cheka* preparation. Grinded flours are sieved, mixed, and then water is added to make dough, which is the first phase in *Cheka* preparation (Fig. 2). After 24 h of fermentation time, the balls (Kurkufa) were prepared from dough by introducing small amount of fresh flour. In the second phase of *Cheka* ingredient preparation, the brewers can break down the knead balls of dough already prepared in the first phase, and cool it to mix with water and malt, and then knead to allow 24 h fermentation. In the third phase, the brewers prepare porridge (semi-liquid material called 'Absite'), that can be mixed with malt and previously fermented products in the second phase and allow the final fermentation process take place for 12 h to obtain pure *Cheka* (Fig. 2) in case of Konso people. In case of Derashe, the process is the similar with Konso people but the difference is that they use vegetables like leaves of taro, cabbage, and moringa (Worku 2016).

Cultural and cellular characterization of lactic acid bacteria from Cheka

Five grams (5 g) of each sample was placed aseptically into a sterile stomacher bag and homogenized in 45 mL of sterile saline solution (0.85% w/v NaCl) to stabilize osmotic pressure using a Stomacher lab blender (Star Blender TM LB 400, France). Then, sequential decimal dilutions of the homogenate were made by serially diluting up to 10^{-6}. A volume of 0.1 mL sample from 10^{-3}, 10^{-4} and 10^{-6} was spread-plated in duplicates on the surface of MRS (de-Mann, Rogosa, and sharp) agar (Sisco Research Laboratories Pvt. Ltd, India) plates. Then inoculated plates were incubated anaerobically using anaerobic jars (Terumo Europe N.V., Leuven, Belgium) at 37°C for 24 to 48 h. Then, growths of different colonies were observed on the plate containing MRS agar medium. The isolates were characterized morphologically, biochemically, and physiologically by considering basic criteria to identify LAB. Morphological consideration was based on Gram's staining, colony size, color, edge, form, arrangements, and texture as described elsewhere (Angelescu et al. 2019) with slight modification. The most important biochemical tests conducted were catalase,

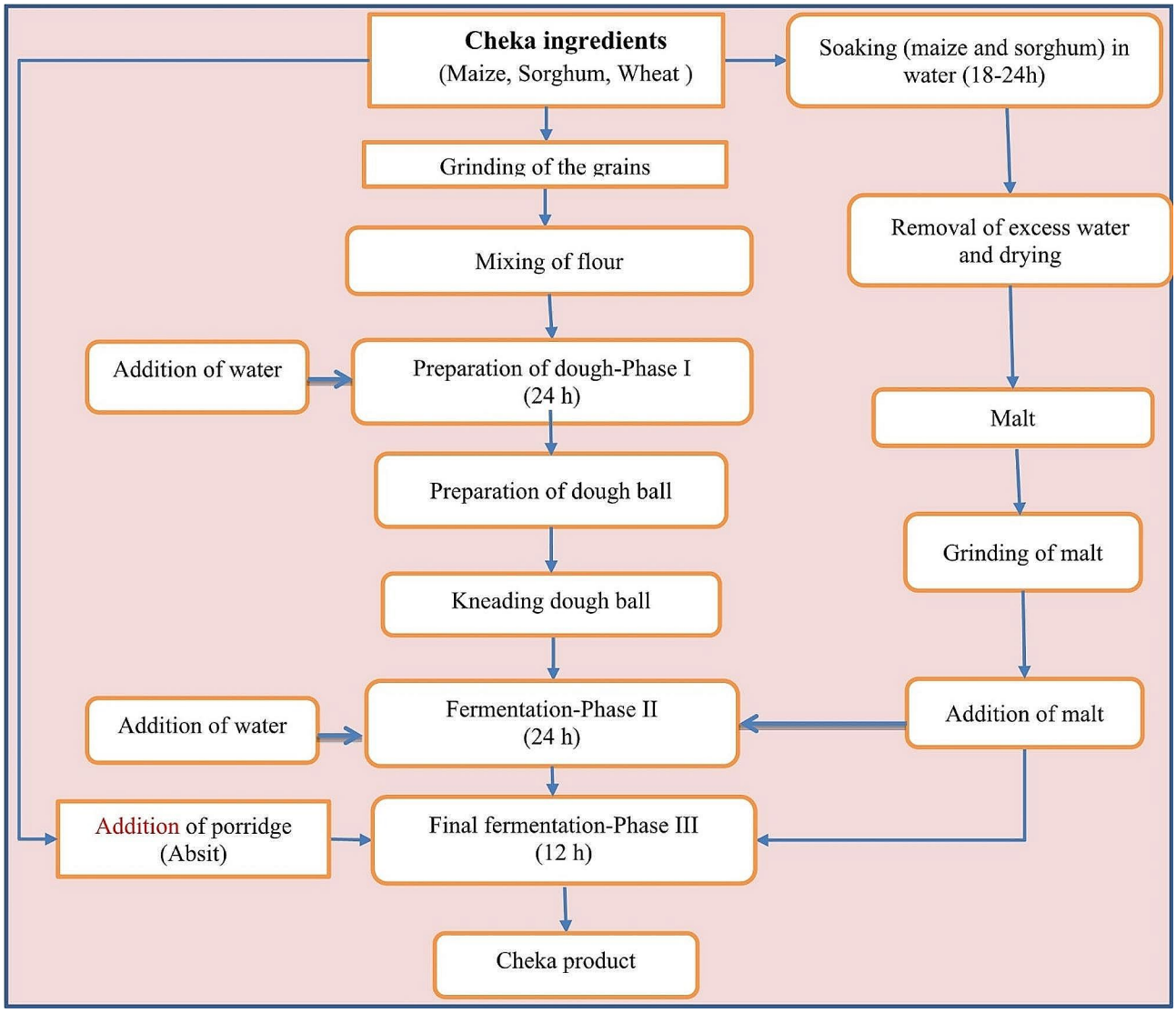

Fig. 2 The overall *Cheka* fermentation process

motility, spore-staining oxidase, and indole production as described in (Amenu and Bacha 2023). Acid and gas formation from modified MRS broth with 5% glucose and sugar fermentation test containing Durham's tube was based on another previous study (de Almeida Júnior et al. 2015).

Eco-physiological characterizations of LAB from Cheka

Eco-physiological characterizations were done to ensure the isolates tolerate different environmental conditions. Briefly, to ensure isolates temperature tolerance, test tubes with modified MRS broth and (0.12 g/L) bromecresol purple indicator were used and the tubes were incubated at distinguishing temperatures (10, 15, 37, and 45°C) in accordance with Mulaw et al. (2019) with minor modification. To investigate the NaCl tolerance of the isolates, test tubes containing MRS broth and bromecresol purple indicator at a concentration of 0.12 g/L were adjusted with different NaCl (w/v) concentrations (2.0, 4.0, 6.5, and 10.0%) (Amenu and Bacha 2024) with slight modification. Briefly, five (5 mL) of modified MRS broth containing bromecresol purple indicator with the above salt concentrations were prepared and transferred into test tubes. All test tubes containing MRS broth with bromecresol purple indicator with each salt concentration were sterilized at 121°C for 15 min. After sterilization, each test tube was inoculated with equal concentration of the fresh overnight culture of 50 μL of the isolates and incubated at 37°C for 3–5 days. During this incubation time, the change of the color from purple to yellow was considered as the tolerance of the isolates to different temperature and salt stress condition. Each experiment in this study was done in triplicate.

Source of test microorganisms

The test organisms (*Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853 and *Listeria monocytogenes* ATCC19115) were collected from the Ethiopian Public Health Institute (EPHI), National Clinical Bacteriology and Mycology Reference Laboratory, Addis Ababa, Ethiopia, and used for evaluation of the antagonistic properties of the isolates.

In vitro characterization of probiotics properties

The isolates of LAB were evaluated in vitro probiotic properties with low pH (2.0, and 3.0) and also bile salt concentrations (0.3 and 0.5%) for 3, and 6 h (Mulaw et al. 2019). Production of antimicrobial substances was done using the agar well diffusion method as indicated elsewhere (Amenu and Bacha 2023). Briefly, the fresh culture of LAB isolates on MRS broth was centrifuged separately at 5000 revolution per minute (rpm) for 10 min at 4 °C. The cell-free supernatant from each separate culture was collected as a crude extract for the antagonistic study against selected food-borne bacterial pathogens and adherence to stainless steel plate (El-Jeni et al. 2015). In all cases the survival rate (%) of the isolates was calculated using the following formula (Eq. 1):

$$\text{Survival rate}\,(\%) = \frac{\text{Biomass at time}\ (t)}{\text{Biomass at initial}\ (0)} \times\ 100 \quad (1)$$

Safety assessment of the isolates

The bacterial susceptibility test of the isolates to six different commercial antibiotic discs were done according to clinical and Laboratory Standard Institutes tables (Kassim et al. 2016). Based on these disc result the isolates were grouped in three categories: resistant (R), intermediate (I), and sensitive (S).Hemolytic activity was done on blood agar medium added with 5% defibrinated sheep blood (Amenu and Bacha 2023). Gelatin hydrolysis was also done as described by Bouguerra et al. (2020). The DNase activity was also done on DNase agar medium supplemented with 0.5 g/L methyl blue as described elsewhere in the literature (Amenu and Bacha 2023) with slight modifications. Each experiment in this research work was done in triplicate.

Bacterial genomic DNA extraction

The procedure for extracting DNA was as follows: 1.5 mL sterilized microtubes having the bacterial pellet were filled with 250 µL of TE (10 mM Tris HCl pH 7.6; 1 mM EDTA); these were then followed by the addition of 100 µL of 5 M NaCl and 100 µL of CTAB, which had been preheated to 65 °C. After agitating the suspension for ten seconds, it was incubated for ten minutes at 65 °C in a water bath. After that, 750 µL of phenol/chloroform (1:1) was added, and the mixture was spined after being gently agitated for 10 s by inversion (Brito et al. 2022). About 200 µL of chloroform-isoamyl alcohol (24:1) was applied before centrifugation and then followed by washing. To precipitate the DNA, concentrated ethanol with 1/10 volumes of 3 M sodium acetate was used. Subsequently, it undergoes inversion to homogenize and was incubated at -20°C overnight. After that, 500 µL of room-temperature 70% ethanol was added, and the mixture was centrifuged for 20 min at 15,000 rpm, and then removing the supernatant. The pellet was again cleaned with 70% ethanol and heated at 56 °C to dry it out. The DNA was dissolved in 50 µL of molecular-grade water after the ethanol had evaporated. After waiting 20 min at room temperature, the tubes were placed in storage at -20 °C. Spectrophotometric analysis was used to evaluate the DNA's yield and purity (Spectrophotometer code, Company, Country where made).

16 S rRNA gene amplification, sequencing and phylogenetic analysis

Thermocycler (code, Company, Country where made) amplified the extracted DNA using reverse (1492R) primer, (GGTTACCTTGTTACGACTT), and the forward (27 F primer, (AGAGTTTGATCMTGGCTCAG) (Prabhavathy et al. 2013). Using an automated sequencer, the amplified 16 S rDNA PCR result was sequenced. An ABI 3730xl sequencer was used in South Africa to sequence the 16s rRNA gene.

Using BioEdit 7.2, the 16 S rRNA gene sequences were aligned. The BLAST-NCBI website was used to examine the sample sequences (Chentouf et al. 2023). The sequences were used for a gene homology quest, and the 16 S rRNA sequences were identified to the generic level using the public databases from BLAST (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, USA). The 16 S rRNA sequences of the isolated strains were aligned with sequences of similar organisms that were received from GenBank using the CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France) (Prabhavathy et al. 2013). The MEGA X 11 was used for the phylogenetic analysis, and TreeView was used to create phylogenetic trees using the neighbor-joining technique. A bootstrap analysis was carried out to confirm the branching pattern's reproducibility (Priya et al. 2014).

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 software. Every experiment was conducted in triplicate, and data was evaluated using one-way ANOVA at a 95% level of significance before being statistically compared. The probability value at $p<0.05$ was regarded

as statistically significant. The information was displayed in triplicate as mean values ± standard deviation.

Results and discussions

Indigenous knowledge and practice on *Cheka* preparation

The majority (64%) of the respondents use sorghum and maize as the main ingredient to prepare *Cheka*. Sorghum, maize and millet also used as ingredient to prepare *Cheka* (Table 1). However, very few (3%) respondents said barley used as ingredient in addition to maize and sorghum for Cheka preparation. According to respondents, the *Cheka* ingredients were obtained from open market (77%), farmland (17%), and sources not identified (6%). As indicated in Table 1, the respondents prefer to prepare malt from sorghum (64%) followed by maize (23%), and wheat (6%). However, 3% of prefer malt preparation using small percentage of *Cheka* from previously fermented.

Respondents prepare *Cheka* for both home use and money source (67%), and market (23%) purpose (Table 1). They also prepare *Cheka* during special festival for enjoyment and entertainment drink. Respondents consider *Cheka* is the best food and drink that didn't go even a day without drinking it. In their everyday activities, all of the interviewees were able to produce and consume *Cheka*. Overall, *Cheka* has found to be the most frequently used and have greater acceptance value for consumption and production in the study area.

As the information given by the respondents, the overall *Chek*a fermentation time can take 3–4 days (80%) to get quality product. Once *Cheka* is ready to consume, it can be consumed around 8 h (47%) for the market purpose, but it can be used for longer time for home consumption purpose.

Morphological and biochemical characterization of the LAB from *Cheka*

A total of 27 different colonies were isolated from 16 *Cheka* samples eight from each area. Among these, 17 were found to be positive for Gram's reaction, non-spore-forming, cocci, and rod in shape. The study showed that rod-shaped (65%) isolates were dominant over cocci-shaped (35%) isolates. Out of the 17 isolates obtained, 11 isolates that were non-motile and negative to catalase and non-spore-forming were confirmed as LAB and selected for further physiological characteristics. Other important biochemical tests like citrate utilization, oxidase, and

Table 1 Respondent's indigenous knowledge on *Cheka* preparations perception and use

Items	Alternatives	Respondent	
		Frequency	%
1) What types of ingredients you frequently use to prepare *Cheka*?	Sorghum, maize and wheat	4	13
	Sorghum and maize	20	67
	Sorghum, maize and millet	5	17
	Sorghum, maize, barely	1	3
2) Where do you get the ingredients to prepare *Che*ka?	Open market	23	77
	Farmland	5	17
	Other area	2	6
3) What type of malt you use to prepare *Cheka*?	Sorghum	19	64
	Maize	7	23
	Wheat	3	10
	Previously fermented *Cheka*	1	3
4) For what purpose you prepare *Cheka*?	Home consumption	2	7
	Market	7	23
	Festivals/ceremonies	1	3
	Both home and market purpose	20	67
5) For how long engaged working in this Cheka preparation job (year)?	< 2	1	3
	2–3	3	10
	4–5	5	17
	>5	21	70
6) For how long you allow fermentation to get quality *Cheka* (hour)?	< 24	0	0
	24–48	3	10
	49–96	24	80
	> 96	3	10
7) Once *Cheka* is ready, for how long you consume it (hour)?	≤ 8	14	47
	9–12	12	40
	13–24	3	10
	25–48	1	3

Table 2 Biochemical characterization results of the isolates

Isolate code	Different tests							
	Catalase	Motility	Citrate	Arginine	MR	VP	Oxidase	Indole
ChK-3		Non	+	+	+			
ChK-4		Non	+		+		+	
ChK-7		Non			+			
ChK-11		Non			+		+	
ChK-19		Non	+		+			
ChK-23		Non			+			
ChD-2		Non	+	+	+		+	
ChD-5		Non			+			
ChD-8		Non	+		+			
ChD-17		Non		+	+			
ChD-25		Non	+		+		+	

- = indicates negative for the test, + = indicates positive for the test, Non = not motile

Table 3 Sugar fermentation properties of the isolates and acid/gas production from glucose

Isolate code	Acid and gas-Glucose	Different sugar fermentation test						
		Glucose	Fructose	Lactose	Sucrose	Maltose	Manitol	Sorbitol
ChK-3	Homo	+	-	+	+	+	+	+
ChK-4	Homo	+	+	+	-	+	-	-
ChK-7	Homo	+	+	+	+	-	+	+
ChK-11	Homo	+	+	+	-	+	+	+
ChK-19	Hetero	+	+	+	+	+	+	-
ChK-23	Hetero	+	+	+	+	+	+	-
ChD-2	Homo	+	+	+	-	+	+	+
ChD-5	Hetero	+	+	+	+	+	+	+
ChD-8	Homo	+	+	+	+	-	+	-
ChD-17	Hetero	+	+	+	+	-	-	+
ChD-25	Homo	+	+	+	-	+	+	-

Homo = homofermentative, Hetero = heterofermentative, += positive for the test, - = negative for the test

indole tests were done to characterize the isolates, which have been shown in (Table 2).

In a related investigation, Divisekera et al. (2019) found that 38 out of 57 bacterial colonies were isolated from three types of fermented finger millet flour and were found to be non-spore-forming Gram-positive cocci and bacilli. LAB is a Gram-positive, non-spore-forming, rod- or cocci-shaped, catalase-negative, non-motile bacterium, as previously described by other researchers (Putra et al. 2018).

Sugar fermentation properties of the isolates and acid/gas production from glucose

In the present study, the homofermentative isolates were dominant over the heterofermentative and other sugar fermentation test (Table 3). Seven (64%) isolates were classified as homofermentative based on their ability to produce acid and gas from 5% glucose, while the remaining four (36%) isolates were classified as heterofermentative due to their ability to produce acid and gas from glucose in the presence of Durham tube. Sugar fermentation ability of the each isolate was varied but all 11 isolates were positive for fermenting glucose and lactose. This study is in agreement with other previous studies (Dessalegn and Ashenafi 2010; Negasi et al. 2017) that have reported homofermentative were dominant over heterofermentative on the basis of their glucose fermentation property.

Eco-physiological characterizations of LAB

The particular range of temperature to which the isolates are able to grow is a significant physiological characteristic used for the identification of LAB (Todorov et al. 2017). Accordingly, all the isolates ($n=11$) were tested for growth at different temperatures (10, 15, 37, and 45°C) (Table 4). In this study, better tolerance was observed at the temperature range between 15-45°C. Generally, growth declines when the temperature increases, suggesting that higher temperature might cause thermal damage and stress can disrupt the bonds and hydrophobic interactions, which results in loss of proteins and nucleic acid functional structure. The growth has been observed as variable depending on the isolates that show a yellow color change in the presence of a bromecresol purple indicator.

Table 4 Physiological characterizations of the isolates

Isolate code	Temperature tolerance (°C)				Salt tolerance test (% of NaCl, w/v)			
	10	15	37	45	2.0	4.0	6.5	10.0
ChK-3	+	+	+	-	+	+	-	-
ChK-4	-	-	+	-	+	-	-	-
ChK-7	-	+	+	+	+	+	+	+
ChK-11	+	+	+	+	+	+	+	-
ChK-19	-	+	+	-	+	-	-	-
ChK-23	-	+	+	+	+	+	+	+
ChD-2	+	+	+	-	+	+	+	-
ChD-5	-	+	+	-	+	+	+	+
ChD-8	-	+	+	-	+	-	-	-
ChD-17	-	+	+	+	+	+	+	-
ChD-25	-	+	+	-	+	+	-	-

+ = indicates growth, - = indicates not growth

Table 5 Grouping of the LAB isolates into different genera

Isolate code	Cell shape	Arrangement	Growth T° (°C)	Salt tolerance (%)	Acid and gas production	Tentative classification*
ChK-3	Cocci	Spherical	10–37	2 & 4	Homo	*Lactococcus*
ChK-4	Rod	Pairs or chains	37	2	Homo	*Lactobacillus*
ChK-7	Rod	Pairs or chains	15–45	2–10	Homo	*Lactobacillus*
ChK-11	Rod	Pairs or chains	10–45	2-6.5	Homo	*Lactobacillus*
ChK-19	Cocci	Pairs or tetrads	15–37	2	Homo	*Pediococcus*
ChK-23	Rod	Pairs or chains	15–45	2-6.5	Hetero	*Lactobacillus*
ChD-2	Cocci	Pairs or chains	10–37	2-6.5	Homo	*Lactococcus*
ChD-5	Cocci	Pair or short chain	15–37	2–10	Hetero	*Leuconostoc*
ChD-8	Cocci	Pairs or tetrads	15 & 37	2	Homo	*Pediococcus*
ChD-25	Rod	Pairs or chains	15&37	2 & 4	Homo	*Lactobacillus*
ChD-17	Rod	Pairs or chains	15–45	2-6.5	Hetero	*Lactobacillus*

*= tentatively classification at genus, Home = homofermentative, Hetero = heterofermentative, T°= temperature

Similar reports were also found in previous studies (Divisekera et al. 2019; Putra et al. 2018) that the strains obtained from fermented food were grown at 10, 15, 30, and 37 °C incubating temperatures, but the growth rate was decreased at 45 °C. All of the isolates ($n=11$) considered to be LAB were subjected to different (2–10%) NaCl concentrations whether the isolates were able to withstand or not various salt concentrations. Results showed that the majority of isolates changed the medium's color from purple to yellow, suggesting improved tolerance to salt concentrations between 4 and 6.5%. This could be due to cell development at various salt concentrations. In general, the growth of LAB isolates showed a decreasing trend with increasing salt concentrations, and vice versa, which is in line with another study (Jampaphaeng et al. 2017). Relatively better LAB growth in 6.5% NaCl concentration was also reported by different scholars (Putra et al. 2018; Samedi and Charles 2019).

Grouping of the LAB isolates into different genera

There are several groups of LAB genera that may vary significantly in morphological, and physiological properties (Mokoena et al. 2016). In the current study, 11 isolates were tentatively grouped into four different LAB genera based on Bergey's manual of systematic bacteriology that considers colony characteristic, cell shape, and fermentation capability of glucose (Table 5). *Lactobacillus* (6 isolates, 55%), *Pediococcus* (2 isolates, 18%), *Lactococcus* (2 isolates, 18%), and *Leuconostoc* (1 isolates, 9%) were LAB genera identified in the current study. From these results, *Lactobacilli* were the most frequently dominant genus observed. This might be due to that this genus has more species than the other genera and has been the most frequently known genus found in fermented food and used as probiotic LAB. As also reported in previous studies (Benavides et al. 2016; Negasi et al. 2017), LAB is identified from different fermented foods and beverages with *Lactobacillus* dominant over other LAB genera.

Screening of LAB isolates for probiotic properties

Antimicrobial activity of LAB isolates against test strains

The probiotic potential of LAB isolates obtained in the present study was evaluated for antimicrobial properties using identified test pathogenic bacteria. Among 11 pure isolates, only 6 isolates showed antibacterial activity on test pathogens with varying inhibition zones and were selected for further study as shown in (Table 6).

Table 6 Antimicrobial activity of LAB isolates

Isolate code	Inhibition zone (mm) against pathogens			
	S. aures	*E.coli*	*L. monocytogenes*	*P. aeruginosa*
ChK-4	12.5 ± 0.25	10.5 ± 0.75	14.3 ± 1.50	15.7 ± 1.25
ChK-7	15.3 ± 0.60	11.0 ± 0.75	16.6 ± 0.65	12.3 ± 1.75
ChK-11	17.0 ± 0.50	10.8 ± 0.50	16.0 ± 0.50	13.7 ± 0.42
ChK-19	5.0 ± 0.25	2.2 ± 0.02	3.5 ± 0.67	2.5 ± 1.25
ChD-5	16.5 ± 0.25	9.0 ± 0.10	16.7 ± 0.35	11 ± 0.75
ChD-8	11.7 ± 0.75	7.0 ± 0.12	15.0 ± 0.10	12.0 ± 0.35

Results are expressed in mean ± SD from the triplicate data

The isolates in this investigation exhibited average inhibition zones in diameter from nine to seventeen mm, which effectively hindered the growth of test bacterial pathogens. The highest antagonistic activity against *S. aures* ATCC25923 (17 mm) and *L. Monocytogenes* ATCC19115 (16 mm) was obtained by ChK-11 followed by ChD-5 with an inhibition zone of 16.7 and 16.5 mm against *L. Monocytogenes* ATCC19115 *and S. aures* ATCC25923, respectively. These antagonistic activities observed by the isolates might be due to the production of bacteriocin and also other inhibitory substances by the LAB (Amenu and Bacha 2024). Similarly, other scholars (Mokoena et al. 2016; Putra et al. 2018) reported that LAB isolates from naturally fermented foods investigated for antimicrobial activity and only a few isolates showed antimicrobial activity against test bacteria. According to Amenu and Bacha (2024) out of 956 presumptive LAB isolated from different Ethiopian fermented foods, only 583 (61%) isolates showed antibacterial activities against test pathogens.

Resistance to low pH, bile salt, and adherence test

In the present study, two growth medium pH values (2.0 and 3.0) were used to test the pH tolerance of the isolates at 3 and 6 h. For this reason, isolates confirmed LAB (n=6) were checked for their ability to tolerate pH 2.0 and 3.0 at 3 and 6 h fermentation time (Fig. 3a).

Except for ChK-19, the five best isolates showed better tolerance to low pH values and were selected for further study. Better tolerance was observed by most LAB isolates at pH 3.0 than pH 2.0, but also the isolates in general showed probiotic potential since better survival at pH 2.0. Among five isolates, isolate ChK-11 showed the highest tolerance with a survival rate of 65% at pH 2.0 for 3 h and 97% at pH 3.0 for 6 h. According to Amenu and Bacha (2023), only four of the 11 LAB isolates from 54 isolates survived at pH 2, 2.5, and 3 at the end of the 3rd hour of incubation, which confirms the current study. The findings of this study also agree with other previous findings (Feng et al. 2017; Samedi and Charles 2019) that stated the LAB cell viability is higher at pH 3 than at pH 2. The properties of the isolates tolerating this much lower acidity (i.e., many other bacteria could not tolerate it) may contribute the isolates to colonizing the human gut (stomach) which is characterized by a lower acidic value (Feng et al. 2017; Mokoena et al. 2016).

In addition to the extreme acidity in the stomach area, the high bile salt concentration in the intestine is also the most important factor that may affect the subsistence of probiotics (Feng et al. 2017). The relevant functional bile salt concentration in the human body ranges from 0.3 to 0.5%. In the present study, all five isolates that showed better tolerance to acidic pH were tested for their tolerance to concentrations of bile (0.3-and 0.5%) for 3 and 6 h. From five isolates, three isolates (ChK-7, ChK-11, and ChD-5) showed the highest tolerance to the given bile salt concentrations (Fig. 3b).

In this regard, isolate ChK-11 showed the highest tolerance with survival rates of 94 and 70% at concentrations of bile salt at 0.3%, and 67.6 and 56.6% at 0.5% followed by ChK-7 with survival rates of 80 and 66% at bile salt of 0.3%, and 62.5% and 45% at bile salt of 0.5% for 3 and 6 h, respectively. Based on this, the three best performed isolates namely (ChK-7, ChK-11 and ChD-5) were selected for further adherence tests. Merely four out of the 56 LAB isolates were found to have survived at a vitality rate of over 90% with 0.3% bile salt, as reported by Mulaw et al. (2019). The survival rates of the isolates generally decreased as the concentration of bile salt increased. Comparable research by Sami and Charles (2019) found that 0.3% bile salt concentration improved cell growth viability than 0.5% bile concentrations.

Once the LAB isolates are successfully entered into the human gastrointestinal track, their ability to colonize and survive in the intestinal epithelial cells is crucial to exert their efficient probiotic properties (Cai et al. 2022). In the present study, three LAB isolates that showed better survival at low pH and bile salt were evaluated for their capability to adhere on the surface of the stainless steel plate and showed different adherence rates as shown in (Fig. 3c).

The highest adherence rate (37%) was observed by ChK-11 followed by ChK-7 with an adherence rate of 35%. The current study's findings demonstrated that the three LAB isolates had strong in vitro adhesion characteristics to a stainless steel plate, suggesting that these

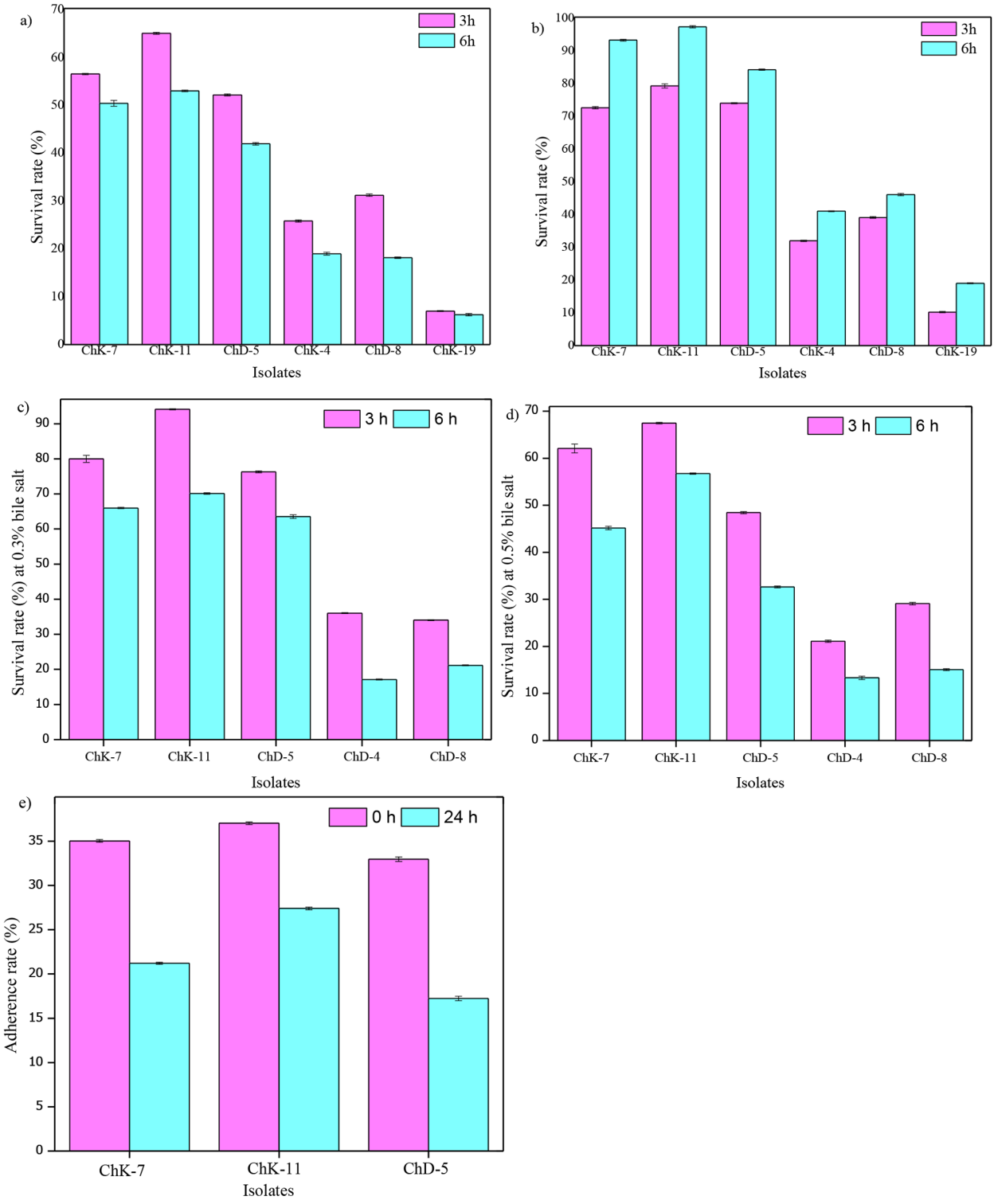

Fig. 3 Survival rate (**a**) pH-2.0, (b) pH-3.0, (**c**) 0.3% bile salt, (**d**) 0.5% bile salt concentration, (**d**) adhesion rate of the isolates to stainless steel plates

isolates would be able to survive the stomach's gastrointestinal tract mucosa. The finding is in line with another study (Mulaw et al. 2019) that all 4 probiotic LAB isolates from *Teff injera* dough, *Ergo*, and *Kocho* foods showed adherence ability ranging from 32.75 to 36.30% on stainless steel plates. According to El-Jeni et al. (2015), four LAB isolates from Tunisian freshwater fishes showed adhesion in vitro to the stainless-steel plates with

different survival rates between 32 and 35%. The current result showed better performance to adhere on the surface of stainless steel with ranges from 33 to 37% as compared with the previous study, suggesting that the nature of the source sample and its composition support abundant probiotic LAB with better performance.

Assessment of the isolates' safety in vitro

Susceptibility of the isolates to commercial antibiotic

In this study, three selected LAB isolates were exposed to six commercial antibiotic discs to ensure their susceptibility challenging using the disc diffusion method. All three LAB isolates were susceptible to six commercial antibiotics with varying zones of inhibition except for ChD-5, which showed resistance to TE (30 µg/mL) (Table 7).

The results showed that isolates obtained were susceptible to almost all tested antibiotic discs, which is one of the most important selection criteria for probiotic LAB to ensure the safety of the isolates. Consistent with the current study, Cai et al. (2022) investigated the 14 LAB strains and showed sensitivity to different commercial antibiotic discs.

Test for hemolytic activity

A criterion for selecting probiotic strains is the lack of hemolytic activity, which guarantees that these bacteria are not virulent (Casarotti et al. 2017). All the three LAB isolates were tested for hemolytic activity on blood agar medium added with (5%, v/v) sheep blood and were all confirmed non-hemolytic, which is prerequisite for safety of the LAB isolates and good indication to be used as potential probiotics. Due to this, the three LAB isolates were selected as probiotics LAB and evaluated for DNase and gelatinase activity. Negative for hemolytic activity confirming that the isolates are safe as LAB for human use (El-Jeni et al. 2015; Jampaphaeng et al. 2017).

Gelatinase activity

This test is also the significant test for the safety consideration of probiotic potential of the LAB isolates. The selected three LAB isolates were tested for gelatinase activity and showed no clear zones observed on gelatin agar medium when incubated anaerobically at 35°C for 24–48 h. When compared with ChD-5, ChK-11, and ChK-7 showed better performance. Hence, the two isolates (ChK-11 and ChK-7) were selected for further characterization. As stated by another scholar (Gupta and Sharma 2017), probiotics LAB should be negative for gelatinase activity because it could harm the lining of mucoid. In line with the current study, Marroki and Bousmaha-Marroki (2014) stated that if the LAB isolates are negative to gelatinase enzyme production, it indicates that these bacterial isolates are non-virulent and as such are suitable to be used as probiotics.

DNase activity

In the current study, both isolates (ChK-11 and ChK-7) were tested for DNase activity and showed negative for the activity of the enzyme DNase production. The isolate ChK-11 showed better performance than ChK-7 and were selected for16S rRNA gene sequencing. According to Amenu and Bacha (2023), isolates with the best probiotic properties were lastly selected by mass spectrometry for identification at the species level. Divisekera et al. (2019) also obtained 15 LAB from finger millet flour, and seven LAB isolates were found to be free from DNase activity in vitro possessing one of the most important safety attributes, and were selected as probiotic candidates.

Phylogenetic analysis and 16 S rRNA gene amplification

According to different reports (Chentouf et al. 2023; Pabari et al. 2020), 16 S rRNA gene amplification and sequencing can be used to identify and characterize bacterial isolates. Based on the 16 S rRNA gene analysis, isolate ChK-11 and ChK-7 revealed the highest sequence similarities with genus *Weissella* and *Leuconostoc spp*, respectively. Phylogenetic analysis using 16 S rRNA nucleotide sequences and BLAST revealed that *Weissella paramesenteroides* with a sequence similarity of 99% was most closely related to isolate ChK-11 (Fig. 4). Similarly, ChK-7 has shown 91% sequence similarity with *Leuconostoc pseudomesenteroides.* The molecular sequences were deposited in National Center for Biotechnology Information (NCBI) with Genebank accession number of OQ820222.1 and OQ820237.1 for isolates ChK-11 and ChK-7, respectively.

Leuconostoc pseudomesenteroides isolated from natural pickles were believed to have probiotic qualities and anticancer activities, and they were detected by doing the 16 S rRNA sequence analysis (Alan et al. 2022). Another study by Wang et al. (2018) identified *L. pseudomesenteroides*, which served as a probiotic isolate and chosen

Table 7 Antibiotic susceptibility of LAB isolates against standard antibiotics (µg/mL)

Isolate code	Te (30)	Va (30)	Ge (10)	Ce (30)	Er (15)	Do (30)
ChK-11	S	R	S	S	S	S
ChD-5	S	R	S	S	S	S
ChK-7	S	S	S	S	S	S

Susceptibility test expressed as R = resistant, I = intermediate, S = sensitive

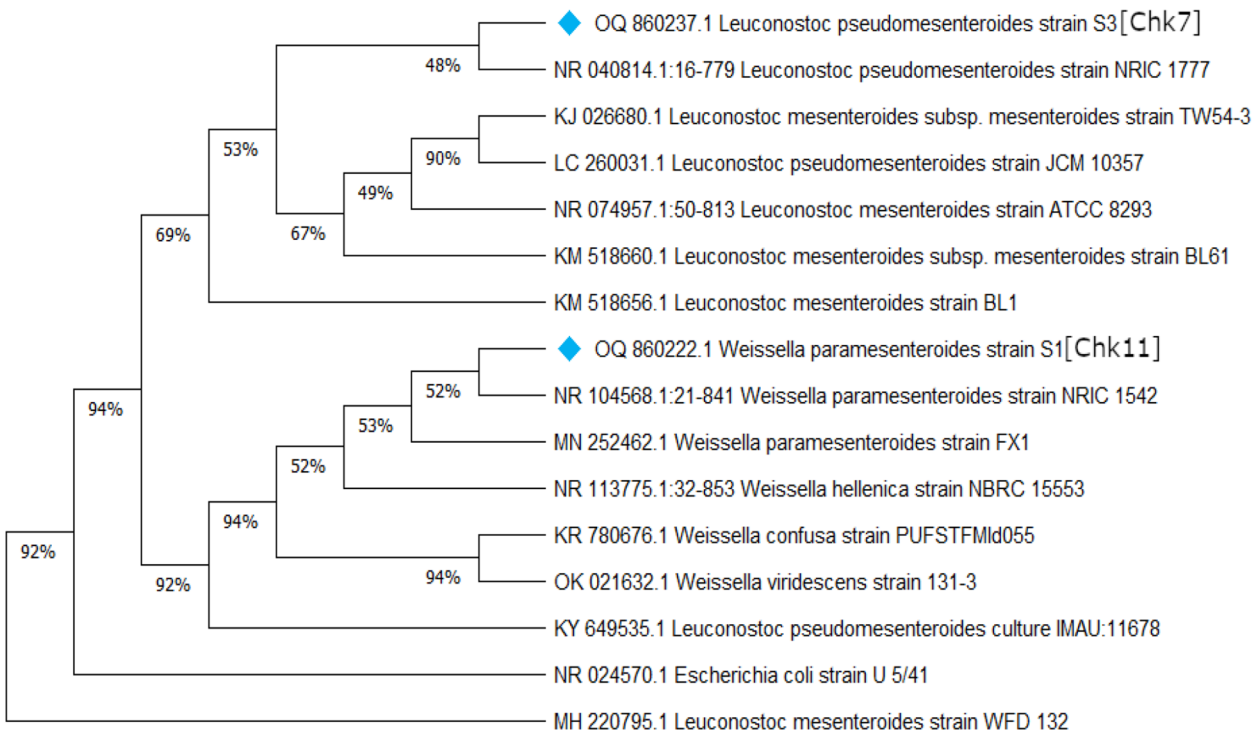

Fig. 4 Phylogenetic tree of *Weissella paramesenteroides* ChK-11 and *Leuconostoc pseudomesenteroides* ChK-7

Table 8 Comparison of various fermented food and beverage with ingredients of *Cheka*

Food type	Major ingredients	FT (day)	pH	Temp (℃)	Microbial Char		Country	References
					Bioch	Mol		
Cheka	Maize, sorghum, finger millet, malt, water	3–4	3.8–4.1	26–28	√	√	Ethiopia	This study
Cheka	Maize, sorghum, millet, malt, water	3–4	4.1–4.6	-	-	-	Ethiopia	Tsegaye et al., 2020
Borde	Maize, barley, wheat, millet, sorghum, water	3–4	3.6–4.1	-	√	√	Ethiopia	Gebre et al. 2023
Shameta	Maize, barley, wheat, sorghum, water	5–7	3.6–4.4	-	√	-	Ethiopia	Kitessa et al. 2022
Tella	Barley, maize, wheat, millet, sorghum, teff, *gesho* (hop), water	5–7	4.8	20–25	√	√	Ethiopia	Yehuala et al. 2024
Korefe	Barley, *gesho*, water	5–8	4.1	25	√	-	Ethiopia	Getnet & Berhanu, 2016
Keribo	Barley, sugar, water	2–3	3.8	-	√	-	Ethiopia	Abawari 2013
Tej	Honey, malt, hop, water	5–6	3.8–4.2	-	√	√	Ethiopia	Fentie et al. 2022
Omegisool	Foxtail, millet powder cakes, nuruk, water	3–5	3.5–3.5	-	√	√	Korea	Oh and Jung 2015
Kimchi	Cabbage, radish, cucumber, scallions, carrots, garlic, ginger, chili flakes, water	50–57	4.2	-	√	√	Korea	Maoloni et al. 2020
Raabadi	Barley, millet, salt buttermilk and coriander	2	-	-	√	√	India	Yadav et al. 2016
Bushera	Millet, sorghum, water	5–6	3.7–4.5	27–30	√	√	Uganda	Muyanja et al. 2003
Kunun-zaki	Millet, wheat, rice, water	4–5	3.9–4.3	28–30	√	√	Nigeria	Daniel et al. 2023

√= Characterized, -= characterization Not done, FT = Fermentation time, Char = Characterization, Bioch = Biochemical, Mol = Molecular

for health industry with the support of more research. Two strains of *W. paramesenteroides* were also reported that their potential of probiotic quality (Pabari et al. 2020).

In order to understand more about the ingredients used, duration of maturation time and other physico-chemical parameters, this study was compared with other previously reported fermented food and beverage results in different parts of Ethiopia, Korea, India, Uganda, and Nigeria (Table 8). Like *Cheka* used as a local fermented beverage in the current study area, *Borde*, *Shameta*, *Tella*, *Korefa*, *Keribo*, and *Tej* are also commonly prepared food in different parts of Ethiopia. *Omegisool* and *Kimchi* in Korea, *Raabadi* in India, *Bushera* in Uganda, and *Kunun-zaki* in Nigeria are also locally prepared fermented foods (Table 8).

The comparison results have shown that the ingredients utilized and the physicochemical parameters of *Cheka* were also found in another fermented beverages used as a source of potential probiotic foods (Table 8). For instance, like *Cheka*, maize, and sorghum are also the ingredients used in *Shameta*, *Borde*, and *Tella* production. In *Bushera* preparation, Ugandans also use maize as the main ingredient. Except for *Kimchi* (prepared from cabbage, radish, cucumber, scallions, carrots, garlic, ginger, and chili flakes) fermented for 50–57 days, most other foods reported in Table 8 showed 3–7 days fermentation. Among fermented foods, *Raabadi* (which is prepared from barley, millet, salt buttermilk, and coriander) have the shortest (2 days) fermentation time for consumption. Almost all the matured fermented ready food for consumption has a pH value ranged from 3.5 to 4.8 (Table 8). *Cheka* which have a comparative physicochemical characteristic with Ethiopian and other countries fermentation food qualifies promotion to be used as a potential probiotic food. The results observed in this study as compared with the previous study indicated that the two molecularly identified LAB isolates from *Cheka* can play a significant role in consumers as probiotics source.

Conclusion

In the present study, probiotic LAB were successfully isolated from *Cheka* samples obtained from Konso and Derashe. The findings of this in vitro study indicated that fermented foods like *Cheka* are the source of LAB with functional properties and able to exhibit probiotic features. The isolates were negative to hemolytic, gelatinase, and DNase activity, which are the most important criteria to ensure the safety of the probiotic LAB in vitro. Among all the isolates, ChK-11 had showed the highest survival rate to acidic pH (2.0 and 3.0) and stomach bile salt concentration (0.3 and 0.5%) for 3 and 6 h, and also highest anti-microbial property and adhesion potential when compared to the others. In general, these important characteristics may enable the isolates to subsist in the stomach and intestine, or even to contend with other pathogenic bacterial groups in this situation and to dominate the gastrointestinal tract of the host. The 16 S rRNA gene sequence analysis revealed that the highest sequence similarities with *Weissella and Leuconostoc spp* were identified for ChK-11 and ChK-7, respectively. Overall, these LAB isolates with better tolerance to acidic pH and bile salt, and capability to attach to the intestinal epithelial cell model in vitro could make them to be considered a potential probiotics LAB.

Abbreviations

LAB	Lactic Acid Bacteria
EPHI	Ethiopian Public Health Institute
ATCC	American type culture collection
CTAB	Cetyltrimethylammonium bromide
NCBI	National center for biotechnology information
ANOVA	Analysis of variance

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

D. A. is the principal investigator, carried out the bacterial isolation and screening in the lab, and made significant contributions to the manuscript's composition. Writing the initial manuscript draft and data curation was done by A.G., N.K., and A.F. Editing and reviewing the manuscript were G.T. and K.A. Every listed author has perused the work and provided their consent for its dissemination.

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Availability of data and material

16 S rRNA gene sequences that corroborate the findings of this paper can be found with accession codes OQ820222.1 and OQ820237.1 in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Declarations

Ethics approval and consent to participate

Not applicable.

Conflict of interest

The writers claim to have no conflicting agendas.

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