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Running title: Probiotic starters from cereal beverages

Abstract

Aims: To test the *in vitro* probiotic potential and starter culture capacity of lactic acid bacteria (LAB) isolated from Naaqe and Cheka, cereal-based Ethiopian traditional fermented beverages.

Methods and Results: 44 strains were isolated from spontaneously fermented Ethiopian cereal-based beverages, Naaqe and Cheka, with 24 putatively identified as LAB and 14 identified up to the species level. The species *Limosilactobacillus fermentum* (6/12; 50%) and *Weissella confusa* (5/12, 41.67 %) were the predominant species identified from Naaqe, while the two Cheka isolates were *Limosilactobacillus fermentum* and *Pediococcus pentosaceus*. Six LAB strains inhibited eight of the nine gastrointestinal indicator key pathogens in Ethiopia, including *Escherichia coli*, *Salmonella enterica* subsp. *enterica* var. Typhimurium, *Staphylococcus aureus*, *Shigella flexneri*, and *Listeria monocytogenes*. Three of the LAB isolates exhibited strain-specific immunostimulation in human monocytes. Based on these probiotic properties and growth, six strains were selected for *in situ* evaluation in a mock fermentation of Naaqe and Cheka. During primary fermentations, *L. fermentum* 73B, *P. pentosaceus* 74D, *L. fermentum* 44B, *Weissella confusa* 44D, *L. fermentum* 82C and *Weissella cibaria* 83E and their combinations demonstrated higher pH-lowering properties and colony-forming unit counts compared to the control spontaneous fermentation. The same pattern was also observed in the secondary mock fermentation by the Naaqe LAB isolates.

Conclusions: In this study, we selected six LAB strains with antipathogenic, immunostimulatory and starter culture potentials that can be used as autochthonous probiotic starters for Naaqe and Cheka fermentations, once their health benefit is ascertained in a clinical trial as a next step.

Significance and Impact of the Study: Improving quality of the fermentation process through LAB-based probiotic starters enhances their ability to fight off food-borne infections.

Key words: Traditional cereal beverages, Naaqe; Cheka, Lactic acid bacteria; Antimicrobial activity; Nuclear factor kappa B; Probiotic starter cultures

1. Introduction

African communities used fermentation of cereals as a food processing and preservation means for millennia (Mokoena *et al.*, 2016; Setta *et al.*, 2020). Sub-Saharan Africa traditions include a wealth of knowledge about cereal fermentations (production processes and fermentation microorganisms), which is largely unexplored and undocumented (Pswarayi and Gänzle, 2022). Millions of African people depend on this technology to preserve and often enhance organoleptic properties, nutritional qualities, digestibility, and acceptability of their traditional foods at costs affordable to the average consumer (Aka Solange *et al.*, 2014; Mokoena *et al.*, 2016; Setta *et al.*, 2020). Important beverages are produced through fermentation of cereals such as maize, barley, millet, wheat and sorghum (Aka Solange *et al.*, 2014). Socially, when served, these drinks show a gesture of hospitality, friendliness and strengthen amicable relationships between individuals (Worku *et al.*, 2016; Setta *et al.*, 2020).

The process is spontaneous, with the procedure of how to make such products passed down from one generation to another. The beverages share common production processes such as cooking/baking/boiling of doughs of single or mixed grain flours along with single or multiple fermentation steps. Malt and a small amount of the beverage from previous fermentation (back-slopping) can also be added (Steinkraus, 1996; Arici and Daglioglu, 2002; Worku *et al.*, 2016; Desta and Melese, 2019). Differences in the fermentation practices and their recipes can make the artisanal beverages either alcoholic or non-alcoholic. Lactic acid bacteria (LAB)-based fermentations are examples of non-alcoholic fermentations. Since starter cultures are not used, these fermentations are largely uncontrolled, with the quality and stability of the products compromised. Selection of appropriate starter cultures is one of the key strategies to make the fermentation processes controllable, predictable and efficient (Fentie *et al.*, 2020; Hotessa and Robe, 2020; Setta *et al.*, 2020). Starter cultures are preparations with a large number of single or multiple types of microbial cells added to the fresh substrate to enhance fermentation (García-Díez and Saraiva, 2021). Specific LAB are of special interest as starter cultures, because they do not produce (large amounts of) alcohol (Hutkins, 2019) and are "generally recognized as safe (GRAS)" for addition to food (Aka Solange *et al.*, 2014; Mokoena *et al.*, 2016; Setta *et al.*, 2020). For dairy fermentations, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii subsp. bulgaricus* are examples of commonly used LAB starter cultures, while *Lacticaseibacillus*

rhamnosus is often added to provide additional functionalities (García-Díez and Saraiva, 2021). For cereal fermentations, starter cultures are commonly isolated from the food product itself (autochthonous) (Edema and Sanni, 2008).

Over the last decades, specific probiotic starter cultures have attracted increasing attention due to their unique ability to combine fermentation capabilities with probiotic properties such as a capacity to inhibit enteric pathogens (Edema and Sanni, 2008; Garriga *et al.*, 2015; Rao *et al.*, 2019; Mathur *et al.*, 2020). Probiotics are “"live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (Hill *et al.*, 2014). Selection of probiotic cultures for food fermentations is primarily based on their antimicrobial activities, resistance to acid and bile, and fermentative activity, among others (Enujiugha and Badejo, 2017; Ogunremi *et al.*, 2017). This is highly relevant for countries such as Ethiopia, as foodborne infections are a major cause of morbidity and mortality. According to the World Health Organization (WHO) estimate in 2010, Africa had the highest burden of foodborne diseases (caused mainly by *Salmonella* spp, *Escherichia coli,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Shigella* spp., *Campylobacter* spp and *Listeria monocytogenes*) per capita, with a median of 2,455 foodborne Disability Adjusted Life Years (DALYs) per 100,000 inhabitants (Havelaar *et al.*, 2015; WHO, 2015; Eshetie *et al.*, 2016; Hussen *et al.*, 2019).

Among the Ethiopian indigenous fermented cereal-based beverages, Naaqe, Borde, Cheka, Tella, Areki, Keribo and Shamita are most often produced and consumed (Lee *et al.*, 2015; Fentie *et al.*, 2020). In this study, Cheka and Naaqe were characterized as potential source of probiotic starter cultures. Cheka is widely consumed in the southwestern parts of Ethiopia, mainly in Konso and Dirashe (Worku *et al.*, 2016; Fentie *et al.*, 2020) and prepared from cereals such as sorghum (*Sorghum bicolor*), maize (*Zea mays*), barley (*Hordeum vulgare*), and finger millet (*Eleusine coracana*), and vegetables such as leaf cabbage (*Brassica* spp.), moringa (*Moringa stenopetala*), and decne (*Leptadenia hastata*) (Worku *et al.*, 2016; Hailemariam, 2017). The production process of Cheka has two fermentation processes running through three phases. The people of Konso mostly use mixture of the cereals as ingredients, while in Dirashe, cabbage and moringa leaves are used in addition to cereals (Hailemariam, 2017). To the best of our knowledge, no research has yet been conducted on the probiotic potential of LAB from Cheka (Hotessa and Robe, 2020). Naaqe is a traditional cereal-based beverage produced and consumed in Arba Minch district, Gamo

Zone, Southern Ethiopia. It is made mainly from maize and barley, but the product has -to the best of our knowledge- not yet been scientifically documented or studied. The processes of Naaqe preparation are relatively simple with two fermentation steps separated by cooking of the primary fermentation product. Naaqe fermentation does not involve the use of malt, so that the product is generally non-alcoholic. In this study, LAB from Naaqe (non-alcoholic) and Cheka (alcoholic) were isolated, screened for *in vitro* probiotic potential (e.g., antimicrobial activity and immunostimulation) and starter culture capacity in laboratory-scale fermentation experiments.

2. Materials and Method

2.1. Isolation, characterization and enumeration of LAB

Two Naaqe samples from Arba Minch district (Ethiopia) and two Cheka samples from Konso (Ethiopia), were aseptically collected in sterile 50 mL tubes, and transported in an ice-box to the Bacteriology laboratory of Armauer Hansen Research Institute (AHRI). The samples were processed on arrival for the isolation of LAB. To isolate LAB, 10 mL of each sample was suspended and homogenized in 90 mL phosphate buffered saline (PBS) (pH 7−7.4). Ten µL of appropriate dilution (mostly the 3rd to 6th) was spread-plated on de Man, Rogosa, and Sharpe (MRS) agar (Hi-Media, Mumbai, India) and then incubated anaerobically (BD BBL™ GasPak™ jars) at 37^{o} C for 24 to 48 h. Plates with 30 to 300 colonies were selected and colonies counted. On average, five morphologically distinct colonies per plate were then randomly selected and purified through 3 successive streaking on MRS agar. Aliquots of the selected isolates were stored at −80°C in MRS broth containing 25% glycerol. The pure isolates were characterized presumptively as LAB based on cell morphology, Gram staining, catalase test, and motility, according to standard procedures as described elsewhere (Silva, 2013). The number of colony forming units (CFU) per milliliter (CFU mL^{-1}) in the collected Naaqe and Cheka was calculated as a function of the number of confirmed LAB colonies and the inoculated dilution using the following formula (Silva, 2013):

CFU mL^{-1} = total colonies present x percent confirmed colonies x dilution.

2.2. Molecular identification of LAB isolates

From the isolates putatively identified as LAB, those selected based on initial antimicrobial screening (Supplementary Table 1) and diversity of sample origin were further identified to the species level by *16S rRNA* gene sequencing. Colony PCR amplification using primers: 27F (5'-

AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'); followed by Sanger sequencing was performed as previously described (Gizachew *et al.*, 2023). The resulting *16S rRNA* gene sequences were then analyzed using the SeqTrace 0.9.0 software and submitted to a search for similarity in the EzBioCloud.net 16S-based ID. Bacterial identification was supposed when the query sequence showed pairwise similarity >98.7% for the *16S rRNA* gene sequence (Lagier *et al.*, 2018).

2.3. Antagonistic activity of LAB isolates against indicator pathogens

Spot overlay and radial diffusion assays were employed to examine the antagonistic activity of LAB strains against selected foodborne pathogens.

2.3.1. Spot overlay assay

Spot overlay assay was performed twice, following protocols as described previously (Gizachew *et al.*, 2023). The indicator foodborne pathogens used in the initial antimicrobial assay were *Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 obtained from the Ethiopian Public Health Institute, and a clinical isolate of MRSA obtained from Tikur Anbessa Specialized Hospital, Addis Ababa University, Ethiopia. In the second broader antimicrobial assay, *L. monocytogenes* MB2022 isolated from Wijnendaele cheese, *Salmonella. enterica* subsp. *Enterica* var. Typhimurium NTCT 13347 and *E. coli* O157:H7 BRMSID188 lacking pathogenicity *stx* genes (for biosafety reasons) isolated from bovine (Van Beeck *et al.*, 2020), *Shigella flexneri* LMG 10472, and *S. aureus* MI/1310/1938 – methicillin sensitive were used as indicator pathogenic strains.

The spot overlay test was performed as described elsewhere (Gizachew *et al.*, 2023). Briefly, 2 µL of overnight LAB culture was pipetted onto MRS agar (in the initial antimicrobial assay), or onto Mueller Hinton agar (MHA, for *S. aureus*) or LB agar (for other pathogens) both supplemented with 5 g/L glucose (in the second broader antimicrobial assay). Two µL of chlorhexidine 0.2% or hexetidine (0.1%) were spotted as positive controls while MRS broth was spotted as negative control. The plates were then incubated aerobically at 37°C for 24 h (for LAB spots on MRS agar) and 48 h (for LAB spots on MHA and LB agar). Afterwards, 20 mL of soft agar (0.5% agar) containing each indicator pathogen (overnight culture grown in BHI broth in initial assay, MH

broth for *S. aureus,* and LB broth for others pathogens in the second broader assay) at $5x10^6$ CFU mL^{-1} was poured over the spots and incubated aerobically at 37°C for 24 h. Pathogen growth inhibition zones around each LAB spot were recorded (diameter in mm) as a measure of antagonistic activity. Experiments were run in triplicates.

2.3.2. Radial diffusion assay

This assay was conducted as described previously (Gizachew *et al.*, 2023). Briefly, the cell-free culture supernatants (CFS) of overnight LAB culture were collected by centrifugation (2484 g, 15 min, 4°C) and sterilized by filtering through 0.22 µm filter, with or without pH adjustment to pH 7.4. A volume of overnight growth of indicator pathogens was added to a cooled agar (55° C) to make the final concentration of $5x10^6$ CFU mL^{-1} and poured onto a square plate. CFS (45 µL), pH adjusted (7.4) or non-adjusted, was dispensed into 6 mm diameter wells made using sterile glass Pasteur pipette. The plates were aerobically incubated at 37° C for 24 h. After incubation, the antagonistic activity was recorded as the diameter (mm) of pathogen growth inhibition zones around each well. Hexetidine (0.1%, 45 µL) and MRS broth (45 µL) were used as positive and negative controls, respectively. Experiments were carried out in triplicates.

2.4. Resistance to gastrointestinal conditions *in vitro*

LAB isolates were inoculated in MRS broth and incubated at 37°C overnight. After incubation, the bacterial cells were harvested (4,000 g, 10 min, 4°C), washed twice with PBS, and the number of cells was adjusted to $1.5x10^8$ CFU mL^{-1} by measuring OD at 600 nm. To assess survival of the LAB strains in GI acidic environment, 100 µL of the $1.5x10^8$ CFU mL^{-1} of each LAB strain was added into 900 µL of sterile PBS adjusted to pH 3.0 (using 1M HCl) and then incubated under stirring (150 rpm) at 37°C for 3h, to simulate the time spent by food in the stomach. Following incubation, 50 µL of each bacterial solution was collected, 10-fold serial dilutions were prepared in PBS and plated onto MRS agar in triplicates to count viable CFUs. To test tolerance of LAB strains in bile salt solution, 100 µL of each LAB strain at 1.5×10^8 CFU mL^{-1} was added into 900 µL of sterile PBS (pH 8.0) supplemented with 0.5% (w/v) bile salts. The solution was then incubated at 37°C under stirring (150 rpm) for 4 h, mimicking the time spent by food in the small intestine (Argyri *et al.*, 2013; Garcia *et al.*, 2016; Panya *et al.*, 2016). After incubation, 50 µL of each bacterial solution was collected, 10-fold serial dilutions prepared in PBS, and plated onto

MRS agar in triplicates to count CFUs. Percentage (%) survival of the LAB isolates was calculated using the following formula:

$$\% \text{ of cell survival} = (\log CFU_T / \log CFU_C) \times 100$$

where CFU_C and CFU_T represent the total viable count of LAB isolates before and after incubation under the simulated GI condition (low pH or bile salts), respectively.

2.5. Evaluation of immunostimulatory capacity of LAB isolates

Immunostimulatory capacity of the LAB strains was evaluated in the human THP1-Dual™ reporter monocytes (InvivoGen, San Diego, CA, USA) maintained according to the manufacturer's instructions. Briefly, LAB strains at 10^7 CFU mL^{-1} were first UV-inactivated in a biosafety level 2 cabinet for 90 min with vortexing after each 15 min, and added to THP1-Dual™ cells seeded at 10^6 cells mL^{-1} in 96-well plates. For both bacteria and THP1-Dual™ cells, RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum and Pen-Strep (100 µg mL^{-1}) was used. After co-incubation of LAB with THP1-Dual™ for 24 h at 37 °C and 5% CO_2, activation of nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF) pathways was measured using the Synergy HTX plate reader (BioTek) (Gizachew *et al.*, 2023).

2.6. LAB growth curve analysis

For the analysis of the growth ability of LAB isolates, a growth curve in MRS broth was constructed using MRS broth as control. Ten µL-culture aliquots of each LAB isolate were added to each well of a 96-well microplate containing 190 µL of MRS broth (final viable cell count of approximately 7 log CFU mL^{-1}). Bacteria were allowed to grow at room temperature, and the OD_{600} was measured every 30 min for 48 h using a Synergy HTX multi-mode reader. Each condition was tested in triplicate. OD_{600} data from 0 to 48 h were used to obtain the growth parameters area under the bacterial growth curve (AUC) and intrinsic growth rate within 48 h (r) using the R package Growthcurver (Sprouffske and Wagner, 2016). The lag time, as an adaptation to the growth conditions (Sterniša *et al.*, 2022), was estimated from the growth curve plots of LAB isolates OD_{600} measurements.

2.7. Laboratory-scale fermentation experiments

Based on an overall evaluation of the tested probiotic and growth properties of the LAB isolates (Table 4), 6 LAB isolates were selected as candidate probiotic strains for laboratory-scale fermentation experiments of Naaqe and Cheka. In each of Naaqe and Cheka fermentation experiment, two LAB isolates (e.g., *L. fermentum* 44B and *W. confusa* 44D) singly or their combination were inoculated into a fermentation vessel in duplicates at time point 0. Spontaneous fermentation vessels (N4- for the 44B & 44D batch, N8- for the 82C & 83E batch or N- for the 73B & 74D batch)) served as controls. Inoculum of LAB isolates used were prepared from overnight culture in MRS broth by harvesting (4,000 g, 10 min, 4°C), washing with and resuspending in PBS, and calculating a volume required to make cells final concentration 10^5 CFU mL^{-1} in 300 mL fermentation mix by measuring OD at 600 nm. Contents of the fermentation vessels were mixed by thorough stirring using sterile glass rods. At each time points, appropriate dilutions were plated out on MRS agar, incubated for 24-48 h, at 37 ^{0}C anaerobically and then colonies were counted. The experiments were run for 72 h with sampling and processing at baseline and regular intervals (14, 24, 48 and 72 h). The 14 h time point is a regular time of consumption for Naaqe, hence used as the first sampling time point for Naaqe secondary fermentation. All ingredients used in this laboratory-scale fermentations were collected from their indigenous locale (Konso and Arba Minch District).

2.7.1. Naaqe fermentation

To develop a protocol for the Naaqe laboratory-scale fermentation experiment, indigenous Naaqe preparation techniques including ingredients used, fermentation time, fermentation facilities and related information were gathered through interview of local breweries, onsite observation, and analysis of 2 samples. Details of the indigenous Naaqe preparation method is provided as supplementary information (Supplementary Text 1).

Protocol for Naaqe lab-scale fermentation

Primary Fermentation of Naaqe

At time point 0 h (baseline), maize (Bako Hybrid-660) flour was kneaded thoroughly with water (flour: water; 1:0.75) and a sample was taken from that portion for pH measurement. Ten mL of the mix was also sampled for plating out for CFU- /mL^{-1} counts after serial dilutions. Immediately

after sampling, the mix was divided into 8 parts for 8 different sterile clay pots (duplicates for either Spontaneous (2) or starter culture fermentation (6). Fermentation vessels except for spontaneous fermentation (N4 & N8), were inoculated with respective single strain or combination of strains. The vessels were then kept at room temperature (20-25 ^{0}C) and allowed to ferment and sampled at 24, 48, and 72 h for pH measurement and plating out on MRS medium for CFU mL^{-1} (Figure 1A).

Secondary Fermentation of Naaqe

At time point 0 h (baseline), a spontaneous primary fermentation was set up for 30 h after which the mixture was made in to dough balls and cooked (for 70 min). The cooked dough balls were then allowed to cool (4 h) and smashed into pieces in a tray using a clean bottle, and kneaded with little water and barley flour thoroughly (cooled smashed dough balls:malt:kneading water; 1:0.18:0.12). Water was then added and mixed well to make a soft mass (mix:water; 1:0.33) and a portion was sampled for pH measurement. Ten mL of the mix was also sampled for plating out after serial dilutions. Immediately after sampling, the mix was divided into 8 parts for 8 different sterile clay pots (duplicates for either Spontaneous (2) or starter culture fermentation (6)). Fermentation vessels except for spontaneous fermentation (N4 & N8), were inoculated with respective single strains or combination of strains. The vessels were then kept at room temperature (20-25 ^{0}C) and allowed to ferment and sampled at 14, 48, and 72 h for pH measurement and plating out on MRS medium for CFU mL^{-1} (Figure 1A).

2.7.2. Cheka fermentation

The protocol used in Cheka laboratory scale fermentation was constructed based on available literature (Worku *et al.*, 2016), interviews, onsite observation, and analysis of 2 samples. Details of the indigenous Cheka preparation method is provided as supplementary information (Supplementary Text 2).

Protocol for Cheka lab-scale fermentation

Primary Fermentation of Cheka

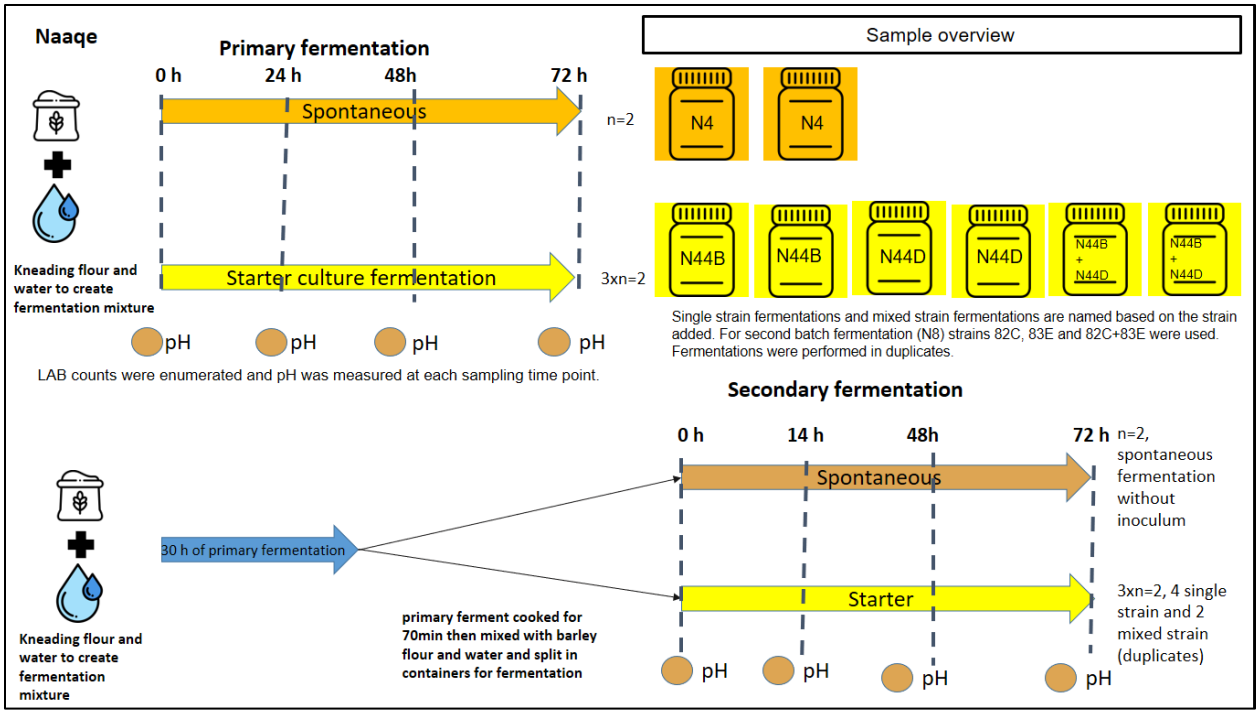
At time point 0 h (baseline), grain (maize:sorghum; 3:1) flour was kneaded thoroughly with water (flour: water; 1.00:0.65) and a portion was sampled to measure the pH and 10 mL sample was taken for plating out. Immediately after sampling, the mix was divided into 8 parts for 8 different

sterile clay pots. Fermentation vessels except for spontaneous fermentation (N) were inoculated with respective single strain or combination of the strains. The vessels were then kept at room temperature (20-25 ^{0}C) and allowed to ferment. The ferment was sampled at 24, 48, and 72 h for pH measurement and plating out on MRS medium for CFU mL^{-1} (Figure 1B).

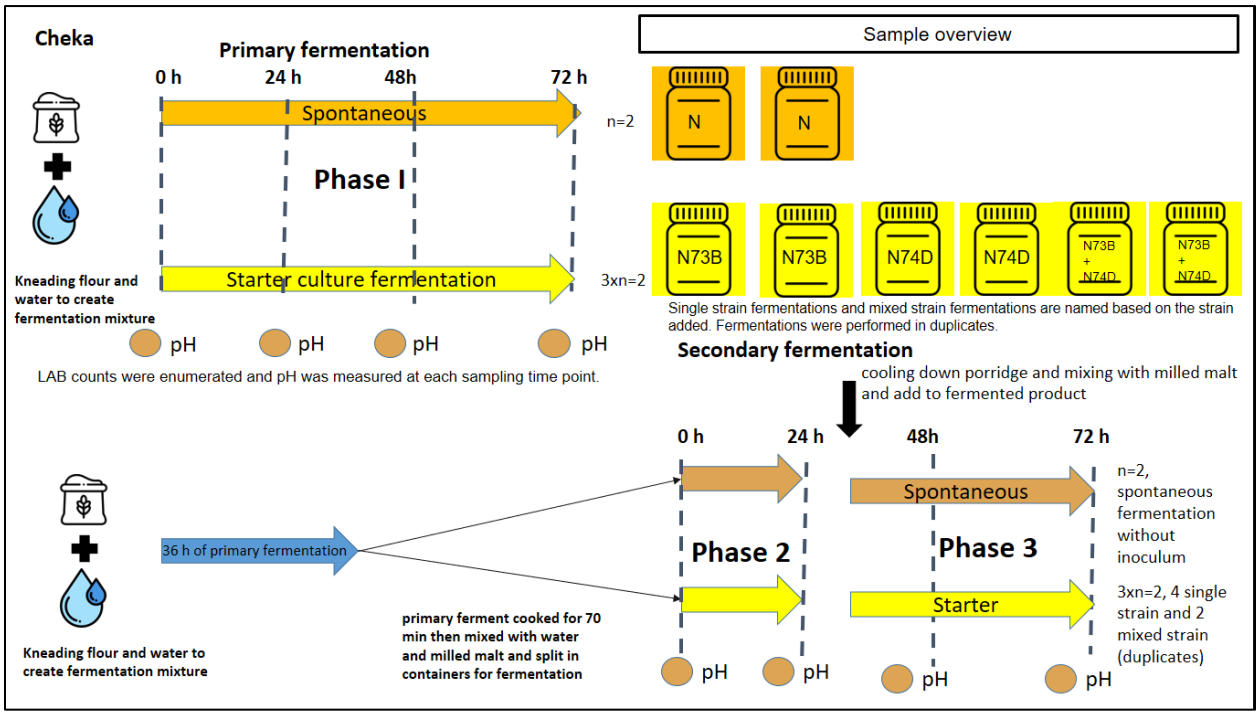
Secondary Fermentation of Cheka

At time point 0 h (baseline), dough balls were made and cooked (for 1 h) from the primary fermentation mix fermented for 36 h. The dough balls were then cooled (4 h) and smashed into pieces in a tray using a clean bottle, and kneaded with little water, mixed with adequate milled malt and kneaded thoroughly (smashed dough balls:malt:kneading water; 1:0.15:0.11). Water was added and mixed well (mix:water; 1:0.33). A portion of the mix was sampled for pH measurement and 10 mL sample was taken for plating out. Immediately after sampling, the mix was divided into 8 parts for 8 different sterile clay pots. Fermentation vessels except for spontaneous fermentation (N) were inoculated with respective single strain or combination of the strains. The vessels were then kept at room temperature (20-25 ^{0}C) to ferment.

At time point 24 h, from the mix allowed to ferment for 24 h, a portion of the mix was sampled for pH measurement and 10 mL of the mix was taken for plating out. In parallel, a very thick porridge was prepared by adding grain flour to boiling water (Grain flour (maize:sorghum; 3:1):boiling water for porridge preparation; 1.00:0.70) and the porridge was then allowed to cool (3-4 h). The cooled porridge was then kneaded thoroughly with milled malt and then mixed with the product in the vessel, and kneaded well. (Mix:porridge:Malt:Water for mixing; 1.00:0.15:0.11: 0.22). The vessels were then kept at room temperature to ferment and sampled at 48, and 72 h for pH measurement and plating out on MRS medium for CFU mL^{-1} (Figure 1B).



A



B

Figure 1: Flow chart of laboratory scale Naaqe (A) and Cheka (B) fermentation experiments: Six LAB isolates (four from Naaqe: 44B, 44D, 82C and 83E and two from Cheka: 73B and 74D) were selected based on their probiotic properties and growth curve data, as indicated in Table 4. In each experiment, two

LAB isolates (e.g., *L. fermentum* 44B and *W. confusa* 44D) individually or their combination were inoculated into fermentation vessels in duplicates at time point 0. LAB isolates were added at 10^5 CFU mL^{-1} (or at $5x10^4$ CFU mL^{-1} per strain when 2 strains were used) in 300 mL fermentation mix. The spontaneous fermentation controls were also included (indicated as N4- for the 44B & 44D batch, N8- for the 82C & 83E batch or N- for the 73B & 74D batch). Icons were obtained from flaticon.com (designed by freepik).

2.8. Statistical Analysis

Results are expressed as mean ± standard deviation. Normal distribution of data was evaluated using Shapiro-Wilk and Kolmogorov-Smirnov normality tests before statistical comparisons. For normally distributed data, one-way ANOVA followed by Dunnett's multiple comparisons test was used. Otherwise, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. If only two groups were compared, t-tests were used. Statistical testing for fermentation experiments involving three variables [time, response (pH or CFU mL^{-1}) and strain] was performed using two-way ANOVA followed by Dunnett's multiple comparisons test. Statistical comparisons were made when applicable using GraphPad Prism version 9.2.0. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Isolation and identification of LAB from Naaqe and Cheka

To isolate LAB from Naaqe and Cheka as model Ethiopian cereal drinks, four spontaneously fermented samples (2 Naaqe and 2 Cheka batches) were plated out on MRS medium. The total colony count on MRS agar (CFU mL^{-1}) and presumptive LAB (based on gram staining, catalase test and motility test) in Naaqe and Cheka samples is shown in Table 1, demonstrating notable variation in LAB and total CFU mL^{-1} between batches.

A process consisting of phenotypic and genotypic methods depicted as a flow chart in Figure 2 was followed to select potential LAB probiotic starter strains from the collected fermentation samples.

Table 1: CFU mL^{-1} of LAB and total colonies counted on MRS agar in spontaneously fermented Ethiopian cereal drink samples.

Cereal drink (batch code)	Confirmed LAB count (CFU mL^{-1})	Total colony count on MRS agar (CFU mL^{-1})
Naaqe (batch 1)	1.04E+09	1.68E+09
Naaqe (batch 2)	5.55E+05	5.55E+05
Cheka (batch 1)	0	9.9E+07
Cheka (batch 2)	3.65E+07	8.65E+07

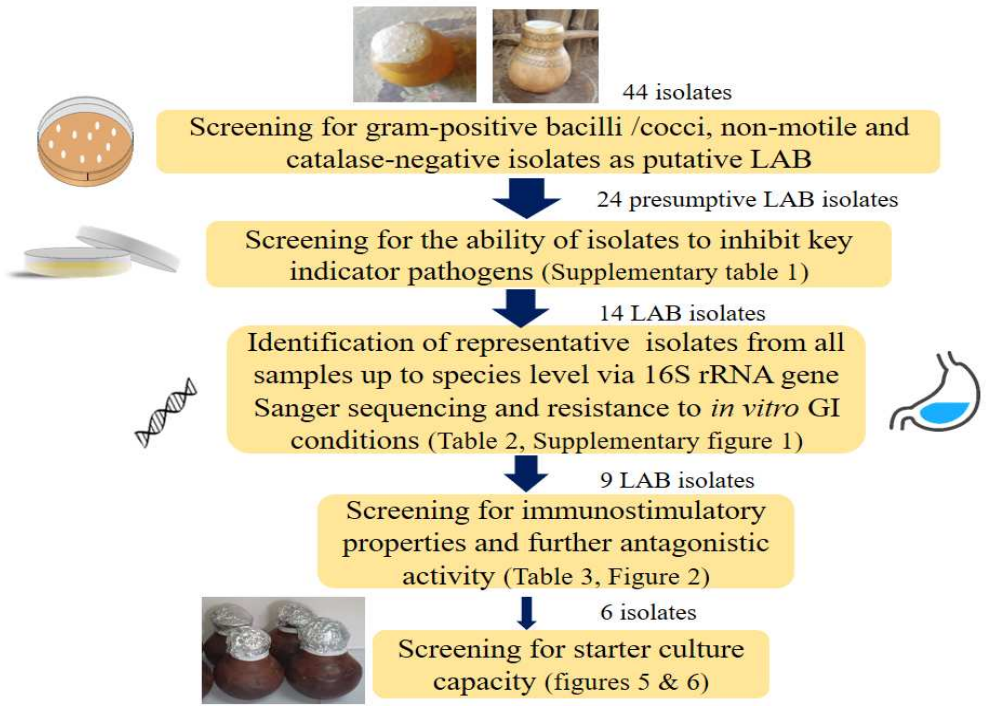


Figure 2. Flow chart used to select potential LAB probiotic starters from Ethiopian cereal beverages, Naaqe and Cheka based on a combination of phenotypic and genotypic methods.

The colonies on MRS agar were checked for morphological characteristics typical for LAB (circular, smooth, and milky colony), ten to fifteen pure colonies were then picked from each sample (Garcia *et al.*, 2016), and subjected to the screening process. From a total of 44 isolates

selected (23 from Naaqe and 21 from Cheka), 24 isolates (19 from Naaqe and 5 from Cheka) were presumptively identified as LAB based on their gram-positivity, catalase-negativity, and non-motility. From these 24 isolates, 14 (2 from Cheka and 12 from Naaqe) were selected based on the degree of antibacterial activity displayed against the key indicator pathogens *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and MRSA in the spot overlay assay (Supplementary Table 1). These 14 LAB isolates were then identified up to the species level by *16S rRNA* gene Sanger sequencing (Table 2). The species *Limosilactobacillus fermentum* (6/12; 50%) and *Weissella confusa* (5/12, 41.67 %) were the predominant species identified from Naaqe and the remaining isolates turned up as *Weissella cibaria* (1/12, 8.33%). The two Cheka isolates were identified as *Limosilactobacillus fermentum* and *Pediococcus pentosaceus,* respectively. Of note, these isolates did only come from one Cheka sample. The other Cheka sample did not contain any putative LAB (Table 1) and was probably an alcoholic (yeast based) fermented beverage.

Table 2. *16S rRNA*-gene based identification of LAB isolates from Ethiopian cereal fermentation products Naaqe and Cheka

Strain (source)	**Identified by *16S rRNA gene* as:**	**Pairwise Similarity (%)**
44B (N)	*Limosilactobacillus fermentum*	100*
44D (N)	*Weissella confusa*	100*
45C (N)	*Weissella confusa*	100
82C (N)	*Limosilactobacillus fermentum*	99.84*
82D (N)	*Weissella confusa*	100*
82E (N)	*Limosilactobacillus fermentum*	98.93
83A (N)	*Weissella confusa*	100*
83B (N)	*Weissella confusa*	100
83C (N)	*Limosilactobacillus fermentum*	99.93
83E (N)	*Weissella cibaria*	100*
84C (N)	*Limosilactobacillus fermentum*	99.84*
84D (N)	*Limosilactobacillus fermentum*	99.85
73B (C)	*Limosilactobacillus fermentum*	99.45*
74D (C)	*Pediococcus pentosaceus*	100*

Note: N, Naaqe; C, Cheka; *Selected for further characterization.

In addition to the initial testing of antibacterial activity against foodborne pathogens (Supplementary Table 1), the *in vitro* GI conditions resistance of the LAB isolates against gastric acid and bile salts was also tested as a key property for potential probiotic candidates for food applications (Supplementary Figure 1). All the 14 LAB isolates showed resistance to 0.5% bile salt after 4 h exposure, whilst 11 of the 14 LAB strains showed resistance to low pH (pH =3) after 3 h exposure. Considering the initial antibacterial activity (Supplementary Table 1) and GI resistance, as well as species variety, nine isolates (2 from Cheka and 7 from Naaqe) were further selected for more probiotic characterization, as depicted in Figure 2.

3.2. Probiotic Properties of LAB Strains from Naaqe and Cheka

3.2.1. LAB isolates show strong antimicrobial activity against foodborne pathogens

The nine LAB isolates from Naaqe and Cheka were selected for further characterization of their potential probiotic properties. This screening included broader antimicrobial assay against five indicator pathogen strains [*L. monocytogenes* MB2022, *S. enterica* subsp. *Enterica* Typhimurium, *E. coli* O157:H7 (-stx genes), *S. aureus* MI/1310/1938-methicillin sensitive, and *S. flexneri* LMG 10472] using the radial diffusion and spot overlay assays (Table 3). A radial diffusion assay was used to specifically investigate the antagonistic activity of secreted LAB metabolites in culture supernatants, while spot overlay assay was used to explore the interaction between pathogen and live potential probiotic starters.

Five of the LAB isolates tested (*L. fermentum* 44B, *W. confusa* 44D and 82D, *P. pentosaceus* 74D, and *W. cibaria* 83E) inhibited the growth of all indicator pathogens in the spot overlay assay, with similar inhibition levels to that of the model probiotics, *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1 (Table 3). In the radial diffusion assay, six of the isolates tested (*L. fermentum* 44B, 73B, 82C and 84C; *W. confusa* 44D, *P. pentosaceus* 74D) and the model probiotics *L. rhamnosus* GG and *L. plantarum* WCFS1 inhibited the growth of all the indicator pathogens except *S. aureus* MI/1310/1938 (Table 3). Three *L. fermentum* strains (73B, 82C and 84C) displayed inhibition activity against only *L. monocytogenes* MB2022 by spot overlay assay. Of note, in the radial diffusion assay, three of the LAB isolates belonging to the genus *Weissella*

(82D, 83A & 83E) showed no antagonistic activity against *L. monocytogenes* MB2022, but showed moderate to high inhibitory activity in the spot assay.

The antagonistic activity of LAB isolates CFS was shown to be pH-dependent, as no inhibition was observed for the neutralized CFS (Supplementary Table 2). *P. pentosaceus* 74D, a strong acidifier with CFS pH = 3.90, showed a significantly higher inhibition ($p<0.05$) compared to *W. confusa* 83A, a weak acidifier with pH = 4.39. It is, however, noted that isolates acidifying the medium to similar pH (Supplementary Table 2) did not always have similar inhibitory activities, suggesting the effect is not merely pH-dependent. Overall, *L. fermentum* (44B, 82C and 73B), *W. confusa* 44D, *W. cibaria* 83E (active against gram-negative pathogens), and *P. pentosaceus* 74D LAB isolates displayed superior antagonistic activity compared to the positive control chlorhexidine 0.2% and to the other LAB isolates tested.

3.2.2. Activation of NF-κB and IRF Pathways

Before making a final selection of strains for mock fermentations, the immunomodulatory performance of the nine selected LAB strains in THP1-Dual™ monocytes was assessed, focusing on their capacity to stimulate the NF-κB and IRF pathways as key factors in the antimicrobial protective mechanisms. Because the target application for these probiotics is inhibiting gastro-intestinal infections and enhancing the host defense against pathogens, a moderate induction of NF-κB was considered a desirable property. Only three LAB isolates (*L. fermentum* 73B, 82C and 84C) significantly ($p<0.0001$) induced NF-κB in human monocytes (Figure 3). *L. fermentum* 44B and *P. pentosaceus* 74D LAB isolates demonstrated a trend towards NF-κB induction, but this trend failed to reach statistical significance in the tested conditions. The NF-κB induction by the tested LAB strains was also remarkably strain-dependent, highlighting that it is important to screen different strains from the same species. For example, while *L. fermentum* 73B led to significant NF-κB activation, *L. fermentum* 44B did not (Figure 3). Interestingly, all the tested isolates of the *Weissella* genera did not display an NF-κB stimulatory activity in the experimental setting of the present study. In contrast to NF-κB, none of the nine tested isolates displayed significant IRF induction (Supplementary Figure 2), though they tended to show an increase in IRF pathway activity. Of note, the NF-κB and IRF induction by all the tested strains was much lower compared

to the pathogenic *S. aureus* MI/1310/1938-MSSA, but was comparable to that of the model probiotics *L. rhamnosus* GG and *L. plantarum* WCFS1.

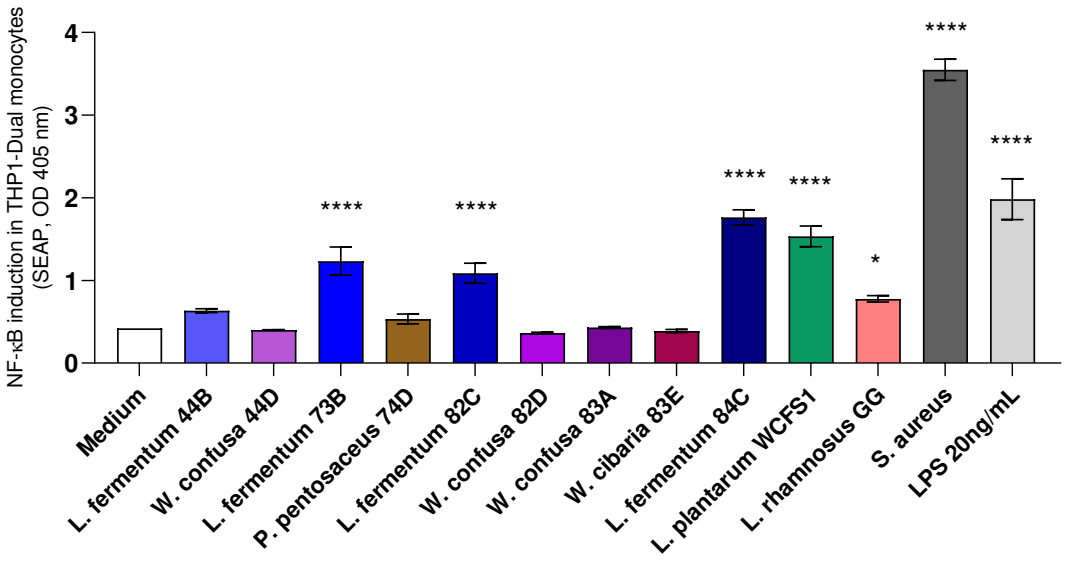


Figure 3: NF-κB pathway activation by LAB strains isolated from Naaqe and Cheka in THP1-Dual human monocytes. Bars depict mean values ± standard deviation per condition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to medium control.

Table 3: Antagonistic activity of the selected nine LAB strains by spot overlay and radial diffusion methods against nine strains indicator foodborne pathogens.

Zone of inhibition (mm)[1], Data are mean values ± SD, (n=3)					**Zone of inhibition (mm)[2],** Data are mean values ± SD, (n=3)									
	L. monocytogenes ATCC 19115	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	methicillin resistant *S. aureus*	*E. coli* O157:H7 BRMSID188		*S. enterica* subsp. *enterica* var. Typhimurium NTCT 13347		*S. flexneri* LMG 10472		*L. monocytogenes* MB2022		*S. aureus* MI/1310/1938	
LAB strain (Source)	Spot overlay	Spot overlay	Spot overlay	Spot overlay	Radial diffusion	Spot overlay	Radial diffusion	Spot overlay	Radial diffusion	Spot overlay	Radial diffusion	Spot overlay	Radial diffusion	Spot overlay
Limosilactobacillus. fermentum 44B (N)	++	–	++	++	++	++	++	+	++	++	+	+++	–	++
W. confusa 44D (N)	++	++	++	++	++	++	++	++	++	++	+	+++	–	+++
L. fermentum 82C (N)	+++	++	++	++	++	–	++	–	++	–	+	++	–	–
W. confusa 82D (N)	–	++	+++	++	++	++	++	+++	++	+++	–	+++	–	+++
W. confusa 83A (N)	–	++	++	++	++	–	++	+	++	–	–	++	–	+
W. cibaria 83E (N)	–	++	+++	+++	++	+	++	+++	++	+++	–	+++	–	+++
L. fermentum 84C (N)	++	++	++	++	++	–	++	–	++	–	+	++	–	–
L. fermentum 73B (C)	++	++	++	+++	+++	–	++	–	++	–	+	+	–	–
P. pentosaceus 74D (C)	+	–	+++	++	++	++	++	+++	++	+++	++	+++	–	+++
Chlorhexidine 0.2%	++	+	+	+										
Lacticaseibacillus rhamnosus GG					+++	+++	++	++	++	+++	+++	+++	–	+++
Lactiplantibacillus plantarum WCFS1					+++	++	+++	++	++	+++	+++	++	–	++
Hexetidine 0.1%					++	++	+	–	++	+	+++	+++	+++	+++

Positive controls, Chlorhexidine 0.2% and Hexetidine 0.1%; Source: C, Cheka; N, Naaqe; Shaded, experiment not conducted; [1]Results of experiments of **initial antimicrobial assays**: –, no inhibition; low, + (9–14 mm); moderate, ++ (14–19 mm), and high inhibition, +++ (>19 mm); [2]Results of experiments of **second broader antimicrobial assay**: for **Radial diffusion assay:** – = no inhibition; low, + (6–8 mm); moderate, ++ (8–11 mm), and high inhibition, +++ (>11 mm); for **Spot assay:** – = no inhibition; low, + (5–7 mm); moderate, ++ (7–10 mm); and high inhibition, +++ (>10 mm)

3.3. *In situ* evaluation of candidate probiotic LAB starter cultures in laboratory-scale fermentations

To further select potential probiotic strains from Naaqe and Cheka that could also function as starter cultures for these traditional fermented foods, we next evaluated the growth potential of the 9 LAB strains in MRS broth under laboratory conditions (Supplementary Figure 3). The analysis revealed that *L. fermentum* 44B (5 h) and *P. pentosaceus* 74D (6 h) had the shortest lag phases and *W. confusa* 82D (14 h) had the longest lag phase. The remaining isolates had an intermediate lag phase, ranging from 7 h (*W. confusa* 83A, *W. cibaria* 83E, *L. rhamnosus* GG) to 11 h (*W. confusa* 44D). The total bacterial growth of the LAB strains was also estimated by area under the growth curve (AUC) analysis (Figure 4A) as it correlates with both the growth rate and maximum possible population size (Sprouffske and Wagner, 2016; Ram *et al.*, 2019). Accordingly, *P. pentosaceus* 74D exhibited the highest AUC values, differing significantly from the values of *L. rhamnosus* GG and *L. plantarum* WCFS1. All the tested LAB isolates, except *W. confusa* 82D, displayed similar or higher AUC values with the control *L. plantarum* WCFS1, indicating they have a sufficient growth capability (Figure 4A). The intrinsic growth rates of *W. confusa* (44D and 82D), *W. cibaria* 83E, and *P. pentosaceus* 74D in MRS broth at 37°C were similar to that of the model probiotic *L. rhamnosus* GG (Figure 4B). All the tested LAB isolates also exhibited similar or higher intrinsic growth rates with *L. plantarum* WCFS1. One should note that the growth performance of the strains in this laboratory experiment (in MRS broth, at room temperature 22.8°C) is different from the growth under spontaneous fermentation conditions. Therefore, to assess the strains' performance in a real-world setting, mock community fermentations were set up in a next phase.

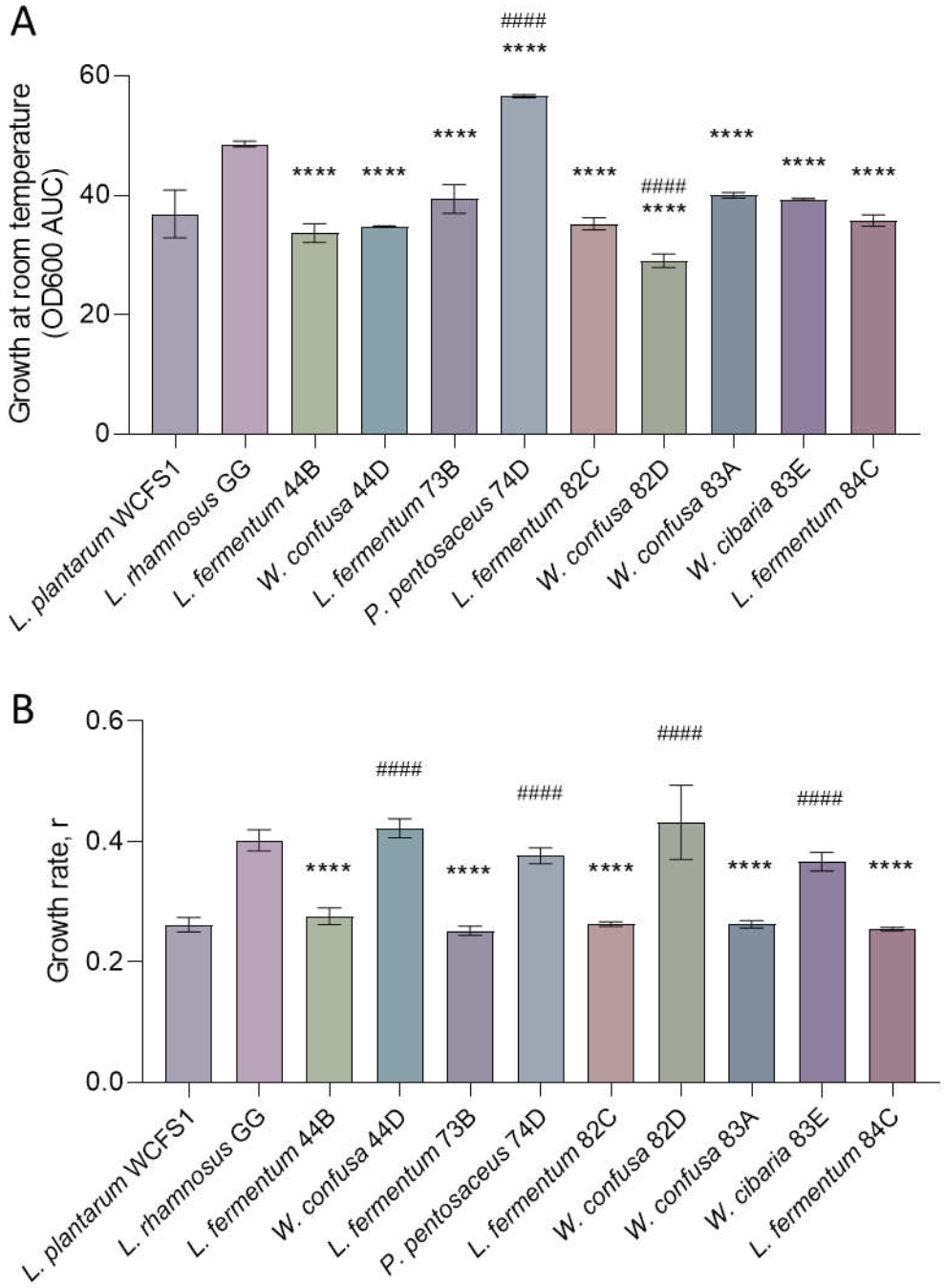


Figure 4: The growth (absorbance at 600 nm/h, OD_{600}) area under the curve (AUC) (A) and intrinsic growth rate, r (B) of the LAB strains in MRS broth at room temperature (average 22.8 °C). Bars depict mean values ± standard deviation per condition (three replicates). ****p<0.0001 compared to *L. rhamnosus* GG, and #### p<0.0001 compared to *L. plantarum* WCFS1.

Table 4: Summary of probiotic properties of the 6 LAB strains from Naaqe and Cheka selected as starter cultures for mock fermentation experiments compared to 3 LAB strains not selected.

Property tested		6 promising candidate probiotic starter LAB strains						Others not selected		
		L. fermentum 44B (N)	*W. confusa* 44D (N)	*L. fermentum* 82C (N)	*W. cibaria* 83E (N)	*L. fermentum* 73B (C)	*P. pentosaceus* 74D (C)	*W. confusa* 82D (N)	*W. confusa* 83A (N)	*L. fermentum* 84C (N)
Antipathogenic activity against	*L. monocytogenes* ATCC 19115	√	√	√	–	√	√	–	–	√
	S. aureus ATCC 25923	–	√	√	√	√	–	√	√	√
	E. coli ATCC 25922	√	√	√	√	√	√	√	√	√
	Methicillin resistant *S. aureus* (MRSA)	√	√	√	√	√	√	√	√	√
	L. monocytogenes MB2022	√	√	√	–	√	√	–	–	√
	S. enterica subsp. *enterica* var. Typhimurium NTCT 13347	√	√	–	√	–	√	√	√	–
	E. coli O157:H7 BRMSID188	√	√	–	√	–	√	√	–	–
	S. aureus MI/1310/1938	√	√	–	–	–	√	–	–	–
	S. flexneri LMG 10472	√	√	–	√	–	√	√	–	–
In vitro GI conditions resistance	pH= 3	√	–	√	√	√	√	√	√	√
	Bile salt 0.5%	√	√	√	√	√	√	√	√	√
NF-κB activation	In human monocytes	√	–	√	–	√	√	–	–	√
Starter culture characteristics	Lag phase	√	–	–	√	–	√	–	√	–
	AUC	–	–	–	√	√	√	–	√	–
	Growth rate (r)	√	√	√	√	–	√	√	–	–

Note: √ = robust/significant/safe; – = no activity/ no ability to withstand/ lower starter culture characteristic compared to *Lacticaseibacillus rhamnosus* GG

3.3.1. Naaqe fermentation with potential probiotic starters

Based on a comprehensive evaluation of the tested probiotic and growth properties of the LAB isolates (Table 4), 6 LAB isolates were selected as candidate probiotic strains for fermentation experiments of Naaqe and Cheka. Specifically, 4 starter cultures (*L. fermentum* 44B and *W. confusa* 44D, and *L. fermentum* 82C and *W. cibaria* 83E) were selected for Naaqe fermentations and 2 starter cultures (*L. fermentum* 73B and *P. pentosaceus* 74D) for Cheka fermentation. The strains and their mixtures were used to ferment Naaqe or Cheka for 3 days and, the fermentative activities (pH and cell viability) of Naaqe and Cheka fermentation were evaluated *in-situ*. The set-up of the fermentation experiments is depicted in Figure 1.

Primary fermentation

A general decrease in pH was observed for all the tested conditions (spontaneous and inoculated fermentations). The inoculated fermentations N44B+44D (*L. fermentum* 44B + *W. confusa* 44D), N83E (*W. cibaria* 83E) and N82C + 83E (*L. fermentum* 82C+*W. cibaria* 83E) had a significantly lower pH after 24 h than the respective spontaneous fermentation controls N4 and N8 ($p<0.05$). pH of the above three inoculated fermentations were recorded below the spontaneous fermentation(N4 and N8) throughout the experiment, indicating increased growth of LAB and subsequent acid production in the fermented product. Although the inoculated fermentations with N44D and N82C exhibited a trend of decline in pH throughout the experiment, it was not statistically significant. Both combinations of starter culture strains produced a lower pH than their individual strain inoculated fermentations after 24 h ($p<0.05$). A pH below 4.6 was achieved for all fermentation after 48h (Figure 5A). This rapid decrease in pH during the first days of fermentation is important for the fermentation and can be linked to a growth of LAB.

For the batches inoculated individually with *L. fermentum* 44B or *W. confusa* 44D or their combination, the LAB count at the start of the spontaneous fermentation was 4.59 ± 0.07 log CFU mL^{-1} and increased to 7.77 ± 0.017 log CFU mL^{-1} during the first 24 h. The initial log CFU mL^{-1} of the inoculated fermentations was 5.14 ± 0.018 log CFU mL^{-1} (Figure 5C), which increased after 24 h of fermentation to 8.42 ± 0.71 log CFU mL^{-1} with N44B, 9.37 ± 0.062 log CFU mL^{-1} with N44D, and 9.37 ± 0.017 log CFU mL^{-1} with N44B+44D. The inoculated conditions N44D and N44B+44D displayed a statistically significant increase in log CFU mL^{-1} ($p<0.05$) compared to the spontaneous fermentation control (N4) after 24 h, which is consistent with the pH data.

Inoculated fermentation with N44B showed a trend towards an increase in log CFU mL^{-1}, although this was not significantly different from that of the control fermentation N4.

The baseline LAB count of the spontaneous fermentation (N8) of the batch which also included inoculated fermentations with *L. fermentum* 82C & *W. cibaria* 83E as starters was 4.57 ± 0.29 log CFU mL^{-1}, while the baseline count in the inoculated fermentations was 5.15 ± 0.08 log CFU mL^{-1}. At the 24 h time point, LAB counts increased to above the 7.84 ± 0.06 log CFU mL^{-1} value of the N8 control in all inoculated fermentations, with a maximum count (9.76 ± 0.12) in the condition N82C+83E (Figure 5C) and a significant difference was observed in inoculated fermentations with N83E and N82C+83E ($p < 0.01$) compared to the control spontaneous fermentation (N8). Inoculated fermentation N82C showed also a trend towards an increase in log CFU mL^{-1}, though this was not significantly different from that of N8.

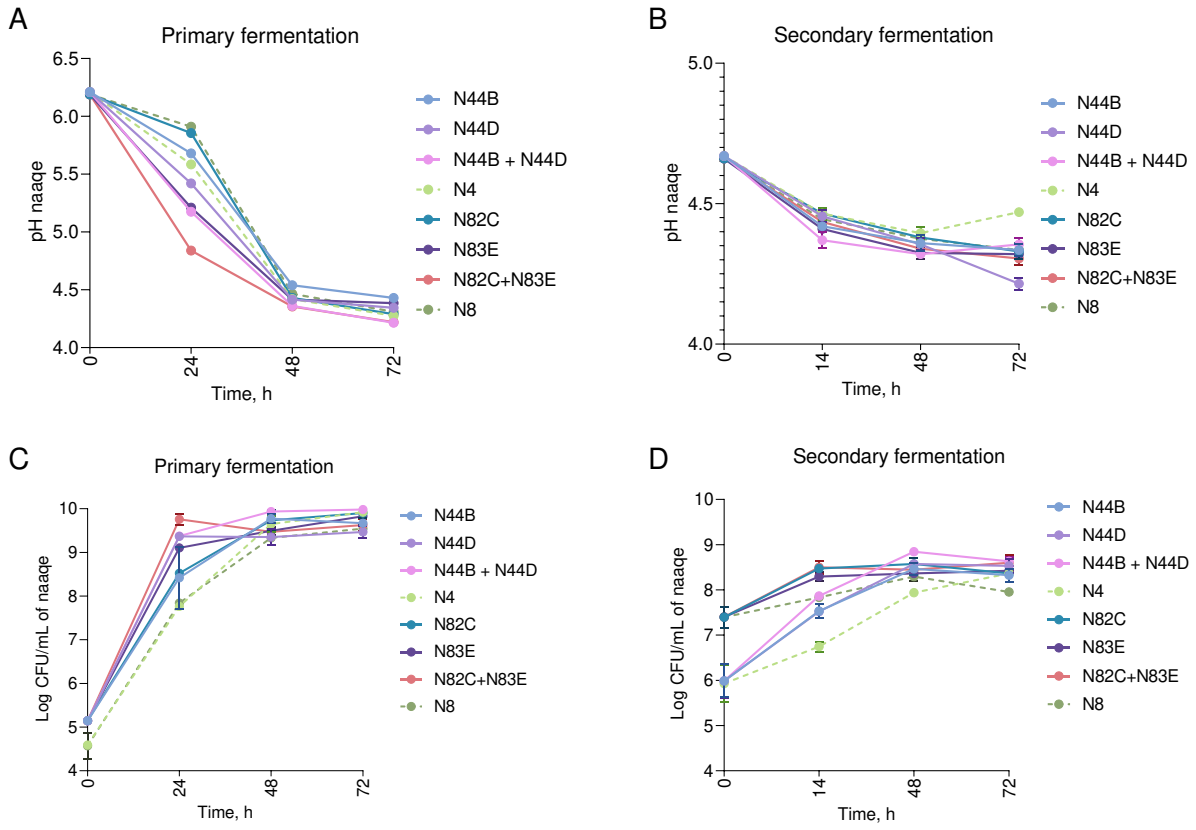


Figure 5. Assessment of the pH (A & B) and viable count of LAB (C & D) during the primary and secondary fermentation of Naaqe as depicted in Figure 1, respectively. Data points are presented as the mean of two experiments ± SD per time point. N = spontaneous fermentation

(N4 = N for 44B & 44D batch; N8 = N for 82C & 83E batch); N44B = inoculated with *L. fermentum* 44B; N44D = inoculated with *W. confusa* 44D; N44B+44D = inoculated with *L. fermentum* 44B & *W. confusa* 44D; N82C = inoculated with *L. fermentum* 82C; N83E = inoculated with *W. cibaria* 83E; N82C+83E= inoculated with *L. fermentum* 82C & *W. cibaria* 83E.

Secondary fermentation

In the Naaqe secondary fermentation (Figure 5B), all the tested conditions (spontaneous and inoculated fermentations) attained the pH 4.6 threshold during the first 14 h time point (regular time of consumption) for both batches. All the inoculated fermentations exhibited a trend towards lowering the pH, although there were no statistically significant differences in the inoculated fermentations compared to control spontaneous fermentations after 14 h and 48 h fermentation. Inoculated fermentations with N44B and N44D showed significant differences in pH at 72 h time point, and N44B+N44D showed a trend towards a decline in pH, compared to the spontaneous control N4. In the Naaqe making process, the duration of the final secondary fermentation to make it ready for consumption is around 14 h. The baseline LAB population was 5.93 ± 0.41 log CFU mL^{-1} and 5.99 ± 0.37 log CFU mL^{-1} in the secondary phase of the spontaneous fermentation N4 and inoculated fermentation *L. fermentum* 44B & *W. confusa* 44D, respectively. This increased above the threshold 6 log CFU mL^{-1} (Marinova *et al.*, 2019) at 14 h time point of the fermentation process in all fermentations (Figure 5D). All the three inoculated fermentations exhibited a trend towards an increase in the log CFU mL^{-1} throughout the experiment, though it was not statistically significant compared to the spontaneous control N4. The baseline LAB population of the secondary spontaneous fermentation control (N8) of the *L. fermentum* 82C and *W. cibaria* 83E batch was recorded as 7.39 ± 0.24 log CFU mL^{-1}, which increased to 7.83 ± 0.05 log CFU mL^{-1} at 14 h time point in the fermentation process. The most increase (8.5 ± 0.14 log CFU mL^{-1}) in LAB load after 14 h fermentation was recorded in the inoculated fermentation N82C+83E (Figure 5D) compared to the control N8. Throughout the experiment, all the three inoculated fermentations displayed a trend towards an increase in LAB load, but it was not significantly different from that of the spontaneous control N8, except for N82C, for which significance ($p = 0.0237$) was seen at 14 h time point.

3.3.2. Cheka fermentation with potential probiotic starters

Two isolates, *L. fermentum* 73B and *P. pentosaceus* 74D were chosen for Cheka fermentation based on their promising probiotic properties and growth kinetics. Subsequently, their capacity to lower the pH and impact the population dynamics of LAB was analyzed for primary and secondary fermentation of Cheka.

Primary fermentation

In the primary fermentation (Figure 6A), the baseline pH value of the mix was 6.24 ± 0.00, which decreased throughout the fermentation. The inoculated fermentations N73B (*L. fermentum* 73B) and N73B+74D (*L. fermentum* 73B+*P. pentosaceus* 74D) resulted in significant decline in pH during the first 24 h compared to the control spontaneous fermentation N with the highest decline for the mixed starter culture ($p < 0.05$). At 48 h time point, all fermentations except the spontaneous control N had a pH below 4.6. The trend of pH reduction was consistent throughout the experiment. Inoculated fermentation N74D was observed to significantly lower the pH at 48 h time point ($p = 0.0329$), while the inoculated fermentation N73B+74D was noted to significantly decrease the pH at the 72 h time point. Plate counting of the corresponding population dynamics of LAB of Cheka primary fermentation revealed that the baseline LAB count of the control spontaneous fermentation (N) was 4.34 ± 0.46 log CFU mL^{-1} while that of the inoculated fermentations was 5.04 ± 0.00 log CFU mL^{-1} (Figure 6C). All the inoculated fermentations recorded an increase in LAB load throughout the fermentation process, but only inoculated fermentation N73B+74D had significant log CFU mL^{-1} increase ($p < 0.05$) at 72 h time point compared to the spontaneous control fermentation N, which was in agreement with the significant lowering of the pH at that time point for the fermentation. After 24 h, the highest LAB count (8.65 ± 0.06 log CFU mL^{-1}) was recorded in inoculated fermentation N73B+74D. All fermentations reached LAB count above 9.5 log CFU mL^{-1} after 72 h, and inoculated fermentation N73B+74D (10.51 ± 0.12 log CFU mL^{-1}) still showed overall higher log CFU mL^{-1}.

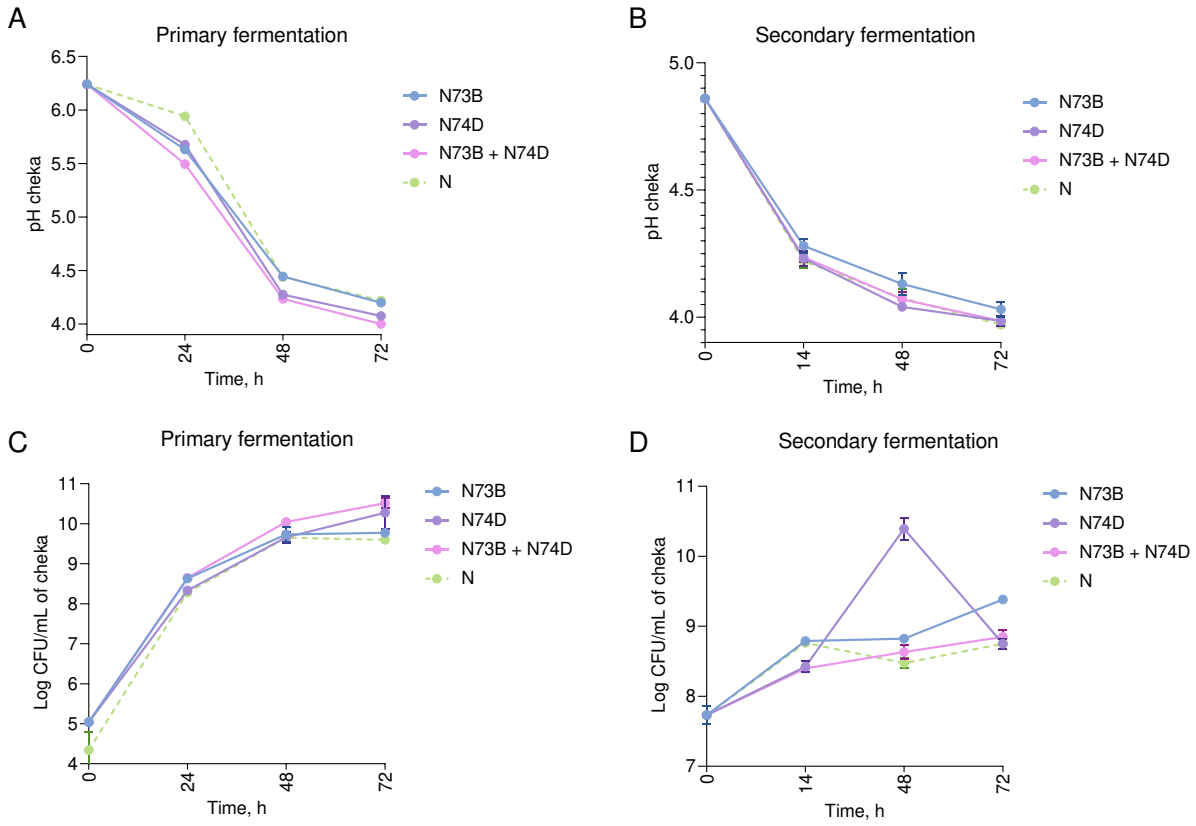


Figure 6. Evaluation of the pH (A & B) and population of lactic acid bacteria (C & D) during the primary and secondary fermentation of Cheka, respectively. Data are presented as the mean of two independent experiments ± SD. Conditions: N = spontaneous fermentation; N73B = inoculated with *L. fermentum* 73B; N74D = *P. pentosaceus* 74D; N73B+N74D = inoculated with *L. fermentum* 73B & *P. pentosaceus* 74D.

Secondary fermentation

In the Cheka secondary fermentation, the baseline pH value of the mix was recorded as 4.86 ± 0.00 and dropped below 4.6 in all fermentations, with no significant difference among the fermentations during the first 24 h, the time at which the fermenting Cheka can be served by the indigenous consumers (Figure 6B). The baseline LAB count (both the spontaneous fermentation control N and inoculated with starter strains) of the secondary Cheka fermentation was found to be 7.73 ± 0.13 log CFU mL^{-1} (Figure 6D). The LAB load increased to 8.76 ± 0.05 for the control spontaneous fermentation N, and to 8.79 ± 0.05, 8.42 ± 0.08, and 8.40 ± 0.04 log CFU mL^{-1} for the inoculated fermentations N73B, N74D, N73B+74D respectively at 24 h time point. Generally,

there was a trend of increased LAB load for the three inoculated groups throughout the fermentation process. However, there was no statistical difference compared to the spontaneous control N except for the inoculated fermentation N74D, which showed a significant difference after 48 h of fermentation compared to the spontaneous control N.

In general, inoculation with the selected candidate autochthonous LAB starter cultures (*L. fermentum* 44B, *W. confusa* 44D, *L. fermentum* 82C and *W. cibaria* 83E for Naaqe fermentations; and *L. fermentum* 73B and *P. pentosaceus* 74D for Cheka fermentation) resulted in higher pH decline and higher LAB load compared to control spontaneous fermentation in the primary Cheka fermentation than in the secondary fermentation.

4. Discussion

People from sub-Saharan Africa produce and consume a wide array of cereal fermented foods and drinks, which are an untapped source of potentially beneficial microorganisms. However, these foods are largely unexplored and not adequately represented in the scientific literature (Pswarayi and Gänzle, 2022). For example, there is no scientifically documented information on the microbiological properties of the fermented cereal beverage Cheka, despite it being a widely consumed fermented beverage in Konso and Dirashe, southern Ethiopia (Hotessa and Robe, 2020). Naaqe has – to the best of our knowledge- never been documented scientifically. These fermented cereal beverages are often spontaneous in nature, and the resulting quality and health benefits of the fermented product could be enhanced by using a well-selected dedicated probiotic starter culture. In the present study, we addressed this research gap by identifying the LAB members of fermented cereal beverages of Naaqe and Cheka, characterizing the *in vitro* beneficial properties as potential probiotics, and the growth and fermentation properties as potential starter cultures.

Out of 23 microbial isolates from Naaqe, 19 were identified as LAB. On the other hand, out of the 21 microbial isolates from Cheka, only 5 isolates were identified as LAB, indicating that spontaneously fermented Cheka was not a good source of LAB. This could be due to yeast-based alcoholic fermentation in Cheka and a high alcohol content, especially for batch one. This is in agreement with values from the literature on rather high alcohol levels (3.04%–8.96% v/v) (Worku *et al.*, 2018). However, the spontaneous batch 2 contained a rather high LAB level and was a good source for LAB isolation. Species level identification of the 14 isolates from Naaqe and Cheka

selected based on the initial antimicrobial activity and representativeness of source samples revealed that *L. fermentum* was the predominant (50 %) species identified from Naaqe, with the other 50 % being *Weissella* spp. The two Cheka isolates were identified as *L. fermentum* and *P. pentosaceus*. Although these species have been documented in other spontaneous African cereal fermented foods such as mahewu (a non-alcoholic drink from Zimbabwe made from fermenting cooked maize porridge with addition of millet or sorghum malt at the household level) (Pswarayi and Gänzle, 2022), to the best of our knowledge, this is the first study describing their isolation from Cheka and Naaqe.

Spontaneously fermented beverages represent a potential source of beneficial bacteria with antimicrobial properties (Enujiugha and Badejo, 2017; Pswarayi and Gänzle, 2022). The isolation and use of these bacteria as probiotic starter cultures could help combat Ethiopia's large burden of foodborne-diseases (WHO, 2015; Misganaw *et al.*, 2017). All the 14 LAB isolates from Naaqe and Cheka showed higher inhibition of *E. coli* ATCC 25922 and MRSA in the spot assay than the antiseptic 0.2% chlorhexidine. This indicates their promising applicability against diarrheal diseases caused by these organisms, which are reported to be the second most important contributors to the total burden of all disease types and the second leading cause of premature death in Ethiopia (Misganaw *et al.*, 2017). The broad spectrum of antagonistic activity against both gram-negative and gram-positive pathogens observed in the present study is in line with findings reported for LAB isolates including *Lactobacillus* spp. (before reclassification) (Zheng *et al.*, 2020), *Pediococcus* spp., and *Weissella* spp. from other cereal beverages, such as Borde and Shamita (Tadesse *et al.*, 2005; Dejene *et al.*, 2021). What makes the present study different is that it reports a broader antipathogenic action by testing the isolated LAB against nine indicator pathogenic strains, including a resistant clinical isolate, and assessing antagonistic activity of both live bacteria (spot assay) and their secreted metabolites (radial diffusion assay).

Besides antimicrobial activity, another key health-promoting property of a potential beneficial bacteria in spontaneous fermentations and starter cultures is the ability of specific LAB strains to modulate the immune system. This mode of action can provide protection against diseases related to immune imbalances, such as allergic diseases (Spacova *et al.*, 2020), inflammatory bowel disease (IBD) (Lorea Baroja *et al.*, 2007) and even COVID-19 (De Boeck *et al.*, 2022). In the

present study, a moderate activation of the immune system was desired, as this can help patients to better protect against invading (gut) pathogens, and more rapidly clear pathogens. NF-κB activation by LAB could help stimulate antipathogenic immune responses and correct development and regulation of immune self-tolerance (Brown *et al.*, 2008; Liu *et al.*, 2017; Grinberg-Bleyer *et al.*, 2018; Miraghazadeh and Cook, 2018). Three (*L. fermentum* 73B, 82C and 84C) of the nine tested LAB isolates from Naaqe and Cheka were capable of activating the key immune transcription factor NF-κB to similar levels as the model probiotic strains *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003) and *L. rhamnosus* GG (Kankainen *et al.*, 2009). Notably, although some specific strains of *W. cibaria* and *W. confusa* have been reported to possess immunomodulatory activity (Ladda *et al.*, 2015; Hong *et al.*, 2016; Park *et al.*, 2020), the LAB isolates belonging to the genus *Weissella* tested here were unable to stimulate NF-κB pathway. Furthermore, all selected LAB isolates from Naaqe and Cheka showed a trend towards activation of IRF, however, no isolate demonstrated statistically significant IRF induction. Our data lend support to the notion that immunostimulatory activity of LAB is strain-specific (Spacova, I *et al.*, 2022), and suggest that selection of appropriately defined starter culture strains is essential to harness the claimed immunomodulatory benefit of functional foods. We hypothesize that all the tested LAB isolates are safe from an immunostimulatory perspective, because they only induced NF-κB pathway in a moderate way: similar or lower level compared to the established probiotics *L. plantarum* WCFS1 and *L. rhamnosus* GG, and lower than the pathogen *S. aureus* MI/1310/1938-MSSA. Lack of excessive immune stimulation in susceptible individuals is one of the safety assessments criteria required by WHO for live microorganisms intended to be added in foods and feeds (FAO/WHO, 2002). Antimicrobial and fermenting LAB isolates of the genus *Weissella* such as *W. confusa* 44D and *W. cibaria* 83E could thus represent safer probiotic starters for vulnerable individuals. Therefore, they were still considered in the starter culture experiments.

To assess the fermentation quality and reproducibility, pH and viable LAB counts were used as important read-out parameters, because acid production along with its associated lowering of pH and containing adequate level of viable probiotic bacteria are one of the key requirements for functional foods (Ogunremi *et al.*, 2017; Marinova *et al.*, 2019). In the present mock fermentation, as expected, viable LAB concentrations increased above the threshold of 6 logs after 48 h of

primary fermentation in all fermentations since the process of cereal fermentations leads to a succession of fermentation organisms with the last organisms being LAB (Pswarayi and Gänzle, 2022). The 6 log CFU mL^{-1} is also suggested as the minimum amount of LAB load need to be detected in probiotic foods to compensate for the loss of bacteria during passage through the GI tract (Marinova *et al.*, 2019). In the primary fermentation processes of Naaqe and Cheka, all the six isolates tested in the laboratory-scale fermentation as starters were shown to enhance the fermentation (pH lowering and colony count on MRS agar) compared to control spontaneous fermentation. Importantly, the tested strains also enhanced fermentation better when combined in a multi-strain mixture. One should note that this is not always the case. For instance, Adebo *et al*. (Adebo *et al.*, 2018) reported that the use of two *L. fermentum* strains combined as starter culture resulted in reduced fermenting performance. This could be due to antagonism, probable competitive inhibition and conflicting modes of similar metabolism and action by the strains. In our study, the fact that combined starter cultures performed better could be due to species differences of the isolates combined. Evidence comes for this assertion from the observation that starters, whether single or mixed, were able to lower pH and increase LAB counts than the spontaneously fermented ones using maize (Edema and Sanni, 2008) and whole grain sorghum (Adebo *et al.*, 2018). In addition to improved fermentation capacity, combining LAB starter cultures with different beneficial modes of action can lead to multiple health benefits of the resulting fermented product (Ogunremi *et al.*, 2017; Min *et al.*, 2019).

In the secondary fermentation processes, pH decreased below 4.6 in all fermentations at the fermentation time point when the product is ready for consumption, which is an important food safety threshold (FDA, 2022). The increase in cell density of Naaqe and Cheka secondary fermentation indicates that dominance and better adaptability of the isolates in the system, which could enable the consumer to ingest live probiotic LAB. Addition of the candidate starter cultures in the Cheka primary fermentation could also help force the fermentation towards a LAB-based fermentation with little to no alcohol produced, instead of an alcoholic fermentation. These laboratory-scale fermentation experiments overall showed that the tested autochthonous LAB isolates could be promising starters (with respect to pH lowering and LAB count) for both Naaqe primary and secondary fermentation processes, however, LAB isolates from Cheka could be promising starters (with respect to pH lowering and LAB count) only for Cheka primary

fermentation process. Both Cheka and Naaqe could be good potential carriers for future documented probiotic strains. Overall, we demonstrated that six (*L. fermentum* 73B, *P. pentosaceus* 74D, *L. fermentum* 44B, *W. confusa* 44D, *L. fermentum* 82C and *W. cibaria* 83E) LAB isolates have promising antimicrobial activities, GI conditions tolerance, and starter culture properties related to fermentation and growth. These two spontaneous fermentation processes could thus benefit from the use of a dedicated starter culture to remove inter-batch differences and be enhanced with specific health promoting properties.

5. Conclusions

The present study describes the identity and properties of LAB isolated from the traditional Ethiopian fermented cereal beverages Naaqe and Cheka. To the best of our knowledge, no other study has yet described and documented Naaqe scientifically, which we showed to be dominated by LAB. Six LAB isolates were selected as potential functional probiotic starter cultures based on their potential antimicrobial and immunostimulatory properties. Furthermore, these LAB strains demonstrated growth performance and tested *in situ* in mock laboratory scale Naaqe and Cheka fermentations, resulting in a faster acidification and higher LAB counts in the primary fermentation phase. These results indicate that the selected strains are promising autochthonous probiotic starter candidates for use in fermentation of Naaqe and Cheka. They can be considered autochthonous probiotic starter strains once their health benefit is ascertained in a clinical trial as a next step.

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7. Ethical approval

This work was approved by Ethics Committee of the School of Pharmacy, College of Health Sciences, Addis Ababa University with reference number ERB/SOP/15/10/2018

8. Author contributions

Concept: SG., EE. Experimental design: SG., WVB., SL., IS. Experimental work: SG., WVB., MD., AA. Data analysis: SG., WVB., IS. Writing— original draft: SG. Writing— review and editing: SG., WVB., SL., IS., MD., WMW., EE. All authors read and approved the final submission text.

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10. Conflict of Interest

The authors declare that they have no competing interests related to this work. SL is an academic board member of the International Scientific Association on Probiotics and Prebiotics (ISAPP) and co-founder of YUN. However, these organizations were not involved in this work.

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Supplementary materials

Supplementary Text 1.

Indigenous Naaqe preparation method

Indigenously, Naaqe is prepared by simple procedures with no use of malt and other steps in order to make the product non-alcoholic. The procedure has two fermentation steps. In the primary fermentation, grain (commonly, maize (usually Bako Hybrid-660 variety) alone or mixed with barley) flour is kneaded with water and allowed to ferment for 24-48 h. The fermenting mix is then kneaded with barley flour to make dough balls that are cooked for 45 to 90 min. The dough balls are then allowed to cool and smashed into particles, kneaded with barley flour and added into fermentation vessel. Water is added to the produce and kneaded, and then the mix is allowed to ferment for 8 to 14 h (secondary fermentation). This product, ready for consumption, is called Naaqe.

Supplementary Text 2.

Indigenous Cheka preparation method

Indigenously, most Cheka preparation methods involve three major phases and two fermentation steps. In phase I, grain flour (usually maize:sorghum; 3:1) is thoroughly kneaded with water in plastic/wooden tray and allowed to ferment for 36-40 h (primary fermentation). In phase II, the fermenting material is made into dough balls, the dough balls are then cooked for about 1 h, allowed to cool, smashed and kneaded with water, mixed with milled malt and allowed to ferment overnight (13-16 h, secondary fermentation starts here). In Phase III, a very thick porridge is prepared, allowed to cool and kneaded with malt. The porridge is then added into the fermentation vessel containing the produce; sufficient water is added and thoroughly mixed. The Cheka is ready for consumption after 6-12 h of fermentation (secondary fermentation) (Worku *et al.*, 2016).

Supplementary Table 1: Initial Antimicrobial activity of potential probiotic starter LAB strains by spot overlay method against the pathogens *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, methicillin resistant *S. aureus*. Chlorhexidine 0.2% = control antimicrobial condition. Source: (N) = Naaqe, (C) = Cheka

Zone of inhibition (mm)[1], Data are mean values ± SD, (n=3)				
LAB strain (Source)	*L. monocytogenes* ATCC 19115	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	methicillin resistant *S. aureus*
LAB isolates selected for sequencing				
L. fermentum 44B (N)	14.67 ±1.15	0.00 ± 0.00	15.33 ± 1.15	17.00 ± 1.00
W. confusa 44D (N)	16.00 ± 0.00	16.00 ± 2.00	18.00 ± 2.00	16.67 ± 1.15
W. confusa 45C (N)	0.00 ± 0.00	17.00 ± 1.00	14.33 ± 0.58	19.33 ± 1.15
L. fermentum 82C (N)	22.00± 3.46	17.67 ± 0.58	16.67 ± 1.15	18.67 ± 1.15
W. confusa 82D (N)	0.00 ± 0.00	17.33 ± 1.15	21.33 ± 1.15	17.33 ± 1.15
L. fermentum 82E (N)	17.00 ± 1.00	15.33 ± 1.53	15.33 ± 1.15	16.67 ± 1.15
W. confusa 83A (N)	0.00 ± 0.00	18.00 ± 2.00	17.33 ± 1.15	15.33 ± 1.15
W. confusa 83B (N)	13.67 ± 0.58	20.67 ± 1.15	15.67 ± 0.58	20.67 ± 1.15
L. fermentum 83C (N)	0.00 ± 0.00	17.33 ± 1.15	16.33 ± 0.58	17.00 ± 1.00
W. cibaria 83E (N)	0.00 ± 0.00	17.67 ± 0.58	20.67 ± 1.15	19.33 ± 1.15
L. fermentum 84C (N)	16.33 ± 0.58	18.33 ± 0.58	14.67 ± 1.15	17.00 ± 1.00
L. fermentum 84D (N)	13.00 ± 1.00	17.67 ± 0.58	18.00 ± 2.00	19.33 ± 1.15
L. fermentum 73B (C)	17.00 ± 1.00	15.00 ± 1.00	17.67 ± 0.58	19.67 ± 2.52
P. pentosaceus 74D (C)	8.33 ± 0.58	0.00 ± 0.00	20.00 ± 0.00	14.67 ± 1.15
Chlorhexidine 0.2%	18.67 ± 1.53	11.33 ± 0.58	13.33 ± 0.58	10.67 ± 0.58
LAB i**solates not selected for sequencing**				
44C (N)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
45A (N)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
45D (N)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
82A (N)	0.00 ± 0.00	0.00 ± 0.00	14.33 ± 0.57	16.00 ± 0.00
82B (N)	0.00 ± 0.00	0.00 ± 0.00	20.67 ± 1.15	15.00 ± 1.00
83D (N)	15.00 ± 3.00	20 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
84B (N)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

73A (C)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
73E (C)	0.00 ± 0.00	18.67 ± 1.15	16.67 ± 1.15	0.00 ± 0.00
74C (C)	0.00 ± 0.00	0.00 ± 0.00	18 ± 2.00	0.00 ± 0.00

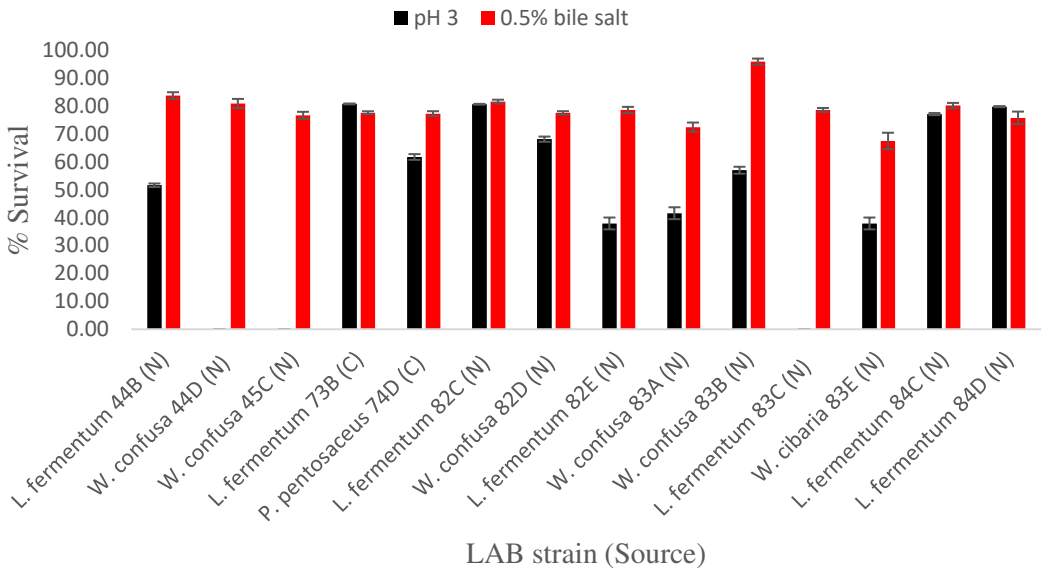
[1]Results of experiments of inhibition of initial antimicrobial assay

Isolates selection for *16S rRNA* gene-based species identification was based on antagonistic activity observed (diameter of inhibition zone, brighter/clearer zones and number of indicator pathogens inhibited) in the initial antimicrobial assay. Isolates represented by numeric and alphabet are those with poor/questionable antagonistic activity and were not selected for sequencing.

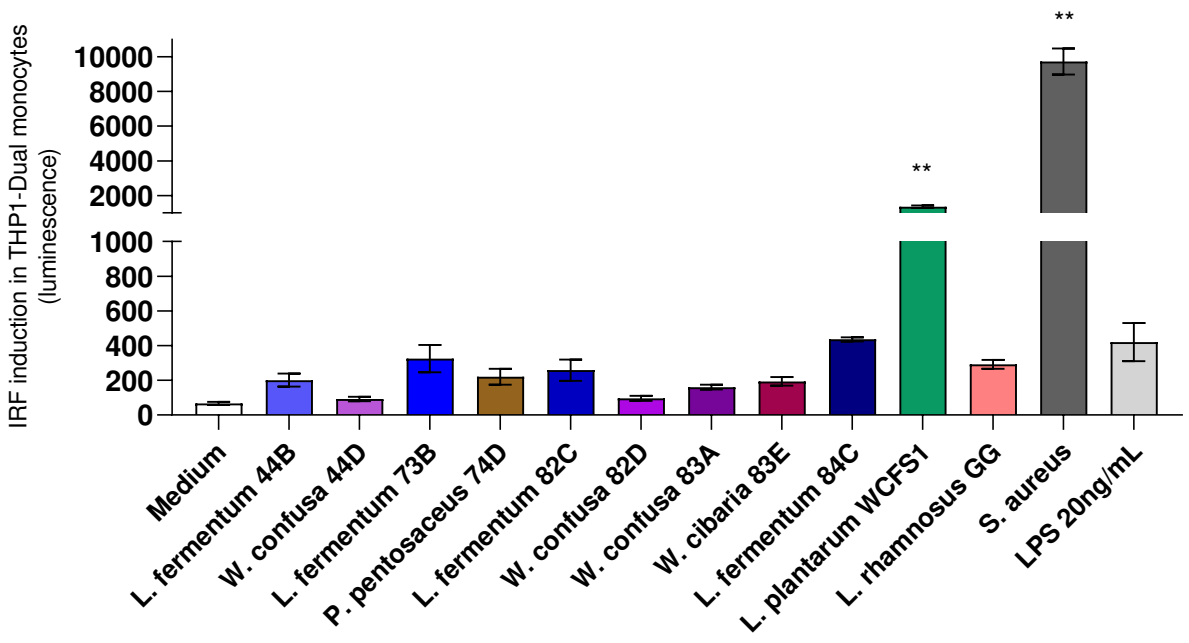
Supplementary Table 2: pH of the LAB isolates cell-free culture supernatants in MRS broth.[1]

Isolate	44B	44D	73B	74D	82C	82D	83A	83E	84C	LGG	WCFS1
pH	4.17 ± 0.007	4.30 ± 0.17	4.35 ± 0.04	3.90 ± 0.09	4.30 ± 0.007	4.34 ± 0.04	4.39 ± 0.007	4.34 ± 0.03	4.31 ± 0.007	3.84± 0.06	3.81± 0.04

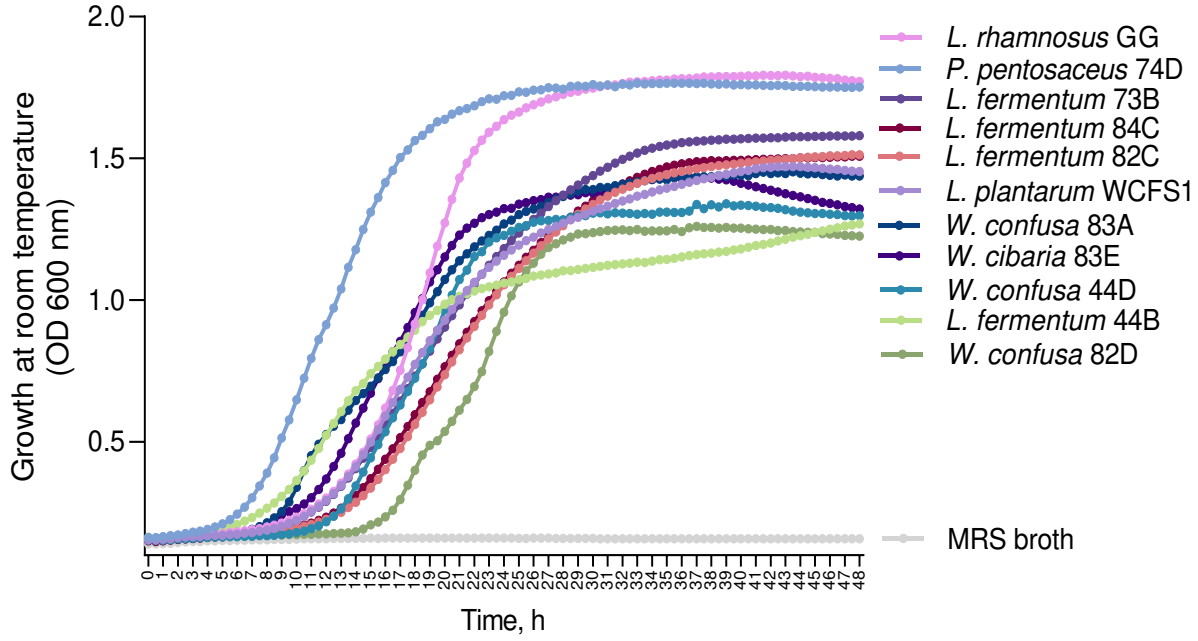
[1] Results expressed in mean ± SD *(n=2)*



Supplementary Figure 1: Percentage of survival of LAB isolates after exposure to acidic environment mimicking the stomach pH and bile salt solution. LAB isolates were exposed to pH 3.0 for 3 h at 37°C, or to 0.5% (w/v) bile salt solution (pH 8.0) for 4 h at 37°C, under stirring (150 rpm). Data expressed as mean ± SD (*n=3*). N = Naaqe, C = Cheka



Supplementary Figure 2: IRF pathway activation by LAB strains from Naaqe and Cheka in THP1-Dual human monocytes. Bars depict means ± standard deviation per condition. **p<0.01 compared to medium control.



Supplementary Figure 3: The growth curve (absorbance at 600 nm/h) of LAB strains compared with MRS broth as a control.