PROBIOTIC AND STARTER CULTURE PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM SELECTED ETHIOPIAN FERMENTED FOODS AND BEVERAGES

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A dissertation submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Pharmacology

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This is to certify that the dissertation prepared by Seyoum Gizachew Adall, entitled "**Probiotic and Starter Culture Properties of Lactic Acid Bacteria Isolated from Selected Ethiopian Fermented Foods and Beverages**", and submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Pharmacology complies with the regulations of the University and meets the accepted standards concerning originality and quality.

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Abstract

Probiotic and Starter Culture Properties of Lactic Acid Bacteria Isolated from Selected Ethiopian Fermented Foods and Beverages

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Lactic acid bacteria (LAB) are a group of bacteria that form the majority of probiotics and are typically found in fermented products. Probiotics are currently accepted as reasonable alternative remedies in the control of infectious diseases and immuno-allergic disorders. Sub-Saharan African knowledge on cereal fermentations is largely unexplored and undocumented. Use of LAB as starter cultures is one of the key strategies to make most of these spontaneous African cereal fermentations of sufficient quality. The criteria for the selection of probiotic/probiotic starter strains include the functional characterization and safety assessments. This work aimed to isolate and assess in vitro probiotic and starter culture capacity of LAB strains from yoghurt, cheese, cottage cheese, Naaqe and Cheka. LAB were isolated by plating on MRS agar. Spot overlay, radial diffusion, and microdilution methods were used to assess antimicrobial activity against pathogens commonly causing foodborne diseases in Ethiopia. Species identification was done by 16S rRNA gene sequencing. Immunostimulatory activity was tested by measuring nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF) pathway activation in THP-1 cell lines. In situ evaluation of starter culture performance of selected isolates from cereal beverages was conducted in a mock fermentation of Naaqe and Cheka. Genomes of three dairy isolates selected based on their potential probiotic properties were analyzed for the secondary metabolites biosynthetic gene clusters, resistome, virulome, and carbohydrate-active enzymes. 27 isolates from the dairy and 14 isolates from the cereal beverage samples were selected and identified to the species level. *Limosilactobacillus fermentum* was found to be the predominant species. Five strains from cottage cheese (L. plantarum 54B, 54C and 55A; L. pentosus 55B, and P. pentosaceus 95E) showed inhibitory activity against indicator pathogens tested. Six cereal beverages origin LAB strains also inhibited eight of the nine gastrointestinal indicator key pathogens tested. Strain-specific NF-κB and IRF

activation was documented for dairy origin strains *L. plantarum* 54B, *L. plantarum* 55A and *P. pentosaceus* 95E. Three of the cereal beverages origin LAB isolates (*L. fermentum* 73B, 82C and 84C) significantly exhibited strain-specific NF-κB induction. During *in situ* 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. Based on the whole genome sequence (WGS) analysis, *Lactiplantibacillus plantarum* 54B and 54C also showed to be closely related but different strains. The analysis also revealed that the three strains do not harbor resistome and virulome and have five classes of carbohydrate-active enzymes with several important functions. Cyclic lactone autoinducer, terpene, Type III polyketide synthases (T3PKS), ribosomally synthesized and post-translationally modified peptides (RiPP)-like gene clusters and complete riboflavin operon have been identified in the *L. plantarum* 55A genome. Overall, five isolates of dairy origin and six isolates of cereal beverages origin showed promising results in all assays and are novel probiotic and probiotic starter candidates of interest, respectively.

Key words: Cheka; Comparative genome analysis; Cottage cheese; Ethiopia; Lactic acid bacteria; Lactiplantibacillus plantarum; Naaqe; Probiotics; Probiotic starters; Safety; Traditional cereal beverages

Dedication

This thesis is dedicated to my beloved wife **Tewedaj Daniel Asress**.

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List of Symbols and Abbreviations

AHRI	Armauer Hansen Research Institute
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
ANI	Average Nucleotide Identity
AUC	Area Under the Curve
CFS	Cell-Free Culture Supernatants
CFU	Colony Forming Unit
CDS	Coding Sequence
DALYs	Disability Adjusted Life Years
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
GIT	Gastrointestinal Tract
GRAS	Generally Regarded as Safe
IFN	Interferon
IRF	Interferon Regulatory Factor
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
LAB	Lactic Acid Bacteria
LAMB	Laboratory of Applied Microbiology and Biotechnology
MAMPs	Microbe-Associated Molecular Patterns
MRSA	Methicillin-Resistant *Staphylococcus Aureus*
M-SHIME	Mucosal-Simulator of the Human Intestinal Microbial Ecosystem
NF-κB	Nuclear Factor Kappa B
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pNPP	p-Nitrophenyl Phosphate

QPS	Qualified Presumption of Safety
RiPPs	Ribosomally Synthesized and Post-Translationally Modified Peptides
RNA	Ribonucleic Acid
SCFAs	Short-Chain Fatty Acids
SD	Standard Deviation
SEAP	Secreted Embryonic Alkaline Phosphatase
SNPs	Single Nucleotide Polymorphisms
SNVs	Single Nucleotide Variants
T3PKSs	Type III Polyketide Synthases
US FDA	United States Food and Drug Administration
VFDB	Virulence Factor Database
WGS	Whole Genome Sequence
WHO	World Health Organization

List of Original Articles

This dissertation is primarily based on the following published and publishable articles.

Paper I: Gizachew, S.; Van Beeck, W.; Spacova, I.; Dekeukeleire, M.; Alemu, A.; Woldemedhin, W.M.; Mariam, S.H.; Lebeer, S.; Engidawork, E. Antibacterial and Immunostimulatory Activity of Potential Probiotic Lactic Acid Bacteria Isolated from Ethiopian Fermented Dairy Products. *Fermentation* 2023, 9, 258. https://doi.org/10.3390/fermentation9030258

Paper II: Gizachew, S.; Van Beeck, W.; Spacova, I.; Dekeukeleire, M.; Alemu, A.; Mihret, W; Lebeer, S.; Engidawork, E. Characterization of potential probiotic starter cultures of lactic acid bacteria isolated from Ethiopian fermented cereal beverages, Naaqe and Cheka. *J Appl Microbiol*. 2023 Nov 1;134(11):lxad237 DOI: 10.1093/jambio/lxad237

Paper III: Gizachew, S.; Van Beeck, W.; Spacova, I.; Dekeukeleire, M.; Lebeer, S.; Engidawork, E. Whole Genome Sequence and Comparative Genome Analysis of the three *Lactiplantibacillus plantarum* isolates: Potential Probiotics from Ethiopian Traditional Fermented Cottage Cheese. (To be submitted)

1. Introduction

1.1 Background

The human-microbiota supraorganism appears to have been associated and co-evolved over the past 15 million years (Moeller et al., 2016). The term 'Microbiota' refers to the collection of microorganisms, including bacteria, viruses and yeasts, cohabit in various sites of the human body regions (gut, respiratory, oral cavity, conjunctiva, vagina and skin) (Hou et al., 2022). The majority is found in the gastrointestinal tract (GIT) (Fekete et al., 2023). The GIT alone is host for the largest number of bacteria with about 3.8×10^{13} (Sender et al., 2016). Looking into the revised estimates for the total number of human ($3.0*10^{13}$) cells and bacterial ($3.8*10^{13}$) cells in the body, the microbiota of our bodies constitute about 50% of the total number of cells associated with our bodies (Sender et al., 2016). The human microbiota also contribute above 150 times more genetic information than that of the whole human genome (Ursell et al., 2014).

The gut microbiota has a key role in maintaining health (Hou et al., 2022). Generally, the gut microbiota is made up of six major bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Fusobacteria*, among which *Bacteroidetes* and *Firmicutes* are the main types (Hou et al., 2022). From the phylum *Firmicutes*, *Lactobacillus* (before reclassification (J. Zheng et al., 2020)), *Streptococcus*, *Enterococcus*, *Clostridium*, and *Ruminococcus* were among the most commonly genera detected in the intestinal microbiota (Ringel et al., 2015). Beside the genera from these two phyla (*Bacteroidetes* and *Firmicutes*), the human colon can also contains primary pathogens, e.g., species such as *Vibrio cholera*, *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enterica* and *Bacteroides fragilis*, but with a low quantity (0.1% or less of entire gut microbiota) (Jandhyala, 2015). There is a continuous flux of bacterial populations of both the symbiotic and pathogenic groups leaving and entering the host. Consumption of fermented foods and beverages is primarily associated with influx of supposedly beneficial bacteria to the host. They constitute mostly of lactic acid bacterial (LAB) species (Giraffa, 2004; Rezac et al., 2018), which are capable of reseeding the gut microbiota during dysbiosis (Peláez et al., 2019).

The term LAB has no approved status in taxonomy. It is a catch-all term for a group of bacteria that are functionally and genetically linked. LAB are generally defined as a group of Gram-positive rods and cocci, non-spore-forming, low % G+C, non-respiring but aerotolerant, which synthesize lactic acid as one of important fermentation products by using carbohydrates during fermentation processes (Ayivi et al., 2020; Hutkins, 2019). They share many physiological, biochemical and genetic properties. Most LAB are acid-tolerant, catalase-negative, non-motile, facultative anaerobes (Hutkins, 2019). As an end product of carbohydrate metabolism, these bacteria produce lactic acid, in addition to organic compounds that contribute to the texture, flavor and scent, resulting in distinct organoleptic features of fermented foods (Bell et al., 2018; Hutkins, 2019).

In 1919, Orla Jensen first published a paper that laid the foundation for categorizing LAB (Ayivi et al., 2020). This classification method was related to specific parameters such as glucose fermentation properties, cell morphology, sugar utilization patterns, and optimal growth temperature range. Based on this classification method, only four LAB genera were thus recognized as *Lactobacillus* (before reclassification (J. Zheng et al., 2020), *Pediococcus*, *Streptococcus* and *Leuconostoc* (Quinto et al., 2014). The number of genera listed in the LAB group has grown because of advances in molecular biological methods such as 16S *rRNA gene* sequencing, random amplified polymorphic DNA profiling, PCR-based fingerprinting and whole genome sequencing (Ayivi et al., 2020; EFSA, 2021a). The current taxonomic classification of the LAB includes groups in the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales* (Quinto et al., 2014). Hence, the LAB includes many genera including the emended genus *Lactobacillus* (J. Zheng et al., 2020), *Lacticaseibacillus*, *Lactiplantibacillus*, *Limosilactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Hutkins, 2019; J. Zheng et al., 2020). Due to their long-time use in many food and feed preparations causing no pronounced adverse effects, many strains of LAB (particularly those belonging to *Lactobacillaceae*) have been granted a generally recognized as safe (GRAS) status by the United States Food and Drug Administration (US FDA) (Dejene et al., 2021) and many species a “Qualified Presumption of Safety” (QPS) by the European Food Safety Authority (EFSA, 2021b). Over the last

decades, LAB use as probiotics has increased and they form the group of bacteria to which most probiotics belong (Sadiq, 2022). Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host' (Hill et al., 2014).

Food fermentation is an important component of human civilization, serving as a method to preserve and prolong the shelf-life; enhance the texture, flavor, nutritional value, taste and functional properties of food (Bell et al., 2018; Hutkins, 2019). Fermented products are foods and beverages produced through desired microbial growth and enzymatic conversions of food components (Marco et al., 2021). Africa is taken as the continent with the richest variety of fermented foods (Koricha et al., 2020). Specially, Ethiopia is a culturally diverse country, in which each culture has its own variety of fermented foods and beverages (Ashenafi, 2008). Fermented foods and beverages commonly produce and consumed in Ethiopia include fermented dairy products (e.g., *yoghurt* (Ergo), *cheese*, *cottage cheese* (Ayib)), fermented plants (e.g., *Kotcho*, *Enjerra*), fermented beverages (e.g., *Cheka, Borde, Naaqe*), and fermented condiments (e.g., *Datta, Siljo, Awaze*) (Ashenafi, 2008; Fentie et al., 2020; Lee et al., 2015). Most of these traditional fermented foods are prepared on a small-scale level, commonly for household consumption and, occasionally, sold by local vendors (Ashenafi, 2008; Fentie et al., 2020). Nowadays, in Ethiopia, there is a fast rise in the number of industrially produced fermented foods and beverages in urban areas, specially for dairy products (Minten et al., 2020). The microbiology and potential health benefits of these fermented foods and beverages, however, are not yet widely studied (Ashenafi, 2008; Fentie et al., 2020).

For millennia, human cultures have consumed fermented dairy products, utilizing the biochemical effect of microorganisms naturally found in raw milk, microorganisms on the surface of containers in which the dairy foods are contained, or through a process known as back-slopping, in which some of the material from a preceding fermentation is used to inoculate fresh substrate (Macori & Cotter, 2018). In the last century, large-scale industrial production of fermented foods have brought about the usage of starter and adjunct culture(s) (Macori & Cotter, 2018; Peláez et al., 2019). Recently, the health claims of various fermented dairy products have been under intense investigation. To mention some,

studies demonstrating reduced risk or symptoms of many GI disorders such as ulcerative colitis and irritable bowel syndrome, viral or bacterial infections (Wilkins & Sequoia, 2017), improvements in bone mineral density, reduced risk of type 2 diabetes and impact on the function of brain regions that control sensation and emotion, are among others (Macori & Cotter, 2018). Additionally, during dysbiosis, fermented dairy products could be carriers for reseeding the gut microbiota (Peláez et al., 2019). Dysbiosis, non-optimal or unwanted compositional and functional changes of gut microbiota, is associated with a whole range of diseases of civilization (Hrncir et al., 2019). Dysbiosis is actually very difficult to define and there is currently no consensus, but it is typically characterized by the following three major features: (1) a decrease in microbiota diversity that is associated with various chronic inflammatory diseases, (2) a reduction or complete loss of beneficial microorganisms, and (3) an increased number of potentially pathogenic microorganisms (pathobionts) (Hrncir et al., 2019). Outgrowth of pathobionts, such as *Enterobacteriaceae*, is often seen in many immune-mediated and infectious diseases (Hrncir et al., 2019; Stecher et al., 2013).

Most African communities have employed cereal fermentation as a food processing and preservation means since the dawn of human civilization thousands of years ago (Mokoena et al., 2016; Setta et al., 2020). Sub-Saharan African traditions include a wealth of knowledge about cereal fermentations (production processes and fermentation microorganisms), which is largely unexplored and undocumented (Pswarayi & Gänzle, 2022). Millions of Africans depend on this technology to preserve and often enhance organoleptic properties, nutritional qualities, digestibility and acceptability of their traditional foods at costs available to the average consumer (Aka et al., 2014; Mokoena et al., 2016; Setta et al., 2020). In Africa, important beverages are produced by fermenting cereals such as wheat, maize, barley, sorghum and millet (Aka et al., 2014). Socially, when served, these drinks show friendliness, a gesture of hospitality and also to strengthen good relationships between individuals (Setta et al., 2020; Worku et al., 2016). They are also consumed during farm work, in ceremonies such as funerals and marriage and as supplement food (e.g., weaning food for babies) and as food replacement by both adults and children. The sale of fermented cereal beverages has also economic relevance by

providing income to households (Setta et al., 2020; Worku et al., 2016), however, its production is largely uncontrolled.

Most African traditional fermented cereal beverages fermentation process is spontaneous, with the procedure of how to produce such products passed on from one generation to another. Generally, African traditional fermented cereal beverages share common production processes such as cooking/baking/boiling of doughs of a single or mixed grain flours along with a single or multiple fermentation steps. Malt and small amount of the beverage from previous fermentation (back-slopping) can also be added (Arici & Daglioglu, 2002; Desta & Melese, 2019; Steinkraus, 1996; Worku et al., 2016). Differences in fermentation processes and their recipes can make an artisanal beverage either alcoholic or non-alcoholic. Addition of malt and fermentation for longer time are the main component of the fermentation process that make a beverage alcoholic (Arici & Daglioglu, 2002; Desta & Melese, 2019; Okaru & Lachenmeier, 2022; Steinkraus, 1996).

Among the Ethiopian indigenous fermented cereal based beverages Naaqe, Borde, Areki, Cheka, Tella, Keribo and Shamita are produced and consumed (Fentie et al., 2020; Lee et al., 2015). Cheka is considered to be a model fermented cereal, because it is consumed rather widely in southwestern parts of Ethiopia mostly in Konso and Dirashe (Fentie et al., 2020; Worku et al., 2016). Cheka is mostly prepared from cereals such as maize, sorghum (*Sorghum bicolor*), barley and finger millet (*Eleusine coracana*) and vegetables such as moringa (*Moringa stenopetala*), leaf cabbage (*Brassica* spp.), and decne (*Leptadenia hastata*) (Hailemariam, 2017; Worku et al., 2016). The Cheka preparation processes are complex as it has two fermentation processes running through three phases by employing different raw materials (cereals and vegetables) and vary among localities and households. The people of Konso mostly use mixture of the cereals as ingredients to prepare Cheka, while others like Dirashe, use cabbage and moringa leaves as ingredients in addition to cereals (Hailemariam, 2017). There is no research conducted on probiotic potential of LAB of Cheka to date (Hotessa & 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 (*Zea mays*) and barley (*Hordeum vulgare*), but the product has not yet been documented or studied scientifically.

Since starter cultures are not used for most African traditional fermented cereal beverages, these fermentations are largely uncontrolled, with the stability and quality of the products compromised. Selection of appropriate starter cultures for different cereal fermented products is one of the key strategies to make the fermentation processes controllable, predictable and efficient (Fentie et al., 2020; Hotessa & Robe, 2020; Setta et al., 2020). Specially, LAB are of interest as starter cultures, because they do not produce (large amounts of) alcohol (Hutkins, 2019) and are GRAS (Aka et al., 2014; Mokoena et al., 2016; Setta et al., 2020). The production methods of Ethiopian fermented beverages are different from place to place and from product to product, so that selection of the potential probiotic starter cultures can be anticipated to differ for each specific product.

Contaminated or naturally harmful foods or drinks usually cause foodborne diseases. A hazard is anything in food that can harm users' health. They are usually classified as chemical hazards which may be artificial or natural; biological hazards, which are pathogenic organisms and toxins they produce and, physical hazards such as foreign objects in food (Grace et al., 2018). The World Health Organization (WHO) Foodborne Disease Epidemiology Reference Group provided the most comprehensive estimates of the health burden of foodborne diseases in African countries (Havelaar et al., 2015), which estimated that foodborne disease burden is comparable to that of HIV/AIDS, malaria or tuberculosis. Most of this burden is come from low-income countries and most of it is due to biological hazards (Havelaar et al., 2015), with diarrheal disease agents being the most important contributor to overall foodborne disease burden in African region E (which includes Ethiopia) (Havelaar et al., 2015). The estimate reported that Africa have the highest burden of foodborne disease per capita, with a median of 2,455 foodborne Disability Adjusted Life Years (DALYs) per 100,000 inhabitants (Havelaar et al., 2015). From these DALYs, 26.6% were attributed to *Salmonella* spp., 11.2% to enteropathogenic *Escherichia coli*, 8.6% to enterotoxigenic *E. coli*, 5.7% to *Campylobacter* spp., 0.08% to

Listeria monocytogenes, and 0.004% to Shiga-toxin producing *E. coli* (Havelaar et al., 2015; WHO, 2015b).

Diarrheal diseases have been reported to be the second most important contributor to the total burden of all disease types and the second leading cause of premature death in Ethiopia (Misganaw et al., 2017). Two meta-analyses on the burden of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Shigella* species in Ethiopia also provided a pooled prevalence of 32.5% (Eshetie et al., 2016) and 6.6% (Hussen et al., 2019), respectively. Another systematic review and meta-analysis work also showed that *Salmonella*, *Listeria monocytogenes*, *E. coli*, *Campylobacter* spp. and *Shigella* are among the most common food borne pathogens reported from Ethiopia (Belina et al., 2021). Antimicrobial resistance has also increased worldwide, causing an enormous clinical and public health burden, demanding the search for alternatives to deal with the emerging risk of resistant pathogens (WHO, 2015a). Probiotics could be a valuable approach to decrease the burden of foodborne diseases in a cost-efficient manner, since they can target different steps in the infection processes via a multifactorial mode of action (Jubeh et al., 2020).

According to an expert panel of the Food and Agriculture Organization (FAO)/WHO guideline (FAO & WHO, 2006), the criteria for the selection of probiotic/probiotic starter strains include the functional characterization (i.e., tolerance to GI harsh conditions, ability to adhere to the GI mucosa and cell lines, antimicrobial activity against pathogenic bacteria, and competitive exclusion of pathogens), and safety assessments (lack of systemic infections, deleterious metabolic activities, gene transfer, and lack of excessive immune stimulation in susceptible individuals). In the development process of new probiotics or probiotic starters, their isolation, identification and characterization are of paramount importance. Moreover, whole genome sequence (WGS) analysis provides better understanding of the relation between their phenotypic and genotypic profiles and thus are increasingly required to better understand strain features (Qureshi et al., 2020). Although most LAB can obtain a GRAS status, there are rare emergence of some infections by certain probiotics and antibiotic resistance (Senan et al., 2015). Hence, live microorganisms intentionally added to foods need to be strictly regulated and monitored for safety, both

before and after release on the market. In addition to the routine *in vitro*, *in vivo*, and clinical safety studies conducted on candidate probiotics, EFSA requires WGS analysis in order to assess risks (EFSA, 2021a). The data obtained from WGS analysis of the microorganism/s can provide valuable information on the unequivocal taxonomic identification of the strains and characterization of their potential traits of concern, which may include resistance to antimicrobials, virulence factors and production of known toxic metabolites (EFSA, 2021a).

1.2 Statement of the Problem

Given the diversity of food and beverage items in the country, the microbiology and potential health benefits of a variety of Ethiopian traditional foods and drinks remain to be studied (Ashenafi, 2008; Fentie et al., 2020). Food-borne LAB in most traditional foods are natural and contain strains uncharacterized with respect to their biodiversity, probiotic potential, antimicrobial susceptibility, and possible (opportunistic) virulence properties (Sanders et al., 2010). As with any bacteria, antimicrobial resistance occurs among some LAB, including probiotic microbes. This resistance genes may be related to chromosome or mobile genetic elements. There is a concern over the use of probiotic bacteria in foods that contain specific drug resistance genes. Bacteria, which contain transmissible drug resistance genes should not be used in foods as probiotics (FAO & WHO, 2006).

Many probiotic health claims have not yet been well characterized by experimental evidence (Bermudez-Brito et al., 2012). Not well-characterized LAB or even designated probiotics may pose risks such as potential pathogenicity or toxin production. For example, *E. coli* Nissle 1917, which has been in use as a probiotic for many decades for intestinal disorders such as Crohn disease, irritable bowel syndrome and ulcerative colitis, was shown to have side effects and undesirable effects in clinical (Guenther et al., 2010) and in laboratory conditions (such as induction of DNA double strand breaks in eukaryotic cells) that are inseparable from their probiotic effect (Olier et al., 2012). Nevertheless, the largest body of evidence suggests that most probiotic strains are safe to use in most individuals with a proper functioning immune system and gut barrier function, such as the general population (Katkowska et al., 2021). Foodborne bacterial and viral infections in Africa,

and particularly in Ethiopia, are important causes of morbidity and mortality, demanding an efficient remedy to tackle these public health hurdles (WHO, 2015b). Antimicrobial resistance has also been alarmingly increasing worldwide, posing an enormous clinical and public health burden, demanding the search for alternatives to tackle the emerging risk of resistant pathogens (WHO, 2015a). The prevention of foodborne bacterial and viral infections in Africa is thus a valuable target for probiotic and fermented food applications. Clearing foodborne and viral infections requires a proper functioning of the immune system, both the innate and adaptive immune system. Therefore, probiotic strategies that can improve the innate immune capacity to enhance pathogen killing are of great interest.

1.3 Hypothesis and Research Questions

We hypothesized that selected Ethiopian fermented foods and beverages could be a valuable source of promising LAB candidate probiotics and probiotic starters that can have activities against foodborne pathogens, and help improve fermentation processes of traditional cereal beverages production.

Based on the hypothesis, the following research questions were formulated:

- Can the selected Ethiopian fermented dairy products and cereal beverages be a valuable source of LAB?
- What are important LAB species contained in the selected Ethiopian fermented dairy products and cereal beverages?
- Can we select LAB isolates from the selected Ethiopian fermented dairy products and cereal beverages that have *in vitro* antimicrobial activity against the key indicator pathogens causing gastro-intestinal infections in Ethiopia?
- Can we select LAB isolates from the selected Ethiopian fermented dairy products and cereal beverages that can induce key immunostimulatory pathways in human monocytes *in vitro*?
- Can we select LAB isolates from the selected Ethiopian fermented dairy products and cereal beverages that can withstand the harsh GI conditions by simulating these conditions *in vitro*?

- Can we select LAB isolates from traditional fermented cereal beverages that are promising autochthonous probiotic starters for more controlled cereal fermentations?
- Can we select LAB isolates from the selected Ethiopian fermented dairy products and cereal beverages with promising probiotic and probiotic starters safe with regard to antibiotic resistance?
- Do the genomes of selected promising LAB isolates harbour any functional genes of interest and lack virulence or resistance determinant genes of concern?

1.4 Objectives

1.4.1 General Objective

To isolate, identify and characterize *in vitro* probiotic and starter culture properties of LAB present in the selected Ethiopian fermented foods and beverages.

1.4.2 Specific Objectives

- To isolate and identify LAB present in selected Ethiopian fermented dairy products and cereal beverages.
- To evaluate probiotic properties of LAB isolated from Ethiopian fermented dairy products, yoghurt and cheese.
- To characterize probiotic starter culture potential of LAB isolated from Ethiopian traditional fermented cereal beverages, Naaqe and Cheka.
- To perform comparative genome *in silico* analysis of selected promising probiotic/starter LAB isolates.

1.5 Significance of the Study

Fermented foods and beverages are among commonly served food items in Ethiopia and contain uncharacterized microbial species. This fact leads to transient or longer-lasting colonization of consumers by these microbes. Foodborne LAB in most Ethiopian traditional foods and beverages are natural and contain uncharacterized strains with respect

to biodiversity, health benefits, antimicrobial susceptibility, and possible (opportunistic) virulence properties. The efforts made in this study enable us to select candidate probiotics that could be used in the prevention and treatment of foodborne diseases, which Ethiopia has a large burden (Misganaw et al., 2017; WHO, 2015b), caused by *Salmonella enterica* subsp. *enterica* var. Typhimurium, *E. coli*, *Shigella flexneri*, *Staphylococcus aureus* and *Listeria monocytogenes*. Probiotics could be a promising approach to decrease the burden of these foodborne diseases in a cost-efficient manner (Jubeh et al., 2020).

Given the significance of antimicrobial resistance (AMR) as a health burden (WHO, 2015a), probiotics are also expected to contribute to reduction of the spread and/or evolution of AMR by assisting antibiotics, reducing the need for antibiotics in some cases, modulating immunity, and increasing antibiotic adherence (Jubeh et al., 2020). Furthermore, the results of this study would provide an impetus to start a larger *in vivo* and clinical probiotic research.

Since selection of appropriate starter cultures is one of the key strategies to improve the fermentation processes (Fentie et al., 2020; Hotessa & Robe, 2020; Setta et al., 2020), this research could also enable selection of autochthonous potential LAB probiotic starter cultures for Naaqe and Cheka fermentations. Especially LAB are of interest as starter cultures, because they do not produce (large amounts of) alcohol (Hutkins, 2019) and are GRAS (Aka et al., 2014; Mokoena et al., 2016; Setta et al., 2020).

The benefits of this research also accrue to consumers, fermented foods and beverage processing firms, government, and the scientific society in general through improved understanding of the biodiversity, characteristics, probiotic and probiotic starter potential of bacterial populations of fermented foods and beverages. In terms of scientific contribution, the results of the research relate to advancement of theoretical and practical understanding of the probiotic properties and safety (with respect to antibiotic resistance) of LAB of Ethiopian fermented foods origin. By combining different fields of research, e.g., Pharmacology, microbiology, and molecular biology, the research provided all-

inclusive understanding of the topic. Having a cross-disciplinary approach, the research project had the potential to discover the whole new openings in the area of the research.

1.6 Scope of the Study

The study focused on searching for candidate probiotics and probiotic starters among LAB population of Ethiopian fermented dairy and cereal foods and beverages. As such, it made use of microbiologic, cell culture and molecular methods to isolate, identify and characterize the LAB from fermented foods and beverages collected from Addis Ababa and Arba Minch district, southern Ethiopia, for their probiotic and probiotic starter properties. The scope of the study is limited to using *in vitro* screening methods to select promising candidate probiotics and probiotic starters.

1.7 Limitations of the Study

In the *in vitro* GI conditions resistance assay, acidified phosphate buffered saline (PBS) and PBS containing bile salts were used instead of simulated gastric and intestinal fluid, respectively. It would be good if the simulated gastric and intestinal fluid had been used. However, acidified PBS and PBS containing bile salts also work fine as reported by many authors (Argyri et al., 2013a; Garcia et al., 2016a; Touret et al., 2018; Yusuf et al., 2020; Zoumpopoulou et al., 2008). Detailed GIT conditions resistance test is important and could be further expanded in follow up works including *in vitro* gastrointestinal models (e.g., M-SHIME) or *in vivo* studies. In the present laboratory-scale fermentation study, measurement of different parameters such as alcohol content, sensorial properties and nutritional values were not performed, since they were not the focus areas of the present work. Again, in the follow up study, these parameters will be studied.

2. Literature Reviews

2.1 Ethiopian Fermented Foods and Beverages as sources of Lactic Acid Bacteria

Most probiotics belong to the LAB group, for which the human GIT and fermented dairy products such as cheese, fermented milk and yoghurt form the two major sources (Touret et al., 2018). However, other sources, such as plant-based foods represent valuable alternatives, since strains isolated from these foods may be more viable and useful for application in similar, non-dairy based probiotic products (Fontana et al., 2013; Peres et al., 2012). Various authors reported isolation and identification of LAB from various fermented dairy products (Colombo et al., 2018; Girma & Aemiro, 2021; Obioha et al., 2021; Rezac et al., 2018; Taye et al., 2021). Although there is no literature documenting LAB derived from the cereal beverages Naaqe and Cheka, other cereal-based foods and beverages have also been reported as an alternative source of LAB (Dejene et al., 2021; Pswarayi & Gänzle, 2022; Tadesse et al., 2005).

Girma and Aemiro (Girma & Aemiro, 2021) isolated LAB from fermented Ethiopian dairy products ("Metata Ayib," "Ergo," and "Ayib") and reported that, from a total of 97 LAB isolates 33 were *Lactobacillus* (before reclassification), 20 *Lactococcus*, 17 *Leuconostoc*, 12 *Pediococcus*, 10 *Streptococcus*, and 5 *Enterococcus* spp, with *Lactobacillus* seen to be the most dominant genus. Nigatu et al. also reported that from a total of 60 LAB isolates of Ergo (traditional Ethiopian fermented milk), the selected 6 isolates were identified as *Lactobacillus* spp (before reclassification), indicating the dominance of *Lactobacillus* spp in the Ergo samples (Nigatu et al., 2015). Mulaw et al. has also reported that the selected 2 isolates from Ethiopian Ergo turned out to be *Lactobacillus plantarum* and *Lactobacillus paracasei* (Mulaw et al., 2019). Another LAB isolation and identification work by Taye et al. from different dairy products (cheese, raw cow milk and yogurts) revealed that the majority (24.38%) of the LAB isolated from the different dairy products was found to be *Lactobacillus*, followed by *Lactococcus* (21.94%), *Streptococcus* (19.51%), *Leuconostoc* (14.64%), *Bifidobacteria* (12.19%), and *Pediococcus* (7.31%) spp (Taye et al., 2021). The work also enumerated LAB and showed that *Lactobacillus* spp. were presumptively

counted as 4.5×10^7, 7.7×10^7, and 2.3×10^8 colony forming unit (CFU)/mL from raw cow's milk, cheese, and yogurt, respectively.

A study reported that *Lactobacillus viridescens*, *Weissella confusa*, *Lactobacillus brevis*, *Pediococcus pentosaceus* and *P. pentosaceus* subsp. *intermedius* were identified throughout borde (Ethiopian cereal beverage) fermentation as dominant LAB (Abegaz, 2007). Tadesse et al. isolated LAB genera of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* spp., from Borde and Shamita, the Ethiopian traditional cereal beverages (Tadesse et al., 2005). Dejene et al. also reported isolation of *Enterococcus*, *Leuconostoc*, and *Weissella* from borde (Dejene et al., 2021). Another recent study also identified eleven LAB isolates, belonging to seven *Pediococcus pentosaceus*, two *Pediococcus acidilactici*, and two *Lactococcus lactis* from four Ethiopian traditional fermented foods and beverages (*Bulla*, *Kotcho*, *Ergo*, and *Bukuri*) (Amenu & Bacha, 2023).

2.2 Probiotic Properties of Lactic Acid Bacteria

Important probiotic properties of microorganisms include the functional traits (i.e., tolerance to GI harsh conditions, ability to adhere to GI mucosa and cell lines, competitive exclusion of pathogens, antimicrobial activity against potentially pathogenic bacteria), and safety issues (lack of systemic infections, deleterious metabolic activities, gene transfer and lack of excessive immune stimulation in susceptible individuals) (FAO & WHO, 2006). Many authors reported antagonistic activities of LAB isolated from Ethiopian dairy products (Girma & Aemiro, 2021; Goa et al., 2022; Nigatu et al., 2015) and cereal beverages (Akalu et al., 2017; Dejene et al., 2021; Tadesse et al., 2005). The mechanisms behind the beneficial effects of LAB as probiotics are largely unknown but are likely to be multifactorial (Bermudez-Brito et al., 2012; Reid, 2016). Several proposed mechanisms related to the antipathogenic effects of probiotics on various microorganisms may include the following: competitive adherence to the mucosa and epithelium, secretion of antimicrobial substances, gut epithelial barrier strengthening, and the immune system modulation (Bermudez-Brito et al., 2012; Reid, 2016). As an alternative strategy of the commensals and probiotics to outcompete the pathogenic microbes, these microbes may, preferentially consume nutrients that are required by pathogenic bacteria for their growth.

For instance, commensal *E. coli* competes with Enterohemorrhagic *E. coli* for amino acids (especially proline) and carbohydrates (Fabich et al., 2008; Momose et al., 2008a). The commensal bacteria cause starvation of the competing pathogens by consuming the usually available limited nutrient resources. In addition to competition for sources of carbon and energy, invading pathogenic bacteria compete with the microbiota for trace metals. For example, the probiotic *E. coli* Nissle 1917 strain reduces mice intestinal colonization by pathogenic *Salmonella typhimurium* through competition for the limiting nutrient iron (Deriu et al., 2013).

Probiotics and especially LAB also directly inhibit the growth of bacterial, fungal and even viral pathogens, via their capacity to produce substances with antimicrobial effects including the broad-acting antimicrobial molecule lactic acid, short-chain fatty acids (SCFAs), secondary bile acids and more species- or strain-specific antimicrobials such as bacteriocins (Lebeer et al., 2008). Butyrate, an SCFA, downregulates expression of several virulence genes, mainly genes localized to the *Salmonella* pathogenicity island 1 in the *Salmonella* serovars Enteritidis and Typhimurium (Gantois et al., 2006). Acetate and lactate produced by the microbiota eliminates *E. coli* O157:H7 from mice intestine by suppressing motility of the pathogen under anaerobic conditions (Momose et al., 2008b). Deconjugated primary bile acids can be converted into the two main products, deoxycholic acid and lithocholic acid, by a few bacteria, mostly *Clostridium* spp. (*C. hylemonae*, *C. hiranonis*, *C. absonum*, and *C. scindens*), via 7α-dehydroxylation through a complex biochemical pathway. Although the exact mechanisms of colonization resistance against *C. difficile* are unknown, there is increasing evidence that gut microbiota derived secondary bile acids play a key role (Ducarmon et al., 2019; Winston & Theriot, 2016). Secondary bile acid deoxycholic acid is believed to be bactericidal to many bacteria, including *Clostridium difficile* and *S. aureus* by disrupting membrane and subsequently causing leakage of cellular content. Lactic acid, the main product of LAB carbohydrate metabolism (Mokoena, 2017), is widely used to inhibit the growth of important microbial pathogens. A study reported that 0.5% lactic acid completely inhibited the growth of *E. coli, Salmonella* Enteritidis and *L. monocytogenes* cells, through leakage of proteins of the bacterial cells via disruption of cytoplasmic membrane (C. Wang et al., 2015). Commensal

microbes can also produce certain metabolites that can directly inhibit specific microbial members of bacterial species. Bacteriocins are short, toxic peptides produced by specific bacterial species that can prevent the colonization and growth of other microbes. Their modes of action are diverse, including disrupting DNA and RNA metabolism and killing cells via formation of pores in the cell membrane (Ducarmon et al., 2019). A bacteriocin produced by a strain of *Latilactobacillus curvatus* displayed antibacterial activity against some selected foodborne pathogens, including *L. monocytogenes*, *S. aureus*, and *E. coli* (Heidari et al., 2022).

Most enteropathogenic bacterial species, such as *E. coli*, *Bacillus cereus*, and *C. difficile*, require optimal pH to grow (Ceuppens et al., 2012; Ducarmon et al., 2019; Gantois et al., 2006; Khan et al., 2021). To inhibit pathogenic bacterial growth, commensals evolve mechanisms that alter the gut local physiological environment by modifying pH, preventing pathogen colonization and lowering the risk of intestinal infectious diseases (Ducarmon et al., 2019; Gantois et al., 2006). SCFAs such as butyric acid, propionic acid, and acetic acid, are major metabolic products of most gut microbial fermentation by certain commensal bacteria, modulate the gut pH, and prevent the proliferation of certain intestinal pathogenic bacteria (Ducarmon et al., 2019; Shin et al., 2002). Various studies demonstrated that antipathogenic activity of LAB against different pathogens is dependent on the ability of LAB to lower pH (Reuben et al., 2020; Spacova et al., 2020; van den Broek et al., 2018). Although commensal microbes withstand pathogen colonization and lower the risk of pathogens associated intestinal infections via modulating the gut pH, the underlying molecular mechanism of the effect is partially or completely unexplored (Ducarmon et al., 2019; Khan et al., 2021).

Another key mechanism of action of probiotics is immunomodulation, whereby probiotics can activate the host cells to produce antimicrobial molecules or cellular activities (Shanahan, 2010; B. Wang et al., 2017). This activity is generally mediated by microbe-associated molecular patterns (MAMPs) expressed by the probiotics, which can interact with different immune receptors on the host cells, such as Toll-like receptors (Lebeer et al., 2010). This interaction leads to activation of nuclear transcription factors such as NF-κB

that plays a key signaling role in the activation of immune responses following a variety of stimuli such as with MAMPs (Jensen et al., 2015; Wells et al., 2010). Usually, NF-κB induces a number of genes mainly involved in pro-inflammatory cascades at sites of infection to kill pathogens, the intestinal epithelium generally does not trigger inflammatory responses against commensal bacteria, but rather induces tolerance towards the commensal microorganisms. Yet, some of the signals induced by commensals and probiotics may result in a better alertness and more rapid clearance of incoming pathogens. Aghamohammad et al. recently reported that a probiotic cocktail showed anti-inflammatory effects on HT-29 cells by modulating JAK/STAT and NF-kB pathways (Aghamohammad et al., 2022). Another important signaling pathway in response to microbial stimuli is related to IFN production, which is controlled by IRFs (Kawashima et al., 2013; Spacova et al., 2021). This pathway is necessary for effective antiviral responses and commonly induced by viral MAMPs (Spacova et al., 2021). However, the same group (Spacova, et al., 2023) reported that several selected strains of probiotic lactobacilli can also induce this pathway and boost antiviral responses.

Research has since showed that probiotics can provide a wide range of health benefits specially those directly related to the gut (Islam, 2016; Kechagia et al., 2013). For instance, probiotics can regulate gut microbiota, improve immune system and the bioavailability of nutrients, as well as reduce symptoms of lactose intolerance (M. Zheng et al., 2017). A wealth of evidence emerging from studies also indicates anti-cancer activity of probiotics (Ambalam et al., 2016; E. L. Ma et al., 2010). Other notable health claims include the lowering of viral-associated pulmonary damage (Zelaya et al., 2014) and the decrease in cholesterol level that reduces the risk of cardiovascular diseases (M. Zheng et al., 2017). Spacova et al. (Spacova, et al., 2023) have recently developed a throat spray with probiotic lactobacilli having antiviral action and capable of temporary colonization of the throat. Indeed, the global probiotics market size was estimated at $ 54.77 billion in 2020 and is expected to grow at a compound annual growth rate of 7.2% from 2021 to 2028 (Grand View Research, Inc., 2021).

2.3 Probiotic Starter Potential of Lactic Acid Bacteria

Starter cultures are preparations with a large number of cells, either of a single type or a mixture of two or more microorganisms that are added to the fresh substrate (e.g. dairy for cheese or yoghurt) in order to enhance, speed up or guide the fermentation (García-Díez & Saraiva, 2021). For dairy fermentations, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii subsp. bulgaricus* are examples of commonly used LAB starter cultures (García-Díez & Saraiva, 2021). Cultures for food fermentations are selected primarily on the basis of their antipathogenic activities, resistance to acid and bile, and fermentative activity, among other properties (Enujiugha & Badejo, 2017; Ogunremi et al., 2017). LAB are of interest as starter cultures since they do not produce (large amounts of) alcohol (Hutkins, 2019) and are GRAS (Aka et al., 2014; Mokoena et al., 2016; Setta et al., 2020).

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 pathogens that can cause gastro-intestinal diseases (Edema & Sanni, 2008; Garriga et al., 2015; H. Mathur et al., 2020; Rao et al., 2019). For cereal fermentations, starter cultures are not widely applied. Starter cultures for a specific fermented food product are commonly isolated from the food product itself (autochthonous) (Edema & Sanni, 2008). Although dairy products such as fermented sour milk, yoghurts and cheese remain at the forefront of probiotic food development at present, there are main drawbacks related to them such as lactose intolerance, allergy and their cholesterol content. Furthermore, cultural (strict vegans) and specific religious believes among certain communities may also limit the intake of dairy foods. Hence, many non-dairy probiotic foods such as cereal based fermented products and fruit and vegetable juices are beneficial for the people with such conditions (Enujiugha & Badejo, 2017; Ranadheera et al., 2017).

2.4 Safety of Probiotics

The joint FAO/WHO (FAO/WHO, 2002) guidelines on probiotic evaluation reported that probiotics may theoretically be linked to four specific types of side effects in patients with underlying medical conditions: deleterious metabolic activities, systemic infections; gene

transfer and excessive immune stimulation in susceptible individuals. The at-risk population groups for probiotic side effects are broadly characterized by the weakened immune system, gut dysbiosis and/or impaired intestinal barrier, and therefore, it is important to thoroughly assess the safety associated with deliberate administration of probiotics. In addition, the availability of limited or conflicting evidence on the benefit of many probiotic intervention studies due to variability in the target population, types of probiotic formulations administered, statistical and clinical heterogeneity, limitations of study and small sample size also requires a comprehensive safety assessment for the population at risk groups (Kothari et al., 2019). Bacterial translocation is the passage of viable indigenous bacteria from the GIT to extraintestinal sites (mainly systemic circulation) because of an impaired intestinal barrier, gut prematurity or immunosuppression (Liong, 2008; C. Wang et al., 2019). Bacterial translocation to sterile niches has been seen following stroke, cirrhosis, severe burn, and trauma, among a range of other conditions (Kothari et al., 2019; C. Wang et al., 2019). It has been speculated that the intestinal mucosa adhesion capacity or mucolytic activity of probiotic strains might possess a role in their translocation (Kothari et al., 2019). Predominantly, infections associated with probiotics strains include endocarditis, sepsis, localized infections and opportunistic infections via bacteremia or fungemia (Kothari et al., 2019). The safety of probiotics is mainly studied by the population who consumes them. Generally, they are very well tolerated by healthy population, and the related adverse effects are mild and do not endanger health or life. However, in the higher risk group, there may be episodically severe adverse effects (Katkowska et al., 2021). Sepsis is a clinical syndrome associated with systematic inflammation and circulatory malfunctions following pathogenic infections such as fungemia or bacteremia. Sepsis is one of the most common causes of morbidity and mortality in patients with critical illnesses (Jacobi et al., 2011) and pre-term infants (D'Agostin et al., 2021). The *Lacticaseibacillus rhamnosus* GG strain is the most commonly used as a probiotic and the most frequently isolated bacteria from various infections caused by probiotic strains (Katkowska et al., 2021). Sepsis caused by *Lacticaseibacillus rhamnosus* GG is mainly reported in newborns (Katkowska et al., 2021).

The microbiota may regularly be exposed to a variety of antimicrobials, which are used in the treatment and prevention of human and veterinary bacterial infections (Marshall & Levy, 2011). Therapy with antibiotics may cause secondary effects such as distortion of the homeostasis of gut microbiota and selection for antibiotic-resistant microbes. A large portion of the resistome is carried within chromosomal DNA, though it may also be present on extra-chromosomal replicons like phages and plasmids, which can be transmitted to other bacteria in the gut (including pathogens) through horizontal gene transfer events (Duranti et al., 2017; Ouwehand et al., 2016). A review (Sharma et al., 2014) reported that many commercial probiotics were resistant to common antibiotics. Reuben *et al*. reported that the LAB strains tested showed varying degree of resistance toward some antibacterials; strains from cow milk were resistant to ampicillin, ceftriaxone, erythromycin, oxacillin, chloramphenicol, vancomycin, streptomycin, gentamicin and tetracycline, whereas strains of goat milk origin were resistant to ciprofloxacin, erythromycin, vancomycin, oxacillin and streptomycin (Reuben et al., 2020). A study conducted recently in Ethiopia also showed that all the tested 11 LAB isolates were resistant to ampicillin, vancomycin, gentamicin, kanamycin, clindamycin, and chloramphenicol, while they were susceptible to streptomycin and tetracycline (Amenu & Bacha, 2023).

Lactobacilli are usually intrinsically resistant to trimethoprim and sulphonamides, and quinolones; and commonly susceptible to low concentrations of several protein synthesis inhibitors except aminoglycosides (such as macrolides, chloramphenicol, tetracycline and lincosamides) (Ammor et al., 2007; Gueimonde et al., 2013). Lactobacilli are also generally sensitive to the cell wall-targeting penicillins and β-lactamase inhibitors (combined with penicillins), nonetheless, are more resistant to cephalosporins (Gueimonde et al., 2013; Klare et al., 2007).

Resistance of various species of lactobacilli to glycopeptides (vancomycin) is also considered intrinsic (S. Mathur & Singh, 2005; Nawaz et al., 2011). Generally, *Lactobacilli*, *Leuconostoc* and *pediococci* spp. have been reported to have a high intrinsic resistance to vancomycin, a property that is useful to differentiate them from other Gram-positive bacteria (S. Mathur & Singh, 2005). Vancomycin makes a contact with the

peptidoglycan precursors on the cell wall side of the bacterial cytoplasmic membrane and binds to the D-alanine/D-alanine terminus of the pentapeptide, inhibiting polymerization of the peptidoglycan precursors. In several LAB species, the terminal D-alanine residue is replaced by D-lactate or D-serine in the muramylpentapeptide, precluding vancomycin binding and therefore becoming intrinsically resistant to the agent (Daniali et al., 2020).

Chromosomal mutations leading to antimicrobial resistance phenotypes have been reported in lactobacilli (Gueimonde et al., 2013). The lactobacilli spontaneous mutation rate to kanamycin, streptomycin, and nitrofurazone was found to be high (10^{-4}-10^{-5} frequency/rate) per generation (Curragh & Collins, 1992). Bacterial resistance to streptogramins, lincosamides and macrolides is usually due to efflux systems, target site methylases and inactivating enzymes. However, it can also occur because of mutations in the ribosomal proteins L4 and L22 encoding genes and at several positions in the 23S rRNA gene, the most common of which are mutations in the chromosome changing the erythromycin binding site of the 23S rRNA molecule's V domain (Flórez et al., 2007). This mutation has been described in a number of clinical isolates, including *Helicobacter pylori, Mycoplasma* spp., and *Propionibacterium* spp. (Flórez et al., 2007). Flórez et al. (2007) identified a single mutation in the 23S rRNA gene of a *Lactobacillus rhamnosus* strain reducing the affinity of erythromycin for the ribosome, conferring resistance to macrolides (Flórez et al., 2007). Although the transfer risk is regarded as very low for intrinsic resistance or acquired resistance from chromosomal mutation(s), knowing the antimicrobial resistance phenotypes may still be crucial even in the absence of transferable resistance, since intrinsic resistance might still be relevant for the treatment of *Lactobacillus*-related bacteremia (Cannon et al., 2005).

Chloramphenicol resistance genes (*cat*; chloramphenicol acetyltransferases that transfer an acetyl group to the antibiotic, plasmid located) have been identified in *L. johnsonii* (Mayrhofer et al., 2010) and *L. acidophilus*, *L. delbrueckii* subsp. *Bulgaricus* (Hummel et al., 2007) as well as in *L. plantarum* (Ahn et al., 1992) and *L. reuteri* (Lin et al., 1996). Target modification results in the resistance of macrolide/lincosamide/streptogramin (MLS) and results from a mutation in the 23S rRNA gene or is encoded by erythromycin-

resistant-methylase (*erm*) genes. These *erm* genes, responsible for the MLS resistance phenotype, have been described in several *Lactobacillus species*; the *erm(B)* gene, which encodes an rRNA adenine N-6-methyltransferase, which can methylate adenine on the 23S ribosomal subunit, is the most commonly found of such genes. However, other genes such as *erm(A)*, *erm(C)*, or *erm(T)* have also been identified (Mayrhofer et al., 2010; van Hoek et al., 2008). The presence of macrolide efflux pumps coding genes such as *mef(A)*, genes coding for lincosamide transferase (*lnu(A)*) (Cauwerts et al., 2006) and streptogramin A acetyltransferases (*vat(E)*) (Gfeller et al., 2003; Mayrhofer et al., 2010) have also been detected. Nevertheless, the most common resistance genes found in lactobacilli are the tetracycline resistance genes, which are occasionally found in combination (Ammor et al., 2008).

Tetracycline is a broad-spectrum antibiotic used in human and veterinary medicine, and aquaculture industry, for which at least 40 different resistance genes (*tet*) have been characterized to date and, these genes are involved in either ribosomal protection, active efflux of the drug or enzymatic drug modification (Hedayatianfard et al., 2014). To collectively evaluate tetracycline resistance in LAB, a group of researchers tested the susceptibility patterns of 478 LAB strains by comparing phenotypes with genotypes based on genome-wide annotations (Q. Ma et al., 2021). The group detected five tetracycline resistance genes, *tet*(L), *tet*(M), *tet*(W/N/W), *tet*(S) and *tet*(45) in LAB with *tet*(M) and *tet*(W/N/W) were the most broadly distributed tetracycline resistance genes in LAB. The group also observed that multiple LAB strains not harboring tetracycline resistance genes were also found to be resistant to tetracycline at the currently recommended cutoff values (Q. Ma et al., 2021). It is important to note that many of the genetic determinants mentioned above are occasionally found in mobile genetic elements (plasmids and conjugative transposons), which may spread the antibiotic resistance genes primarily by conjugation mechanisms (Aquilanti et al., 2007).

3. Methodology

3.1 Isolation and Characterization of LAB Strains

Aseptically, one yogurt and one cheese products from two separate dairy industries in Addis Ababa, Ethiopia and two spontaneously fermented traditional cottage cheeses from Arba Minch district, Ethiopia were collected. In addition, four spontaneous cereal fermentation samples, two Naaqe samples from Arba Minch district (Ethiopia) and two Cheka samples from Konso (Ethiopia), were aseptically collected in sterile 50 mL tubes. Samples were transported in an ice-box to the Bacteriology laboratory of Armauer Hansen Research Institute (AHRI) and processed on arrival for the isolation of LAB. To isolate LAB, 10 mL (or g) of each sample was suspended and homogenized in 90 mL PBS (pH 7−7.4). The homogenized sample (1^{st} dilution) was used to prepare 10-fold serial dilutions and ten μL of the appropriate dilution (usually 3^{rd} to 6^{th}) was spread-plated on de Man, Rogosa, and Sharpe (MRS) agar (Hi-Media, Mumbai, India). MRS is a selective medium used to enrich LAB (De MAN et al., 1960). These plates were then incubated at 37^{o}C anaerobically (BD BBL™ GasPak™ jars) for 24 to 48 h. Plates with 30 to 300 colonies were selected and their colonies were counted. On average, five colonies were then randomly selected based on their morphology and purified through three successive streaking on MRS agar, from which aliquots of the selected isolates were stored at −80°C in MRS broth containing 25% glycerol. Eventually, the purified isolates were characterized presumptively as LAB by cell morphology, catalase test, gram staining and motility according to standard procedures (Hutkins, 2019), whereby gram-positive, non-motile and catalase-negative isolates were presumptively identified as LAB. The LAB CFU/mL(g) was calculated as a function of the number of confirmed LAB colonies and the inoculated dilution by the below formula (Silva, 2013)

CFU/mL(g) = total colonies present x percent confirmed colonies x dilution.

3.2 Molecular Identification of LAB Isolates

The selected isolates from putatively identified as LAB were further identified by 16S rRNA gene sequencing. For the identification of LAB strains using 16S *rRNA gene* sequences, the primers used were: 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1492R (5'-GGTTACCTTGTTA CGACTT-3'). The LAB isolates genomic DNA was

extracted using a 16S rDNA colony PCR technique. In brief, a colony was picked, mixed and vortexed in 10 µL molecular grade water. The cells were lysed through microwaving for 1.5 min, 2 times at 800W. The master mix was prepared in clean room containing; 2.5 µL 10xVWR Buffer, 0.5 µL dNTPs (10mM), 2.5 µL 27F (10µM), 2.5 µL 1492R (10 µM), 0.2 µL Taq polymerase and 6.8 µL molecular grade water to make master mix of 15 µL final volume for each sample. This 15 µL master mix was then added to each tube containing 10 µL DNA template, making the final volume 25 µL. Conventional PCR was run under the following conditions: initial activation at 95◦C for 2 min; denaturation step cycles 95°C for 30 s; annealing step at 55°C for 30 s; extension step at 72°C for 1 min and 30 s; and final extension cycle at 72°C for 5 min; for 30 cycles. Five µL of the PCR product was then used to run 1% agarose gel electrophoresis on a gel with 5 µL GelRed dye. Successful samples (bright band at 1500 bps) were sent for Sanger sequencing at the Neuromics Support Facility VIB, UAntwerpen. The resulting sequences were analyzed by using the SeqTrace 0.9.0 software and submitted to a search for similarity in the EzBioCloud.net 16S-based ID. Bacterial species identification was considered when the query sequence showed pairwise similarity of >98.7% for the 16S rRNA gene sequence, as previously described (Lagier et al., 2018).

3.3 Antagonistic Activity of LAB Isolates against Sensitive and drug Resistant Indicator Foodborne Pathogens

The antipathogenic activity of LAB isolates against indicator foodborne pathogens was evaluated by using spot overlay and radial diffusion assays with *Escherichia coli*, *Salmonella* spp., *Shigella* spp and *Listeria* spp., and *Staphylococcus* spp. as indicators of antagonistic activity. Longitudinal liquid culture growth assay against *S. aureus* MI/1310/1938 was also performed.

Spot overlay assay: This assay was performed both at AHRI, Addis Ababa, Ethiopia and the Laboratory of Applied Microbiology and Biotechnology (LAMB), University of Antwerp, Antwerp, Belgium. The indicator foodborne pathogens used in AHRI were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *L. monocytogenes* ATCC 19115; obtained from the Ethiopian Public Health Institute, and a clinical isolate of methicillin resistant *S.*

aureus (MRSA) obtained from Tikur Anbessa Specialized Hospital, Addis Ababa University, Ethiopia. At the LAMB, *E. coli* O157:H7 BRMSID188 lacking pathogenicity stx genes (for biosafety reasons) isolated from bovine (Van Beeck et al., 2020), *L. monocytogenes* MB2022 isolated from Wijnendaele cheese, *S. enterica* subsp. *Enterica* var. Typhimurium NTCT 13347, *S. flexneri* LMG 10472 and *S. aureus* MI/1310/1938 – methicillin sensitive (MSSA) were used as indicator pathogenic strains. In the spot overlay assay, 2 µL from each LAB isolate overnight (20–24h) culture cultivated under micro-aerobiosis in MRS broth, was spotted on the surface of agar media (AHRI: MRS agar for all pathogens tested; LAMB: Mueller Hinton agar (MHA) (1.5% agar) supplemented with 5 g/L glucose for *S. aureus* and LB agar (1.5% agar) supplemented with 5 g/L glucose for other pathogens, as described previously (van den Broek et al., 2018). After spotting, the plates were incubated aerobically at 37°C for 24 h (for spots on MRS agar) and 48 h (for others). A volume of overnight (18–22h) growth of each indicator pathogen required to make final concentration of $5x10^6$ CFU/mL was mixed with 20 mL of soft agar (0.5% agar) and uniformly poured over the spot inoculated square plate (7 mL/round petri dish). The plates were then incubated aerobically at 37°C for 24 h. The antipathogenic activity was measured as the diameter (mm) of the inhibition zones. Two µL of Hexetidine (0.1%) or chlorhexidine 0.2% were spotted as positive controls while MRS broth was spotted as negative control. Experiments were conducted in triplicates and the average values were calculated and recorded.

Radial diffusion assay: This assay was performed by using the method described previously (van den Broek et al., 2018) using the same indicator pathogens, final concentration of the indicator pathogen inoculum, and media as described in the spot overlay assay conducted at the LAMB. The LAB strains were first cultivated overnight (20 – 24 h) in MRS broth at 37°C. The supernatants of these cultures (ca. 10^9 CFU/mL) were collected by centrifugation (2484 g, 15 min, 4°C) and then filter sterilized by 0.22 µm filter, with or without pH adjustment to pH 7.4. An adequate volume of overnight growth of indicator pathogens were added to a cooled (55°C) agar (50 mL) and mixed well to make the final concentration of $5x10^6$ CFU/mL and poured onto a square plate. LAB cell-free culture supernatants (CFS) (45 µL), pH adjusted (7.4) or non-adjusted, were dispensed into

6 mm diameter wells drilled using sterile glass Pasteur pipette. The plates were aerobically incubated at 37◦C for 24 h. After the incubation period, the antimicrobial activity was measured as the diameter (mm) of growth inhibition zones around each well. In this assay, MRS broth (45 µL) and hexetidine (0.1%, 45 µL) were used as negative and positive controls, respectively. Experiments were conducted in triplicates and the average values were calculated recorded.

Time-course analysis of the antimicrobial activity of cell-free culture supernatants: This assay was also performed as described previously (van den Broek et al., 2018). Briefly, 190 µL of a diluted (in LB medium) overnight (20-24h) culture of *S. aureus* MI/1310/1938 (ca. 10^5 CFU/mL) was added to the wells of a microplate supplemented with 10 µL CFS of LAB strains (obtained in the same way as in the radial diffusion assay) to get a total volume of 200 µL. Ten µL 0.1% hexetidine and 10 µL MRS and LB medium were used as a positive and negative control, respectively. The indicator bacteria were grown at 37ºC and the optical density (OD) was measured at 600 nm (OD_{600}) each 30 min for 24 h using a Synergy HTX multi-mode reader. Each test was measured in triplicate and the average $OD6_{00}$ was recorded.

3.4 Resistance of LAB Strains to Gastrointestinal Conditions *in vitro*

LAB strains were inoculated in MRS broth and incubated at 37°C overnight (18-22 h). 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 by measuring OD at 600 nm. To assess survival of the LAB strains in stomach acidic environment, 100 µL of the $1.5x10^8$ CFU/mL 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. After 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 the LAB strains tolerance in bile salt solution, 100 µL of each LAB strain at 1.5×10^8 CFU/mL 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, to mimic the time spent by food in the small intestine

(Argyri et al., 2013b; Garcia et al., 2016b; Panya et al., 2016). 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. Percent (%) 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.

3.5 Evaluation of Immunostimulatory Activity of LAB Strains

Immunostimulatory activities of the LAB strains were assessed by measuring NF-κB and IRF pathways activation in the human THP1-Dual™ reporter monocytes (InvivoGen, San Diego, CA, USA) as previously described (Spacova, et al., 2023). The cells were maintained according to the manufacturer's instructions in growth medium containing RPMI 1640, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 µg/mL Normocin™ and Pen-Strep (100 U/mL;100 µg/mL). The bacterial cells were UV-inactivated in a biosafety level 2 cabinet for 90 min with vortexing after each 15 min before co-incubation with THP1-Dual™ cells. In the immunostimulation assay, UV-inactivated bacterial cells (final concentration 10^7 CFU/mL before inactivation) were added to THP1-Dual™ cells (final concentration 10^6 cells/mL) and co-incubated for 24 h at 37 °C and 5% CO_2. For the assessment of NF-κB pathway induction, secreted embryonic alkaline phosphatase (SEAP) activity in the THP1-Dual™ monocyte supernatant after addition of a p-nitrophenyl phosphate (pNPP) solution was measured at absorbance of 405 nm using a BioTek Synergy HTX multimode reader according to the manufacturer's instructions. IRF pathway activation was measured by assessing the activity of a secreted luciferase (Lucia) by using QUANTI-Luc buffer, a luciferase detection reagent, based on luminescence using a BioTek Synergy HTX multimode reader according to the manufacturer's instructions.

3.6 Antibacterial Susceptibility Testing of LAB Isolates

The antibiotic susceptibility of test of the selected LAB isolates was determined for ampicillin, chloramphenicol, erythromycin, clindamycin, gentamycin, streptomycin,

tetracycline and kanamycin as per the recommendations of the EFSA (EFSA, 2012), using a broth microdilution test previously described (CLSI, 2012), with minor modifications. In brief, 10 µL of each antibiotic solution was dispensed into each well of a 96-well microplate containing 180 µL of MRS broth. Subsequently, a 10 µL-culture aliquot of each test LAB strain was added to each well (final viable cell count of approximately 7 log CFU/mL). The microplates were sealed with plastic bags in order to prevent bacterial dehydration. The experiments included controls, in particular bacteria alone, MRS broth, and known probiotic control strains *L. rhamnosus* GG (Kankainen et al., 2009) and *L. plantarum* WCFS1 (Kleerebezem et al., 2003), and were done in triplicates. The system was then statically and aerobically incubated at 37◦C for 48 h, and the plates were observed for any visible growth. The strains showed visible growth were considered resistant.

3.7 Growth Curve Analysis of LAB Isolated from Naaqe and Cheka

For the analysis of the growth ability of LAB strains, 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 ca. 7 log CFU/mL). Bacteria were allowed to grow at room temperature, and the OD_{600} was measured every 30 min for 48 h using a BioTek Synergy HTX multi-mode reader. The experiment was run in triplicates. OD_{600} data from 0 to 48 h were employed 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 & Wagner, 2016). The lag time, as an adaptation to the conditions of the growth (Sterniša et al., 2022), was estimated from the growth curve plots of LAB strains OD_{600} measurements.

3.8 Laboratory-Scale Fermentation Experiments Using Selected LAB Isolated Naaqe and Cheka as Starters

Based on a comprehensive evaluation of the tested probiotic and growth properties of the LAB isolated from Naaqe and Cheka (Paper II, Table 4), 6 LAB isolates were selected as candidate probiotic strains for laboratory-scale fermentation experiments. In each of Naaqe and Cheka fermentation experiment, two LAB isolates (e.g., *L. fermentum* 44B and *W. confusa* 44D) individually 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 was 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 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 the ingredients used in these laboratory-scale fermentation experiments were acquired from their indigenous locale (Arba Minch District and Konso).

3.8.1 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. Indigenously, Naaqe is produced by simple procedures with no use of malt and other steps in order to make the product non-alcoholic. The production process has two fermentation steps. In the primary fermentation, grain (commonly maize) 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 pieces, 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.

Protocol for Naaqe lab-scale fermentation

Primary Fermentation of Naaqe

At time point 0 h (baseline), maize (Bako Hybrid-660 variety) 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 spread plating for CFU/mL counts after 10-fold 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 spread plating on MRS medium for colony count.

Secondary Fermentation of Naaqe

At time point 0 h (baseline), a fermentation mix was prepared from a spontaneous primary fermentation that was set up for 30 h, made in to dough balls and cooked (for 70 min), allowed to cool (4 h), smashed into pieces in a tray using a clean bottle, 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). At time point 0 h (baseline), from this soft mass, a portion was sampled for pH measurement. Ten mL of the mix was also sampled for spread-plating after 10-fold serial dilutions. Immediately after sampling, the soft mass 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.

3.8.2 Cheka Fermentation

The protocol used in Cheka laboratory-scale fermentation was developed based on available literature (Worku et al., 2016), interviews, onsite observation, and analysis of 2 samples. 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 made, allowed to cool and kneaded with malt. The porridge is then added into the fermenting produce in the vessel; 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).

Protocol for Cheka lab-scale fermentation

Primary Fermentation of Cheka

At time point 0 h (baseline), grain (maize (Bako Hybrid-660 variety):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 to count colonies. 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 spread-plating on MRS agar for colony count.

Secondary Fermentation of Cheka

At time point 0 h (baseline), dough balls that were made and cooked (for 1 h) from the primary fermentation mix fermented for 36 h were 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).Then, from this produce, a portion was sampled for pH measurement and 10 mL sample was taken for spread-plating. 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 produce allowed to ferment for 24 h, a portion was sampled for pH measurement and 10 mL was taken for spread-plating. 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 spread-plating on MRS medium for colony count.

3.9 Genomic DNA Extraction, Whole Genome Sequencing, Assembly and Annotation

Three LAB strains (*Lactiplantibacillus Plantarum* 54B, 54C, 55A) isolated from Ethiopian traditional cottage cheese were selected based mainly on their performance in the antimicrobial and cell culture assays for the whole genome sequencing and its analysis. Strains were revived from -80°C glycerol stocks on MRS plates and incubated for 48h at 37° C. Single colonies were cultivated in MRS broth for 24h at 37°C. Total DNA content was extracted using a modified protocol based on Alimolaei and Golchin (Alimolaei & Golchin, 2016). Briefly, 1.5 mL of overnight culture were transferred twice to two sterile eppendorf tubes, 1.5µL of ampicillin (100mg/mL) were added and incubated at 37°C for 1h. Then the culture was spun down at 12.000 x g for 3 min to remove supernatant and the pellet was washed 3x with 1mL of NaCl-EDTA. Pellets present in both eppendorfs were pooled in to one eppendorf. The cell pellets were then resuspended in 100µL of NaCl-EDTA, 100µL of lysozyme (10mg/mL) and 1µL Rnase (20mg/mL) were added to the tube and incubated at 37°C with periodic shaking for 1h. Following this, 229µL of NaCl-EDTA, 50µL 10% SDS and 20µL Proteinase K were added, vortexed and incubated at 55°C for 1h. Then 200 µL of cold protein precipitation solution were added and vortexed at maximum speed for 20 sec. The mix was then centrifuged at 12.000 g, 4°C for 3 min after putting on ice for 5 min. The supernatant was transferred to a clean 1.5 mL tube, centrifuged again (12.000 g, 4°C, 3 min) and the supernatant was transferred to clean 1.5 mL tube. The DNA was precipitated with 600 µL ice-cold isopropanol, centrifuged at

12.000 g, 4°C for 3 min to discard the supernatant. The pellet was then washed with 600 µL fresh 70% ethanol, the supernatant discarded and the tube left to air-dry. Finally, the pellet was dissolved in 50 µL H_2O, incubated at 55°C for 5 min and stored at -20°C. DNA concentration (measured with Qubit) in the range of 25-50 ng/µL in minimum 20µL volume were sent for WGS.

High molecular weight genomic DNA of the isolates was then further processed for sequencing using Nextera library preparation and MiSeq sequencing at the lab of Medical Microbiology, University of Antwerp. After sequencing, raw reads were analyzed using in-house pipeline. In short, reads were assembled into contigs using shovill (https://github.com/tseemann/shovill). Quality and completeness were assessed using CheckM (completeness >94% required). Annotation was performed with Prokka (Seemann, 2014).

3.10 Comparative Genomic Analysis

Comparative genomic analysis of the selected genomes was performed by making use of Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (Olson et al., 2023). Comparative phylogenetic trees were constructed in BV-BRC using the "Bacterial Genome Tree" tool, which generates a phylogenetic tree based on using codon tree method. Comparative Systems Service of the BV-BRC was also utilized to compare protein families among the genomes included in the analysis.

3.11 Strains Identification and Average Nucleotide Identity Analysis

The 16S *rRNA gene* was extracted and used for classification using Bayesian lowest-common ancestor (BLCA) taxonomic classification method based on the NCBI taxonomy database. Average Nucleotide Identity (ANI) was used to calculate the orthologous average nucleotide identity of the genome. ANI values were calculated by using FASTANI. ANI is the average value based on the comparisons of all orthologous protein-encoding genes of the pairwise genomes. 'Variation Analysis Service' of the BV-BRC was employed to analyze the genetic variations of single nucleotide polymorphisms (SNPs) between the isolates *L. plantarum* 54B and 54C.

3.12 Prediction of Putative Biosynthetic Gene Clusters (BGCs) of Bioactive Compounds and Carbohydrate-Active Enzyme Analysis

To predict genes coding for many different types of biosynthetic pathways involved in production of secondary metabolites (SMs), antiSMASH 7.0 (Antibiotics and Secondary Metabolite Analysis Shell) was utilized (accessed on 10 August 2023) (Blin et al., 2023). More in-depth analyses were performed in antiSMASH for BGCs encoding non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), and the ribosomally synthesized and post-translationally modified peptides (RiPPs). The annotated genome FASTA file of the isolates were used as an input file and default antiSMASH features were assumed during the analysis. Genes encoding the riboflavin metabolic pathway were predicted through the BV-BRC's 'Comparative Systems' service. Carbohydrate-active enzymes (CAZymes) of the isolates were searched against the CAZy database (http://www.cazy.org/). The database mainly included glycoside hydrolase (GHs), glycosyltransferases (GTs), carbohydrate esterases (CEs), carbohydrate-binding enzymes (CBM), auxiliary active enzymes (AAs), and polysaccharide lyases (PLs) (Mao et al., 2021).

3.13 Prediction of Genes Involved in Safety

The genomes were assessed for safety using several tools recommended in the EFSA (EFSA, 2018). ABRIcate (https://github.com/tseemann/abricate) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/; accessed on 20 October 2021) (Zankari et al., 2012) were employed to identify the restistome in the genomes of the isolates. ABRIcate and VFDB (virulence factor database, http://www.mgc.ac.cn/VFs/main.htm, accessed on 20 October 2021) were also employed to predict putative virulence factors (B. Liu et al., 2019).

3.14 Whole Genome Sequences Data Accession Number

The sequence data for the genomes of *Lactiplantibacillus plantarum* isolates (54B, 54C, and 55A) will be deposited at the European nucleotide archive and the accession number will be acquired (Paper III).

3.15 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) 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 $p < 0.05$.

4. Results

In this work, samples were taken from a typical Ethiopian fermented yoghurt and a representative cheese obtained from different large scale commercial dairy farms in Addis Ababa, Ethiopia. Two typical traditionally fermented cottage cheese samples from Arba Minch district, Ethiopia were also taken. In addition, four spontaneously fermented cereal beverages samples (2 Naaqe and 2 Cheka batches) were obtained from Arba Minch district and Konso, Ethiopia, respectively. The traditionally fermented cottage cheese samples were produced in a similar traditional method at household level by heating a fermented (18 – 24 h) and defatted cow milk. The commercial cheese sample used in this work was a soft cheese type produced from pasteurized milk coagulated by adding a starter culture and rennet, whereas the yogurt sample was prepared commercially by fermenting pasteurized cultured milk. Preparation methods for the cereal beverage samples are described in detail in the materials and method section.

4.1 Isolation of Lactic Acid Bacteria

From the dairy samples, following anaerobic cultivation on MRS agar, 54 microbial isolates were obtained (Paper I): 43 identified putatively as LAB based on morphological characteristics (circular, smooth, and milky colony), and because they were gram-positive bacilli or cocci, catalase-negative and nonmotile. Of these 43 isolates, 27 were selected based on the degree of antibacterial activity displayed (16 showed poor activity, see Paper I Supplemental Table S2) and to cover diversity of the sample origins. The isolates were then subjected to a screening pipeline to select potential probiotic strains depicted in Paper I, Fig. 1. To isolate LAB from Naaqe and Cheka as model Ethiopian cereal drinks, 4 spontaneously fermented samples (2 Naaqe and 2 Cheka batches) were plated out on MRS medium and 10 to 15 pure colonies were then picked from each sample (Garcia et al., 2016b) and subjected to the screening process (Paper II). 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. From these 24 isolates, 14 (2 from Cheka and 12 from Naaqe) were selected based on the degree of the initial antibacterial activity displayed against the key indicator pathogens (Paper II, Supplementary Table 1). These 14 LAB isolates were then identified up to the species level by *16S rRNA* gene Sanger sequencing

(Paper II). A process consisting of phenotypic and genotypic methods depicted as a flow chart in Paper II, Figure 2 was followed to select potential LAB probiotic starter strains from the collected cereal beverages samples. The CFU/mL(g) of the presumed LAB was carried out on the dairy samples with the level of LAB has shown in Paper I (Table S1), with more CFUs confirmed as LAB from traditionally fermented products than industrially fermented products. The total colony count of presumptive LAB in Naaqe and Cheka samples was also shown in Paper II Table 1, demonstrating notable variation in LAB and total CFU/mL between batches.

4.2 Identification of Lactic Acid Bacteria to the Species Level

The selected LAB isolates from diary (27) and cereal beverages (14) sources were further identified up to the species level by 16S rRNA Sanger sequencing. *Limosilactobacillus fermentum* was the predominant species identified in both sources (70.4% from dairy vs. 50% from cereal beverages) (Paper I, Table 1; Paper II, Table 2). This species appeared to be abundant in the yogurt (91.7%) than the cheese (53.33%), and cereal (50%) samples. *Weissella confusa* and *Pediococcus pentosaceus* were identified from both sources. However, whilst *Lactiplantibacillus pentosus*, *Enterococcus lactis*, and *Lactiplantibacillus plantarum* appeared to be restricted to the dairy samples; *Weissella cibaria* was found only in the cereals source, particularly Naaqe. The query sequence showed that the pairwise similarity of all strains was > 98.9 % for the 16S *rRNA gene* sequence of the top hits.

4.3 Resistance to Simulated *In vitro* GI Conditions

For a probiotic to act in the GI tract and exert their beneficial effect on the host, the ingested LAB must withstand the acidic conditions in the stomach and resist bile acids in the small intestine. Therefore, the survival of selected LAB isolates was tested in simplified stomach- and bile-mimicking conditions using a starting absolute number of 1.5 10^8 CFU/mL. All the 27 dairy isolates showed resistance to 0.5% bile salt, with 15 LAB isolates having a viability of more than 80% after 4 h exposure (Paper I, Figure 2). On the other hand, all the 14 cereal LAB isolates also showed resistance to 0.5% bile salt after 4 h exposure, but only 5 isolates had viability of more than 80% (Paper II, Figure S1). As regards to pH exposure, whilst 26 of the 27 (Paper I, Figure 2) and 11 of the 14 (Paper II, Figure S1) LAB

isolates from the diary and cereal samples, respectively, exhibited resistance. Overall, the LAB isolates tested showed better tolerance capacity to 0.5% bile salt exposure than to low pH.

4.4 Antimicrobial Activity of Isolates against Foodborne Pathogens

In the initial antimicrobial activity, 12 (out of 23), 5 (out of 19) and 6 (out of 19) LAB isolates from cottage cheese, yoghurt and Naaqe samples respectively, displayed inhibition activities against all the pathogens tested to varied extent (Paper I, Table S2; Paper II, Table S1).

In the more detailed profiling of antimicrobial activity, six of the 11 dairy origin LAB isolates (*Lactiplantibacillus plantarum* 54B, 54C, 55A; *Lactiplantibacillus pentosus* 55B, *W. confusa* 93A, and *P. pentosaceus* 95E) and five of the nine cereal beverages origin LAB isolates (*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 *(Lactiplantibacillus plantarum* WCFS1 and *Lacticaseibacillus rhamnosus* GG) used as controls (Paper I, Table 2; Paper II, Table 3). In the radial diffusion assay, 8 dairy origin (6 cottage cheese origin) and 6 cereal origin LAB isolates, and the model probiotics inhibited the growth of all the indicator pathogens except *S. aureus* MI/1310/1938 (Paper I, Table 2; Paper II, Table 3).

Nine of the eleven dairy origin LAB isolates and five of the nine cereal beverages origin LAB isolates displayed inhibitory activity against *S. aureus* MI/1310/1938 in the spot overlay method, however, no CFS of the isolates tested (including model probiotics) was able to replicate the activity in the radial diffusion method (Paper I, Table 2; Paper II, Table 3). This prompted us to measure the time-course effect of the selected LAB isolates CFS on the growth of *S. aureus* MI/1310/1938 in a more fine-scale, longitudinal liquid culture growth assay. Stronger longitudinal effects of LAB isolates CFS on the growth of *S. aureus* MI/1310/1938 were observed for four dairy origin LAB isolates (*Lactiplantibacillus plantarum* 54B, 54C, 55A and *Lactiplantibacillus pentosus* 55B) and one Cheka derived isolate *P. pentosaceus* 74D compared to the model probiotics

(*Lactiplantibacillus plantarum* WCFS1 and *Lacticaseibacillus rhamnosus* GG) (Paper I, Figure 3A; Figure 1A). Cottage cheeses derived isolate *P. pentosaceus* 95E and Naaqe derived isolate *L. fermentum* 44B also displayed significant but weaker inhibitory activity on the growth of *S. aureus* MI/1310/1938 (Paper I, Figure 3A; Figure 1A). AUC analysis of *S. aureus* MI/1310/1938 growth curves also revealed that the above active five dairy origin, and 2 cereal origin LAB strains significantly inhibited growth ($p<0.05$) of *S. aureus* MI/1310/1938 compared to MRS medium control (Paper I, Figure 3B; Figure 1B).

To explore medium acidification as an anti-pathogenic mechanism of the LAB isolates, the CFS was neutralized to pH 7.4 and subsequent radial diffusion assay against the 5 indicator pathogens (used in the secondary detailed antimicrobial activity) and, longitudinal time-course analysis against *S. aureus* MI/1310/1938 were done. The assays showed that the antimicrobial activity of the CFS was seen to be pH-dependent, as the inhibition completely disappeared. Strong acidifiers with CFS pH < 4 also showed higher inhibition ($p<0.05$) against the pathogenic strains tested (Paper I, Table S3; Table 1). However, isolates that were able to acidify the medium to similar pH (Table 1) displayed different inhibitory capabilities as can be exemplified by isolates *L. fermentum* 73B, *W. confusa* 82D and *W. cibaria* 83E. Isolate *L. fermentum* 73B displayed activity against *L. monocytogenes* MB2022 in radial diffusion assay, whereas, neither *W. confusa* 82D nor *W. cibaria* 83E showed activity (Paper II, Table 3). Overall, five of the eleven dairy origin LAB strains (*Lactiplantibacillus plantarum* 54B, 54C, 55A, *Lactiplantibacillus pentosus* 55B and *P. pentosaceus* 95E) and, six cereal beverages origin LAB isolates *L. fermentum* 44B, 82C and 73B; *W. confusa* 44D, *W. cibaria* 83E (active against gram-negative pathogens), and *P. pentosaceus* 74D displayed superior antagonistic activity.

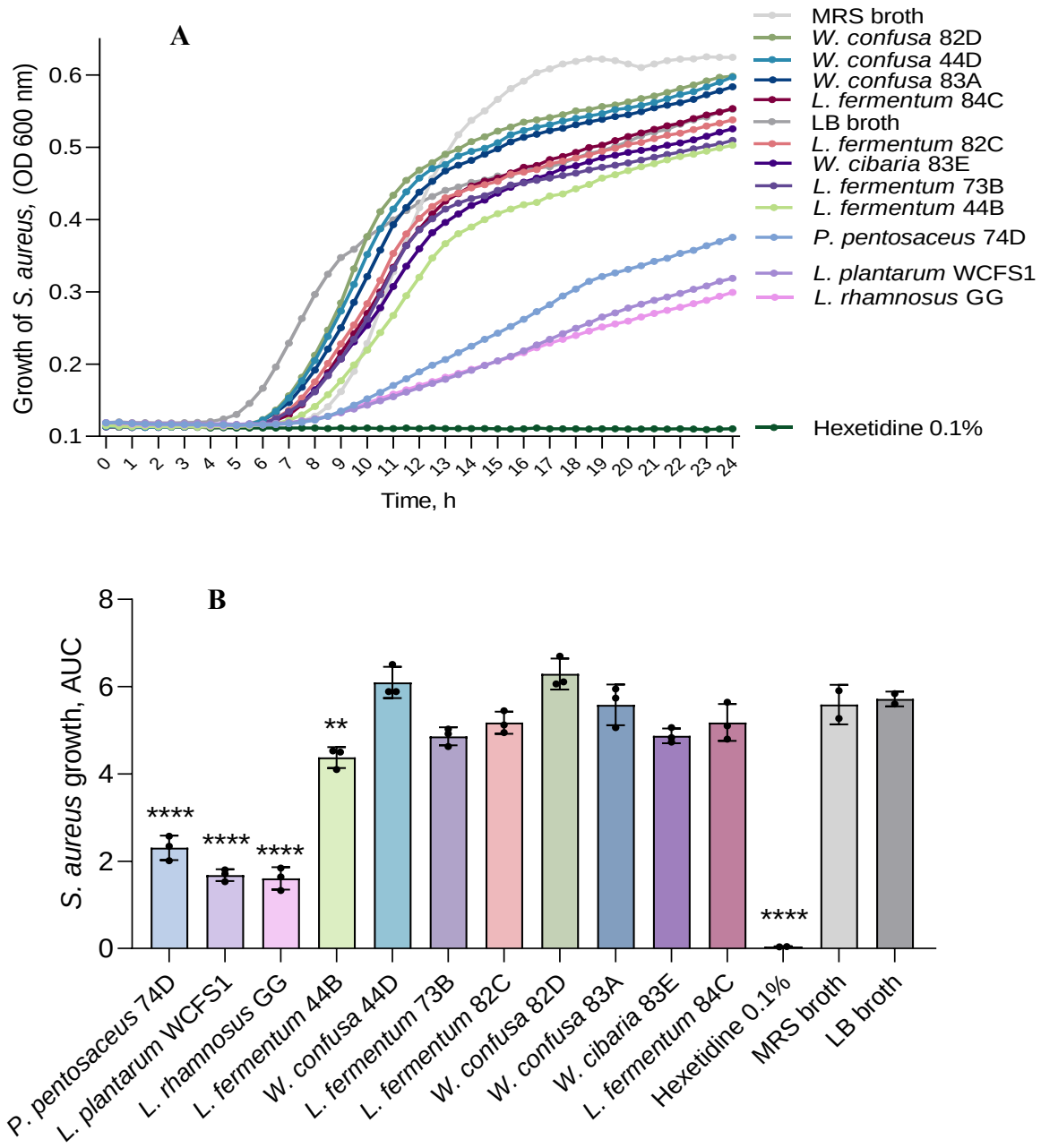

Figure 1: Effect of cereal beverages origin LAB strains cell-free supernatant (CFS) against growth of *S. aureus* in LB broth.

(A) Growth curves of *S. aureus* over the course of 24 h are depicted. Non-inoculated MRS & LB broth and 0.1% hexetidine were used as negative and positive control, respectively. (B) Area under the curve (AUC) of *S. aureus*. Bars depict means ± SD per condition (n = 3). ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 compared to *S. aureus* grown with MRS medium control.

Bacteriocins are significant class of antimicrobial peptides produced by LAB. In the present work, the antiSMASH system predicted four fundamental areas that produce bacteriocins and secondary metabolites in the genome of *Lactiplantibacillus plantarum* 55A (Paper III, Figure 3). The system also predicted three fundamental areas that produce bacteriocins and secondary metabolites in the genome of *Lactiplantibacillus plantarum* 54B and 54C (Paper III, Figure 3). This WGS analysis of the three selected isolates also shows that the genomes studied harbored bacteriocin and secondary metabolites coding genes, partially explaining and emphasizing the isolates additional antipathogenic mechanism (Paper III).

Table 1: pH of the corresponding cereal beverages origin LAB isolates cell-free culture supernatants.[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)*

4.5 Activation of NF-κB and IRF Pathways

Immunomodulation is one of the potential mechanisms of action of probiotics. In this study, the selected eleven dairy origin and nine cereal beverages origin LAB strains were further explored for their capacity to stimulate the NF-κB and IRF pathways as key for antipathogenic defense mechanisms in THP1-Dual™ human monocytes. 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. Nine out of the eleven dairy origin and three of the nine cereals origin LAB isolates tested significantly ($p < 0.05$) induced NF-κB in human monocytes (Paper I, Figure 4A; Paper II, Figure 3). Of note, the tested LAB strains showed variable strain-dependent immunostimulatory capacities. For example, while *L. fermentum* 25A displayed strong NF-κB activation, the other *L. fermentum* strain 55E had a lower activity (Paper I, Figure 4A).

Three of the tested dairy origin isolates, i.e., *Lactiplantibacillus plantarum* 54B and 55A and *P. pentosaceus* 95E also showed significant IRF induction, even higher than the model probiotic *Lactiplantibacillus plantarum* WCFS1 (Paper I, Figure 4B); while, none of the nine tested cereal beverages origin isolates displayed significant IRF induction (Paper II, Figure S2), though they tended to show an increase in IRF pathway activity. 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 (Paper II, Figure 3). Interestingly, no tested isolates of the *Weissella* genera displayed NF-κB stimulatory activity in the experimental setting of the present study. Of note, the NF-κB and IRF induction by all the tested strains was lower compared to the pathogenic *S. aureus* MI/1310/1938-MSSA, but was comparable to that of the model probiotics *Lactiplantibacillus plantarum* WCFS1 and *Lacticaseibacillus rhamnosus* GG, particularly LAB isolates from cereal beverages.

4.6 Safety Profile of Select LAB Isolates as Candidate Probiotic Strains

As per the joint WHO/FAO report, microbial strains to be consumed as probiotics should be safe in the host. Lack of systemic infections and gene transfer of specially antimicrobial resistance genes and virulence factors are listed as one of the potential adverse events associated with the use of probiotics (FAO/WHO, 2002). Hence, it is important to verify that LAB strains to be consumed as a probiotic lack virulence factors and transferable antimicrobial resistance markers on mobile genetic elements prior to considering them safe for human and animal consumption (FAO/WHO, 2002). In the present study, antibacterial susceptibility profile of the selected 11 dairy origin and 9 cereal beverages origin LAB isolates to 8 antibiotics recommended by EFSA (EFSA, 2012) (chloramphenicol, ampicillin, clindamycin, erythromycin, gentamycin, streptomycin, kanamycin and tetracycline) was examined (Paper I, Table 4; Table 2). All the 11 dairy and 9 cereal beverages origin LAB isolates tested showed sensitivity to ampicillin, erythromycin, clindamycin, tetracycline and chloramphenicol at the respective reference concentration (Paper I, Table S4). Dairy strain *Lactiplantibacillus plantarum* 55A was resistant to gentamycin. All the dairy origin LAB strains except *S. thermophilus* 15E and

Lactiplantibacillus pentosus 55B showed resistance to kanamycin. Cereal beverages origin strains *W. confusa* 44D, *W. confusa* 83A, and *W. cibaria* 83E were resistant to streptomycin. Five of the nine cereal beverages origin LAB isolates and model probiotic *Lacticaseibacillus rhamnosus* GG were also showed resistance to gentamycin. All the nine-cereal beverages origin LAB isolates and *Lacticaseibacillus rhamnosus* GG were found to be resistant to Kanamycin. LAB resistance to aminoglycosides such as kanamycin is considered to be natural (Monteagudo-Mera et al., 2012; Szutowska & Gwiazdowska, 2021), therefore, non-transmissible, so that these strains could still be considered safe and counted on for further development.

Table 2: Antibiotic susceptibility profile of potential probiotic strains from cereal beverages

(Amp; ampicillin, Gent; gentamycin, Kana; kanamycin, Strep; streptomycin, Eryth; erythromycin, Clind; clindamycin, TTC; tetracycline and CAF; chloramphenicol)

Isolate	Amp	Gent	Kana	Strep	Eryth	Clind	TTC	CAF
44B	S	R	R	S	S	S	S	S
44D	S	S	R	R	S	S	S	S
73B	S	R	R	S	S	S	S	S
74D	S	R	R	S	S	S	S	S
82C	S	R	R	S	S	S	S	S
82D	S	S	R	S	S	S	S	S
83A	S	S	R	R	S	S	S	S
83E	S	R	R	R	S	S	S	S
84C	S	S	R	S	S	S	S	S
LGG	S	R	R	S	S	S	S	S
WCFS1	S	S	S	S	S	S	S	S

Based on the overall probiotic properties recorded so far, three *Lactiplantibacillus plantarum* strains (54B, 54C, 55A) were selected for WGS analysis and their genomes were evaluated to cover all the safety concerns as recommended by EFSA Guidance for characterization of microorganisms used as food additives in animal feed and as producing organisms (EFSA, 2018). Here in our analysis, both ABRIcate and ResFinder revealed that the genomes of the strains under study harbored no antibiotic-resistant genes. ABRIcate and VFDB analyses also showed that the strains under study harbored no putative virulence factors (Paper III). These findings suggest the strains' potential safety for food and other applications.

4.7 *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 nine LAB strains in MRS broth under laboratory conditions (Paper II, Figure S3). 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, *Lacticaseibacillus rhamnosus* GG) to 11 h (*W. confusa* 44D). The total bacterial growth of the LAB isolates was also estimated by AUC analysis (Paper II, Figure 4A) as it correlates with both the growth rate and maximum possible population size (Ram et al., 2019; Sprouffske & Wagner, 2016). In the analysis, *P. pentosaceus* 74D exhibited the highest AUC values, differing significantly from the values of for the model probiotics, *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1. All the tested LAB isolates, except *W. confusa* 82D, displayed similar or higher AUC values with the control *Lactiplantibacillus plantarum* WCFS1, indicating they have a sufficient growth capability (Paper II, 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 *Lacticaseibacillus rhamnosus* GG (Paper II, Figure 4B). All the tested LAB isolates also exhibited similar or higher intrinsic growth rates with *Lactiplantibacillus plantarum* WCFS1. It should be noted that the strains' growth performance in this laboratory experiment (in MRS broth, at room temperature 22.8°C) differs from growth under natural spontaneous fermentation. Therefore, to assess the strains' performance in a real-world setting, in a next phase, mock community fermentations were set up. Based on a collective evaluation of the tested probiotic and growth properties of the cereal beverage origin LAB isolates (Paper II, Table 4), 6 LAB isolates were selected as candidate probiotic strains for fermentation experiments of Naaqe and Cheka. Specifically, four Naaqe derived starter cultures (*L. fermentum* 44B and *W. confusa* 44D; *L. fermentum* 82C and *W. cibaria* 83E) were selected for Naaqe fermentations and two Cheka derived starter cultures (*L. fermentum* 73B and *P. pentosaceus* 74D) for Cheka fermentation. The selected strains and

their combinations were used to ferment Naaqe or Cheka for 3 days and, the fermentative activities (capacity to lower the pH and their impact on the bacterial population dynamics) of Naaqe and Cheka at *in-situ* fermentation were evaluated. The set-up of the fermentation experiments is depicted in Paper II, Figure 1.

Naaqe fermentation with potential probiotic starters

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 ($p<0.05$). Both combinations of starter culture strains produced a lower pH than their individual strain inoculated fermentations after 24 h ($p<0.05$). 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 batch inoculated individually with *L. fermentum* 44B or *W. confusa* 44D or their combination, the inoculated fermentations N44D and N44B+44D displayed a statistically significant increase ($p<0.05$) in log CFU/mL compared to the spontaneous fermentation control (N4) after 24 h, which confirmed the significantly lowered pH for the groups (Paper II, Figure 5C). Whilst, for the batch that employed LAB isolates *L. fermentum* 82C & *W. cibaria* 83E as starters a significant difference was observed in inoculated fermentations with N83E and N82C+83E ($p < 0.01$) compared to the N8 control, at the 24 h time point (Paper II, Figure 5C).

Secondary fermentation

In the Naaqe secondary fermentation (Paper II, Figure 5B), all the tested conditions (spontaneous and inoculated fermentations) attained the pH 4.6 threshold during the first 14 h time point (regular start 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. For the *L. fermentum* 44B & *W. confusa* 44D batch of fermentation LAB log CFU/mL values increased above the threshold 6 log CFU/mL (Marinova et al., 2019) at 14 h time point of the fermentation

process in all fermentations (Paper II, Figure 5D). All the three inoculated fermentations exhibited a trend towards an increase in the log CFU/mL throughout the experiment, though it was not statistically significant compared to the N4 control. For the batch *L. fermentum* 82C and *W. cibaria* 83E, 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 (Paper II, Figure 5D).

Cheka fermentation with potential probiotic starters

Primary fermentation

The two Cheka derived LAB isolates used in Cheka lab-scale fermentation experiments were *L. fermentum* 73B and *P. pentosaceus* 74D individually or in combination. The inoculated fermentations N73B and N73B+74D resulted in significant decline ($p < 0.05$) in pH during the first 24 h compared to the control spontaneous fermentation N, with the highest decline for the combined starter culture (Paper II, Figure 6A). At 48 h time point, all fermentations except the spontaneous control N had a pH below 4.6. Plate counting for LAB revealed that 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 increase ($p < 0.05$) at 72 h time point compared to the control 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) was recorded in inoculated fermentation N73B+74D (Paper II, Figure 6C)..

Secondary fermentation

The pH was dropped below 4.6 in all fermentations, with no significant difference ($p > 0.05$) among the fermentations during the first 24 h, the time at which the fermenting Cheka can be served by the indigenous consumers (Paper II, Figure 6B). With respect to LAB count, there was a trend of increased LAB load for the all three inoculated groups throughout the fermentation process. However, there was no statistical difference compared to the spontaneous fermentation control N except for the inoculated fermentation N74D, which displayed a significant difference after 48 h of fermentation compared to the spontaneous fermentation 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. These mock fermentation experiments overall also showed that the tested autochthonous Naaqe LAB isolates could be promising starters (with respect to pH lowering and LAB count) for both Naaqe primary and secondary fermentation processes. Both Cheka and Naaqe could be good potential carriers for future documented probiotic strains.

4.8 General Features, Identification and Quality of the Three Genomes Sequenced

The chromosomal properties, quality control statistics and identification to the species level of the three *Lactiplantibacillus plantarum* isolates (54B, 54C, and 55A) sequenced in this study are summarized in Table 1 of Paper III. The raw reads assembly resulted in the generation of bacterial chromosomes each with a size similar to that previously reported for sequenced *Lactiplantibacillus plantarum* isolates (range of 3–3.6Mbp), which is higher compared to other LAB (Surve et al., 2022). The two isolates (54B and 54C) possessed a genome length of 3.39 and 3.37 Mbp, respectively, while, the isolate 55A possessed a genome length of 3.29 Mbp, a little less than the other two. The two isolates (54B and 54C) also possessed approximately the same coding sequence (CDS) (3259 and 3230, respectively) and the same GC content (44.3 %), although isolate 55A contained lower CDS (3108) and relatively higher GC content (44.5 %). However, the number of tRNA, rRNA and tmRNA genes was found to be the same among the isolates (Paper III, Table 1).

The 16S rRNA gene of these isolates was extracted and used for classification based on the NCBI taxonomy database and showed that all the three isolates belong to the *Lactiplantibacillus plantarum* species. The genome sequences with a completeness of higher than 94% passed the quality control requirement, as assessed using CheckM, and further analyzed. Here, the completeness percentage was found to be 99.07 for all the

genomes sequenced. It has also been reported that the median total length of *Lactiplantibacillus plantarum* genome assemblies is 3,253,870 bp, with a median protein (CDS) count of 2926 and median GC content percentage of 44.5 (Nikodinoska et al., 2022). Thus, the sequencing of our isolates produced a genome size (101% – 104%) comparable to the median genome size of the microbe. The calculated ANI value between *Lactiplantibacillus plantarum* 54B and 54C, the number and type of BGCs they harbored, CAZymes and SNPs analyzed showed that they are very closely related but different strains (Paper III, Table 2).

4.9 Comparative Genomic Analysis of the three genomes sequenced

To advance our understanding of the genome diversity and molecular evolution of the isolates, genomic characterization was conducted. Based on the comparative phylogenetic trees and protein families analyses, one of the closest evolutionary relative for all of our isolates was *Lactiplantibacillus plantarum* WCFS1, a single colony isolate from *Lactiplantibacillus plantarum* NCIMB8826, which was originally isolated from human saliva (Kleerebezem et al., 2003) (Paper III, Figure 1).

4.10 Prediction of Carbohydrate-Active Enzymes, Secondary Metabolites and Genes Involved in Safety

The analysis of CAZymes revealed that the *Lactiplantibacillus plantarum* 54B and 54C genomes each contained 91 genes in the five CAZymes gene families (Paper III, Table S1): 36 GT, 41 GH, 2 AA, 3 CBMs, and 9 carbohydrate CE genes. CAZymes analysis on the genome of *Lactiplantibacillus plantarum* 55A also revealed that it contained 90 genes in the five CAZymes gene families (Paper III, Table S1): 31 GT, 47 GH, 2 AA, 2 CBM, and 8 CE genes. We found that the most abundant CAZymes genes in the *Lactiplantibacillus plantarum* strains genomes belonged to the GH family, followed by the GT and CE families. The antiSMASH system predicted four fundamental areas that produce bacteriocins and secondary metabolites (cyclic-lactone autoinducer, RiPP-like, T3PKS and terpenes) in the genome of *L. plantarum* 55A (paper III, Figure 2), while, three fundamental areas (T3PKS, terpene, and Cyclic-lactone-autoinducer) were predicted in the genomes of *L. plantarum* 54B and 54C (Paper III). The genomes under study also harbored a complete

riboflavin operon. The *in silico* analysis also revealed that the genomes of strains under study harbored no antibiotic-resistant and virulence genes.

5. Discussion

Although a large variety of spontaneously fermented foods and beverages exists in Ethiopia, their microbial constituents are largely underexplored. 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 & Robe, 2020). Naaqe has – to the best of our knowledge – never been documented scientifically. Yet, they form an interesting source of potentially novel isolates for applications in fermented foods and beverages, and as probiotics. In this study, we present one of the first dedicated studies on Ethiopian LAB strains isolated from different dairy and cereal beverage sources, evaluating their selected efficacy and safety profile as potential probiotics and probiotic starters. The species *Limosilactobacillus fermentum* appeared to be abundant in the yogurt (91.7%) than the cheese (53.33%), and cereal (50%) samples. This could be due to deliberate inoculation of the yoghurt by the species, since the product is a commercial one. Probiotics intended for oral administration are required to survival in the GI tract. The resistance of our LAB isolates to bile salts and acidic pH was studied *in vitro* to predict bacterial survival after oral administration. The acidic and protease-rich conditions of the stomach are generally the strongest barrier for probiotics (Del Piano et al., 2011). The LAB isolates showed resistance to 4h exposure to 0.5% bile salt and 3h exposure to pH 3, with bile salt tolerance being universal, suggesting good candidates as gastrointestinal probiotics.

In the initial antimicrobial activity, more LAB isolates from cottage cheese (52.17 %) displayed inhibition activities against the all 4 tested indicator pathogens than from Naaqe (31.58 %) and yoghurt (26.33). This could possibly be due to the fact that cottage cheese samples contained *Lactiplantibacillus plantarum* and *pentosus* which were absent in other samples. In the second broader antimicrobial activity assay and longitudinal time-course effect of LAB CFS on the growth of *S. aureus* MI/1310/1938, the trend has also seen to continue that the cottage cheese derived LAB isolates performance was better than LAB isolates from other sources (Paper I, Table 2; Paper II, Table 3). The fact that CFS of all LAB isolates neutralized to pH 7.4 failed to show any antagonistic activity, suggesting that the antimicrobial activity of the isolates is probably mainly due to the production of acidic

substances and LAB isolates from our fermented foods and beverages have similar mechanism of antipathogenic activity. However, cereal beverages origin isolates that were able to acidify the medium to similar pH displayed different inhibitory capabilities, indicating that the effect is not merely pH related. This can partially be explained by fact that LAB isolates could be expressing antimicrobial substances such as bacteriocins and secondary metabolites as the WGS analysis revealed for the three selected and studied genomes (Paper III).

In the immune modulation 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. Our results show that nine of the eleven tested LAB isolates from Ethiopian fermented dairy products and three of the nine tested LAB isolates from cereal beverages were capable of activating the important immune transcription factor NF-κB to similar levels as the model probiotics strains, *Lactiplantibacillus plantarum* WCFS1 (Kleerebezem et al., 2003) and *Lacticaseibacillus rhamnosus* GG (Kankainen et al., 2009). Notably, although some specific strains of *W. cibaria* and *W. confusa* have been reported to possess immunomodulatory activity (Hong et al., 2016; Ladda et al., 2015; Park et al., 2020), the Naaqe derived LAB isolates belonging to the genus *Weissella* tested here were unable to stimulate NF-κB pathway. NF-κB activation by LAB could help stimulate antipathogenic immune responses and correct development and regulation of immune self-tolerance (Brown et al., 2008; Grinberg-Bleyer et al., 2018; T. Liu et al., 2017; Miraghazadeh & Cook, 2018). Furthermore, our selected LAB isolates from dairy products demonstrated activation of IRF, which is especially necessary for host antiviral defenses. For example, activation of IRF by *Lactobacillus acidophilus* (Weiss et al., 2012) or dsDNA of various LAB (Kawashima et al., 2013) has previously been linked to protective IFN-β response induction in host cells. All the selected LAB isolates from cereal beverages also 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, et al., 2023), and highlights the need to select appropriate probiotic and starter culture strains for each envisioned application.

Four of the five strains that demonstrated the most efficient NF-κB and IRF activation similar or higher than the model probiotic *Lactiplantibacillus plantarum* WCFS1, were from cottage cheese and belonged to the genus *Lactiplantibacillus* (*Lactiplantibacillus plantarum* 54B, 54C and 55A, and *Lactiplantibacillus pentosus* 55B), suggesting these strains are promising candidates to induce protective immune responses in the host. This might be specially promising if these strains are used in fermented foods. Due to this immunostimulatory and antagonistic activity recorded, and to identify to strain level, the above three cottage cheese derived *Lactiplantibacillus plantarum* strains were selected for WGS analysis. Of note, a recent systematic review and meta-analysis conducted on the effects of orally administered probiotics on respiratory tract infections in adults specifically showed that infection duration was more efficiently reduced when fermented dairy was used as the delivery matrix for probiotics (Coleman et al., 2022). We hypothesize that all the tested LAB isolates are safe from (especially the cereal beverages derived) an immunostimulatory perspective, because they only induced NF-κB pathway in a moderate way: similar or lower level compared to the established probiotics *Lactiplantibacillus plantarum* WCFS1 and *Lacticaseibacillus 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 considered in the starter culture experiments.

LAB resistance to aminoglycosides (streptomycin, gentamycin, kanamycin or neomycin) and glycopeptide (vancomycin), in most of the cases, is considered to be natural and, therefore, non-transmissible (Monteagudo-Mera et al., 2012; Reuben et al., 2020; Szutowska & Gwiazdowska, 2021). Hence, all tested LAB isolates are assumed to be safe regarding antibiotic resistance. One of the most important findings of the WGS analysis of the three *Lactiplantibacillus plantarum* strains and that strengthens our safety claim was lack of resistome and virulome from the strains studied and this is consistent with another

study that reported the non-pathogenicity of *Lactiplantibacillus plantarum* strain (Aziz et al., 2022).

In the present Naaqe and Cheka laboratory-scale 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 & Gänzle, 2022). The 6 log CFU/mL 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 starter cultures were shown to enhance the fermentation (pH lowering and increasing colony count on MRS agar) compared to spontaneous fermentation control. 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*. reported that the use of two *L. fermentum* strains combined as starter culture resulted in reduced fermenting performance (Adebo et al. 2018). 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 & 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 (Min et al., 2019; Ogunremi et al., 2017).

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 a key food safety threshold (FDA, 2022). The increase in LAB count 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 starter cultures (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 Naaqe and Cheka could be good potential carriers for future documented probiotic strains. Altogether, 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 antipathogenic activities, *in vitro* GI conditions tolerance, met antibiotic resistance recommendations and starter culture properties related to fermentation and growth. These two spontaneous Naaqe and Cheka fermentation processes could thus benefit from the use of a dedicated starter culture to minimize inter–batch differences and be enhanced with specific health promoting properties. We also demonstrated that the five selected (*L. plantarum* 54B, 54C & 55A; *L. pentosus* 55B and *P. pentosaceus* 95E) dairy derived LAB isolates have promising antimicrobial and immunostimulatory properties and are presumed to be safe with respect to antibiotic resistance (Paper I, Table 5) and could thus be considered as promising candidate probiotics for use in fermented foods or as food supplements.

The report of WGS analysis of the draft genome sequence of three *Lactiplantibacillus plantarum* strains (54B, 54C, and 55A), isolated from the Ethiopian traditional cottage cheese sample, focuses on insights into the potential probiotic properties of these strains based on the presence of putative beneficial genes and absence of genes of safety concern. Notably, the food-dwelling *Lactiplantibacillus plantarum* strains analyzed here are representative of isolates that are naturally consumed at very high levels (~10^8 – 10^9 per gram) in cottage cheese (Gizachew et al., 2023), and it is, hence, important to understand the genetic makeup of these strains and their potential impact on the host.

The CAZy data set anticipated five significant classes of CAZymes in the genome of the strains under study, i.e., GTs, GHs, CEs, CBMs and AAs. The existence of these CAZymes help our strains in their survival, competitiveness, and persistence within the host. Because these genes are involved in the metabolism and assimilation of complex non-digestible carbohydrates, they are crucial for the bacteria's adaptation to the GI environment and its interaction with the host (Mehra & Viswanathan, 2021). The presence of fundamental BGCs in the genomes of the isolates to produce bacteriocins, secondary metabolites, and riboflavin shows that our isolates have a potential for being used as a probiotic (Aziz et al., 2022), though the exact beneficial role of these predicted properties remains to be substantiated in follow-up more mechanistic studies.

Based on the genome analysis, the *Lactiplantibacillus plantarum* 54B and 54C are closely related but different strains. This finding meets the regulatory requirement set in the EFSA Guidance document (EFSA, 2018) and the EFSA's statement (EFSA, 2021a) for an unequivocal taxonomic identification at the strain level. Finally, one of the key findings of this study was lack of resistome and virulome from the strains studied and this is consistent with another study that reported the non-pathogenicity of *Lactiplantibacillus plantarum* strain (Aziz et al., 2022). Overall, the lack of resistome and virulome in addition to the previously studied and confirmed *in vitro* functional capabilities of the strains (Gizachew et al., 2023) opens an avenue for a wide spectrum of research with regard to human health-related applications of the bacteria.

6. Conclusions and Recommendations

The present study describes the identity and properties of LAB isolated from the selected Ethiopian fermented dairy products and traditional fermented cereal beverages. *Limosilactobacillus fermentum* was the predominant species identified in both dairy and cereal beverage samples. Five and six LAB isolates from traditional cottage cheese and cereal beverages, respectively showed *in vitro* broad-spectrum antimicrobial activities against nine strains of foodborne pathogens from five species and/or stimulated key immune pathways in human monocyte cells. All the selected LAB isolates complied with antibiotic resistance recommendations. These findings show that the selected five LAB strains are promising probiotic candidates. The six selected cereal beverages derived LAB strains demonstrated growth performance and tested *in situ* in 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 Naaqe and Cheka probiotic starter candidates. The traditional fermented cottage cheese was observed to be the best source of novel probiotic bacteria among the samples studied. To the best of our knowledge, no other study has yet described and documented Naaqe scientifically, which we showed to be dominated by LAB.

The WGS study also reported the genome sequences of three *Lactiplantibacillus plantarum* strains isolated from Ethiopian traditional cottage cheese. The genomic analysis of the strains revealed the presence of putative gene clusters coding for RiPP-like, cyclic lactone autoinducer, terpenes and T3PKS gene clusters, evidencing their role as probiotics. Moreover, none of the strains evaluated proved to have resistome or virulome, which suggests their potential safety for probiotic applications. Collectively, the *in vitro* probiotic properties demonstrated, results from laboratory-scale fermentation experiments and the genomic information from the genomes studied guarantee the selected LAB isolates as safe and effective candidate probiotics and probiotic starters once their health benefits are documented in a clinical trial as a next step. Furthermore, additional *in vitro* and *in vivo* studies on vitamin production, lipid lowering properties, and antihyperglycemic activities of the selected LAB isolates are warranted to broaden the isolates applicability to other ailments.

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Antibacterial and Immunostimulatory Activity of Potential Probiotic Lactic Acid Bacteria Isolated from Ethiopian Fermented Dairy Products

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Abstract: Lactic acid bacteria (LAB) form a group of bacteria to which most probiotics belong and are commonly found in fermented dairy products. Fermented foods and beverages are foods made through desired microbial growth and enzymatic conversions of food components. In this study, 43 LAB were isolated from Ethiopian traditional cottage cheese, cheese, and yogurt and evaluated for their functional and safety properties as candidate probiotics. Twenty-seven isolates, representative of each fermented food type, were selected and identified to the species level. *Limosilactobacillus fermentum* was found to be the predominant species in all samples studied (70.4%), while 11.1% of isolates were identified as *Lactiplantibacillus plantarum.* All 27 isolates tested showed resistance to 0.5% bile salt, while 26 strains were resistant to pH 3. The LAB isolates were also evaluated for antagonistic properties against key pathogens, with strain-specific features observed for their antimicrobial activity. Five strains from cottage cheese (*Lactiplantibacillus plantarum* 54B, 54C, and 55A, *Lactiplantibacillus pentosus* 55B, and *Pediococcus pentosaceus* 95E) showed inhibitory activity against indicator pathogens that are key causes of gastrointestinal infections in Ethiopia, i.e., *Escherichia coli*, *Salmonella enterica* subsp. *enterica* var. Typhimurium, *Staphylococcus aureus*, *Shigella flexneri,* and *Listeria monocytogenes*. Strain-specific immunomodulatory activity monitored as nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF) activation was documented for *Lactiplantibacillus plantarum* 54B, 55A and *P. pentosaceus* 95E. Antibiotic susceptibility testing confirmed that all LAB isolates were safe concerning their antibiotic resistance profiles. Five isolates (especially *Lactiplantibacillus plantarum* 54B, 54C, and 55A, *Lactiplantibacillus pentosus* 55B, and *P. pentosaceus* 95E) showed promising results in all assays and are novel probiotic candidates of interest for clinical trial follow-up.

Keywords: traditional fermented dairy products; lactic acid bacteria; antimicrobial activity; NF-κB; interferon regulatory factors; probiotics; Ethiopia

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1. Introduction

Food fermentation forms an essential element of human civilization, serving as a means to preserve and enhance shelf-life, flavor, texture, taste, nutritional value, and functional properties of food [1,2]. Fermented foods and beverages are foods made through desired microbial growth and enzymatic conversions of food components [3]. Africa is considered to be a continent with the richest variety of fermented foods [4]. Especially, Ethiopia is a country rich in cultural diversity, with each cultural group having its own variety of fermented food and beverages [5]. Fermented food items commonly consumed in Ethiopia include fermented dairy products (e.g., cottage cheese (Ayib), yogurt (Ergo)),

fermented plants (e.g., Enjerra, Kotcho), fermented beverages (e.g., Borde, Cheka), and fermented condiments (e.g., Siljo, Awaze, Datta) [5–7]. Most of these traditional fermented foods are produced on a fairly small-scale level, usually for household consumption and, at times, sold by local vendors from their homes [5,6]. However, their microbiology and potential health benefits are not yet widely studied. Moreover, there is a rapid rise in the number of industrially processed fermented products in urban areas, especially dairy products [8].

Most fermented dairy products harbor a microbial community characterized by a dominance of lactic acid bacteria (LAB) that can ferment carbohydrates to produce lactic acid. This group of bacteria includes several genera, such as the emended genus *Lactobacillus* [9], *Lactiplantibacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Weissella* [1,9]. Because of their long-time use in various food and feed preparations without pronounced adverse effects, many species of LAB (especially those belonging to *Lactobacillaceae*) have been granted a "generally recognized as safe" (GRAS) status by the US FDA [10] and "Qualified Presumption of Safety" (QPS) by the European Food Safety Authority (EFSA) [11]. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" [12]. According to this definition, the health benefit must be supported by at least one positive human clinical trial conducted according to generally accepted scientific standards [13]. Over the last decades, LAB use as probiotics has increased because specific LAB strains can confer a wide range of health benefits through mechanisms including enhancement of gut barrier function, competitive exclusion of pathogens, production of antimicrobial substances [14], and modulation of immune functioning [15]. These mechanisms of action can result in clinical benefits such as those documented for specific strains in specific clinical trials, especially for reducing the risk or symptoms of various gastrointestinal (GI) disorders such as irritable bowel syndrome, ulcerative colitis, and bacterial or viral infections [16].

Foodborne bacterial and viral infections are an important cause of morbidity and mortality and a significant barrier to the socio-economic development of all nations. In 2010, based on a World Health Organization (WHO) estimation, Africa was reported to have the highest burden of foodborne diseases per capita, with a median of 2455 foodborne Disability Adjusted Life Years (DALYs) per 100,000 inhabitants [17]. Of these, 26.6% were attributed to *Salmonella* spp., 11.2% to enteropathogenic *Escherichia coli*, 8.6% to enterotoxigenic *E. coli*, 0.08% to *Listeria monocytogenes*, 5.7% to *Campylobacter* spp., and 0.004% to Shiga-toxin producing *E. coli* [18,19]. In Ethiopia, diarrheal diseases have been reported to be the second most important contributor to the total burden of all disease types and the second leading cause of premature death [19]. Meta-analyses on the burden of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Shigella* species in Ethiopia provided a pooled prevalence of 32.5% [20] and 6.6% [21], respectively. Antibiotic resistance has also increased worldwide, posing an enormous clinical and public health burden, necessitating the search for alternatives to deal with the emerging risk of resistant pathogens [22]. Probiotics could form a valuable approach to decrease the burden of foodborne diseases in a cost-efficient manner because they can target different steps in the infection processes through multifactorial modes of action [23].

One mode of action of probiotics, and especially LAB, is their capacity to directly inhibit the growth of bacterial, fungal, and even viral pathogens via their capacity to produce the broad-acting antimicrobial molecule lactic acid and more species- or strain-specific antimicrobials such as bacteriocins [24]. Another key mode of action of probiotics is modulation of the mucosal immune system, whereby probiotics can activate the host cells to produce antimicrobial molecules or cellular activities [25,26]. This activity is generally mediated via microbe-associated molecular patterns (MAMPs) expressed by the probiotics, which can interact with various immune receptors on the host cells, such as Toll-like receptors [27]. This interaction leads to activation of nuclear transcription factors such as NF-κB that play a key signaling role in induction of immune responses following a variety of stimuli, such as with MAMPs [28,29]. While NF-κB induces a number of genes

mainly involved in pro-inflammatory cascades at sites of infection to kill pathogens, the intestinal epithelium generally does not trigger inflammatory responses against commensal bacteria but induces tolerance towards the commensal microorganisms. However, some of the signals induced by commensals and probiotics could result in better alertness and more rapid clearance of incoming pathogens. Another important signaling pathway in response to microbial stimuli is related to interferon (IFN) production, which is regulated by interferon-regulatory factors (IRFs) [30,31]. This pathway is necessary for efficient antiviral responses and is generally induced by viral MAMPs [30]. Spacova et al. [32] also found that several selected strains of probiotic lactobacilli can induce this pathway and boost antiviral responses. However, this mechanism has not been widely explored for LAB isolated from traditional fermented foods.

Most probiotic strains are selected without a detailed investigation of the underlying modes of action. Thus, there is a high demand for new strains with specific therapeutic modalities against infectious and other diseases [14]. In this study, we aimed to mine the microbial diversity of fermented foods and beverage items in Ethiopia for novel potential probiotic strains. Interesting isolates were characterized and evaluated for specific antimicrobial and immunological properties.

2. Materials and Method

2.1. Isolation and Characterization of LAB Strains

One yogurt and one cheese product from two different dairy industries in Addis Ababa, Ethiopia, and two traditional cottage cheeses from the Arba Minch district in Ethiopia were aseptically collected. The process of fermentation used to produce traditional cottage cheeses is spontaneous and uncontrolled. To isolate LAB, 10 mL (g) of each sample was suspended and homogenized in 90 mL phosphate-buffered saline (PBS) (pH 7−7.4). The homogenized sample (1st dilution) was used to prepare ten-fold serial dilutions, and 10 µL of the appropriate dilution (mostly the 3rd to 6th) was spread-plated on de Man, Rogosa, and Sharpe (MRS) agar (Hi-Media, Mumbai, India), a selective medium used to enrich LAB [33]. These plates were then incubated anaerobically (BD BBL™ GasPak™ jars) at 37 °C for 24 to 48 h. Plates with 30 to 300 colonies were selected, and colonies were counted. Five colonies were then randomly selected based on their differing appearance and purified through three successive streaking on MRS agar, in which aliquots of the selected isolates were stored at −80 °C in MRS broth containing 25% glycerol. Finally, the pure isolates were characterized presumptively as LAB by cell morphology, Gram staining, catalase test, and motility according to standard procedures [1], whereby Gram-positive, catalase-negative, and non-motile isolates were presumptively identified as LAB. The number of colony-forming units per milliliter/gram (CFU/mL(g)) was calculated as a function of the number of confirmed LAB colonies and the inoculated dilution using the following formula [34]:

$$\text{CFU/mL} = \text{total colonies present} \times \text{percent confirmed colonies} \times \text{dilution}. \quad (1)$$

2.2. Molecular Identification of LAB Isolates

The selected isolates presumptively identified as LAB (Gram-positive, catalase-negative, and non-motile) were further identified through 16S rRNA gene sequencing. For the detection of LAB strains using 16S rRNA gene sequences, the following primers were used: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTA CGACTT-3′). The bacterial genomic DNA was extracted using a 16S rRNA gene colony PCR technique. In brief, a colony was picked, mixed up, and vortexed in 10 µL molecular grade water. The cells were lysed through microwaving for 2 × 1.5 min at 800 W. The master mix was prepared in a clean room and contained 2.5 µL 10×VWR Buffer, 0.5 µL dNTPs (10 mM), 2.5 µL 27F (10 µM), 2.5 µL 1492R (10 µM), 0.2 µL Taq polymerase, and 6.8 µL molecular grade water to make a master mix of 15 µL final volume for each sample. This 15 µL master mix was then added to each tube containing a 10 µL DNA template. PCR was

performed under the following conditions: initial activation at 95 °C for 2 min; denaturation step cycles at 95 °C for 30 s; annealing step at 55 °C for 30 s; extension step at 72 °C for 1 min and 30 s; and final extension cycle at 72 °C for 5 min; for 30 cycles. A total of 5 µL of the PCR product was used to run 1% agarose gel electrophoresis on a gel with 5 µL GelRed dye. Successful samples (bright band at 1500 bps) were sent for sequencing (Sanger sequencing at Neuromics Support Facility VIB, Uantwerpen). The resulting sequences were analyzed using SeqTrace 0.9.0 software and submitted to a search for similarity in the EzBioCloud.net 16S-based ID. Bacterial species identification was assumed when the query sequence showed pairwise similarity of >98.7% for the 16S rRNA gene sequence, as previously described [35].

2.3. Resistance of LAB Isolates to Gastrointestinal Conditions In Vitro

LAB isolates from overnight (18 h) cultures (in MRS broth at 37 °C) were harvested (4000× *g*, 10 min, 4 °C), washed twice with PBS, and adjusted to 1.5×10^8 CFU/mL. To determine survival of the LAB strains in acidic conditions mimicking the GI tract, 100 µL of 1.5×10^8 CFU/mL of each LAB strain was added to 900 µL of sterile PBS adjusted to pH 3.0 (using 1M HCl) and then incubated under stirring (150 rpm) at 37 °C for 3 h, mimicking the time spent by food in the stomach. After incubation, 50 µL of each bacterial solution was collected, and 10-fold serial dilutions were prepared using sterile PBS and spread plated onto MRS agar in triplicates for enumeration of viable cells. To determine survival of the LAB strains in bile salt solution, 100 µL of 1.5×10^8 CFU/mL was added into 900 µL of sterile PBS (pH 8.0) supplemented with 0.5% (*w*/*v*) bile salts. The bacterial solution was then incubated at 37 °C under stirring (150 rpm) for 4 h, mimicking the time spent by food in the small intestine [36–38]. The percentage (%) of cell survival was calculated as shown below:

$$\% \text{ cell survival} = (\log \text{CFU}_T / \log \text{CFU}_C) \times 100$$

where CFU_C and CFU_T represent the total viable count of LAB isolates before and after, respectively, incubated under the simulated GI condition (low pH or bile salts). The starting absolute number was 1.5×10^8 CFU/mL, and the experiment's limit of detection was 10^3 CFU/mL.

2.4. Antagonistic Activity of LAB Isolates against Indicator Pathogens

Antagonistic activity of the LAB isolates against the foodborne pathogens was evaluated via spot overlay and radial diffusion assays with *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Listeria* spp., and *Staphylococcus* spp. as indicators of antimicrobial activity. In addition, a longitudinal liquid culture growth assay was performed using *S. aureus* MI/1310/1938.

2.4.1. Spot Overlay Assay

This assay was performed at both Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia, and the Laboratory of Applied Microbiology and Biotechnology (LAMB), University of Antwerp, Antwerp, Belgium. The indicator pathogenic bacteria used in AHRI were *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 19115), and *E. 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. At the LAMB, *L. monocytogenes* MB2022 isolated from Wijnendaele cheese, *S. enterica* subsp. *Enterica* var. Typhimurium NTCT 13347, *E. coli* O157:H7 BRMSID188 lacking pathogenicity *stx* genes (for biosafety reasons) isolated from bovine [39], *S. aureus* MI/1310/1938—methicillin-sensitive (MSSA), and *S. flexneri* LMG 10472 were used as indicator strains. For the spot overlay assay, 2 µL from each LAB isolate, cultivated overnight (20–24 h) in MRS broth under microaerobiosis, was spotted on the surface of agar media (AHRI: MRS agar for all pathogens tested; LAMB: Mueller Hinton agar (MHA) (1.5%) supplemented with 5 g/L glucose for *S. aureus* and LB agar (1.5%) supplemented with 5 g/L glucose for other pathogens) as described previously [38,40]. After spotting, the plates were 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). A volume of overnight growth of each indicator pathogen required to make a final concentration of 5 × 10^6 CFU/mL was mixed with 20 mL of soft agar (0.5% agar) and uniformly poured over the spot inoculated square plate (7 mL/round Petri dish). The plates were then incubated aerobically at 37 °C for 24 h. The antagonistic activity was recorded as the diameter (mm) of zone of inhibition. A total of 2 µL of hexetidine (0.1%) or chlorhexidine 0.2% were spotted as positive controls, while MRS broth was spotted as negative control. Experiments were run in triplicates and the average values were recorded.

2.4.2. Radial Diffusion Assay

This assay was performed as described elsewhere [40] using the same indicator pathogens, media, and final concentration of the pathogen inoculum as mentioned in the spot overlay assay performed at LAMB. LAB strains were first cultivated overnight (20–24 h) in MRS broth micro-aerobically (non-shaken) at 37 °C. The supernatants of these cultures (ca. 10^9 cfu/mL) were collected through centrifugation (at 2484× *g*, 15 min, 4 °C) and filter sterilized with a 0.22 µm filter, with or without pH adjustment to pH 7.4. An adequate volume of overnight growth of indicator pathogens was added to cooled agar (55 °C) and mixed well to produce a final concentration of 5 × 10^6 CFU/mL and poured onto a square plate. LAB cell-free culture supernatants (CFS) (45 µL), pH adjusted (7.4) or non-adjusted, were dispensed into 6 mm diameter wells drilled using a sterile glass Pasteur pipette. The plates were aerobically incubated at 37 °C for 24 h. After incubation, antagonistic activity was recorded as the diameter (mm) of growth inhibition zones around each well. In this assay, MRS broth (45 µL) and hexetidine (0.1%, 45 µL) were used as negative and positive controls, respectively. Experiments were run in triplicates and the average values were recorded.

2.4.3. Antimicrobial Activity Screening of Cell-Free Culture Supernatants in Liquid Culture Assays

This assay was also performed as described previously [40]. Briefly, 190 µL of a diluted overnight (20–24 h) culture of *S. aureus* MI/1310/1938 (ca. 10^5 cfu/mL) was added to the wells of a microplate supplemented with 10 µL CFS of LAB strains (obtained in the same way as in the radial diffusion assay) to obtain a total volume of 200 µL. A total of 10 µL 0.1% hexetidine and 10 µL MRS and LB medium were used as positive and negative control, respectively. Bacteria were grown, and optical density (OD) was measured at 600 nm (OD_{600}) each 30 min for 24 h using a Synergy HTX multi-mode reader. Each test was measured in triplicates, and the average $OD6_{00}$ was calculated.

2.5. Assessment of Immunostimulatory Activity of LAB Isolates

Immunostimulatory activity of the LAB strains was assessed by measuring activation of the NF-κB pathway and IRF pathway in human THP1-Dual™ reporter monocytes (InvivoGen, San Diego, CA, USA), as previously described [32]. The cells were maintained according to the manufacturer's instructions in growth medium containing RPMI 1640, 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, 100 µg/mL Normocin™ and Pen-Strep (100 U/mL;100 µg/mL). The bacterial cells were UV-inactivated in a biosafety level 2 cabinet for 90 min with vortexing after each 15 min before co-incubation with THP1-Dual™ cells. In the immunostimulation assay, UV-inactivated bacterial cells (final concentration 10^7 CFU/mL before inactivation) were added to THP1-Dual™ cells (final concentration 10^6 cells/mL) and co-incubated for 24 h at 37 °C and 5% CO_2. For assessment of the NF-κB pathway activation, secreted embryonic alkaline phosphatase (SEAP) activity in the THP1-Dual™ monocyte supernatant after addition of a p-nitrophenyl phosphate (pNPP) solution was measured (absorbance) at 405 nm according to the manufacturer's instructions. IRF pathway induction was measured by assessing the activity of a secreted luciferase (Lucia) by using QUANTI-Luc buffer, a luciferase detection reagent,

based on luminescence using a BioTek Synergy HTX multi-mode reader according to the manufacturer's instructions.

2.6. Antibacterial Susceptibility Testing of LAB Isolates

Antibacterial susceptibility of selected LAB strains was determined for ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, and tetracycline as per the recommendations of EFSA [41], using a broth microdilution test previously described [42], with minor modifications. In brief, 10 µL of each antibacterial solution was dispensed into each well of a 96-well microplate containing 180 µL of MRS broth. Subsequently, a 10 µL-culture aliquot of each test LAB isolate was added to each well (final viable cell count of approximately 7 log CFU/mL). The microplates were sealed with plastic bags to prevent bacterial dehydration. The experiments included controls, in particular bacteria alone, MRS broth, and known probiotic control strains, *Lacticaseibacillus rhamnosus* GG [43] and *Lactiplantibacillus plantarum* WCFS1 [44], and were performed in triplicates. The system was then aerobically and statically incubated at 37 °C for 48 h, and the plates were observed for any visible growth. The strains that showed visible growth were considered resistant.

2.7. 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 employed. Otherwise, the Kruskal–Wallis's test, followed by Dunn's multiple comparisons test, was used. Statistical comparisons were made when applicable using GraphPad Prism version 9.2.0. Differences were considered statistically significant at $p < 0.05$.

3. Results

In this study, samples were taken from a representative fermented yogurt and a typical cheese obtained from different large-scale commercial dairy farms in Addis Ababa, Ethiopia. In addition, two representative traditionally fermented cottage cheese samples from the Arba Minch district, Ethiopia, were taken. The traditionally fermented cottage cheese samples were prepared in a similar traditional method at the household level by heating a fermented (18–24 h) and defatted cow milk. The commercial cheese sample used in this study was a type of soft cheese produced from pasteurized milk coagulated by adding a starter culture and rennet, whereas the yogurt sample was produced commercially by fermenting pasteurized cultured milk. Following anaerobic cultivation on MRS agar, 54 microbial isolates were obtained; 43 were identified putatively as LAB based on morphological characteristics because they were Gram-positive bacilli or cocci, catalase-negative and non-motile. Of these 43 isolates, 27 were selected based on the degree of antibacterial activity displayed (16 showed poor activity, see Supplemental Table S2) and to cover diversity of the sample origins. The samples were then subjected to a screening pipeline to select potential probiotic strains, as depicted in Figure 1. The LAB load of the dairy samples in CFU/mL(g) is presented in Supplemental Table S1. The data revealed that more CFU were obtained than LAB from traditionally fermented products than industrially fermented products.

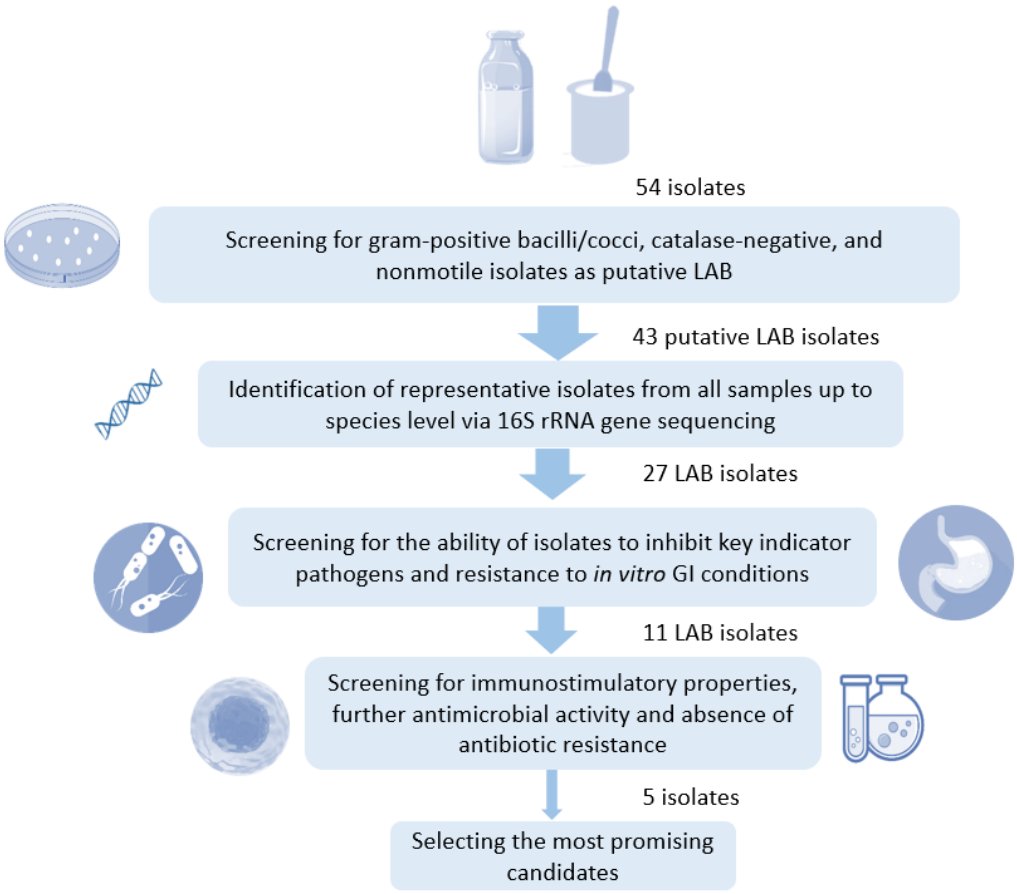

Figure 1. Strain selection flow chart used to select potential probiotic strains from Ethiopian yogurt and cheese products based on a combination of phenotypic and genotypic methods. Fifty-four isolates were obtained from Ethiopian yogurt and cheese-based products. Of these, 43 were classified as putative LAB. After initial antimicrobial analysis, 27 isolates were selected (taking into account origin) for 16S rRNA analysis. Of these 27 isolates, 11 were selected based on initial antimicrobial screening and species diversity for in-depth characterization of their probiotic potential. Of these, 5 isolates scored the best on all tests and were selected as the most promising probiotic candidates.

3.1. Selected LAB Isolates from Ethiopian Fermented Dairy Products Predominantly Belong to the Genus Limosilactobacillus

The 27 selected isolates were identified up to species level with 16S rRNA gene Sanger sequencing (Table 1). *Limosilactobacillus fermentum* showed to be the predominant species (19/27; 70.4%) identified, while 11.1% of isolates were identified as *Lactiplantibacillus plantarum*. Eleven of the twelve selected LAB isolates from the yogurt sample were identified as *Limosilactobacillus fermentum*. Seven of the fourteen selected isolates from spontaneously fermented cottage cheese samples were also identified as *Limosilactobacillus fermentum*, while the remaining isolates were identified as probably *Lactiplantibacillus plantarum* (three isolates), *Weissella confusa* (93A), *Pediococcus pentosaceus* (95E), *Lactiplantibacillus pentosus* (55B), and *Enterococcus lactis* (54A). The query sequence showed that the pairwise similarity of all strains was >99.7% for the 16S rRNA gene sequence of the top hits.

Table 1. *16S rRNA*-gene-based identification of LAB isolates from Ethiopian dairy products.

Source	Strain	Identified by 16S rRNA as:	Pairwise Similarity (%)	Selected (Yes)
Commercially fermented yogurt	12A	*Limosilactobacillus fermentum*	99.92	Yes
	12D	*Limosilactobacillus fermentum*	100	
	12E	*Limosilactobacillus fermentum*	100	
	13A	*Limosilactobacillus fermentum*	99.91	
	13C	*Limosilactobacillus fermentum*	100	
	13E	*Limosilactobacillus fermentum*	100	
	14C	*Limosilactobacillus fermentum*	100	
	14D	*Limosilactobacillus fermentum*	100	
	15B	*Limosilactobacillus fermentum*	100	
	15C	*Limosilactobacillus fermentum*	100	
	15D	*Limosilactobacillus fermentum*	100	
	15E	*Streptococcus thermophilus*	99.92	Yes
Commercially fermented cheese	25A	*Limosilactobacillus fermentum*	99.92	Yes
Spontaneously fermented cheese	54A	*Enterococcus lactis*	99.77	
	54B	*Lactiplantibacillusplantarum*	100	Yes
	54C	*Lactiplantibacillusplantarum*	100	Yes
	55A	*Lactiplantibacillus plantarum*	100	Yes
	55B	*Lactiplantibacillus pentosus*	100	Yes
	55E	*Limosilactobacillus fermentum*	100	Yes
	93A	*Weissella confusa*	100	Yes
	93B	*Limosilactobacillus fermentum*	99.92	
	93E	*Limosilactobacillus fermentum*	99.85	
	94C	*Limosilactobacillus fermentum*	99.85	
	94D	*Limosilactobacillus fermentum*	99.84	
	94E	*Limosilactobacillus fermentum*	99.84	Yes
	95A	*Limosilactobacillus fermentum*	99.85	
	95E	*Pediococcus pentosaceus*	100	Yes

3.2. Selected Isolates Show High In Vitro GI Resistance

In order to act as a probiotic in the GI tract and exert their beneficial effect on the host, the ingested LAB must survive the acidic conditions in the stomach and resist bile acids in the small intestine. Therefore, the survival of the selected LAB isolates was investigated in simplified stomach- and bile-mimicking conditions using a starting absolute number of 1.5×10^8 CFU/mL (Figure 2). All 27 LAB isolates tested showed resistance to 0.5% bile salt, with 15 LAB isolates having viability of more than 80% after 4 h exposure. Exposure to low pH (pH = 3) for 3 h, simulating the time spent by food in the stomach, revealed that 26 of the 27 LAB isolates exhibited resistance. Overall, the LAB isolates tested showed better tolerance capacity to 0.5% bile salt exposure than to low pH.

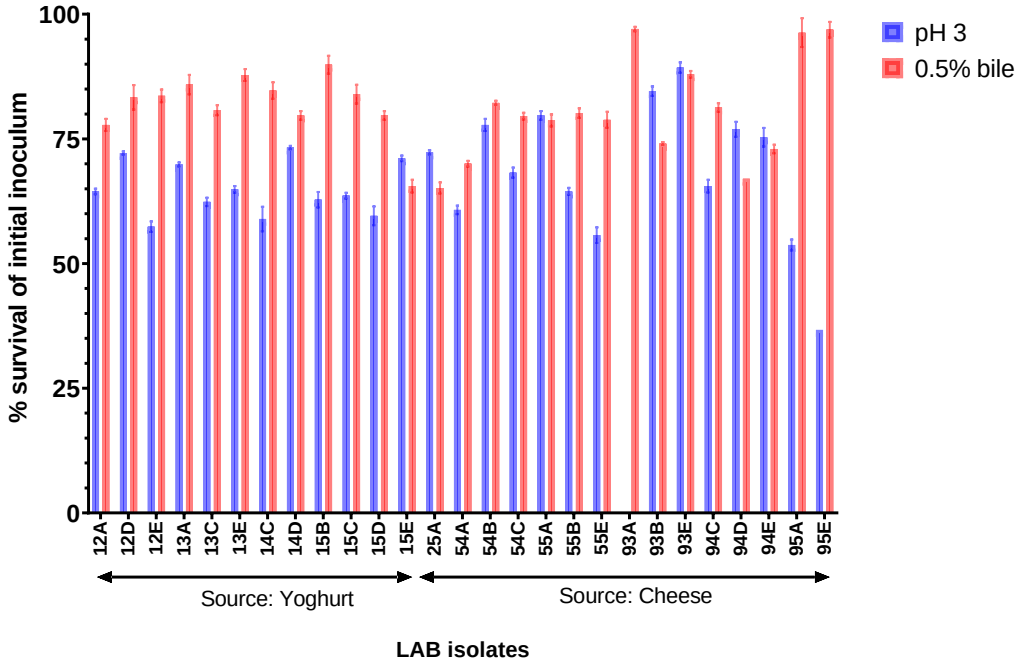

Figure 2. Percentage of survival (from initial inoculum) of the selected LAB isolates after exposure to acidic pH and bile salt solution. Isolates were exposed to pH 3.0 for 3 h at 37 °C and 0.5% (*w*/*v*) bile salt solution (pH 8.0) for 4 h at 37 °C under stirring (150 rpm). Data are expressed as mean ± SD per condition ($n = 3$).

3.3. LAB Isolates from Ethiopian Fermented Foods Inhibit Indicator Foodborne Pathogens

Antagonistic activity of the 27 selected isolates was evaluated against four indicator pathogens, i.e., *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and a clinical MRSA via spot overlay assay. The 27 isolates tested were found to inhibit these pathogens at varying degrees (Supplemental Table S2). A total of 18 LAB isolates (5 from yogurt, 12 from cottage cheese) displayed inhibition activities against all the pathogens tested to a varied extent (Supplemental Table S3). A total of 18 of the 27 isolates tested also showed a wider inhibition zone against indicator pathogens compared to the positive control (chlorhexidine 0.2%). However, eight of the LAB isolates (all from yogurt) failed to show activity against MRSA.

Subsequently, based on the spot assay results and species variety, 11 isolates were selected for more detailed characterization. First, more detailed profiling of their antimicrobial activity was performed against more pathogens using both radial diffusion and spot overlay assays (Table 2). In a radial diffusion assay, the activity of the secreted LAB metabolites was studied, while a spot assay investigated the activity of the live LAB. Five indicator pathogens, including pathogens that are among the key causes of GI infections in Ethiopia (*S. enterica* subsp. *enterica* Typhimurium, *E. coli* O157:H7 (-*stx* genes), *S. aureus* MI/1310/1938, MSSA, *S. flexneri* LMG 10472, and *L. monocytogenes* MB2022) were studied. Six (*L. plantarum* 54B, 54C, 55A, *Lactiplantibacillus pentosus* 55B, *W. confusa* 93A, and *P. pentosaceus* 95E) of the eleven LAB strains tested were effective against *E. coli* O157:H7, *S. enterica* subsp. *enterica* Typhimurium, and *S. flexneri* LMG 10472 using spot overlay assay, with similar levels of inhibition as the model probiotics (*Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1) used as controls. In the radial diffusion assay, CFS of all the LAB isolates displayed inhibitory activity against *E. coli* O157:H7 and *S. enterica* subsp. *enterica* Typhimurium. Eight LAB isolates tested showed inhibitory activity against *S. flexneri* LMG 10472 using radial diffusion assay (Table 2). All LAB isolates except for *S. thermophilus* 15E were effective against *L. monocytogenes* MB2022 using spot overlay assay with similar levels to that of the positive control and model probiotics, while nine of the isolates tested were also effective in the radial diffusion method.

Table 2. Antagonistic activity of the selected potential probiotic LAB strains by spot overlay and radial diffusion methods against 9 strains indicator food-borne 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 12A (1)	+++	+	+++	+++	++	−	++	−	+	−	+	++	−	+
Streptococcus thermophilus 15E (1)	++	+	++	−	++	−	++	−	−	−	−	−	−	−
L. fermentum 25A (2)	++	++	+++	++	++	−	++	−	+	−	++	++	−	++
Lactiplantibacillus plantarum 54B (5)	++	+	++	++	++	++	++	++	+++	++	+++	+++	−	+++
L. plantarum 54C (5)	++	+	++	++	++	++	++	++	++	+++	++	+++	−	++
L. plantarum 55A (5)	++	++	++	++	+++	++	++	++	+++	++	+++	+++	−	++
Lactiplantibacillus pentosus 55B (5)	+++	++	++	++	++	++	++	++	++	++	+	+++	−	+++
L. fermentum 55E (5)	++	++	++	+++	++	−	++	−	−	−	++	++	−	+
Weissella confusa 93A (9)	−	+	++	++	++	++	++	++	−	++	−	+++	−	++
L. fermentum 94E (9)	++	+++	++	++	+++	−	++	−	++	−	++	++	−	−
Pediococcus pentosaceus 95E (9)	+++	++	+++	++	++	++	++	++	++	+++	++	+++	−	++
Chlorhexidine 0.2%	++	+	+	+										
Lacticaseibacillus rhamnosus GG					+++	+++	++	++	++	+++	+++	+++	−	+++
L. plantarum WCFS1					+++	++	++	++	++	+++	+++	++	−	++
Hexetidine 0.1%					++	++	+	−	++	+	+++	+++	+++	+++

Chlorhexidine 0.2% and hexetidine 0.1% = Positive controls. Source: 1 = Commercially fermented, yogurt; 2 = Commercially fermented, cheese; 5 = Spontaneously fermented, cheese; 9 = Spontaneously fermented, cheese; 10 = Industrially fermented, probiotic yogurt. [1] Results of experiments of inhibition at AHRI: –no inhibition; low, + (9–14 mm); moderate, ++ (14–19 mm), and high inhibition, +++ (>19 mm). [2] Results of experiments of inhibition at LAMB: 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).

Nine of the eleven LAB isolates displayed activity against *S. aureus* MI/1310/1938 in the spot overlay method, but no CFS of the isolates tested (including model probiotics) could replicate the activity in the radial diffusion method (Table 2). Subsequently, the time-course effect of the 11 LAB isolates CFS on the growth of *S. aureus* MI/1310/1938 was measured in a more fine-scale, longitudinal liquid culture growth assay. Stronger longitudinal effects of LAB isolates CFS on the growth of *S. aureus* MI/1310/1938 were observed for four active LAB isolates (*Lactiplantibacillus plantarum* 54B, 54C, 55A, and *Lactiplantibacillus pentosus* 55B) compared to the model gastrointestinal probiotics (*Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1) (Figure 3A). *P. pentosaceus* 95E displayed significant inhibitory activity comparable to the model probiotics but lower than the four isolates (Figure 3A). The growth curve's area under the curve (AUC) estimates total bacterial growth as it correlates with both the growth rate and maximum density [45]. Consequently, AUC analysis of *S. aureus* MI/1310/1938 growth curves also revealed that five of the eleven LAB strains (*Lactiplantibacillus plantarum* 54B, 54C, 55A, *Lactiplantibacillus pentosus* 55B, and *P. pentosaceus* 95E) and model probiotics significantly inhibited growth ($p < 0.0001$) of *S. aureus* MI/1310/1938 compared to MRS medium control (Figure 3B). Although the differences in mean AUC of the LAB isolates 55E and 94E were statistically significant ($p < 0.05$) compared to that of the MRS medium control, these isolates were shown to be weak inhibitors, as they had overlapping growth curves with the medium (Figure 3A) and larger AUC values (Figure 3B). MRS broth (used as negative control) only induced a small delay in growth of the indicator pathogen.

To explore medium acidification as an antipathogenic mechanism of the LAB isolates, the CFS (Supplemental Table S3) was neutralized to pH 7.4, and subsequent radial diffusion assay against all indicator pathogens and longitudinal time-course analysis against *S. aureus* MI/1310/1938 were performed. The assays showed that antimicrobial activity of the CFS was pH-dependent, as the inhibition completely disappeared. Strong acidifiers (*Lactiplantibacillus plantarum* 54B, 54C, and 55A, *Lactiplantibacillus pentosus* 55B, *P. pentosaceus* 95E, *Lacticaseibacillus rhamnosus* GG, and *Lactiplantibacillus plantarum* WCFS1) with CFS pH < 4 also showed higher inhibition ($p < 0.05$) against the pathogenic strains tested.

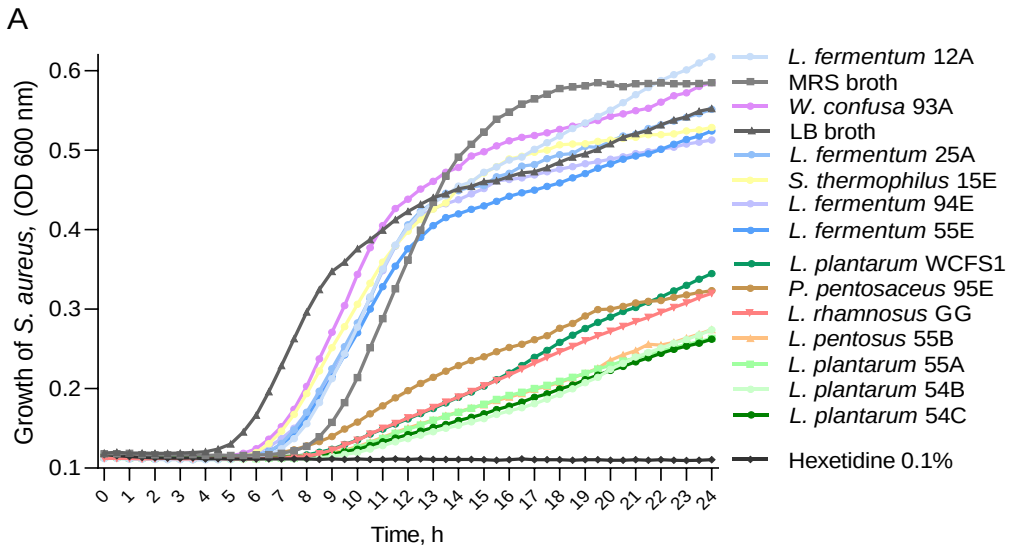

Figure 3. *Cont.*

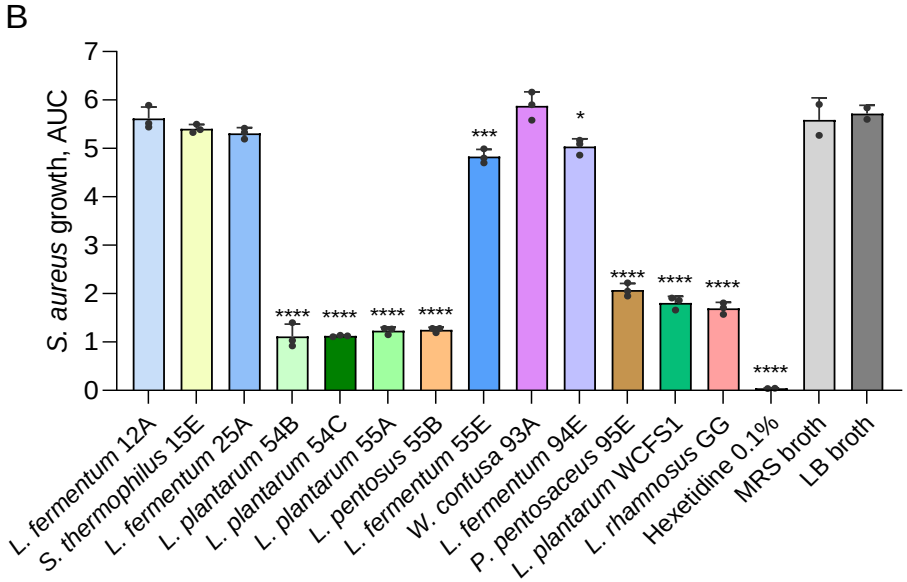

Figure 3. Effect of the LAB strains cell-free culture supernatant (CFS) against the growth of *S. aureus* MI/1310/1938 in LB broth: (**A**) Growth curves of *S. aureus* over the course of 24 h, non-inoculated MRS and LB broth and 0.1% hexetidine were used as negative and positive control, respectively. Curves of the most active four LAB strains (*L. plantarum* 54B, 54C, 55A, and *L. pentosus* 55B) are below the curves for the model probiotics (*L. rhamnosus* GG and *L. plantarum* WCFS1), indicating isolates were strong inhibitors. Curves for 95E and model probiotics are overlapping since 95E showed comparable inhibitory activity against the pathogen as model probiotics. (**B**) Area under the curve (AUC) of *S. aureus* growth curves. Bars depict AUC means ± SD per condition ($n = 3$). 55E and 94E have large AUC since they are weak inhibitors. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ compared to *S. aureus* grown with MRS broth control. *L. plantarum*, *Lactiplantibacillus plantarum*; *L. pentosus*, *Lactiplantibacillus pentosus*; *L. fermentum*, *Limosilactobacillus fermentum*; *P. pentosaceus*, *Pediococcus pentosaceus*; *S. thermophilus*, *Streptococcus thermophilus*; *W. confusa*, *Weissella confusa*.

3.4. Selected Ethiopian Dairy LAB Isolates Activate NF-κB and IRF Pathways in Human Monocytes

Immunomodulation is one of the potential mechanisms of action of probiotics. In this study, the eleven selected LAB strains were further explored for their capacity to stimulate the NF-κB and IRF pathways as key for antipathogenic defenses in human monocytes. Nine out of the eleven tested LAB isolates significantly ($p < 0.05$) induced NF-κB, while *S. thermophilus* 15E and *W. confusa* 93A did not (Figure 4A). Of note, the tested LAB strains demonstrated variable strain-dependent immunostimulatory capacities. For example, while *Limosilactobacillus fermentum* 25A showed strong NF-κB activation, the other *Limosilactobacillus fermentum* strain 55E had a lower activity (Figure 4A). Three of the tested isolates, i.e., *Lactiplantibacillus plantarum* 54B and 55A and *P. pentosaceus* 95E, also displayed significant IRF induction, even higher than the model probiotic *Lactiplantibacillus plantarum* WCFS1 (Figure 4B). Several tested isolates demonstrated a trend towards IRF induction, including *Limosilactobacillus fermentum* 25A and 94E, *Lactiplantibacillus plantarum* 54C, and *Lactiplantibacillus pentosus* 55B, but this was not statistically significant in the tested conditions.

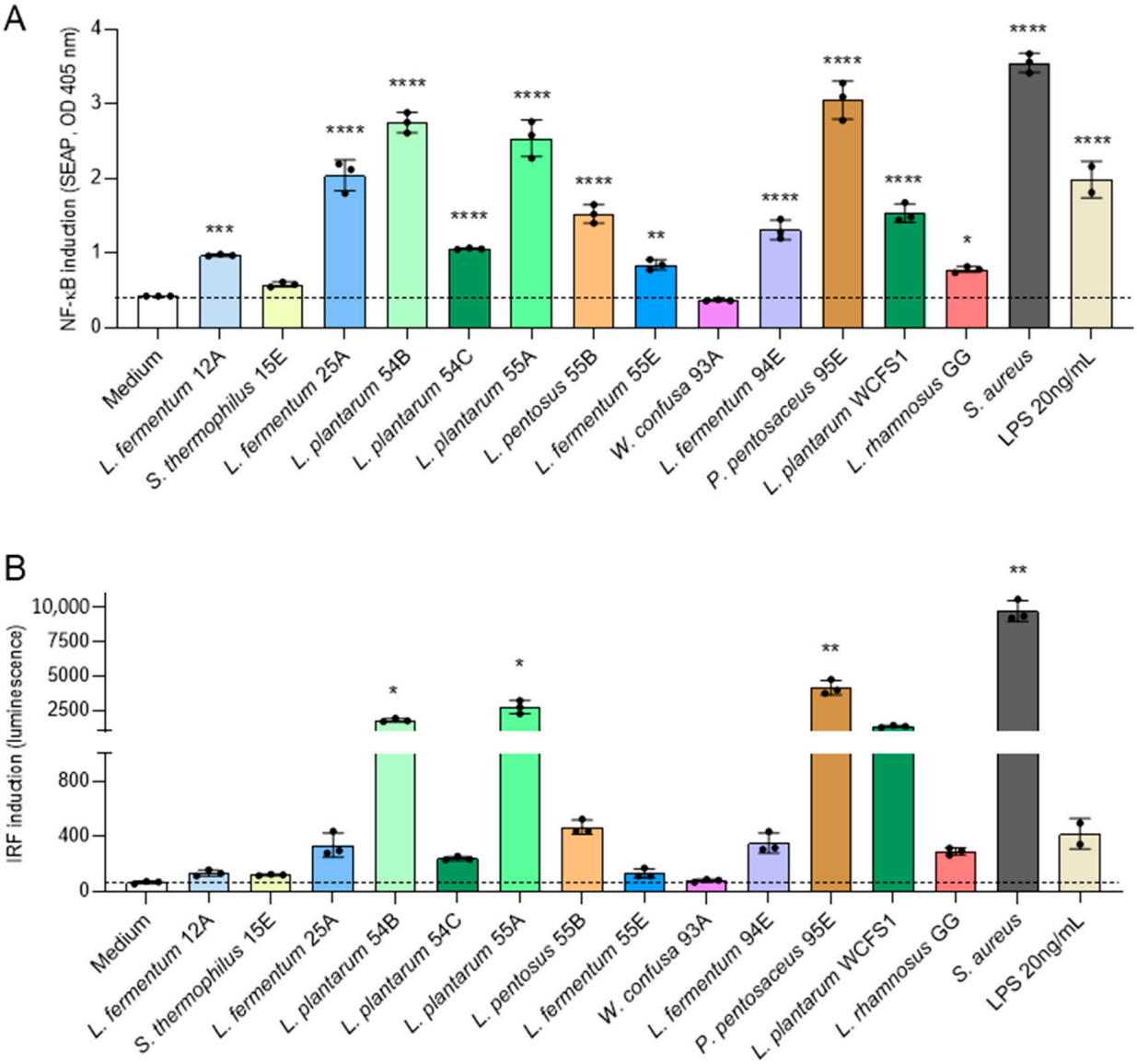

Figure 4. Immunostimulatory (**A**) NF-κB and (**B**) IRF activation by LAB strains in THP1-Dual human monocytes. *S. aureus* MI/1310/1938 was used as a Gram-positive pathogenic control strain. Bars depict mean ± SD per condition ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to medium control without bacteria (indicated by dotted line); *L. plantarum*, *Lactiplantibacillus plantarum*; *L. pentosus*, *Lactiplantibacillus pentosus*; *L. fermentum*, *Limosilactobacillus fermentum*; *P. pentosaceus*, *Pediococcus pentosaceus*; *S. thermophilus*, *Streptococcus thermophilus*; *W. confusa*, *Weissella confusa*.

3.5. Antibiotic Susceptibility Profile of Select LAB Isolates as Candidate Probiotic Strains

According to a 2002 report jointly released by the WHO and FAO of the United Nations, microbial strains to be used as probiotics should be safe in the host, with gene transfer of especially antibiotic resistance markers listed as one of the potential adverse events associated with probiotic use [46]. Therefore, it is important to verify that LAB strains to be consumed as a probiotic lack transferable antimicrobial resistance markers on mobile elements prior to considering them safe for human and animal consumption [46]. In the present study, antibacterial susceptibility profile of the 11 LAB isolates to 8 antibiotics recommended by EFSA [41] (ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, and tetracycline) was examined (Table 3). All 11 LAB isolates tested showed sensitivity to ampicillin, erythromycin, clindamycin, and chloram-

phenicol at the respective reference concentration (Supplemental Table S4). Strain *Lactiplantibacillus plantarum* 55A was resistant to gentamycin. All LAB strains except *S. thermophilus* 15E and *Lactiplantibacillus pentosus* 55B showed resistance to kanamycin. LAB resistance to aminoglycosides such as kanamycin is considered to be natural [47,48] and, therefore, non-transmissible, so these strains could still be considered for further development.

Table 3. Antibiotic susceptibility profile of potential probiotic strains from dairy products.

Isolate	Amp	Gent	Kana	Strep	Eryth	Clind	TTC	CAF
L. fermentum 12A	S	S	R	S	S	S	S	S
S. thermophilus 15E	S	S	S	S	S	S	S	S
L. fermentum 25A	S	S	R	S	S	S	S	S
L. plantarum 54B	S	S	R	n.r	S	S	S	S
L. plantarum 54C	S	S	R	n.r	S	S	S	S
L. plantarum 55A	S	R	R	n.r	S	S	S	S
L. pentosus 55B	S	S	S	n.r	S	S	S	S
L. fermentum 55E	S	S	R	S	S	S	S	S
W. confusa 93A	S	S	R	S	S	S	S	S
L. fermentum 94E	S	S	R	S	S	S	S	S
P. pentosaceus 95E	S	S	R	S	S	S	S	S
L. rhamnosus GG	S	R	R	S	S	S	S	S
L. plantarum WCFS1	S	S	S	S	S	S	S	S

(Amp: ampicillin; Gent: gentamycin; Kana: kanamycin; Strep: streptomycin; Eryth: erythromycin; Clind: clindamycin; TTC: tetracycline; CAF: chloramphenicol; n.r.: not required). *L. plantarum*, *Lactiplantibacillus plantarum*; *L. pentosus*, *Lactiplantibacillus pentosus*; *L. fermentum*, *Limosilactobacillus fermentum*; *P. pentosaceus*, *Pediococcus pentosaceus*; *S. thermophilus*, *Streptococcus thermophilus*; *W. confusa*, *Weissella confusa*.

4. Discussion

Although a large variety of spontaneously fermented foods exist in Ethiopia, their microbial constituents are largely underexplored. However, they form an interesting source of potentially novel isolates for applications in fermented foods and as probiotics. Isolating and characterizing LAB strains directly from widely consumed fermented foods is a particularly promising approach because of their applicability to fermented foods and their increased probability of being safe for oral consumption. In this work, we present one of the first dedicated studies on Ethiopian LAB strains isolated from different dairy sources, evaluating their efficacy and antibiotic susceptibility profile as potential probiotics.

A total of 27 LAB isolates were identified from Ethiopian yogurt and cheeses with *16S rRNA* gene Sanger sequencing: *Limosilactobacillus* (19), *Lactiplantibacillus* (4), *Streptococcus* (1), *Enterococcus* (1), *Pediococcus* (1), and *Weissella* (1) spp. The presence of these genera is consistent with Girma et al. [49], who isolated LAB (*Lactobacillus* (current reclassification as *Lactobacillus*, *Lacticaseibacillus*, and *Lactiplantibacillus* [9]), *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus* spp.) from other fermented Ethiopian traditional dairy products (Ergo, Ayib, and Metata Ayib). Colombo et al. [49] also reported that *Lactobacillus* (current reclassification as *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, and *Schleiferilactobacillus* [9]), *Pediococcus* spp., and *Weissella paramesenteroides* were the species isolated from a Brazilian dairy production environment. *Limosilactobacillus fermentum* was the predominant (70.4%) species in our samples, and this is in line with the report of Taye et al. [50] from cow milk and milk products from Ethiopia. However, the fact that *Limosilactobacillus* is isolated so often in fermented Ethiopian dairy products is of particular interest and extends the habitats of this genus because it seems to be different from other

geographical regions, where *Limosilactobacillus* is not often linked to dairy but rather to chicken and animal hosts [51].

Survival in the GI tract is a desirable property required for probiotics intended for oral administration. The tolerance of our LAB isolates to bile salts and acidic pH was studied in vitro to predict bacterial survival after oral administration. The acidic and protease-rich conditions of the stomach are generally the strongest barrier for probiotics [52]. The LAB isolates showed resistance to 4 h exposure to 0.5% bile salt and 3 h exposure to pH 3, with bile salt tolerance being universal, indicating good candidates as gastrointestinal probiotics.

Probiotics can exert their beneficial properties through many different mechanisms [46]. One of the potential probiotic properties of strains is antimicrobial activity. Ethiopia has a large burden of foodborne diseases [17,19], for which probiotics could be a good alternative to traditional antibiotic treatment. In the present study, three approaches were utilized to assess antipathogenic activity: radial diffusion, a spot overlay assay, and antimicrobial activity screening of CFS in liquid culture assays for the main causes of infection in Ethiopia (*E. coli*, *S. enterica* subsp. *enterica* var. Typhimurium, *S. aureus* (including MRSA) and *S. flexneri* and *L. monocytogenes*). In the radial diffusion assay, all CFS of the tested LAB isolates—containing secreted metabolites—displayed inhibition activities against *E. coli* O157:H7 and *S. enterica* subsp. *enterica* Typhimurium, while, because of the specificity of the spot overlay tests [53], only six of the eleven strains tested were effective against these two pathogens using a spot overlay assay that monitors more the live interaction between pathogen and potential probiotics (Table 2). To further explore and confirm the antimicrobial activity of the CFS of LAB isolates against *S. aureus* MI/1310/1938, we performed an inhibition experiment with the CFS and monitored the growth of *S. aureus* MI/1310/1938 for 24 h. A confirmed inhibitory activity was recorded for the five of eleven LAB isolates CFS (Figure 3). Three of the five isolates that showed antimicrobial activity against all nine strains of indicator pathogens using all methods and protocols tested belonged to the genus *Lactiplantibacillus plantarum*. Al-Madboly and Abdullah [54] detected and reported five potent antibacterial *Lactiplantibacillus plantarum* isolates recovered from fermented milk samples in Egypt, which were able to inhibit all the eight tested pathogenic bacterial strains from five pathogenic species (*S. aureus*, *E. faecalis*, *E. coli*, *S. flexneri*, and *S. enterica* subsp. enterica serovar *Typhi*). The LAB CFS neutralized to pH 7.4 failed to show any antagonistic activity, indicating that antimicrobial activity of the isolates is probably mainly due to the production of acidic substances. Similarly, Van den Broek et al. [40], Spacova et al. [55], and Reuben et al. [53] reported a loss of antagonistic activity by most LAB CFS tested against selected pathogens after neutralizing the supernatant, but these previous studies did not use native Ethiopian isolates.

In addition to their antipathogenic and adaptation properties, probiotics capable of modulating the immune system are highly promising for application against diseases related to immune imbalances, such as allergic diseases [56], inflammatory bowel disease [57], and even COVID-19 [58]. Our results demonstrate that nine of the eleven tested LAB isolates from Ethiopian fermented dairy products were capable of activating the key immune transcription factor NF-κB to similar levels as the model probiotic strain, *Lactiplantibacillus plantarum* WCFS1 [44]. The latter strain was recently successfully implemented as part of a throat spray in COVID-19 patients [58]. NF-κB activation by LAB could help stimulate antipathogenic immune responses and correct the development and regulation of immune self-tolerance [59–62]. Furthermore, our selected LAB isolates demonstrated activation of IRF. IRF is especially necessary for host antiviral defenses. For example, activation of IRF by *Lactobacillus acidophilus* [63] or dsDNA of various LAB [31] has previously been linked to protective IFN-β response induction in host cells. Importantly, we observed that the immunostimulatory activity of LAB was strain-specific. This supports previous results on LAB that immunostimulatory activity is strain-specific [32] and highlights the need to select appropriate probiotic strains for each envisioned application. Three of the eleven tested strains belonging to *Lactiplantibacillus plantarum* (54B, 54C, and 55A), *Lactiplantibacillus pentosus* 55B, and *P. pentosaceus* 95E demonstrated the most efficient NF-κB and IRF activation

similar or higher than the model probiotic *Lactiplantibacillus plantarum* WCFS1, suggesting these strains are promising candidates to induce protective immune responses in the host. This might be especially promising if these strains are used in fermented foods. Of note, a recent systematic review and meta-analysis focusing on the effects of orally administered probiotics on respiratory tract infections in adults specifically demonstrated that infection duration was more efficiently reduced when fermented dairy was used as the delivery matrix for probiotics [64].

To assess the prospective application of the selected LAB strains as probiotics or in food/feed, we next considered the recommendations by EFSA [41] regarding antibiotic resistance. LAB can serve as a reservoir for antibiotic-resistant genes and transfer them to other microorganisms, including pathogens [65]. A probiotic candidate should be verified for lack of acquired transferrable resistances. Therefore, susceptibility to the recommended antibiotics should be assessed for all potential probiotic strains [41]. LAB resistance to aminoglycosides (gentamycin, kanamycin, streptomycin, or neomycin) and glycopeptide (vancomycin), in most cases, is considered to be natural and, therefore, non-transmissible [47,48,53]. Hence, all tested LAB isolates are presumed to be safe regarding antibiotic resistance. Although LAB strain *P. pentosaceus* 95E had a lower survival rate at low pH, it is one of the best performers in antagonistic activity and immunostimulatory assays. As there is no clear cut-off value for in vitro GI conditions resistance and proof of benefit can be established in further in vivo and human studies, it can be taken as a promising probiotic candidate. Overall, we demonstrated that five (*Lactiplantibacillus plantarum* 54B, 54C, and 55A, *Lactiplantibacillus pentosus* 55B, and *P. pentosaceus* 95E) select LAB isolates have promising antimicrobial and immunostimulatory properties and are presumed to be safe with respect to antibiotic resistance (Table 4) and could, thus, be considered as promising candidates for use in fermented foods or as food supplements.

Table 4. Summary of results of probiotic properties of LAB strains to select as candidate probiotics.

Property Tested		Good Candidate LAB Strains						Poor candidate LAB Strains				
		***L. plantarum* 54B**	***L. plantarum* 54C**	***L. plantarum* 55A**	***L. pentosus* 55B**	***P. pentosaceus* 95E**	***W. confusa* 93A**	***L. fermentum* 12A**	***S. thermophilus* 15E**	***L. fermentum* 25A**	***L. fermentum* 55E**	***L. fermentum* 94E**
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		√	√	√	√	√	–	√	–	√	√	√
IRF induction		√	ns	ns	ns	√	–	–	–	ns	–	ns
AST		√	√	√	√	√	√	√	√	√	√	√

√ = robust/significant/safe; AST = antibiotic susceptibility test; – = no activity.

5. Conclusions

In this study, five LAB isolates from traditional cottage cheese showed in vitro broad-spectrum antimicrobial activities against nine strains of foodborne pathogens from five species and stimulated key immune pathways in human cells. All five LAB isolates complied with antibiotic resistance recommendations. These findings indicate that the selected LAB strains are promising probiotic candidates for use in fermented foods and food supplements and highlight the potential of traditional fermented dairy products as a source of novel probiotic bacteria. They can be considered probiotic strains once their health benefits are documented in a clinical trial as a next step.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030258/s1, Table S1: LAB load of dairy samples; Table S2: Antagonistic activity of the selected 43 potential probiotic LAB strains by spot overlay method against; *L. monocytogenes* (ATCC 19115), *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), methicillin resistant *S. aureus* (MRSA); Table S3: pH of the corresponding LAB isolates cell-free culture supernatants; Table S4: Concentration of antibiotics used for determination of antibacterial susceptibility test.

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Data Availability Statement: All pertinent data are included in the article. Other data can be obtained from the corresponding authors on reasonable request.

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Characterization of potential probiotic starter cultures of lactic acid bacteria isolated from Ethiopian fermented cereal beverages, Naaqe and Cheka

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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

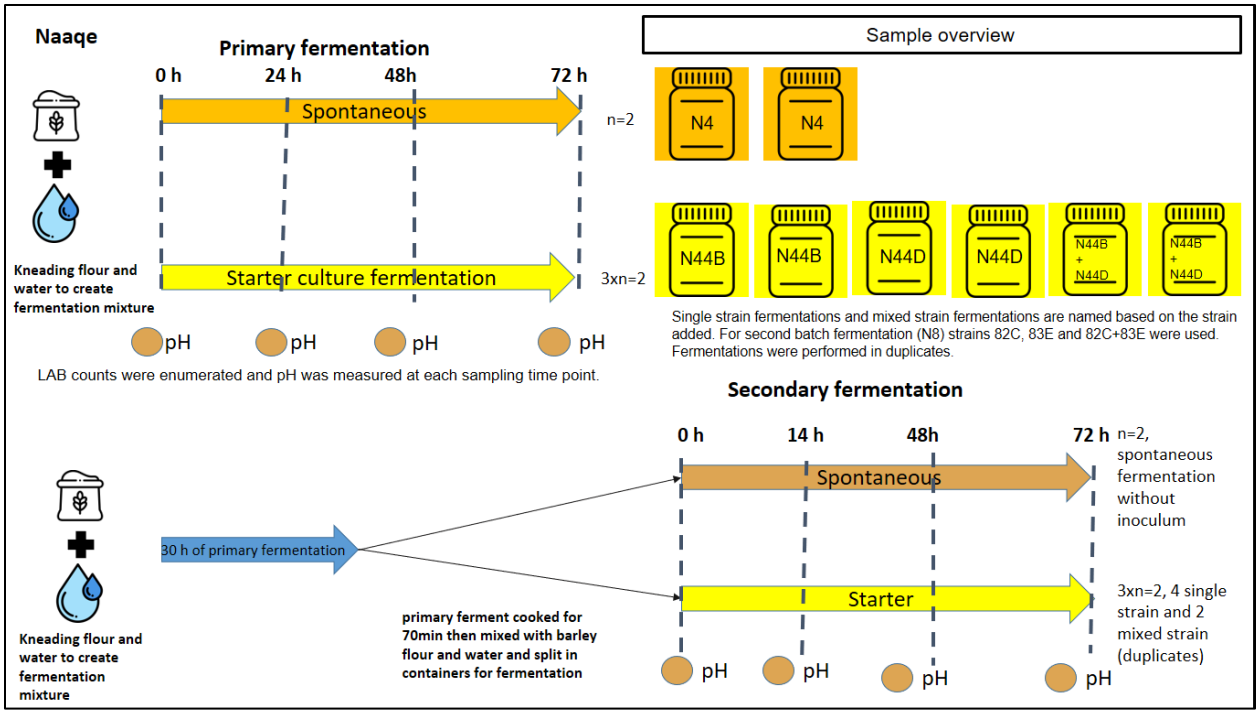
At time point 0 h (baseline), grain (maize:sorghum; 3:1) flour was kneaded thoroughly with water (flour: water; 1.00:0.65) and a portion was sampled to measure the pH and 10 mL sample was taken for plating out. Immediately after sampling, the mix was divided into 8 parts for 8 different

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).

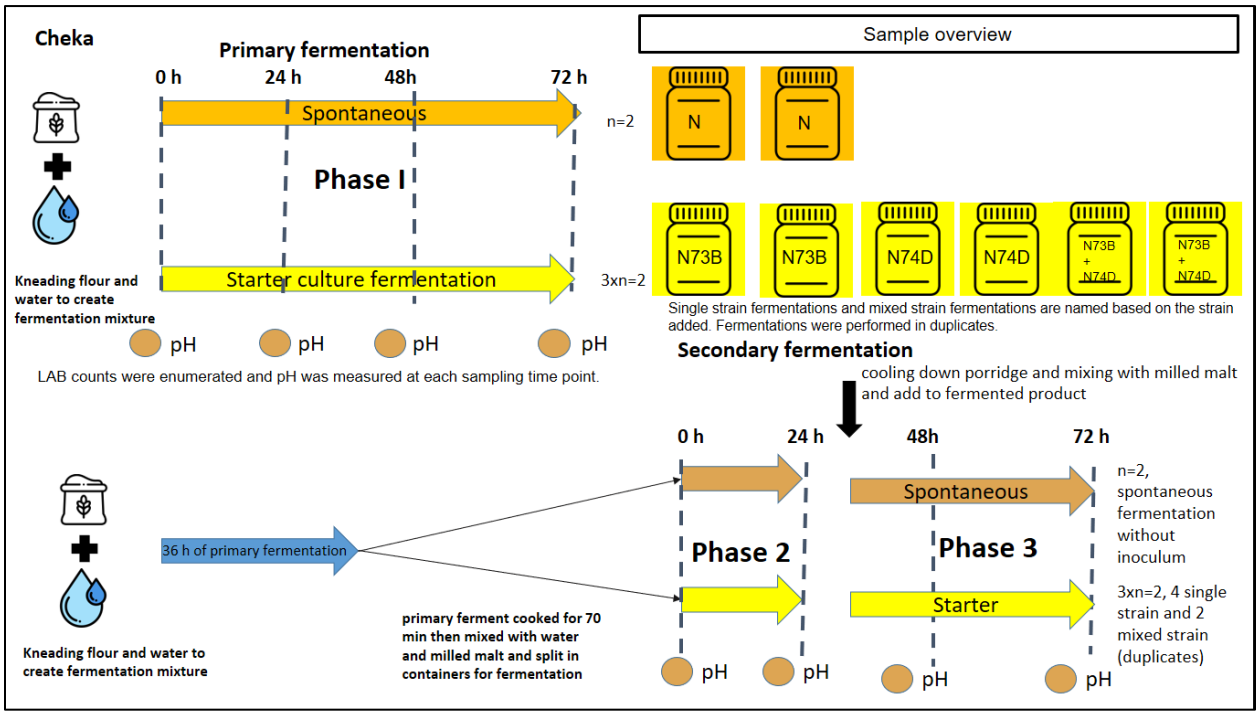
Secondary Fermentation of Cheka

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

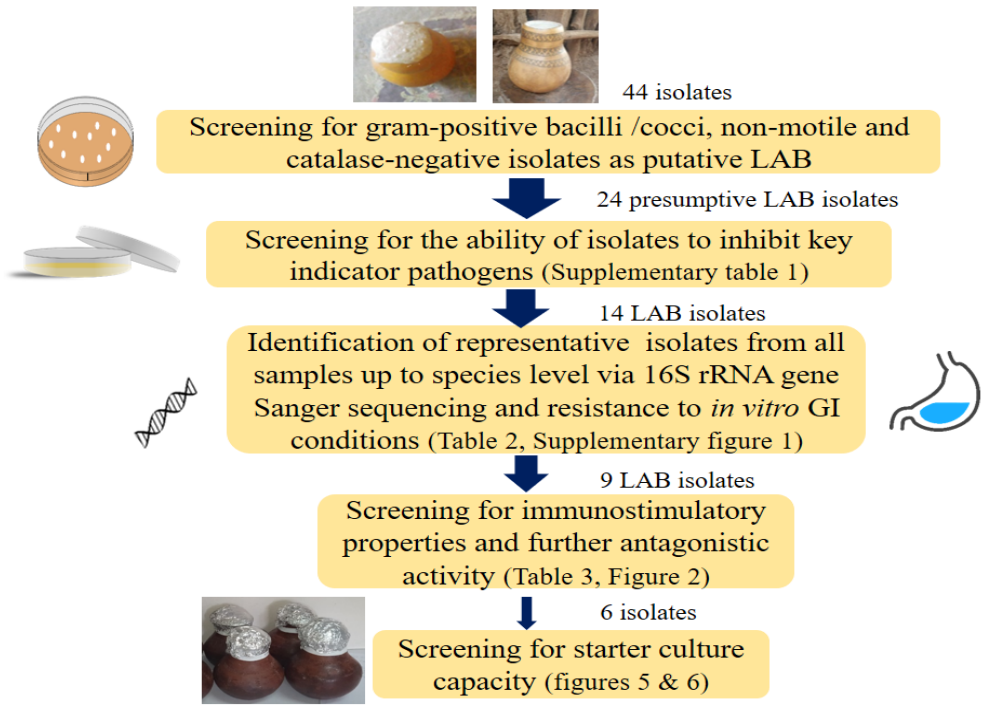


Figure 2. Flow chart used to select potential LAB probiotic starters from Ethiopian cereal beverages, Naaqe and Cheka based on a combination of phenotypic and genotypic methods.

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.

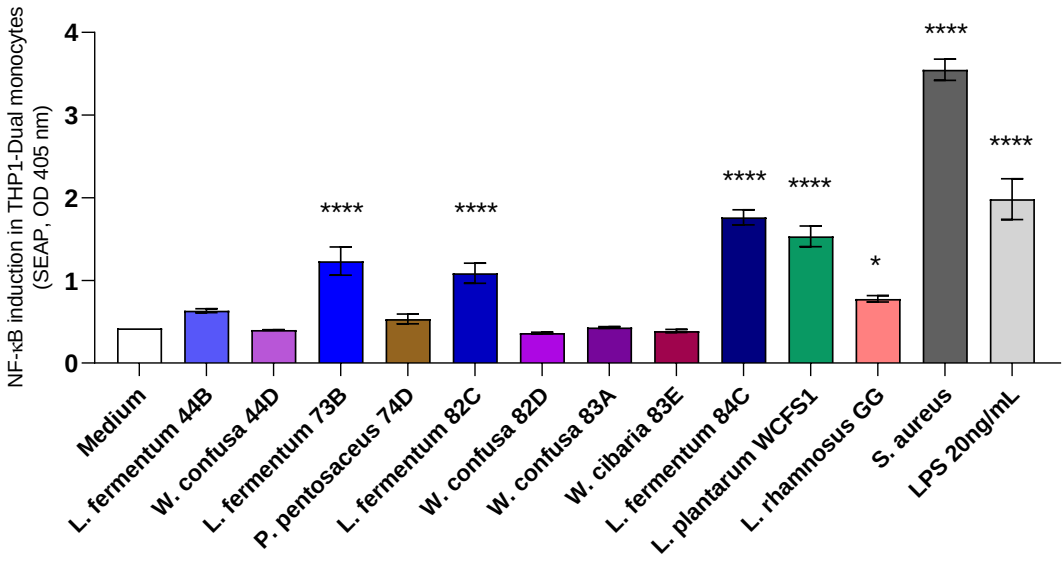


Figure 3: NF-κB pathway activation by LAB strains isolated from Naaqe and Cheka in THP1-Dual human monocytes. Bars depict mean values ± standard deviation per condition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to medium control.

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.

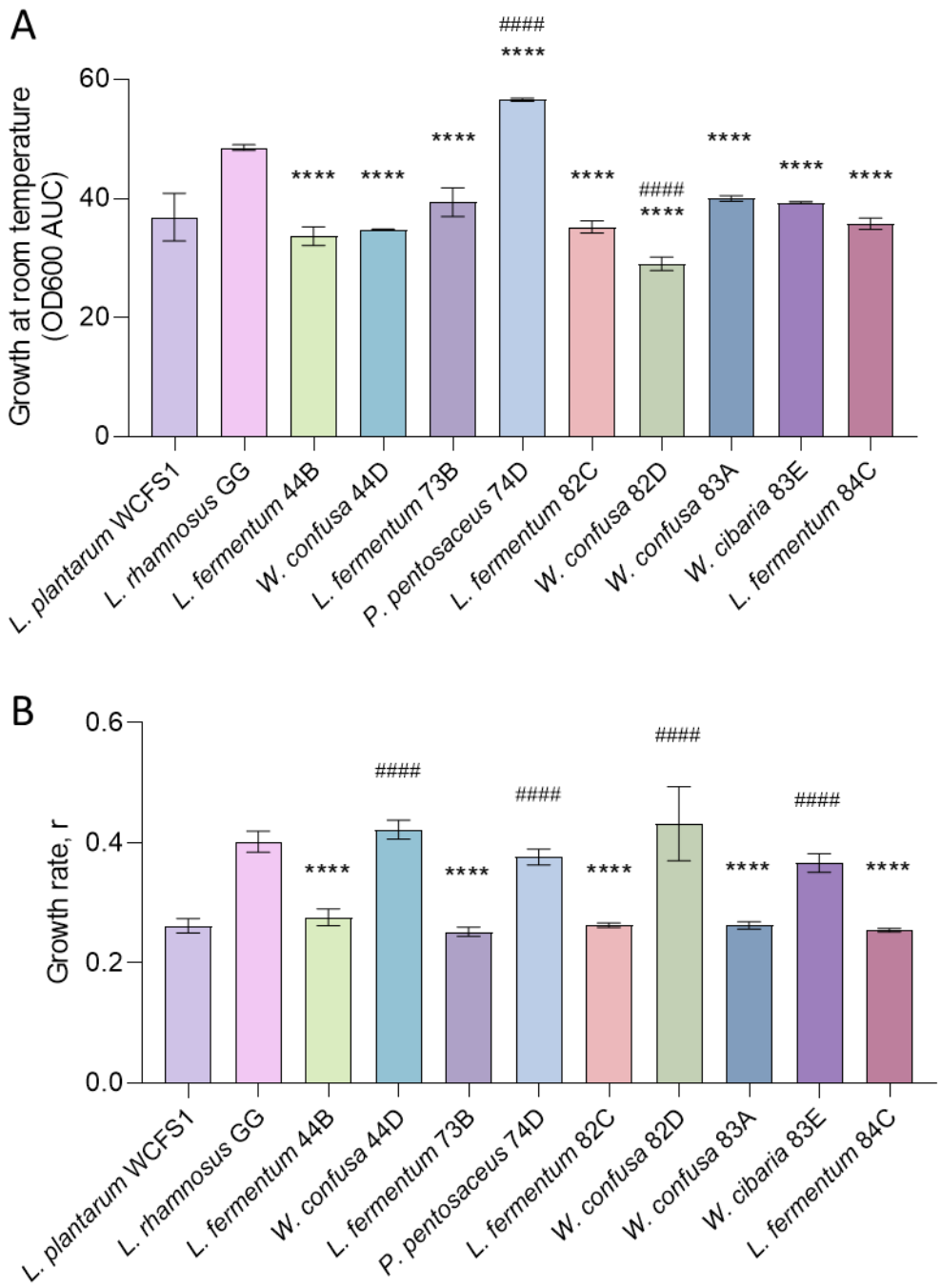


Figure 4: The growth (absorbance at 600 nm/h, OD_{600}) area under the curve (AUC) (A) and intrinsic growth rate, r (B) of the LAB strains in MRS broth at room temperature (average 22.8 °C). Bars depict mean values ± standard deviation per condition (three replicates). ****p<0.0001 compared to *L. rhamnosus*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

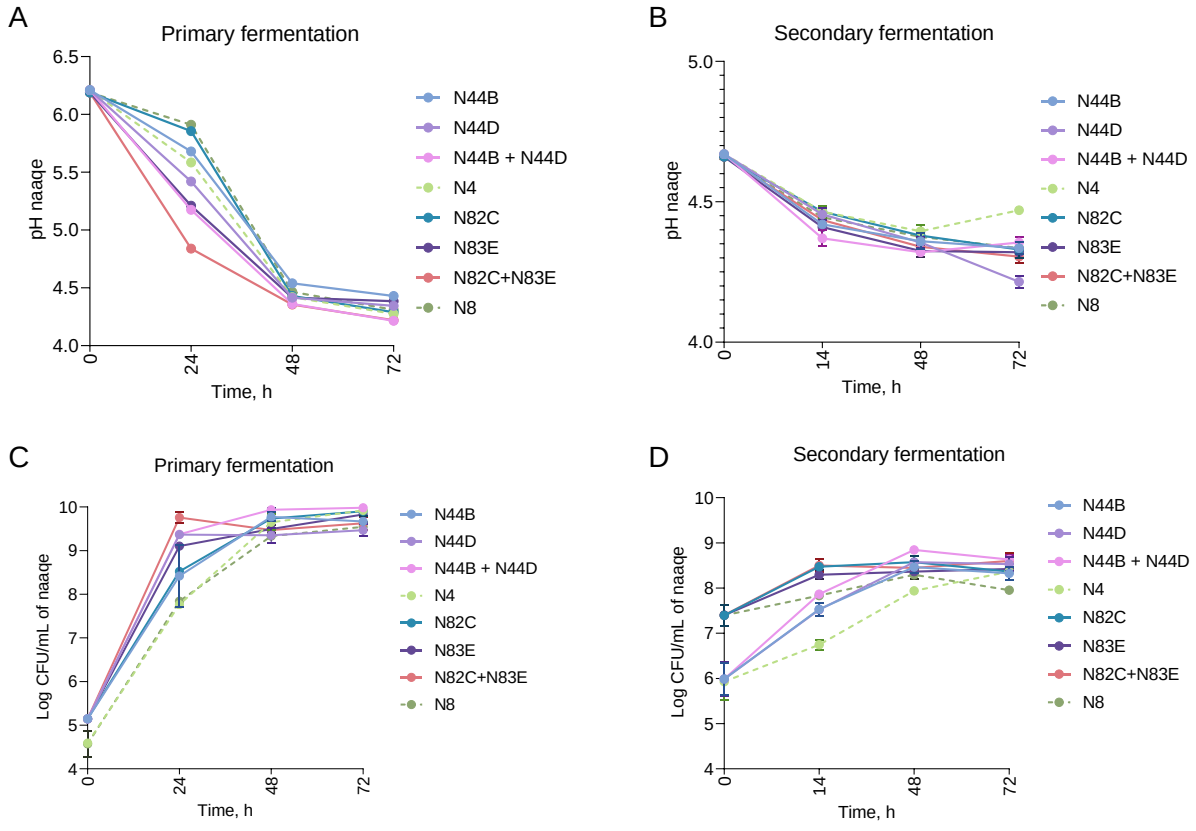


Figure 5. Assessment of the pH (A & B) and viable count of LAB (C & D) during the primary and secondary fermentation of Naaqe as depicted in Figure 1, respectively. Data points are presented as the mean of two experiments ± SD per time point. N = spontaneous fermentation

(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}.

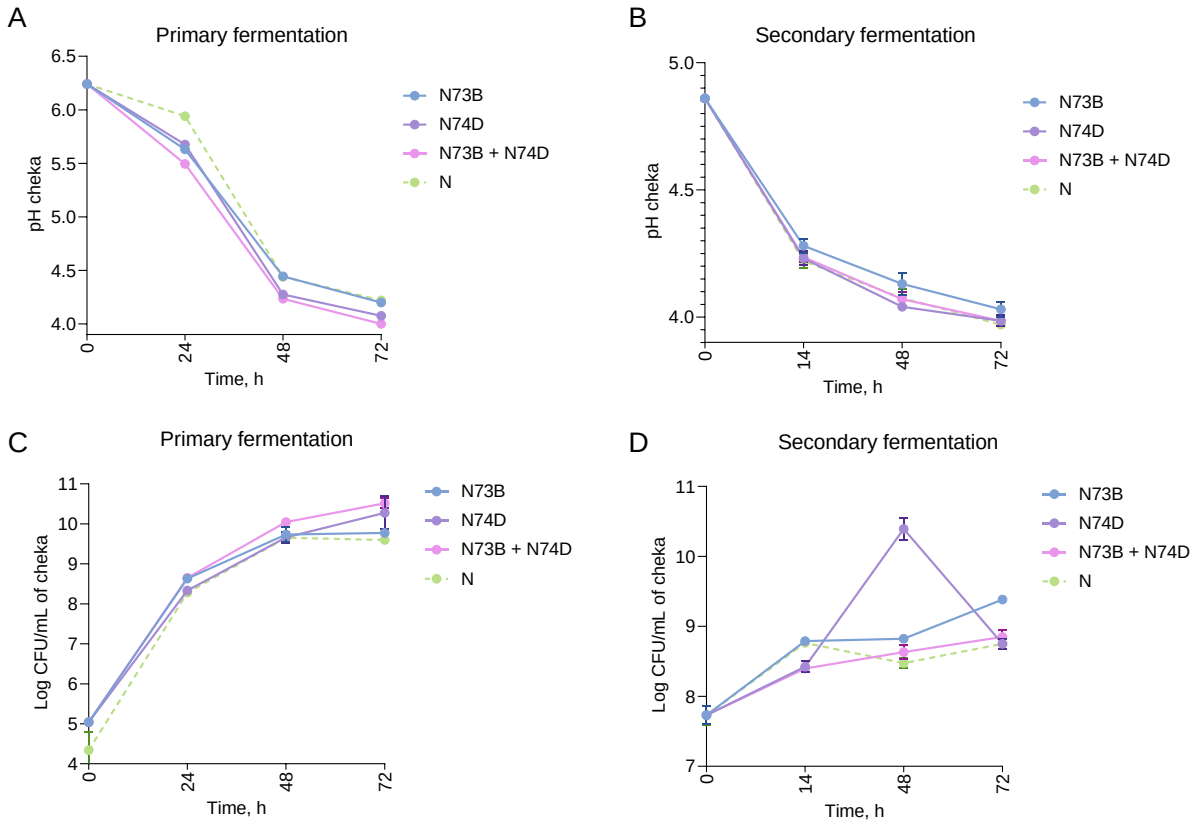


Figure 6. Evaluation of the pH (A & B) and population of lactic acid bacteria (C & D) during the primary and secondary fermentation of Cheka, respectively. Data are presented as the mean of two independent experiments ± SD. Conditions: N = spontaneous fermentation; N73B = inoculated with *L. fermentum*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

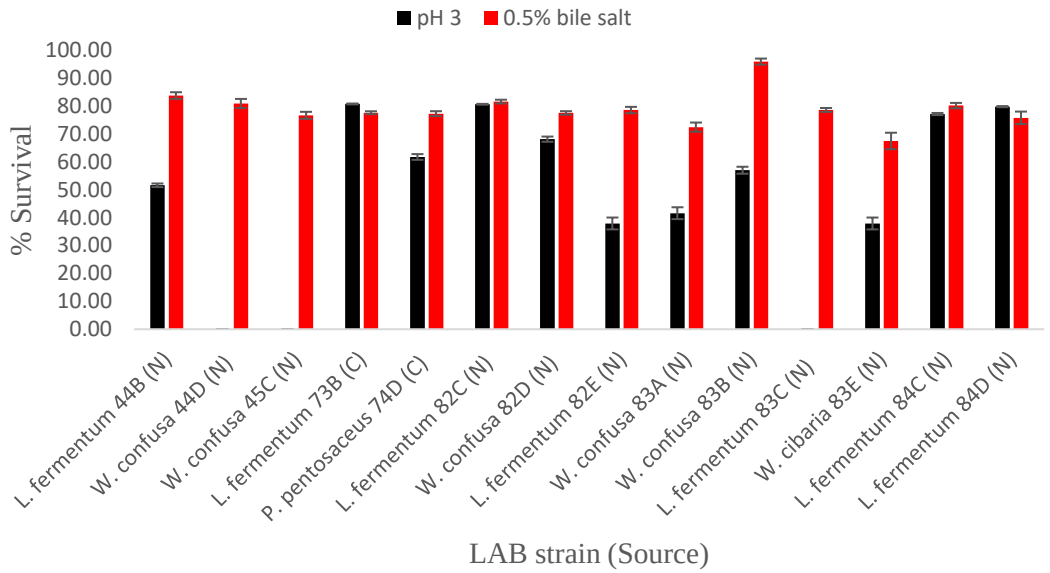
[1]Results of experiments of inhibition of initial antimicrobial assay

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.

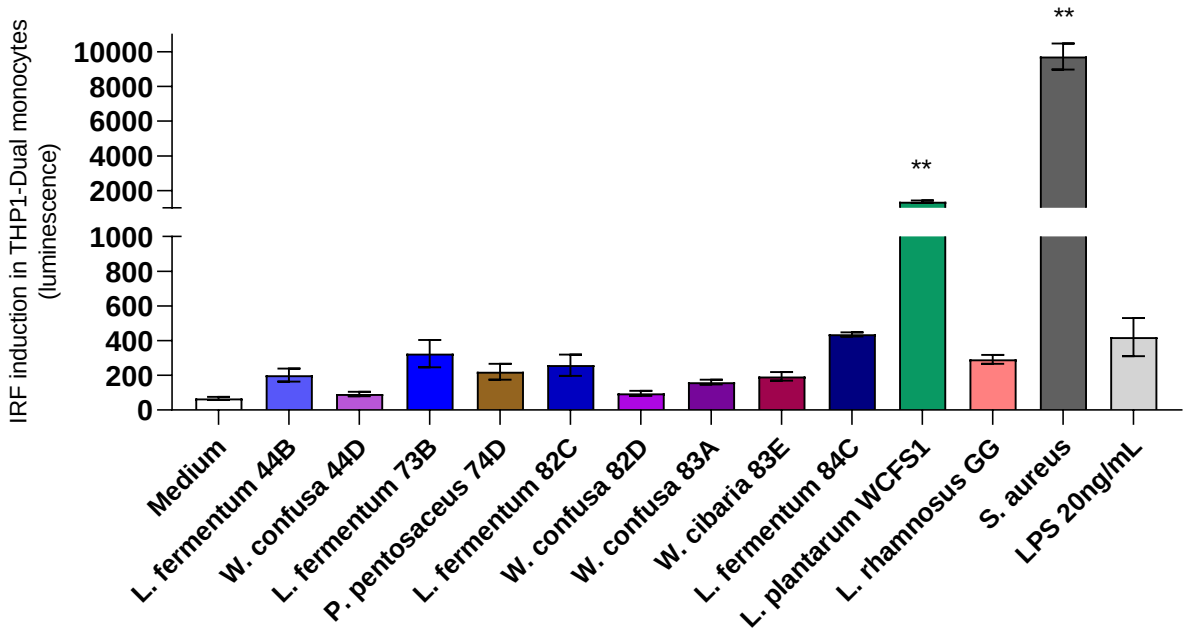
Supplementary Table 2: pH of the LAB isolates cell-free culture supernatants in MRS broth.[1]

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

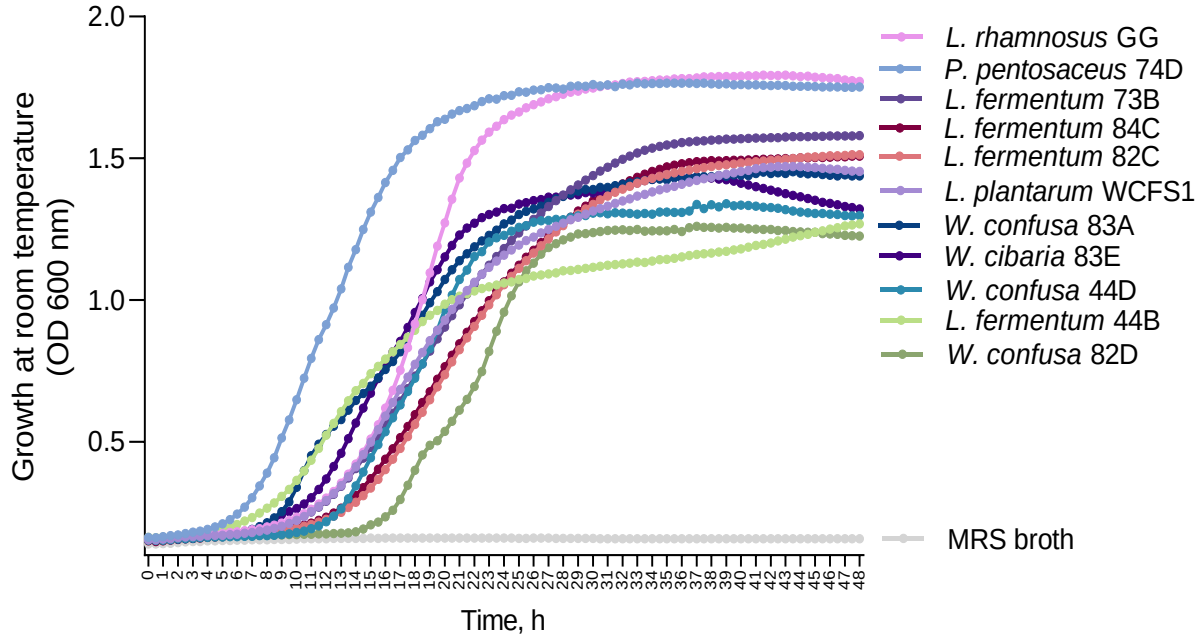
[1] Results expressed in mean ± SD *(n=2)*



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.

Whole Genome Sequence and Comparative Genome Analysis of the three *Lactiplantibacillus plantarum* isolates: Potential Probiotics from Ethiopian Traditional Fermented Cottage Cheese.

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Abstract

Background: *Lactiplantibacillus* (*L.*) *plantarum* is a versatile species found in a wide range of ecological niches, including dairy products (traditional cottage cheese, yoghurt,) and vegetables, and it may also occur naturally in the human gastrointestinal tract (GIT). Although Lactic acid bacteria (LAB) are generally regarded as safe, there are rare emergence of some infections and antibiotic resistance by certain probiotics. The precise mechanisms underlying the beneficial properties of these microbes on their host are also remain obscure.

Objective: The *in silico* genome analysis of putative probiotic bacteria was set up to identify strains, predict desirable functional properties, and identify potentially detrimental antibiotic resistance and virulence genes.

Methods and results: In this study, we characterized the genomes of three *L. plantarum* strains isolated from Ethiopian traditional cottage cheese. Whole-genome sequencing (WGS) was performed using Illumina MiSeq sequencing. We have assessed completeness and quality of the *L. plantarum* strains genome through CheckM. The phylogenetic analyses suggested the highest relatedness between our strains and the model probiotic, *L. plantarum* WCFS1. Analyses results showed that *L. plantarum* 54B and 54C are closely related but different strains. This study reveals that the genomes studied do not harbor resistance and virulence factors and have five classes of carbohydrate-active enzymes with several important functions. Cyclic lactone autoinducer, terpenes, Type III polyketide synthases (T3PKS), ribosomally synthesized and post-translationally modified peptides (RiPP)-like gene clusters and complete riboflavin operon have also been identified in the strain *L. plantarum* 55A evidencing its promising probiotic.

Conclusions: Combined, the lack of resistome and virulome and their previous functional capabilities have rendered these strains important. The results also provide better insights into the probiotic potential and safety of these three strains and indicate avenues for further mechanistic studies using these isolates.

Key words: *Lactiplantibacillus plantarum*; genome sequencing; comparative genome analysis; safety, secondary metabolites.

1. Introduction

Lactic acid bacteria (LAB) are a diverse group of bacteria producing lactic acid as the main end-product of carbohydrate fermentation (Seddik et al., 2017) and ubiquitously distributed in nature (Tenea & Ortega, 2021). They include several genera such as the emended genus *Lactobacillus* (Zheng et al., 2020), *Lactiplantibacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Weissella* (Hutkins, 2019; Zheng et al., 2020). They are being continuously researched due to probiotic attributes and potential health benefits. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (Hill et al., 2014). LAB as probiotics are used to prevent and/or treat gastrointestinal disorders and a wealth of evidence emerging from studies also indicates their anti-cancer activity (Ambalam et al., 2016; Ma et al., 2010).

Lactiplantibacillus (*L.*) *plantarum* (Zheng et al., 2020) is a non-motile, gram-positive, non-spore-forming, microaerophilic, heterofermentative, and mesophilic bacterium that belongs to the LAB (Fiocco et al., 2010; Garcia-Gonzalez et al., 2022). It is one of the most adaptable LAB species, as evidenced by its ability to colonize a wide range of niches such as the gastrointestinal, vaginal and urogenital tracts, meat, fish, fermented vegetables, wine, and dairy products (Garcia-Gonzalez et al., 2022; Mao et al., 2021; Seddik et al., 2017). It is widely used in industrial fermentation and as probiotics since it is "Generally Recognized as Safe" (GRAS) and has Qualified Presumption of Safety (QPS) status (EFSA (BIOHAZ), 2022; Guidone et al., 2014; Setta et al., 2020).

Throughout the last century, documented health-promoting and functional properties of *L. plantarum* strains have generated attention for their applications (Garcia-Gonzalez et al., 2022). Beneficial properties attributed to *L. plantarum* are diverse, varying from its use in fermentation of dairy products such as cheese, kefir, sauerkraut, fermented meat products, fermented vegetables, and beverages to cholesterol-lowering activity, enhancement of the intestinal barrier, immunomodulation, and prevention of bacterial and viral infections (Seddik et al., 2017; Spacova, et al., 2023). Its antibacterial properties are also interesting for food safety as in the biopreservation technology (Seddik et al., 2017). In particular, the three isolates evaluated in the current work (54B, 54C and 55A), obtained from Ethiopian traditional cottage cheese, have shown potential health benefits in our previous study (Gizachew et al., 2023), demonstrating overall superior *in*

*vitr*o antagonistic activities against foodborne bacterial pathogens, immunostimulatory activity, *in vitro* potential to survive in the harsh gastrointestinal condition than other isolates included in the study and safety with regard to antibiotic resistance.

According to the European Food Safety Authority (EFSA) statement on the requirements for whole genome sequence (WGS) analysis of microorganisms intentionally used in the food chain, an unequivocal taxonomic identification at the strain level has to be performed for all microorganisms (EFSA, 2021). Even within the same species, differences between strains may be significant and the properties assigned to one strain cannot necessarily be extrapolated to another (Gizachew et al., 2023). For the development of new probiotics or probiotic starters, their isolation, identification and characterization is of profound importance. Moreover, WGS analysis provides better understanding of the relation between their genotypic and phenotypic profiles and thus, required to better understand strain features (Qureshi et al., 2020). Although LAB are GRAS, there are rare emergence of some infections and antibiotic resistance (Senan et al., 2015). For this reason, data obtained from WGS and WGS-based data analysis are a requirement for the unequivocal taxonomic identification of the strains, and can provide valuable information regarding the characterization of the potential functional traits of these strains as well as information related to virulence factors, resistance to antimicrobials, and the production of toxic metabolites (EFSA, 2021). Previous analysis of existing *L. plantarum* genome sequences demonstrated a high level of genomic variety, plasticity, and adaptability, which may permit extremely successful adaptation of its strains to diverse habitats (Siezen & Van Hylckama Vlieg, 2011). This genome-driven adaptation capacity is also attributable to *L. plantarum* genomes' ability to acquire the so-called carbohydrate utilization islands, which are gene clusters that allow growth on specific carbohydrates present in specific niches (Kleerebezem et al., 2003).

Because of the increased interest with regard to the impact of *L. plantarum* on human health and the necessity to identify genetic determinants associated with probiotic properties, the analysis of the whole genome sequences of the three isolates isolated from cottage cheese was performed. Taxonomic identification as well as genetic exploration of each isolates individually and comparatively for coding probiotic and immunomodulatory traits was performed to inform future mechanistic studies. Moreover, an analysis of the factors related to safety, such as mobile antibiotic

resistance loci or potential virulence factors, was also carried out through a genomic approach, in accordance with the EFSA recommendations (EFSA, 2021).

2. Materials and Method

2.1. Bacterial Strains, Growth Conditions and Genomic DNA extraction

Three isolates (*L. Plantarum* 54B, 54C, 55A) originally isolated from Ethiopian traditional cottage cheese were selected based mainly on their performance in the antimicrobial and cell culture assays. The strains were identified through standard morphological, biochemical, and physiological tests, and by 16S *rRNA gene* sequencing. Strains were revived from -80°C glycerol stocks on MRS plates and incubated for 48 h at 37° C. Single colonies were cultivated in MRS broth for 24 h at 37°C. Total DNA content was extracted using a modified protocol based on Alimolaei and Golchin (Alimolaei & Golchin, 2016). Briefly, 1.5 mL of overnight culture were transferred twice to two sterile Eppendorf tubes, 1.5 µL of ampicillin (100 mg/mL) were added and incubated at 37°C for 1 h. The culture was then spun down at 12.000 x g for 3 min to remove supernatant and the pellet was washed 3x with 1 mL of NaCl-EDTA. Here, pellets present in both Eppendorf's were pooled into one Eppendorf. The cell pellets were resuspended in 100 µL of NaCl-EDTA, 100 µL of lysozyme (10 mg/mL) and 1 µL Rnase (20 mg/mL) were added to the tube and incubated at 37°C with periodic shaking for 1h. Following this, 229 µL of NaCl-EDTA, 50 µL 10% SDS and 20 µL Proteinase K were added, vortexed and incubated at 55°C for 1h. Then, 200 µL of cold protein precipitation solution were added and vortexed at maximum speed for 20 sec. The mix was then centrifuged at 12.000g, 4°C for 3 min after putting on ice for 5 min. The supernatant was transferred to a clean 1.5 mL tube, centrifuged again (12.000g, 4°C, 3 min) and the supernatant was transferred to clean 1.5 mL tube. The DNA was precipitated with 600 µL ice-cold isopropanol, centrifuged at 12.000g, 4°C for 3min to discard supernatant. The pellet was then washed with 600 µL fresh 70% Ethanol, supernatant discarded and the tube left to air-dry. Finally, the pellet was dissolved in 50 µL H_2O, incubated at 55°C for 5 min and stored at -20°C. DNA samples in the range of 25-50 ng/µL (measured with Qubit), with a minimum volume of 20µL were sent for WGS.

2.2. Genome sequencing, assembly and annotation

High molecular weight genomic DNA of the isolates was then further processed for sequencing using Nextera library prep and MiSeq sequencing (Illumina) at the lab of Medical Microbiology, University of Antwerp. After sequencing, raw reads were analyzed using in-house pipeline. In short, reads were assembled into contigs using shovill (https://github.com/tseemann/shovill). Quality and completeness were assessed using CheckM (completeness >94% required). Annotation was performed with Prokka (Seemann, 2014).

2.3. Comparative genomic analysis

Comparative genomic analysis was performed by making use of Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (Olson et al., 2023). Comparative phylogenetic trees were constructed in BV-BRC using the "Bacterial Genome Tree" tool, which generates a phylogenetic tree based on using codon tree method. The Codon Tree pipeline generates bacterial phylogenetic trees by using the amino acid and nucleotide sequences from defined number of the BV-BRC global Protein Families (PGFams), which are picked randomly, to build an alignment, and then generate a tree based on the differences within those selected sequences. Comparative Systems Service of the BV-BRC was also utilized to compare protein families among the genomes included in the analysis.

2.4. Strains Identification and Average Nucleotide Identity (ANI) Analysis

The 16S *rRNA gene* was extracted and used for classification using Bayesian lowest-common ancestor (BLCA) taxonomic classification method based on the NCBI taxonomy database. ANI was used to calculate the orthologous average nucleotide identity of the genome. We calculated ANI by using FASTANI. ANI is the average value based on the comparisons of all orthologous protein-encoding genes of the pairwise genomes. 'Variation Analysis Service' of the BV-BRC was used to measure genetic variations of single nucleotide polymorphisms (SNPs) between the isolates *L. plantarum* 54B and 54C.

2.5. Prediction of Putative biosynthetic gene clusters of bioactive Compounds

To predict genes coding for many different types of biosynthetic pathways involved in production of secondary metabolites (SMs), antiSMASH 7.0 (Antibiotics and Secondary Metabolite Analysis

Shell) was utilized (accessed on 10 August 2023) (Blin et al., 2023). More in-depth analyses were performed in antiSMASH for biosynthetic gene clusters (BGCs) encoding non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), the ribosomally synthesized and post-translationally modified peptides (RiPPs) and RiPP-like molecules. The annotated genome FASTA file of the isolates were used as the input file and default antiSMASH features were assumed during the analysis. Riboflavin metabolism pathway encoding genes were predicted by utilizing the BV-BRC's 'Comparative Systems' service.

2.6. Carbohydrate–Active Enzyme Analysis

Carbohydrate metabolism is gaining attention as a property supporting the probiotic potential of LAB. Carbohydrate metabolism is the primary source of metabolic energy in LAB and is critical to *Lactobacilli* survival and fitness in their ecological niche by contributing to cellular processes such as energy production and stress response (Mehra & Viswanathan, 2021). Carbohydrate-active enzymes (CAZymes) are key in carbohydrates metabolism, especially in the biosynthesis, binding, and catabolism of sugars. Glycosyltransferases (GTs), glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and auxiliary activity (AA) are the different classes of CAZymes (Tarrah et al., 2020). CAZymes of the isolates were searched against the CAZy database (http://www.cazy.org/). The database mainly included GHs, GTs, CEs, carbohydrate-binding enzymes (CBM), AAs, and PLs (Mao et al., 2021).

2.7. Virulome and resistome predictions

The genomes were assessed for safety using several tools recommended in the (EFSA, 2018). ABRIcate (https://github.com/tseemann/abricate) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/; accessed on 20 October 2021) (Zankari et al., 2012) were employed to identify the resistome in the genomes of the isolates. ABRIcate and VFDB (virulence factor database, http://www.mgc.ac.cn/VFs/main.htm, accessed on 20 October 2021) were also employed to predict putative virulence factors (Liu et al., 2019).

2.8. Complete Genome Sequences Data Accession Number

The sequence data for *L. plantarum* isolate (54B, 54C, and 55A) genomes has been deposited at the European nucleotide archive under the accession number--------, --------, and ------ respectively.

3. Results

3.1. General features, identification and quality of the genomes

The chromosomal properties, quality control statistics and identification to the species level of the three *L. plantarum* isolates (54B, 54C, and 55A) sequenced in this study are summarized in Table 1. The raw reads assembly resulted in the generation of bacterial chromosomes each with a size similar to that previously reported for sequenced *L. plantarum* isolates (range of 3–3.6Mbp) (Surve et al., 2022). The two isolates (54B and 54C) possessed a genome length of 3.39 and 3.37 Mbp, respectively, while, the isolate 55A possessed a genome length of 3.29 Mbp, a little less than the other two. The two isolates (54B and 54C) also possessed approximately the same coding sequence (CDS) (3259 and 3230, respectively) and the same GC content (44.3 %), although isolate 55A contained lower CDS (3108) and relatively higher GC content (44.5 %). However, the number of tRNA, rRNA and tmRNA genes was found to be the same among the isolates (Table 1).

The 16S rRNA gene of these isolates was extracted and used for classification based on the NCBI taxonomy database and showed that all the three isolates belong to the *L. plantarum* species. The genome sequences with a completeness of higher than 94% passed the quality control requirement, as assessed using CheckM, and further analyzed. Here, the completeness percentage was found to be 99.07 % for all the genomes sequenced. It has also been reported that the median total length of *L. plantarum* genome assemblies is 3,253,870 bp, with a median protein (CDS) count of 2926 and median GC content percentage of 44.5 (Nikodinoska et al., 2022). Thus, the sequencing of our isolates produced a complete genome (101% – 104%), a little larger size than the median genomic parameters for the microbe.

Table 1: Comparison of chromosomal properties of *L. plantarum* isolates.

Isolate	**54B**	**54C**	**55A**
Completeness (%)	99.07	99.07	99.07
Genome size (bp)	3,398,069	3,372,323	3,299,167
N50 (contigs)	105,937	131,701	169,607
Contigs	156	148	77
GC content (%)	44.3	44.3	44.5
# ambiguous bases	100	100	0
Coding sequence (CDS)	3259	3230	3108
Genes	3335	3306	3185
tmRNA	1	1	1
rRNA	7	7	7
tRNA	68	68	69
Species	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*

3.2. Comparative Genomic Analysis

To advance our understanding of the genome diversity and molecular evolution of the isolates, genomic characterization was conducted. Comparative phylogenetic trees were constructed to reveal the evolutionary relationship between the three *L. plantarum* strains (54B, 54C, and 55A) and other *Lactobacilli* spp. The tree was implemented in BV-BRC using the "Bacterial Genome Tree" tool, which generates a phylogenetic tree based on the amino acid and nucleotide sequences from defined number of the BV-BRC PGFams between 15 reference genomes and our genomes. The phylogenetic tree showed that our isolates were grouped together with other *L. plantarum* strains but were distinguishable from other strains of the *Lactiplantibacillus pentosus* strains (Figure 1). The strains 54B and 54C showed a high degree of similarity with each other. The strain 55A displayed a high degree of similarity with the strain *Lactiplantibacillus plantarum* ERR9969084_bin.1_MetaWRAP_v1.3_MAG.

In the pangenome exploration (via 'Comparative systems service of bv-brc.org), we found 3258, 3226, and 3124 distinct protein family members from the genomes of *L. plantarum* 54B, 54C and 55A, respectively. The corresponding figure for selected reference genomes were 3119, 3017 and 1894 for the genomes *L. plantarum* WCFS1, *L. plantarum* SK151, and *Lactobacillus acidophilus* La-14, respectively. This indicates that one of the closest evolutionary relative for all of our isolates was *L. plantarum* WCFS1, a single colony isolate from *L. plantarum* NCIMB8826, which was originally isolated from human saliva (Kleerebezem et al., 2003). This degree of similarities was remarkable, especially in light of the relative poor similarity in the comparison with other *lactobacilli* strains compared.

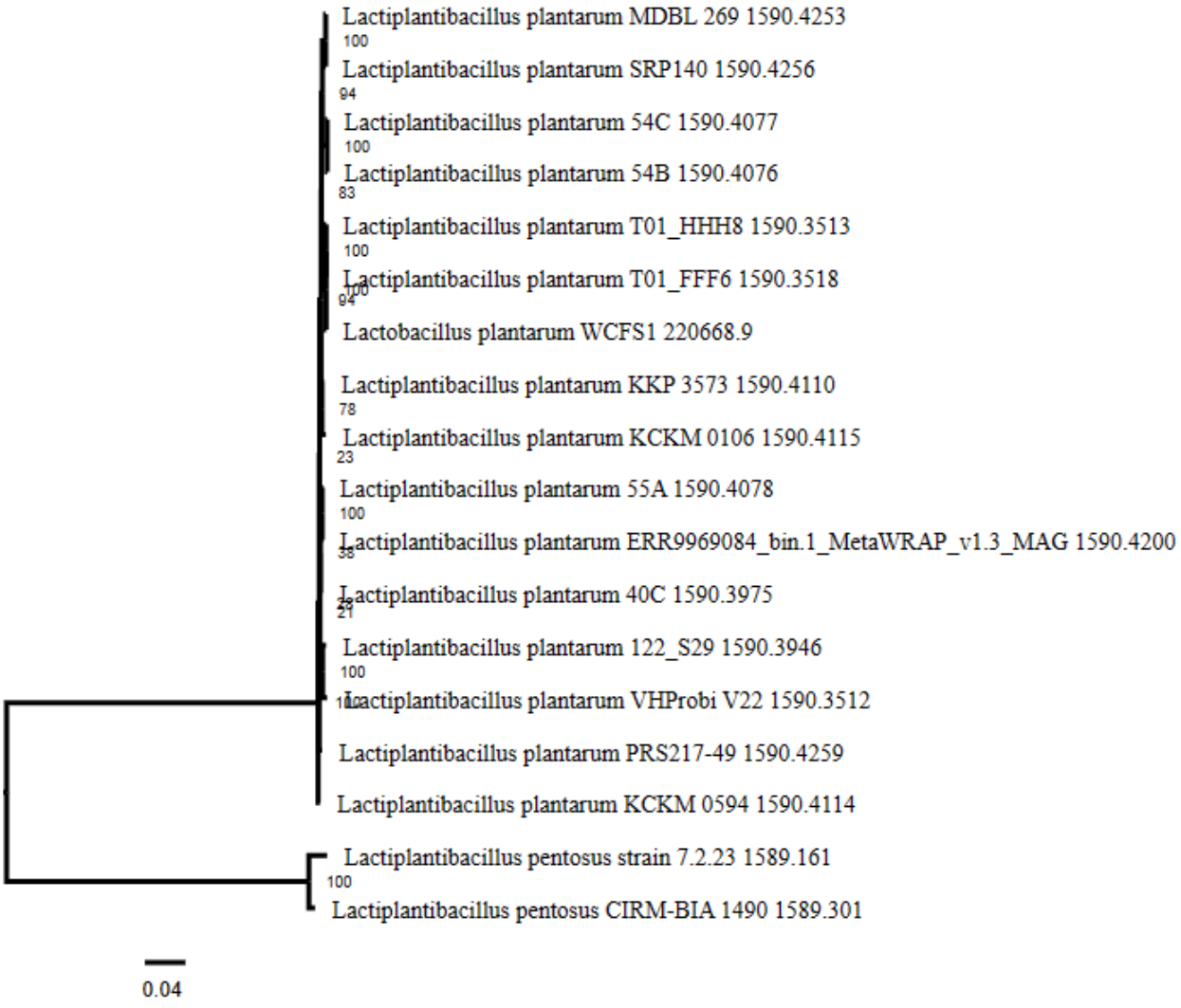

Fig. 1. Phylogenetic analysis of *L. plantarum* 54B, 54C and 55A with 15 other LAB reference genomes.

3.3. Average Nucleotide Identity and Single Nucleotide Polymorphism Analysis

We calculated the ANI value for our three isolates among themselves and between them and *L. plantarum* WCFS1 (Table 2). The ANI value for the *L. plantarum* 54B vs 54C was found to be 99.9941 %, indicating that they are the most related strains. The analysis of genetic variations of SNPs between closely related isolates *L. plantarum* 54B and 54C indicated that there are 111 single nucleotide variations (SNVs) between the genomes.

Table 2: Average nucleotide values of the strains

Strains compared	**ANI values (%)**
54B Vs 54C	99.9941
54B Vs WCFS1	98.7873
54B Vs 55A	98.6644
54C Vs WCFS1	98.7732
54C Vs 55A	98.7297
55A Vs WCFS1	98.8804

3.4. Prediction of Secondary metabolites and Bioactive Products

Bacteriocins constitute a significant class of antimicrobial peptides produced by LAB. In this study, the antiSMASH system predicted four fundamental areas that produce bacteriocins and secondary metabolites in the genome of *L. plantarum* 55A; region 1.1 (RiPP: cyclic-lactone autoinducer; location: 67,119 - 87,824 nt; total: 20,706 nt); region 2.1 (RiPP: RiPP-like; location: 104,505 - 116,655 nt; total: 12,151 nt); region 17.1 (PKS: T3PKS; location: 1 - 28,896 nt; total: 28,896 nt) and region 25.1 (terpene: terpene; location: 2,330 - 23,211 nt; total: 20,882 nt) **(Figure 2)**. The system also predicted three fundamental areas that produce bacteriocins and secondary metabolites in the genome of *L. plantarum* 54B; region 2.1 (PKS: T3PKS; location: 1 - 34,310 nt; total: 34,310 nt), region 4.1 (terpene; terpene; location: 140,604 - 161,485 nt; total: 20,882 nt), and region 40.1 (RiPP; Cyclic-lactone-autoinducer; location: 1,239 - 17,049 nt; total: 15,811 nt). The genome of *L. plantarum* 54C was also observed to have exactly the same bacteriocins and

secondary metabolites profile with *L. plantarum* 54B, the minor differences included location on PKS region and its total nucleotide (**Table 3**).

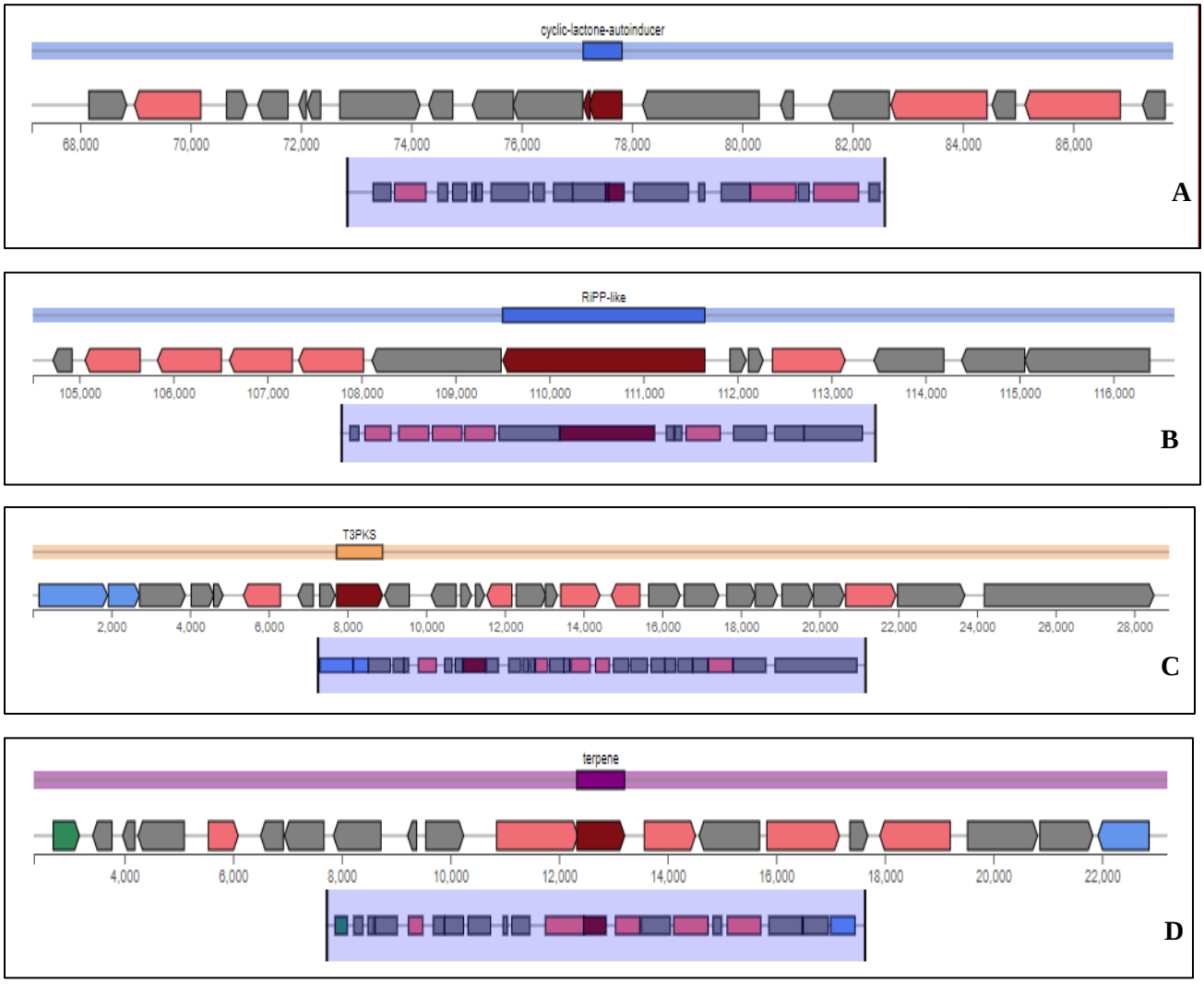

Figure 2: Bacteriocins and secondary metabolite producing regions in the genome of *L. Plantarum* 55A: Red (core biosynthetic genes), pink (additional biosynthetic genes), blue (transport-related genes), green (regulatory genes), grey (other genes), and black (resistance). (A) Cyclic lactone autoinducer; (B) RiPP-like; (C) Type III PKS; (D) Terpene,

Riboflavin, also called vitamin B2, is a water-soluble vitamin that serves as the precursor of the two essential coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are essential in the redox reactions within the cell. Humans are not able to synthesize vitamin B2 and so it must be incorporated it in their diets (Wang et al., 2021). Riboflavin can be produced by many microorganisms including fungi (such as yeast) and bacteria. In LAB, riboflavin synthase coding genes are clustered on a *rib* operon, and its products (RibC, RibB, RibA, and RibH) can catalyze the conversion of GTP and 5-phosphate ribose into riboflavin (Wang et al., 2021). Here, we report that a complete riboflavin operon was observed in all of our *L. plantarum* genomes.

Table 3: characteristics of biosynthetic gene clusters of the isolates *L. Plantarum* 54B and 54C

Secondary metabolite 1: PKS					
isolate	region	BGC	Core gene	Location (nt)	Total nucleotide (nt)
54B	2.1	PKS	T3PKS	1 - 34,310 nt	34,310 nt
54C	2.1	PKS	T3PKS	175,611 - 209,976 nt	34,366 nt
Secondary metabolite 2: terpene					
54B	4.1	terpene	terpene	140,604 - 161,485 nt	20,882 nt
54C	4.1	terpene	terpene	26,228 - 47,109 nt	20,882 nt
Secondary metabolite 3: RiPP					
54B	40.1	RiPP	Cyclic-lactone-autoinducer	1,239 - 17,049 nt	15,811 nt
54C	41.1	RiPP	Cyclic-lactone-autoinducer	1 - 15,811 nt.	15,811 nt

3.5. Carbohydrate-active enzymes

The analysis of CAZymes revealed that the *L. plantarum* 54B and 54C genomes each contained 91 genes in the five CAZymes gene families: 36 GT, 41 GH, 2 AA, 3 CBMs, and 9 carbohydrate CE genes. CAZymes analysis on the genome of *L. plantarum* 55A also revealed that it contained 90 genes in the five CAZymes gene families: 31 GT, 47 GH, 2 AA, 2 CBM, and 8 CE genes. We found that the most abundant CAZymes genes in the *L. plantarum* strains genomes belonged to the GH family, followed by the GT and CE families.

3.6. Prediction of Antibiotic Resistance Genes and Virulence Factors

Although *L. plantarum* is a species with QPS status, aiming to the use of the strains under study in food applications, their genomes were evaluated to cover all the safety concerns as recommended by EFSA Guidance for characterization of microorganisms used as food additives

in animal feed and as producing organisms (EFSA, 2018). Here in our analysis, both ABRIcate and ResFinder revealed that the genomes of strains under study harbored no antibiotic-resistant genes. ABRIcate and VFDB analyses also showed that the strains under study harbored no putative virulence factors. These findings suggest the strains potential safety for food and other applications.

4. Discussion

This study reports the draft genome sequence of three *L. plantarum* strains (54B, 54C, and 55A), isolated from the Ethiopian traditional cottage cheese sample, with insights into the potential probiotic properties of these strains based on the presence of putative beneficial genes and absence of genes of safety concern. Importantly, the food-dwelling *L. plantarum* strains analyzed are representative of isolates that are naturally consumed at very high levels (~10^8 ─ 10^9 per gram) in cottage cheese (Gizachew et al., 2023), and it is, therefore, important to understand the genetic makeup of these strains and their potential impact on the host. Through this genomic analysis, we aimed to obtain insights into the key genes and predict the functionality and concerns of safety of these strains to foster future phenotypic studies and applications.

The raw reads assembly resulted in the generation of bacterial chromosomes each with a size similar to that previously reported for sequenced *L. plantarum* isolates (range of 3–3.6 Mbp), which is higher compared to other LAB (Surve et al., 2022). With the completeness percentage of 99.07 for all the genomes sequenced and the sequences produced 101 % – 104 % of the median total length of *L. plantarum* genomes, we report a genome sequence of comparable size with other genomes of the bacterium. Consistent with the results of the 16S rRNA gene comparison, phylogenetic tree analysis of the three genomes studied showed close relatedness with other *L. plantarum* strains including strain WCFS1. The protein families' analysis of the strains versus the reference genomes revealed a higher-level of similarity with the model probiotic *L. plantarum* WCFS1. These results suggest the potential benefits of our strains may have.

The CAZy data set anticipated five significant classes of sugars in the genome of the strains under study, i.e., GTs, GHs, CEs, CBMs and AAs. The existence of these CAZymes help our strains in their survival, competitiveness, and persistence within the host. Because these genes are involved

in the metabolism and assimilation of complex non-digestible carbohydrates, they are crucial for the bacteria's adaptation to the gastrointestinal environment and its interaction with the host (Mehra & Viswanathan, 2021). GTs are essential for the catalysis of the transfer of sugars from activated donor molecules to specific acceptors and are important for the formation of surface structures, which are recognized by host immune systems (W. Zhang et al., 2019). GTs can also produce structures similar to mucins by making O-linked glycosylations on the serines (Jia et al., 2017). CBMs can enhance the activity of the catalytic activity of the CAZymes on the substrate by binding to the substrate of the CAZymes (Jia et al., 2017). Hence, we assume that the existence of these CAZymes help our strains in their survival, competitiveness, and persistence within the host.

Four fundamental regions in the genome of *L. plantarum* 55A were identified to produce bacteriocins and secondary metabolites including cyclic-lactone autoinducer (postulated to have an effect in quorum sensing to assess their cell density to regulate the production of adhesins used for biofilm formation as well as enzymes involved in the utilization of different sugars) (Mull et al., 2018), RiPP-like molecules (exhibit antibacterial activity) (D. Zhang et al., 2023), T3PKS (produce secondary metabolites with diverse biological activities, including antimicrobials) (Navarro-Muñoz & Collemare, 2020), and terpenes (have antimicrobial, antiparasitic, antiallergenic, antispasmodic, antihyperglycemic, antiinflammatory, and immunomodulatory properties) (Ajikumar et al., 2008). The identification of these four categories of compounds led to the notion that *L. plantarum* 55A does indeed have a potential for being used as a probiotic (Aziz et al., 2022), although the exact beneficial role of these predicted properties remains to be ascertained in follow-up more mechanistic studies. Our findings are in agreement with previous studies such as Mull et al. who discussed that cyclic peptides, similarly to the cyclic lactone autoinducer peptide, govern critical pathways of signal transduction, further targeting the polysaccharide biosynthesis and sugar utilization enzymes (Mull et al., 2018). Aziz et al. also reported the same four regions from *L. plantarum* 13-3 genome that were identified to produce four bacteriocins as those identified in the *L. plantarum* 55A genome (Aziz et al., 2022). Similarly, strains *L. plantarum* 54B and 54C had three identical regions in their genomes to produce bacteriocins and secondary metabolites (T3PKS, terpene and cyclic-lactone autoinducer).

A generally applicable operational definition of strain with a strong biological basis has not been defined and may not exist (Van Rossum et al., 2020). In theory, genomes with as little as one SNV difference may be considered to be different strains. Nonetheless, because of the overwhelming amount of strains that would result from metagenomic data, this method is not usually recommended (Van Rossum et al., 2020). There are no standards governing how many SNVs constitute a different strain or whether such SNVs must be fixed in the population or affect phenotype (Van Rossum et al., 2020). Some authors set a cut-off of less than or equal to two SNV differences (Kong et al., 2019), while others set ANI value greater than 98 % (Brooks et al., 2017), for isolates to be considered to come from the same natural strain.

The genomes of our two closely strains *L. plantarum* 54B and 54C (isolated from the same fermentation and the same plate) showed to have the same (number, family) CAZymes profiles, three identical BGCs, and ANI value of 99.9941 %. However, the genomic analyses have shown that there are differences in the location of BGCs and have high SNV differences (111), indicating that these isolates are closely related but different strains. This finding also meets the regulatory requirement set in the EFSA Guidance document (EFSA, 2018) and the EFSA's statement (EFSA, 2021) for an unequivocal taxonomic identification at the strain level.

LAB can also enhance the nutritional content of fermented foods by producing vitamins and cofactors, which contribute to functional food. The genomes analyzed in the present work harbored the complete *rib* operon, suggesting these isolates potential as probiotics. Amoranto *et al*. reported complete genome sequence of *L. plantarum* SK151 isolated from kimchi, which harbored a complete *rib* operon (Amoranto et al., 2018). Phujumpa et al., also reported that *L. fermentum* KUB-D18 genome contained genes majorly involved in metabolism of cofactors and vitamins including riboflavin (Phujumpa et al., 2022). Finally, one of the most important findings of this study was lack of resistome and virulome from the strains studied and this is consistent with another study that reported the non-pathogenicity of *L. plantarum* strain (Aziz et al., 2022). Overall, the lack of resistome and virulome in addition to the previously confirmed *in vitro* functional capabilities of the strains (Gizachew et al., 2023) opens an avenue for a wide spectrum of research with regard to human health-related applications of the bacteria.

5. Conclusions

This study reported the genome sequences of three *L. plantarum* strains isolated from Ethiopian traditional cottage cheese, a rich source of the LAB strains. The results obtained in this study, with the previous *in vitro* works performed, show the potential of cheese-origin *L. plantarum* strains as probiotics. The phylogenetic analysis demonstrated that the strains were closest to the strain isolated from human saliva, reflecting a lack of niche adaptation. The genomic analysis of the strains revealed the presence of putative gene clusters coding for cyclic lactone autoinducer, terpenes, T3PKS, and RiPP-like gene clusters; and a complete *rib* operon, evidencing their role as probiotics. Moreover, none of the strains evaluated proved to have antibiotic resistance genes or virulence factors, which suggests their potential safety for probiotic applications. Collectively, the genomic information guarantees the safe use of these strains as probiotics and opens new possibilities to exploit the health promoting potential of the strains.

6. References

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