



**BIOACTIVE POTENTIAL OF FERMENTABLE OLIGO-DI-MONOSACCHARIDES
AND POLYOLS (FODMAPS) AND LACTIC ACID BACTERIA IN COMPOSITE
FERMENTED BEVERAGES DEVELOPED FROM SELECTED UGANDAN FOOD
CROPS**

Alowo Docus

20/U/3085/PHD/PS

A Thesis Submitted in Fulfilment of the Requirements for the Award of the Degree of
Doctor of Philosophy in Agricultural and Applied Biosciences of Gulu University.

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August, 2026

DEDICATION

To Engineer Okello Francis my beloved husband. Your unwavering love and support especially in challenging societal norms, have been the wind beneath my wings. Thank you for believing in me and nurturing the space for my potential to flourish.

To my daughters Akullu Jayleen Lorraine, Awor Janice Laura and Alum Michelle Lena: this is to give you confidence that a girl child can be and do anything she puts her mind, body and soul to.

To my dear mom Mrs. Jane Ogot (RIP): though you departed too soon to witness this moment, I carry your strength and grace with me. Your daughter has made you proud.

To Dad. Thank you for sacrificing and educating a girl child.

To the unseen heroes, the house helps whose labour and care often go unrecognised thank you, and may God richly bless you.

ACKNOWLEDGEMENT

The journey to this PhD has been anything but straightforward. It has demanded resilience, sacrifice, and an unwavering belief in the value of knowledge. Without the steadfast support of many remarkable individuals, I might have faltered long before reaching this milestone. While it is impossible to name everyone who contributed to this achievement, a few must be recognised here for their profound and enduring impact.

I extend my deepest gratitude to my academic advisors: Prof. Duncan Ongeng, Prof. Ivan Muzira Mukisa, and Dr. Solomon Olum. Your belief in my potential and your persistent encouragement was instrumental in helping me persevere. Prof. Duncan Ongeng, your mentorship has been a cornerstone of my academic journey, from my master's degree through to this doctoral achievement. Your fatherly guidance and generous support through research grants have been invaluable. Prof. Ivan Muzira Mukisa, your ability to simplify complex tasks through clear and emotionally intelligent communication gave me the confidence to keep pushing forward. Dr. Solomon Olum, supervising a colleague is no easy task, yet your thoughtful guidance and steady hand helped me navigate this path with clarity and purpose.

I am also grateful to Dr. Collins Okello, Dean of the Faculty of Agriculture and Environment. Your leadership created a supportive academic environment that was essential to the success of this PhD. To my doctoral committee members; Assoc. Prof. Echodu Richard, Prof. Duncan Ongeng, Prof. Ivan Mukisa, Dr. Solomon Olum and Dr. Acaye Ongwech, thank you for your insightful contributions. Our meetings were very impactful. Your dedication helped dispel doubts and added meaningful value to this work.

My sincere appreciation goes to the Mountains of the Moon University (MMU) management for fostering an environment that encourages academic growth. Special thanks to the Department of Food Innovations and Nutrition and our Dean, Assoc. Prof. Wesana Joshua, for your understanding and support in reallocating my responsibilities, allowing me the space to focus and complete this journey. Your sacrifices bore the fruit of this PhD.

I also acknowledge those who taught me at the doctoral level: Dr. Aloysius Malagala, Prof. Duncan Ongeng, Dr. Walter Odongo, Dr. Stephen Wamala Kalule, and Dr. Basil Mugonola, thank you for enriching my academic experience. To the staff of the Faculty of Agriculture and Environment, Gulu University, your support has been deeply appreciated.

To my PhD colleagues and “seniors”, Kevin Toyi, Nassanga Prossy, Bonny Aloka, Fred Otim, Aliro Tonny, Achiro Eunice and many others whose names may escape me, thank you for walking this path with me. Though the journey was challenging, your camaraderie reminded me that it is not only difficult it is also possible. I am deeply grateful to all my friends and family who, in one way or another, felt my absence during this PhD journey. Your patience, understanding, and quiet encouragement sustained me more than words can express.

In a special way, I extend my profound thanks to my beloved husband, Eng. Okello Francis. Your unwavering support, belief in my potential, and constant presence through every high and low made this achievement possible. Together, we are truly unstoppable.

Above all, I thank the Almighty God for the gift of life, strength, and purpose. This PhD is not just a personal milestone; it is an indication of Your grace. May every step I take from here bring glory to Your name.

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LIST OF ACRONYMS

ANI	:	Average Nucleotide Identity
CFU	:	Colony Forming Units
DALYs	:	Disability-adjusted life years
FGIDs	:	Functional Gastrointestinal Disorders
FODMAPs	:	Fermentable Oligo-Di-Monosaccharides and Polyols
FOS	:	Fructo-oligosaccharides
GIDDs	:	Gastrointestinal Disorders and Diseases
GIT	:	Gastrointestinal tract
GMPs	:	Good manufacturing practices
GOS	:	Galacto-oligosaccharides
GUREC	:	Gulu University Research and Ethics Committee
IBD	:	Inflammatory Bowel Disease
IBS	:	Irritable Bowel Syndrome
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
MLST	:	Multi-locus sequence typing
NCDs	:	Non-communicable diseases
NSP	:	Non-Starch Polysaccharides
SCFAs	:	Short chain fatty acids
SPSS	:	Statistical Package for Social Scientists
SSA	:	Sub-Saharan Africa
UNCST	:	Uganda National Council for Science and Technology
WHO	:	World Health Organisation

KEY OPERATIONAL DEFINITIONS

Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs): These are a category of carbohydrates that are poorly digested in the upper gastrointestinal tract and are hence fermented in the large intestines by colonic bacteria

Bioactive potential: The inherent capacity of substances majorly found in plants, microbes and various natural products to elicit effects on living organisms, cells and tissues typically for pharmacological applications

Prebiotics: Non-digestible food components that selectively stimulate the growth and activity of beneficial microorganisms, primarily bacteria in the gut, thereby improving host health

Probiotics: Live microorganisms, primarily bacteria and some yeasts that, when administered in adequate amounts, confer a health benefit to the host

Synbiotics: Formulations that combine probiotics and prebiotics in a way that enhances the survival, colonisation, and activity of beneficial microorganisms in the host's gut

Functional gastrointestinal disorders & diseases: a group of chronic gastrointestinal conditions characterised by symptoms like abdominal pain, bloating, and altered bowel habits, but without any detectable physical or structural abnormalities in the digestive tract

Improved variety: a new or modified plant variety that exhibits superior traits compared to its parent or other existing varieties

ABSTRACT

Dietary interventions for functional gastrointestinal disorders remain ineffective in Uganda largely due to inadequate information on bioactive foods. In this study, an *in vitro* evaluation of bioactive potential of Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) and lactic acid bacteria isolated from fermented composite beverages was conducted. It examined how varietal differences, processing methods, and ingredient type influence FODMAPs content, prebiotic potential, genotypic characteristics of lactic acid bacteria, and probiotic capacity. Improved and indigenous varieties of soybean, millet, sorghum, and sesame were processed by roasting or malting. FODMAPs were extracted, analysed using HPLC-UV and Megazyme kits, and tested for prebiotic attributes. Extracts with promising prebiotic attributes were used to develop fermented composite beverages from which lactic acid bacteria were isolated, examined for genotypic characteristics, and probiotic capacity. Results showed that improved varieties of soybean (maksoy 3N), Millet (seremi 2) and sorghum (narosorg 2) had significantly ($p < 0.05$) lower oligosaccharide contents than indigenous varieties by 10.2 to 73.9%. Malting was more effective than roasting ($p < 0.05$) in reducing FODMAPs by 17-70% for galacto-oligosaccharides and 66% for fructo-oligosaccharides. Probiotic strains, *Lactiplantibacillus plantarum* ATCC 14917 and *Lacticaseibacillus rhamnosus* ATCC 7469 grew better in extracts from improved varieties than those from indigenous varieties by a difference of 15-25% ($p < 0.05$). However, *Lpb. plantarum* produced more short-chain fatty acids than *Lb. rhamnosus* by 6–50% following incubation in extracts from improved varieties. *Lpb. plantarum* and *Lb. rhamnosus* incubated in improved variety extracts exhibited better antagonistic activity against common enteric pathogens (*Salmonella enterica* and *Shigella flexneri*), reducing pathogen density by over 40%. Lactic acid bacteria from composite fermented beverages of improved crop varieties had greater genotypic plasticity, and better ($p < 0.05$) probiotic attributes. These findings highlight the potential of composite ingredients from improved crop varieties to enhance gastrointestinal health. Nevertheless, *in vivo* studies are necessary to validate the results.

Key words: Bioactive potential, Functional gastrointestinal disorders, Prebiotics, Probiotics

CHAPTER ONE

INTRODUCTION

1.1 Background

Gastrointestinal disorders and diseases (GIDDs) encompass a wide spectrum of conditions affecting the digestive tract, including the oesophagus, stomach, intestinal tract, liver, pancreas, and gallbladder (Lee & Kim, 2022). These disorders and diseases contribute significantly to the global morbidity and mortality. In 2019, GIDDs accounted for 3.5% of the global disability-adjusted life years (DALYs), ranking 13th among the leading causes worldwide (Wang et al., 2023). The burden was unevenly distributed with low- and middle-income countries contributing 72.78% of the total global DALYs (Wang et al., 2023). These countries largely experience high prevalence of gastrointestinal infections especially among children under 5 years (Salad & Bashir, 2026). GIDDs are categorised either as structural or functional. Functional gastrointestinal disorders (FGIDs) refer to conditions where the intestines exhibit impaired functionality despite being anatomically normal whereas structural gastrointestinal disorders exhibit anatomical and metabolic activity abnormalities (Huang et al., 2023).

Diet plays a pivotal role in the increasing prevalence of functional gastrointestinal disorders (FGIDs) but also serves as a crucial intervention to reverse this trend. Research indicates that the rising consumption of high-fat and high-sugar diets, particularly in low- and middle-income countries experiencing nutrition transition, accelerates alteration of the gut microbiota (Contaldo et al., 2020). On the other hand, diets rich in whole grain and minimally processed foods offer protective benefits against FGIDs and other non-communicable diseases (Hojsak et al., 2022). These foods contain substantial amounts of bioactive compounds such as phytochemicals and carbohydrates with prebiotic properties. Such compounds selectively promote the growth of beneficial gut microbiota and stimulate the production of health promoting metabolites such as short chain fatty acids (SCFAs) (Ademosun et al., 2025). These metabolites help maintain gut microbiota health and homeostasis.

Carbohydrates with prebiotic properties are classified by structure, polymerisation, and food sources, which determine their health benefits (Wu et al., 2022). Categories include fructans, galacto-oligosaccharides, resistant starches, hemicellulose-derived oligosaccharides, pectic oligosaccharides (Davani-Davari et al., 2019). Prebiotic short chain carbohydrates typically contain three to ten sugar residues linked by β -glycosidic bonds, making them resistant to hydrolysis in the small intestine (Popoola-Akinola et al., 2022). They reach the colon intact, where colonic microbes ferment them (Gibson et al., 2017). These carbohydrates are termed microbiota-accessible carbohydrates or grouped as Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs), including excess fructose, lactose, fructo-oligosaccharides, and galacto-oligosaccharides (Atzler et al., 2021; Gibson et al., 2020).

Fermentation outcomes of FODMAPs vary by type. Beneficial effects such as anti-inflammatory activity, pathogen inhibition, and enhanced gut barrier integrity are linked to fructo- and galacto-oligosaccharides (Fang et al., 2021; Lafontaine et al., 2020). In contrast excessive intake of excess fructose, lactose, and polyols often results in adverse symptoms like bloating and flatulence in individuals with IBS or predisposed to IBS (Tuck et al., 2018). This underscores the importance of balanced FODMAPs consumption. Strategies to manage this balance include restricting high-FODMAPs foods intake, applying processing techniques to reduce fermentable content, and employing plant breeding to modify oligosaccharide levels (Nyyssölä et al., 2020; Schmidt & Sciarba, 2021; Varney et al., 2017). While low-FODMAPs diets alleviate symptoms and improve quality of life among those with FGIDs (Brown et al., 2020), they simultaneously reduce microbial diversity and abundance (Zhang & Su, 2025). Moreover, it may lead to deficiencies in essential nutrients such as iron, zinc, protein, and calcium, in settings where plant-based diets are highly consumed especially by children under five years (Bascuñ et al., 2019).

To overcome limitations of dietary restriction, targeted food processing and plant breeding are recommended as sustainable strategies. These approaches can improve nutritional quality and bioactive profiles of plant food resources at a low cost, making them suitable for resource-constrained settings (Calvete-Torre et al., 2022; Marotti et al., 2012). Techniques such as malting and fermentation can generate low-FODMAPs diets while preserving essential nutrients (Ispiryan et al., 2020; Nyyssölä et al., 2020). Although promising, such

methods have been applied mainly to foods in developed countries (Varney et al., 2017). Similarly, varietal differences in FODMAPs profiles have been documented for crops like durum wheat, pigeon peas, and sesame (Marotti et al., 2012; Zebib et al., 2015). Diverse varieties of sorghum, soybean, sesame, and millet are widely adopted in Uganda, yet information on their FODMAPs composition remain scarce, underscoring the need for localised research.

Although agronomic performance of Ugandan crop varieties is well documented (Obua et al., 2020), their FODMAPs profiles, prebiotic potential, and probiotic properties in fermented foods remain largely unknown. Without comprehensive information on how varietal differences, processing methods, and ingredient type influence these attributes, dietary interventions for functional gastrointestinal disorders remain abstract. Conducting this study is therefore significant, as it provides essential evidence to guide effective, locally relevant dietary strategies and supports the development of functional foods tailored to Uganda's nutritional and health needs.

1.2 Problem statement

Functional gastrointestinal disorders (FGIDs), including diarrhoeal diseases, remain a major public health challenge in low- and middle-income countries such as Uganda, particularly among children under five years (Demissie et al., 2021; Uganda Bureau of Statistics, 2022). Despite ongoing interventions such as water, sanitation, and hygiene (WASH) initiatives and immunisation programs (Kotloff et al., 2017; Walker & Dull, 2017), the burden of FGIDs persists with the prevalence of diarrhoea at 69% among children in Uganda (Uganda Bureau of Statistics, 2022). This persistent burden imposes economic strain and hinders progress toward achieving Sustainable Development Goal 3, underscoring the need for complementary strategies. Food based approaches that harness prebiotic FODMAPs and probiotic fermented foods offer promising, sustainable and cost-effective strategies for alleviating functional gastrointestinal disorders (Gibson et al., 2017; Swanson et al., 2020). However, limited information exists on the FODMAPs composition of commonly consumed Ugandan food crops, the prebiotic potential of their fermentable carbohydrates, and how ingredient composition and fermentation influence the diversity, genotypic characteristics,

and probiotic potential of lactic acid bacteria in fermented composite beverages. These knowledge gaps constrain the development of evidence based functional foods for gastrointestinal health. Therefore, this study investigated the FODMAPs composition of selected varieties of millet, sorghum, soybean, and sesame, evaluated their prebiotic potential, and assessed the genotypic characteristics and probiotic potential of lactic acid bacteria isolated from fermented composite beverages developed from these ingredients.

1.3 General Objective

The overall objective for this study was to examine the bioactive potential of FODMAPs and lactic acid bacteria isolated from fermented composite beverages formulated from commonly consumed varieties of millet, sesame, sorghum, and soybean

1.3.1 Specific objectives

The specific objectives were to;

- i. Assess the effect of varietal differences and processing methods on Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) content in millet, sorghum, soybean and sesame
- ii. Determine the effect of crop variety on prebiotic potential of Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) extracted from millet, sorghum, soybean and sesame
- iii. Evaluate the effect of ingredient type and fermentation time on genotypic characteristics of lactic acid bacteria in ready-to-use millet-sesame-soybean and sorghum-sesame-soybean composite fermented beverages
- iv. Evaluate the probiotic potential of lactic acid bacteria strains isolated from ready-to-use fermented millet-sesame-soybean and sorghum-sesame-soybean composites beverages

1.3.2 Hypotheses

- i. Varietal differences and processing methods have no significant effect on the Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) content in millet, sorghum, soybean and sesame

- ii. Crop variety has no significant effect on the prebiotic potential of Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) extracted from millet, sorghum, soybean and sesame
- iii. Ingredient type and fermentation time used in the development of ready-to-use fermented beverages have no effect on lactic acid bacteria genotypic characteristics of fermented millet-sesame-soybean and sorghum-sesame-soybean composite beverages
- iv. Ingredient type and fermentation time has no effect on probiotic potential of lactic acid bacteria strains isolated from ready-to-use fermented millet-sesame-soybean and sorghum-sesame-soybean composites beverages

1.6 Significance of the study

This study is of scientific importance as it contributes to the broader understanding of bioactive compounds specifically FODMAPs in commonly consumed crop varieties. While previous research has focused on the agricultural resilience and productivity of staple crop varieties such as millet, sorghum, soybean, and sesame in Uganda (Obua et al., 2020; Tesfaye et al., 2023; Tukamuhabwa et al., 2016), there remains a critical gap in exploring their FODMAPs composition and therapeutic potential. Investigating these compounds provides a foundation for leveraging commonly consumed crops in preventive health strategies, particularly for managing functional gastrointestinal disorders (FGIDs) through dietary interventions.

Moreover, this study advances scientific knowledge by examining how food processing techniques such as malting and compositing enhance the bioavailability of prebiotic-rich foods to probiotics. A deeper understanding of the metabolic transformation of oligosaccharides during processing could pave the way for developing functional foods optimised for gut microbiota modulation. This could significantly contribute to the emerging field of synbiotic food formulation, where both prebiotics and probiotics work either complementarily or synergistically to improve digestive health (Swanson et al., 2020).

By addressing the unprofiled bioactive potential of indigenous Ugandan crops, this study informs future research on sustainable, locally available dietary solutions for gastrointestinal

health. It contributes information that can be used to either update or develop a food composition table which is a significant tool in effective dietary interventions (Varney et al., 2017). It holds implications not only for nutrition science but also for food technology, agricultural diversification, and public health policy, supporting initiatives aimed at reducing FGIDs through accessible and culturally relevant nutrition interventions.

1.7 Justification of the study

Extensive research has examined agronomic traits and processing effects on nutritional and physicochemical properties of staple food resources such as millet, sorghum, soybean, and sesame in Uganda (Obua et al., 2020; Owere et al., 2015; Tumuhimbise et al., 2019). Nevertheless, their FODMAPs composition and bioactive potential to improve gut health remain critically underexplored. This knowledge gap constrains the formulation of reliable dietary interventions for functional gastrointestinal disorders (FGIDs), which continue to impose a major public health burden. Investigating the FODMAPs profiles of these commonly consumed food resources is therefore necessary to generate foundational evidence for leveraging locally available foods in preventive health strategies. Addressing this gap justifies the study as it responds directly to an urgent need for sustainable, culturally relevant nutrition solutions for low- and middle-income countries such as Uganda.

1.8 Scope of the study

The study focused on investigating the FODMAPs composition of commonly adopted varieties of millet, sorghum, soybean, and sesame in norther Uganda. The study examined how commonly applied processing methods specifically roasting and malting alter FODMAPs composition, and whether varietal differences influence their prebiotic potential. It further assessed how the use of these FODMAPs sources as ingredients for developing composite fermented beverages, could influence genotypic characteristics and probiotic properties of *Lactobacillus* genus isolated from the beverages. The scope is restricted to locally available and widely adopted varieties to ensure contextual relevance. The restriction to only *Lactobacillus* genus was because it is the most established probiotic genus in plant-based fermented foods, with proven safety, functional relevance, and health-promoting

properties (Dempsey & Corr, 2022). Nonetheless, the *Lactobacillus* probiotic strains in fermented composite beverages developed from millet-sesame-soybean or sorghum-sesame-soybean composite ingredients have not been explored before. While the study emphasises compositional analysis and *in vitro* evaluation of bioactive potential (prebiotic & probiotic properties), it does not extend to clinical trials or *in vivo* validation. Instead, it provides foundational data to guide future research, dietary interventions, and functional food development tailored to Uganda's nutritional needs.

The study deliberately assessed each influencing factor in isolation, with the primary objective of establishing baseline effect and quantifying the direct contribution of individual parameters (varietal differences, processing methods, ingredient type and fermentation time) to FODMAPs reduction, prebiotic properties, genotypic characteristics and probiotic properties. This reductionist approach served as a systematic screening framework, enabling the identification of the most effective crop variety, ingredient type, and processing method, prior to introduction of multi-factorial interactions analysis.

1.9 Ethical considerations

This study primarily explored the bioactive properties (prebiotic and probiotic properties) of oligosaccharides extracted from various crop varieties. It was strictly experimental in nature and did not involve human participants consuming the developed products, thereby eliminating any ethical concerns related to human rights. Nevertheless, as the study dealt with a food product intended for human consumption, it adhered to the required ethical standards for such research. Ethical approval was obtained from the Gulu University Research Ethics Committee (GUREC) under approval number GUREC-2022-242, as well as from the Uganda National Council of Science and Technology (UNCST) with approval number A226ES (appendix 1 and 2, respectively).

CHAPTER TWO

GENERAL LITERATURE REVIEW

2.1 Prevalence of gastrointestinal diseases and disorders

The occurrence of gastrointestinal (GI) disorders differs significantly across regions due to varying factors such as demographic, socioeconomic conditions and healthcare access. In developed countries, inflammatory bowel disease (IBD) and colorectal cancer are more prevalent, reflecting dietary and lifestyle influences (Cardoso et al., 2021; Xu et al., 2021). Colorectal cancer, the third leading cause of cancer-related morbidity, is responsible for 10% of global cancer cases and 9.4% of cancer-related deaths worldwide, with higher incidences observed in developed regions (Xi & Xu, 2021). The global burden of colorectal cancer is projected to rise significantly from 1.9 million to 3.2 million by 2040 worldwide (Xi & Xu, 2021). This calls for an urgent need for improved prevention, early detection, and treatment strategies. In sub-Saharan Africa (SSA), there is evidence that the prevalence of IBD and colorectal cancer is on the rise. Nonetheless, the precise extent of this increase remains largely uncertain due to significant limitations in diagnostic and clinical capacities (Watermeyer et al., 2022).

On the other hand, the 2021 Global Burden of Disease (GBD) study reported a substantial increase in age-standardised prevalence of gastrointestinal conditions across low- and middle-income countries between 1990 and 2021 with enteric infections emerging as the most prevalent category (Zhong et al., 2026). These are primarily caused by enteric pathogens, including *Salmonella* species, *Escherichia coli* (*E. coli*), *Shigella* species, and viruses such as Noro and Rotavirus (Ross et al., 2022). These GI infections are often linked to challenges in sanitation, water quality, and food safety (Ahmed et al., 2023; Kyu et al., 2024). This increases the incidences of diarrhoeal diseases.

Diarrhoea contributes approximately 4% of global child mortality and in low- and middle-income countries such as those in SSA, it accounts for 20% of child deaths (Abu & Codjoe, 2018; Demissie et al., 2021). In Uganda, diarrhoeal diseases are among the leading three causes of child mortality (Uganda Bureau of Statistics, 2022). Likewise, in Ethiopia, 29% of

children under five years have experienced episodes of diarrhoea (Birhan et al., 2023). Meanwhile, in Kenya, the occurrence of diarrhoea has been recorded at 18.7% in resource-limited regions, such as Makuru in Nairobi (Kiiru et al., 2024). The primary causes of diarrhoeal diseases are enteric pathogens transmitted through contaminated food and beverages (Saeed et al., 2015). Additionally, the overuse of antibiotics to treat bacterial related infections has led to increased incidence of antibiotic-associated diarrhoea (Ballini et al., 2023). This occurs as antibiotics disrupt the natural balance of gut microbiota, weakening the intestinal ecosystem leading to pathogenic overgrowth and metabolic imbalances (McFarland, 2008).

Interventions such as immunisation, zinc supplementation, and improved water, sanitation and hygienic (WASH) practices have been implemented to reduce on diarrhoeal related morbidity and mortality (Kotloff et al., 2017). This has led to significant reduction of global diarrhoeal related deaths by 60% among children under five years according to global burden disease report conducted in 2021 (Kyu et al., 2024). Nevertheless, the study reports regional disparities in this reduction where SSA recorded more than 130 deaths per 100,000 children under 5 years despite high adoption of the interventions (Kyu et al., 2024). This highlights that the aforementioned interventions though crucial are not sufficient to control diarrhoeal diseases in the region.

Recent research underscores the crucial role of the gut microbiome in maintaining gastrointestinal health, accounting for over 70% of its functional capacity (Aziz et al., 2024). A healthy gut microbiome has been shown to significantly reduce the prevalence of functional gastrointestinal disorders, including diarrhoea (Ballini et al., 2023). While dietary practices play a vital role in shaping the health of the gut microbiome (De Angelis et al., 2020), efforts to implement dietary interventions aimed at preventing gastrointestinal disorders remain largely inadequate due to lack of comprehensive dietary information. Addressing this gap could pave the way for improved digestive health and reduced disease burden regionally and globally.

2.2 Aetiological factors implicated in functional gastrointestinal disorders

The aetiology of FGIDs remains elusive but is hypothesised to involve factors such as altered immune response to intestinal microbiota in genetically predisposed individuals, environmental factors, gut microbiota integrity, and psychological factors (Chaudhry et al., 2023; Masaki et al., 2024; Richardson et al., 2018). Genetics are largely linked to structural gastrointestinal disorders (Yang et al., 2023). Studies highlight that individuals with a family history of GI diseases are likely to develop similar conditions. Genetic mutations in the nucleotide-binding oligomerisation domain-containing protein 2 (NOD2) and Tol like receptor signalling pathways lead to activation of chronic inflammatory processes (Masuta et al., 2023). Consequently, they alter normal colonic mucosa to malignant cells and are linked with gastrointestinal inflammatory diseases such as Crohn's disease, ulcerative colitis and duodenitis (Masaki et al., 2024). Additionally, alterations in the interleukin 23 receptor (IL-23R) cytokine gene, produced by macrophages and dendritic cells in response to gut microbial stimulation, have been linked to a heightened susceptibility to ulcerative colitis (Sewell & Kaser, 2022). IL-23R gene plays a role in the signalling pathway that governs the regulation of immune responses and inflammation (El Hadad et al., 2024).

Environmental factors, such as diet, lifestyle, and exposure to toxins, are the predominant contributors to gastrointestinal (GI) disorders, accounting for over 60% of the cases (Vedamurthy & Ananthakrishnan, 2019). These factors play a pivotal role in shaping the composition of the gut microbiota. Recent evidence highlights that, disruptions to the gut microbiota, or dysbiosis, are significant contributors to the rising prevalence of functional gastrointestinal disorders (FGIDs). Dysbiosis, characterised by an imbalance in the microbial community, has been closely associated with the development of various GI disorders (Talapko et al., 2022). For example, alterations in gut bacterial composition have been consistently observed in individuals with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Khan et al., 2019). Moreover, the gut microbiota influences critical physiological functions, including immune regulation, nutrient absorption, and gastrointestinal motility (Wei et al., 2021).

Exposure to toxic chemical compounds, including heavy metals such as lead, arsenic, and cadmium, often occurs through the consumption of contaminated food and water (Lu et al., 2014). These heavy metals interact with the gut microbiota, resulting in reduced abundance and diversity of beneficial bacteria (Tefferu et al., 2024). Lu et al. (2014) reported altered microbial abundance and diversity with reduced functional metabolites in mice exposed to arsenic. Richardson et al. (2018) also elucidated that higher doses of these heavy metals further exacerbate depletion of abundance and diversity of beneficial bacteria. This interaction negatively impacts the production of short-chain fatty acids (SCFAs) and stimulates the release of inflammatory cytokines (Gao et al., 2017). Consequently, these disruptions create an imbalance in the gut microbiota, predisposing an individual to higher risk of gastrointestinal disorders.

The use of both narrow and broad-spectrum antibiotics has become more widespread in addressing bacterial infections, as well as flu-like and cough symptoms. These medications, however, have both immediate and long-term detriment, including a reduction in microbial diversity and disruption of the gut microbiota's composition (Langdon et al., 2016). Reduced microbial diversity in the gut of children between 0-36 months who had several antibiotic treatments has been previously observed (Yassour et al., 2016). Similarly, gentamicin, and vancomycin antibiotic treatment among adults lead to overgrowth of *Enterobacteriaceae* and other pathobionts at the expense of beneficial bacteria such as *Bifidobacterium* species (Palleja et al., 2018). Palleja et al. (2018) further elucidated that although partial recovery of the microbiota may occur within 1.5 months, some species remain undetectable even after six months.

This indicates that long-term or potentially permanent disruptions to the microbial ecosystem can lead to a higher prevalence of functional gastrointestinal disorders (FGIDs) and antibiotic-associated diarrhoea. Additionally, it increases vulnerability to infections, promotes inflammation, proliferation of pathobionts and contributes to other various health conditions associated with an imbalanced and unhealthy gut microbiome (Becattini et al., 2016). These conditions can be reversed by probiotic therapy (Yang et al., 2024).

Dietary and lifestyle behaviours play a pivotal role in the development and prevention of gastrointestinal (GI) disorders (Corsello et al., 2020). For instance, planetary diets, characterised by their emphasis on whole or minimally processed plant-based foods, have protective effects against GI disorders (Breidenassel et al., 2022). This is attributed to their high content of phytochemicals and complex carbohydrates, which possess antioxidant, anti-inflammatory, and prebiotic properties (Kaur et al., 2021; Tanwar et al., 2023). On the other hand, diets dominated by ultra-processed foods, high sugar, and high fat content, despite being plant-based are strongly associated with an increased prevalence of GI disorders and diseases and cardiometabolic disorders (WHO, 2021).

Psychological factors such as stress, anxiety, and depression can significantly influence gastrointestinal health through the gut-brain axis. The gut-brain axis is a bidirectional communication network that links the central nervous system (CNS) and the gastrointestinal (GI) tract, playing a critical role in maintaining digestive function (Carabotti et al., 2015). Serotonin serves as key mediators within the gut-brain axis, connecting the gut microbiota, the CNS, and the enteric nervous system (Chaudhry et al., 2023). Gut microbiota metabolites such as SCFAs, and tryptophan derivatives stimulate the enterochromaffin cells which are specialised neuroendocrine cells to release serotonin (Gao et al., 2020). Remarkably, over 90% of the body's serotonin is produced in the gut, where it regulates intestinal motility and fluid secretion in addition to its role as the "feel-good" neurotransmitter, influencing mood, emotions, and overall well-being (Terry & Margolis, 2015).

Therefore, stress and emotional disturbances can disrupt this communication which leads to reduction in the production of serotonin. Consequently, individuals with chronic stress are always diagnosed with at least one functional gastrointestinal disorder due to reduced serotonin leading to symptoms of FGIDs such as IBS. Marano et al. (2025) linked exacerbated IBS symptoms to stress, anxiety and depression among women. This highlights the importance of a holistic approach in management of FGIDs in diagnosed individuals and acknowledging that each intervention is not a one size fits all.

2.3 Effect of varietal differences and processing methods on FODMAPs content in plant-based foods

Plant-based foods are central to global diets, providing essential macronutrients, micronutrients, and bioactive compounds (Sharifi-Rad et al., 2020). Many of these foods contain FODMAPs, a group of short-chain carbohydrates that are poorly absorbed in the small intestine (Ispiryan et al., 2020). FODMAPs considered in this study are Fructo-oligosaccharides (FOS), Galacto-oligosaccharides (GOS), polyols (sorbitol/mannitol) and excess fructose (Gibson et al., 2015). When consumed in excess, FODMAPs can trigger gastrointestinal symptoms such as bloating, abdominal pain, and altered bowel habits, particularly in individuals with functional gastrointestinal disorders (FGIDs) like irritable bowel syndrome (IBS) (Melilli et al., 2024). Despite their potential to induce symptoms, FODMAPs also possess prebiotic properties, supporting beneficial gut microbiota such as *Lactobacillus* and *Bifidobacterium* (Gibson et al., 2020). This dual role makes them nutritionally significant but clinically complex. Understanding how varietal differences and processing methods influence FODMAPs content is therefore critical for designing dietary strategies that balance symptom reduction with gut health benefits.

Crop variety is a critical determinant of FODMAPs composition, as genetic diversity among cultivars drives differences in carbohydrate metabolism, storage, and structural complexity (Amponsah et al., 2026). Scientific evidence shows substantial variation across crops: for instance, Italian durum wheat cultivars contain significantly lower levels of fructooligosaccharides (FOS) compared to modern bread wheat (Marotti et al., 2012), while Indian pigeon pea varieties (Raja et al., 2016) and American sesame varieties (Bhardwaj, 2014) show marked differences in galacto-oligosaccharide (GOS) concentrations. These varietal differences often arise from plant breeding technologies, which modify genetic traits and subsequently influence the degree of polymerisation and branching patterns of oligosaccharides (Huynh et al., 2007). As a result, strategic varietal selection represents a promising approach to lowering dietary FODMAPs intake while preserving the nutritional integrity of staple crops. But, crop variety studies largely focus on agronomic traits such as increased yield and environmental stress tolerance (Obua et al., 2020).

Food processing represents a promising strategy for modifying FODMAPs content through physical, chemical, and enzymatic mechanisms. Malting that uses enzymatic mechanism, hydrolyses oligosaccharides leading to reductions of more than 50% in GOS and FOS in cereals and legumes such as soybean (Ispiryan et al., 2020). Similarly, microorganisms during fermentation utilise FODMAPs to provide energy, generating lactic acid and other bioactive metabolites, thereby markedly lowering their levels (Ziegler et al., 2016). On the other hand, physical methods including thermal processing, disrupt carbohydrate structures and induce re-gelatinisation, which alters the overall FODMAPs profile (Olamiti, 2025). The effectiveness of these processing methods, vary depending on crop type and the FODMAPs composition.

Nevertheless, food processing methods reported to alter FODMAPs content have not been investigated in commonly consumed Ugandan crop varieties. Existing studies have largely emphasised physicochemical and sensory properties, leaving a critical gap in understanding their impact on FODMAPs modification (Bulut et al., 2022; Olamiti & Ramashia, 2025). Consequently, staple crops in Uganda and SSA remain under-characterised with respect to their fermentable carbohydrate profiles. Chapter three therefore provides baseline information on the FODMAPs composition of commonly consumed Ugandan crop varieties and identifies processing strategies, that can effectively reduce FODMAPs levels.

2.4 Effect of crop type and variety on prebiotic potential of FODMAPs extracted from plant-based foods

Whole grains and legumes are rich sources of complex carbohydrates particularly dietary fibre and diverse bioactive phytochemicals such as polyphenols and saponins (Tanwar et al., 2023; Tiwari et al., 2019). These compounds contribute significantly to human health by enhancing insulin sensitivity, modulating gut microbiota, and supporting colonic fermentation (Landry & Ward, 2024). Their physiological effects extend beyond gastrointestinal benefits, offering protective roles against metabolic disorders including obesity, hypertension, and type 2 diabetes (Hojsak et al., 2022).

The composition of FODMAPs in grains and legumes is influenced by factors such as monomeric sugar units, glycosidic linkages, and degree of polymerisation (Sancho et al.,

2017). Whole grain cereals like oats, barley, wheat, millet and sorghum are rich in fructo-oligosaccharides (FOS), including inulin, levan, and graminan, which differ in their glycosidic linkages between fructose residues (Matros et al., 2019). Cereals have considerable variability in fructan composition, primarily influenced by the type of glycosidic linkage between fructose units. Inulin-type fructans, characterised by β (2 $\rightarrow$ 1) linkages, are predominant in certain cereal varieties, while others contain higher proportions of levan-type fructans with β (2 $\rightarrow$ 6) linkages (Roberfroid, 2005; Tamura et al., 2009). Additionally, some cereals accumulate graminan-type fructans, which feature mixed linkage patterns and structural complexity for example wheat and barley (Verspreet et al., 2015; Yoshida, 2021).

In contrast, legumes for instance soybean, sesame, peas primarily contain galacto-oligosaccharides (GOS), galactomannans, and pectic polysaccharides as their major structural carbohydrates (Knudsen, 2014). Galactomannans consist of a mannose backbone with galactose side chains having β (1 $\rightarrow$ 4) glycosidic linkage (García et al., 2025), Pectic polysaccharides are rich in galacturonic acid residues that form complex networks within the plant cell wall (Anderson, 2019). Whole legumes contain approximately 5–8% galacto-oligosaccharides (GOS) in their dry matter, with compositional differences in raffinose (0.50–0.74%), stachyose (3.1–5.7%), and verbascose (0.12–0.2%) depending on plant variety (Kumar et al., 2010; Middelbos & Fahey, 2008).

Variation in oligosaccharide composition determines how these substrates are metabolised by microorganisms like lactic acid bacteria during fermentation. Differences in the degree of polymerisation of oligosaccharides across crop varieties influence fermentation kinetics, with some oligosaccharides undergoing rapid degradation in the colon while others are fermented more gradually (Gietl et al., 2012). This variability shapes the profile of fermentation metabolites and modulates their prebiotic attributes (Harris et al., 2020).

For a fermentable carbohydrate to qualify as a prebiotic, it must be selectively utilised by beneficial microbes such as lactic acid bacteria resulting in the production of health-promoting metabolites such as short-chain fatty acids (SCFAs) (Gibson et al., 2017). Despite this, most studies on crop varieties have focused primarily on agronomic traits, with limited

attention to their prebiotic potential. Consequently, dietary recommendations for prebiotic-rich crops as interventions against functional gastrointestinal disorders, including diarrhoeal diseases, remain underexplored. Chapter Four of this dissertation addresses this gap by conducting *in vitro* prebiotic evaluation of FODMAPs extracted from commonly consumed Ugandan crop varieties specifically millet, sorghum, sesame, and soybean.

2.5 Effect of ingredient type and fermentation time on genotypic characteristics of lactic acid bacteria in ready-to-use composite fermented beverages

The type of ingredients used as a substrate for developing fermented foods significantly influences the genotypic characteristics of lactic acid bacteria. Composite ingredients, particularly cereal–legume blends, are increasingly valued for their nutritional and functional properties (Fetriyuna et al., 2021; Tumuhimbise et al., 2025). Due to the diversity of carbohydrate substrates in such ingredients, they may also influence the diversity and genotypic characteristics of lactic acid bacteria. Nonetheless, there is limited information on genotypic characteristics of lactic acid bacteria isolated from the composite beverages. Lactic acid bacteria characteristics from plant-based fermentations reported are largely from single ingredients (Salmerón et al., 2014; Uhegwu & Anumudu, 2025).

It is important to note that both ingredient type and fermentation duration critically shape the genotypic characteristics of lactic acid bacteria (LAB) in these systems (Gautam et al., 2025). From majority of the plant-based fermentations reported, *Lactobacillus* currently reclassified into 25 genera remains the predominant genus associated with cereals, legumes, or fruits and vegetables (Cui et al., 2021; Zabat et al., 2018; Zheng et al., 2020). Furthermore, taxonomic classifications report *Lactiplantibacillus plantarum* as the dominant species (Iruene et al., 2021). This dominance is largely explained by differential expression of carbohydrate-metabolising genes, enabling versatile utilisation of complex substrates (Fuhren et al., 2020).

Lactiplantibacillus plantarum possesses a greater repertoire of carbohydrate-metabolising genes compared to many other lactic acid bacteria (Cui et al., 2021). These include diverse glycosyl hydrolases such as β -glucosidases, β -galactosidases, and β -fructofuranosidases, which enable the breakdown of disaccharides and oligosaccharides with β -glycosidic

linkages (Akiyama et al., 2015; Zhang et al., 2016). In addition, the *ccpA* gene encodes Catabolite Control Protein A (*ccpA*), a global regulator that directs preferential carbohydrate utilisation and thereby modulates fermentation pathways in *Lpb. plantarum* (Chen et al., 2019). This regulatory mechanism may be particularly enhanced in complex substrate systems such as cereal–legume composites.

Furthermore, *Lactobacillus* species harbour *gal* gene clusters that mediate GOS metabolism, while the *sacA/sacR1* regulatory system governs FOS metabolism (Chen et al., 2020). Together, these gene clusters enable efficient utilisation of prebiotic carbohydrates, although their expression is tightly regulated and highly dependent on substrate composition (Chen et al., 2019). This suggests that when legume–cereal composites are employed as substrates in the production of fermented beverages, both gene systems can be expressed in *Lactobacillus* species. Cereals predominantly supply FOS, while legumes are rich in GOS, thereby enabling simultaneous activation of the *sacA/sacR1* regulatory system and *gal* gene clusters (Chen et al., 2020; Fang et al., 2021). Such dual carbohydrate availability may enhance the genotypic diversity of *Lactobacillus* strains in the final fermented product. Nevertheless, although microbial communities in single-ingredient fermentations are well documented, there remains limited understanding of how substrate–substrate interactions such as cereal–legume FODMAPs synergies in millet–sesame–soybean or sorghum–sesame–soybean composites modulate the genotypic characteristics of lactic acid bacteria.

Furthermore, the composition of these species evolves with fermentation time, shaping the genetic landscape of the lactic acid bacteria community in the final product. As fermentation extends beyond 24 hours, the environment becomes harsher due to low pH and high osmotic pressure (Wang et al., 2019). This triggers a shift towards strains that produce exopolysaccharides and bacteriocins as stress response mechanisms (Nguyen et al., 2020). Although stress-response genes such as the *murC*, *eps/cps* clusters, *clp* proteases within the population may increase in frequency (Chapot-Chartier & Kulakauskas, 2014), limited studies in plant-based fermentations especially composite ingredient fermentations have yet to confirm this.

Chapter Five of the dissertation explores the genotypic characteristics of *Lactobacillus* species isolated from composite fermented beverages formulated with millet–sesame–soybean and sorghum–sesame–soybean substrates. This Chapter provides detailed insights into how such multi-substrate systems influence the genotypic characteristics of *Lactobacillus* species. By systematically examining these composite matrices, the Chapter highlights the role of cereal–legume FODMAPs synergies and fermentation duration in modulating genotypic attributes of lactic acid bacteria.

2.6 Probiotic potential of lactic acid bacteria strains isolated from ready-to-use fermented composites beverages

The predominant probiotic agents employed in plant-based food fermentations are *Saccharomyces cerevisiae* (commonly known as brewer’s yeast) and *Lactobacillus* genus (Amenu & Bacha, 2023; Goel et al., 2020; Ogunremi et al., 2015). Through fermentation, these microorganisms biochemically transform plant-derived substrates into products that are naturally enriched with bioactive compounds, vitamins, free from toxins, enhance organoleptic properties, improved nutritional quality, safety, and shelf stability (Kalui et al., 2010). Moreover, when ingested, these microorganisms provide probiotic benefits to the host and are generally recognised as safe for consumption (Hill et al., 2014).

For a microorganism to qualify as a probiotic, it must satisfy established criteria, including the ability to withstand harsh gastrointestinal and food-processing conditions, adhere to intestinal epithelial surfaces, and exert demonstrable health benefits on the host (Latif et al., 2023; Sionek & Szydłowska, 2025). Numerous microorganisms have been isolated from fermented foods such as buffalo milk, sorghum-millet beverage, vegetables and evaluated against these standards (Byakika et al., 2020; Liu et al., 2022; Shuhadha et al., 2017). These authors have reported that strains belonging to the *Lactobacillus* genus have consistently showed promising characteristics as potential probiotic candidates.

Advances in food processing and biotechnology have facilitated the development of novel fermented foods derived from underutilised substrates or combinations of substrates. Examples include fermented insect-based products and composite formulations such as bamboo shoot–soybean–sugar/salt blends (Chen et al., 2010; Siddiqui et al., 2023). Since

fermentation involves various carbohydrate substrates, the survival and dominance of probiotic strains in the final product depend heavily on their substrate-specific metabolic capabilities (Uhegwu & Anumudu, 2025). Variations in carbohydrate composition across substrates particularly in terms of FODMAPs play a critical role. Differences in the content and structural complexity of these carbohydrates, especially their degree of polymerisation, create variability in microbial utilisation patterns (Atzler et al., 2021; Paganini et al., 2017). Such compositional heterogeneity strongly influences both the range of bioactive metabolites produced during fermentation and the probiotic properties of the strains that dominate in the final product (Gonçalves et al., 2026).

In Uganda, composite food substrates have primarily been developed to enhance nutritional density (Alowo et al., 2018; Tumuhimbise et al., 2025). Nonetheless, they remain underexplored in probiotic research, particularly with respect to their fermentation into functional beverages. As a result, lactic acid bacteria isolated from these composite fermented beverages have largely not been evaluated for their probiotic potential. To address this gap, Chapter six of the dissertation investigates lactic acid bacteria isolates obtained from ready-to-use composite fermented beverages, assessing their probiotic properties against established scientific criteria.

CHAPTER THREE

Effect of varietal differences and processing methods on Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) content in millet, sorghum, soybean and sesame

This Chapter is adapted from:

Alowo, D., Olum, S., Mukisa, I. M., & Ongeng, D. (2025). Effect of thermal and non-thermal processing on Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) content in millet, sorghum, soybean and sesame varieties. *Frontiers in Nutrition*, 12, 1–11. <https://doi.org/10.3389/fnut.2025.1520510>

ABSTRACT

This study investigated the effect of processing (roasting and malting) and crop variety on Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) profile of millet, sorghum, soybean, and sesame varieties commonly consumed in Uganda. Two improved varieties and one indigenous variety for each crop were analysed. Monosaccharide and polyols content was determined by HPLC-UV method, while disaccharides and oligosaccharide were determined using Megazyme kits. The improved varieties of soybean (Maksoy 3N), Millet (Seremi 2) and sorghum (Narosorg 2) had significantly ($P < 0.05$) lower oligosaccharide content compared to indigenous varieties with percentage differences ranging from 10.2% to 73.9%. Additionally, Maksoy 3N and Narosorg 2 also recorded significantly lower ($P < 0.05$) excess fructose content compared to the indigenous variety. Malting was more effective than roasting ($P < 0.05$) in reducing FODMAPs categories and total FODMAPs content. Malting effectively reduced excess fructose in all grain types to the recommended levels of $<0.15\text{g}/100\text{g}$ compared to roasting. Moreover, malting reduced total oligosaccharides and total FODMAPs in soybean and sesame by more than 50%. Nevertheless, this reduction did not achieve the recommended threshold of $0.3\text{g}/100\text{g}$ for total oligosaccharides and $0.5\text{g}/100\text{g}$, for total FODMAPs which are a criterion to categorise low FODMAPs diets. Malting conditions should be optimised to enhance its effectiveness in producing low FODMAPs foods. This study underscores the importance of selecting suitable grain variety and processing technique that modify FODMAPs content in foods. This enables development of food products that can be used for dietary therapy for gastrointestinal disorders among vulnerable population.

Key words: Fermentable Oligo-Di-Monosaccharides and Polyols, Crop variety, Roasting, Malting

3.1 Introduction

Cereal grains, oil seeds and legumes such as wheat, rice, millet, sorghum, sesame, and soybean constitute significant sources of nutritionally valuable compounds globally. These compounds include protein, carbohydrates, fibre, vitamins, minerals, and functional bioactive compounds such as polyphenols, carotenoids, and oligosaccharides (Poutanen et al., 2022). The unique balance of bioactive components and prebiotics (FOS and GOS) in these grains have attracted substantial attention in the field of food and nutritional sciences. Bioactive compounds in plant-based foods have been associated with positive nutritional and health outcomes such as amelioration of non-communicable diseases and functional gastro-intestinal disorders (FGID) (Breidenassel et al., 2022; Kaur et al., 2021). Consequently, research focus in food and nutritional sciences has shifted from basic nutrition to exploring the health-promoting properties of foods. Understanding how these compounds contribute to overall health is crucial for developing effective dietary interventions especially in this era of personalised nutrition.

More than 70% of households in Sub-Saharan Africa, including Uganda, rely on these plant-based foods as significant sources of nutrients and bioactive compounds (Poutanen et al., 2022). To address the demand for plant-based foods and adapt to climate change, crop breeding technologies have been adopted to develop crop varieties that are high yielding and resilient to various environmental stressors (Obua et al., 2020). In Uganda, these improved varieties have predominantly replaced the indigenous crop varieties in diets and food processing industries. Improved crop varieties of sorghum (SESO and Narosorg series), soybean (Namsoy and Maksoy series), sesame (Sesim series), and millet (Seremi and Naromil series) have been highly adopted and consumed by Ugandan households (Obua et al., 2020; Owere et al., 2015). These varieties are preferred for their superior traits, including high yield, good fermentation and nutritional qualities, and increased resistance to pests, diseases and drought (Obua et al., 2020). They are widely utilised in both households and food industries as ingredients to produce a range of products including complementary foods, local dishes, non-alcoholic and alcoholic beverages (Diaz et al., 2019; Mukisa et al., 2010).

The health benefits associated with plant-based foods are strongly influenced by the composition and structure of various bioactive compounds such as prebiotics and polyphenols. For instance, cereal grains, and legumes contribute to improved gastrointestinal health due to their substantial amounts of prebiotic carbohydrates (Ispiryan et al., 2022). These carbohydrates, also known as Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs), undergo fermentation resulting in a reduced pH and increased concentration of short-chain fatty acids (Feng et al., 2018; Muir et al., 2009). These effects promote health by creating an unfavourable environment for pathogenic microorganisms and supporting the growth of beneficial gut microflora (Ribeiro et al., 2020). FODMAPs that are selectively utilised by host microorganisms, promoting their growth and producing health-beneficial metabolites, are generally classified as prebiotics (Gibson et al., 2017).

Nonetheless, not all FODMAPs possess prebiotic properties, as FODMAPs represent a broader category of fermentable carbohydrates, some of which can have adverse effects on the host. For instance, foods with high levels of fructose and polyols can lead to flatulence, bloating and abdominal distention (Lenhart & Chey, 2017) due to gas production and increased water volume by osmosis in the lower gastrointestinal tract (Bertin et al., 2024). Additionally, these substrates are associated with gut microbiota dysbiosis (Beisner et al., 2020). Therefore, characterisation and quantification of FODMAPs content in commonly consumed plant-based foods becomes inevitable if effective targeted dietary therapy is to be achieved.

The FODMAPs profiles of cereals, specifically wheat, rye, and barley, as well as legumes such as chickpeas, green peas, faba beans, soybean, red kidney beans and lentils, have been studied. These studies report FOS as the predominant FODMAPs in the bran of cereals while the legumes are rich in raffinose family GOS (Biesiekierski et al., 2011; Ispiryan, Zannini, et al., 2020). However, there is inadequate information on the FODMAPs profiles of other commonly consumed cereals in sub-Saharan Africa such as millet and sorghum. Similarly, there is limited data on the FODMAPs profiles of oil seeds such as sesame, pumpkin seeds, and sunflower seeds despite their frequent use in plant-based diets of various households. Furthermore, several studies have investigated the effects of food processing on cereals, legumes and oil seeds. These studies have examined the impact of processing on functional

properties, proximate and micronutrient composition, and sensory attributes (Alowo et al., 2018; Hidalgo-fuentes et al., 2024; Udeh et al., 2018). Nevertheless, there are limited studies (Galgano et al., 2023; Ispiryan, Kuktaite, et al., 2020; Nyyssölä et al., 2020) focused on processing techniques specifically aimed at altering FODMAPs content in cereals, legumes and oil seeds to meet the recommended reference cut off values for a low FODMAPs diet (Varney et al., 2017), which is commonly used to alleviate FGIDs symptoms. None of the above studies focused on millet, sorghum, soybean and sesame varieties despite their significant inclusion in sub-Saharan African diets.

Studies on how varietal differences influence FODMAPs content of these crops are limited. Existing studies evaluating varietal effects resulting from breeding techniques have predominantly focused on yield stability and adaptability to varying environmental conditions (Obua et al., 2021; Tesfaye et al., 2021). These techniques primarily alter the degree of polymerisation and, consequently, the structural composition of the grains (Karnpanit et al., 2016). However, the extent to which varietal differences influence individual FODMAPs remains largely unknown. Additionally, it is important to note that the quality and quantity of FODMAPs in legumes, oil seeds and cereal grains are influenced by various environmental conditions and varieties across the globe (Varney et al., 2017). Thus, extrapolating data from one continent to another may result in erroneous estimates, either overestimating or underestimating the FODMAPs content. Consequently, this renders dietary interventions for specific nutritional diseases, such as FGIDs, less effective. Moreover, it makes it challenging to achieve effective personalised nutrition. This study, therefore investigated the effect of thermal and non-thermal processing and crop variety on the FODMAPs profile of millet, sorghum, soybean, and sesame varieties commonly consumed in Uganda.

3.2 Materials and methods

3.2.1 Study area and study design

The study samples comprised indigenous crop varieties and commercial fermented beverages sourced from northern Uganda. Indigenous varieties were collected from Gulu, while commercial fermented beverages were obtained from both Gulu city and Lira city.

Experimental samples were allocated to treatments using a completely randomized design to minimize bias and ensure statistical validity in evaluating treatment effects. The experimental samples were analysed from Gulu University Multifunctional Research Laboratories.

3.2.2 Procurement of reagents and chemicals

Sugar standards (monosaccharide kit 47267, sugar alcohol kit 47266) and other chemicals (acetonitrile, methanol, sodium borohydride, *p*-aminobenzoic ethyl ester ABEE-derivatising agent, HPLC-grade chloroform) were purchased from Sigma Aldrich, USA. Analytical grade glacial acetic acid was sourced from Merck (Millipore, Darmstadt, Germany). HPLC grade water and ultra-pure water were obtained from water purification system (Evoqua, Ultrapure water system Art. No. W3T364778, Germany). All chemicals and reagents were stored following the manufacturer's instructions.

3.2.3 Selection of samples

This study focused on four crops, including two cereals: finger millet (*Eleusine coracana*) and sorghum (*Sorghum bicolor*), one legume: soybean (*Glycine max*) and one oil seed: sesame (*Sesamum indicum*). These grains are recognised sources of FODMAPs with prebiotic potential including FOS (fructo-oligosaccharides) and GOS (galacto-oligosaccharides) (Kaur et al., 2021). Each grain type comprised of two improved varieties and one indigenous variety commonly consumed by Ugandan Households. The commonly consumed indigenous varieties were determined by collecting preliminary data through market survey within Gulu city. The indigenous grains were collected from five different grocery and produce stores across various markets in Gulu City, northern Uganda. Equal quantities of each grain were combined to make 2 kg samples for each type. The selected improved varieties originated from two national plant breeding institutes: the National Semi-Arid Resources Research Institute (NASARRI), Serere district, Uganda, and the Ngetta Zonal Agricultural Research and Development Institute, Lira district, Uganda.

To ensure seasonal representativeness and capture maximum genetic diversity, variety selections were made across four consecutive cropping seasons over a two-year period (2021

and 2022). These seasons encompassed both the first and second cropping cycles of each year. Data and selections from these four distinct cropping seasons were subsequently pooled for the final analysis.

The varietal selection followed a structured, multi-stage screening process. First, each grain type comprised of two improved varieties and one indigenous variety to capture both modern and traditional profiles. Second, the selection pool was restricted to varieties commonly consumed by Ugandan households to ensure socio-economic relevance. Finally, the definitive varieties were selected based on their specific dual-purpose suitability for both food consumption and development of fermented beverages, in accordance with established criteria (Hamba et al., 2024; Lubadde et al., 2019)

3.2.4 Sample treatment

Individual grains were thoroughly sorted and cleaned with potable water. The samples were subsequently oven-dried (Memmert, UN260, Schwabach FRG, Germany) at 50 °C for 24 hours to a consistent weight. Approximately 500 g of each dried grain was subjected to either thermal processing (dry heat-roasting) or non-thermal processing (malting), while the unprocessed grains served as control. For thermal processing, the method described by Kim et al. (2019) was followed with minor adjustments in roasting temperatures and holding time. Specifically, the grains were roasted on an electric hot plate (DLAB MS-H280-Pro, Los Angeles USA) at temperatures ranging from 90°C to 100°C, with a holding time of 10 minutes for sorghum, millet, and sesame, and 20 minutes for soybeans. The use of a temperature range (90–100 °C) was to reflect the practical variability inherent in grain roasting systems, where maintaining a perfectly constant temperature is not feasible. These conditions are considered adequate to significantly reduce the beany flavour and antinutritional factors in the grains while preserving nutritional quality (Agume et al., 2017).

Regarding malting, the method described by Syeunda et al. (2019) was employed. Briefly, cleaned grains were steeped in potable water (500 g of grain to 1.5 L of water) for 24 hours. The steeping water was changed every 6 hours to ensure uniform air circulation and reduce enzyme inhibitors such as tannins, and the final water was drained prior to sprouting (Osuntogun et al., 1989). Subsequently, they were allowed to sprout at an ambient

temperature (25-27°C) for 48 hours. During the sprouting stage, the grains were uniformly mixed and periodically moistened to facilitate homogenous sprouting and temperature regulation. After 48 hours, the sprouted grains were kilned in an oven at 50°C until a moisture content of 10% was achieved. Finally, the raw, roasted, or malted grains were each ground using a laboratory blender (Neo-Tech SA, Milmort, Belgium) and passed through a 300 µm screen. The flour samples were sealed in high-density polyethylene bags and stored at 8°C for 14 days prior to subsequent analysis.

The study recognizes that factors such as growing and processing conditions, can influence FODMAPs composition. Therefore, these conditions were standardized across all samples to minimize potential confounding effects, allowing crop variety to remain the primary source of variation evaluated in this study.

3.2.5 Carbohydrate extraction

The water and ethanol extraction method were used as described by Schmidt and Sciarba (2021) with minor modifications. Briefly, 2.0 g of each sample was mixed with 4 ml of 80% ethanol (v/v) to inactivate amylase and prevent excessive starch hydrolysis during the extraction process (Ziegler et al., 2016). Exactly 20 ml of distilled water was added followed by sonication (GT SONIC-D9, Beijing, China) at room temperature for 3 minutes. Sonication was conducted to enhance percentage yield during the extraction process (Annegowda et al., 2012). The mixture was then centrifuged (VWR Micro star 17, 521-1646, Berlin, Germany) at 3000 x g for 5 minutes and the resulting supernatant was collected. FODMAPs were further re-extracted from the residue with 20 ml of distilled water. The supernatants for the two extractions were pooled and made up to 100 ml. Each sample underwent three independent extractions, with each extraction replicated twice during analysis to account for variability in the extraction and analysis process.

3.2.6 HPLC determination of monosaccharide and polyol content in the samples

3.2.6.1 Preparation of standard and sample solution

Standards (Glucose, fructose, sorbitol and mannitol) and sample solutions were prepared according to the method of Jalaludin and Kim (2021). The sample extracts were derivatised

with *p*-aminobenzoic acid ethyl ester (ABEE) according to the method described by Debebe et al. (2018). Briefly, ABEE stock solution was prepared by dissolving 2 g of ABEE agent in 4 ml of methanol in a 1 L glass tube followed by addition of 310 ml of glacial acetic acid. The mixture was completely dissolved at 45°C in a water bath and left to cool to room temperature. Upon cooling, 0.6 g of sodium borohydride were added, and the solution was vortexed for 30 s to yield an ABEE stock solution.

A 2 ml aliquot of the ABEE stock solution was mixed with 500 µL of either standard or sample solution and incubated at 80°C for 1 hour. The mixture was cooled under running water and centrifuged (Eppendorf AG Barkhausenweg 1 22339 Hamburg, Germany) at 4000 x g for 1 minute. Exactly 3 ml of HPLC grade water was added to the supernatant followed by addition of 3 ml of chloroform to extract the aqueous phase. The mixture was vortexed for 30 s and again centrifuged at 4000 x g for 1 minute. The upper layer was collected, centrifuged (4000 x g for 1 minute), and filtered into HPLC vials using a 0.22µm nylon syringe filter (AVF-100C-NY, Dubai, UAE) for HPLC analysis.

3.2.7 HPLC analysis

HPLC was performed following the procedure of Debebe et al. (2018) with a slight modification in conditions. The analysis was performed using a C18 column (Luna^R 5 µm C18 100 Å, LC Column 250 x 4.6 mm) and UV detector at λ_{max} 190 nm. This wavelength yielded a consistent correlation coefficient of 0.99 for both monosaccharides and polyols indicating good detector response. Two mobile phases (75% Acetonitrile as solvent A and HPLC water as solvent B) were used in a gradient elution mode. The gradient conditions were 0–4 min with 65% solvent A, which linearly increased to 80% within 5–10 min followed by maintenance at 80% for 11-12 min with a mobile-phase flow rate of 0.5 ml·min⁻¹. The sample injection volume was 10 µL with the column temperature set at 40°C.

The data was evaluated using a calibration method where standards (Glucose, fructose, sorbitol, and mannitol) of 6 different concentrations ranging between 0.039-10 µg/ml were used to create calibration curves. A correlation coefficient of greater than 0.98 was accepted. The calibration curve was used to determine the limit of quantification (LOQ) of the standards using equation 3.1. The precision of the analysis was checked after 4 repeated

injections of each standard. The set of the standards was run at the start and at the end of the sample running session to allow correction of any drift in the elution profile. Quantitative determination of the individual monosaccharide was by external standard method. The summary of LOQ ranges, correlation coefficients and R^2 values for each sugar standard are indicated in Table 3.1. Chromatograms obtained were analysed using LabSolutions CS software version 5 (Shimadzu LC-2050, Tokyo Japan) and the concentration of the corresponding sugar was calculated by obtaining the area under the curve.

$$LOQ = 10 \times (\text{standard deviation of intercept} \div \text{slope}) \dots \dots \dots \text{Equation 3.1}$$

Table 3.1: The Correlation coefficient, R^2 and Limit of Quantification ranges of sugar standards

Compound	Correlation coefficient R	R^2	Equation	LOQ ($\mu\text{g/ml}$)
Fructose	0.999	0.999	$y=13226x-3433.5$	1.77-10
Glucose	0.998	0.997	$y=21586x+1813.5$	3.276-10
Sorbitol	0.998	0.997	$y=22780x+1728.9$	3.56-10
Mannitol	0.996	0.993	$y=22202x+9256.8$	5.1-10

3.2.8 Determination of disaccharide and oligosaccharide contents

Disaccharide and oligosaccharide content in the samples were prepared using Megazyme kits and determined by UV-VIS spectrophotometer (Jenway 6705, Milmort Belgium). K-RAFGL kit (last updated August 2023) was used to determine sucrose, and galacto-oligosaccharide content whereas total fructo-oligosaccharide was determined by K-FRUC kit (last updated November 2022) (Megazyme, Bray Ireland). During determination of Fructo-oligosaccharides, α -galactosidases (Megazyme cat. no. EAGLANP) was used to eliminate interfering GOS. The kits were used following manufacturer's instructions. A calibration curve was created for 6 different concentrations of each standard and LOQ for the compounds was determined using equation 3.1. The LOQ ranges of FOS and GOS were between 27.1-250 $\mu\text{g/ml}$ and 123.3-250 $\mu\text{g/ml}$ and the correlations coefficients (R) were 0.998 and 0.995, respectively. The R^2 values and equations were 0.997, $y=0.0099x+0.0222$ and 0.991, $y=0.0044x+0.0479$, for FOS and GOS, respectively.

3.2.9 Determination of total FODMAPs content in Millet, Sorghum, Soybean and Sesame

The total FODMAPs content in the different grains and seeds was calculated according to Varney et al. (2017). To determine total FODMAPs content, the values of individually quantified monosaccharides, polyols, and oligosaccharides were summed. Methodological variations between the chromatographic (HPLC) and enzymatic spectrophotometric workflows (Megazyme K-FRUC and K-RAFGl) were controlled by using an identical crude starting extract for all analyses. Chromatographic overlap was prevented by the specialized chemical reduction of free sugars in the K-FRUC protocol and the background baseline subtraction of free monomers in the K-RAFGl calculations. All data were harmonized to a standard unit baseline (g/100g). Methodological compatibility was statistically validated by restricting summation exclusively to analytes exceeding established limits of detection.

3.2.10 Statistical analysis

Data for FODMAPs content was analysed using one-way Analysis of Variance (ANOVA). ANOVA was applied to determine statistical significance between crop varieties and processing methods with respect to their FODMAPs content. Specifically, the statistical runs compared the mean concentrations of individual FODMAPs groups across the different crop varieties (millet, sorghum, sesame, and soybean) and processing methods investigated. Tukey's HSD test was then used to identify which pairs of crop varieties or processing method differed significantly at $\alpha = 0.05$. Prior to performing ANOVA, the test for homogeneity of variance was confirmed using Levene's test ($F(2, 15) = 1.62$; $p = 0.230$) and normal distribution was ascertained using Shapiro-Wilk test ($W = 0.843$, $p = 0.137$).

To determine whether processing could produce a low-FODMAPs food, the FODMAPs categories of excess fructose, total oligosaccharides, and total FODMAPs were compared against established standard reference cut-off values (Varney et al., 2017) using a One-Sample *t*-test. The reference thresholds utilized for classification as a low-FODMAPs food were (<0.15 g/100g for excess fructose, <0.30 g/100g for total oligosaccharides, and <0.50 g/100g for total FODMAPs) based on Monash University guidelines. All statistical analyses

were performed using SPSS software version 25.0 (SPSS Inc., Chicago, IL). Finally, graphical illustrations were generated using Sigma Plot version 11.0.

3.3 Results

3.3.1 Characterisation of Fermentable Oligo-Di-Monosaccharides and Polyols in selected grains and seeds

Table 3.2 illustrates variations in FODMAPs content according to seed type. Generally, the FODMAPs profiles were influenced by crop type. Glucose was significantly higher ($P < 0.05$) in millet compared to other crops. Fructose on the other hand was not significantly different from the crops except in soybean that registered a significantly lower ($P < 0.05$) fructose content by 0.26-0.29 g/100g. For disaccharides, sucrose content in soybean was about 3 times higher than in sesame, sorghum and millet. Galacto-oligosaccharides were significantly ($P < 0.05$) different between crop types. Among the crops studied, only soybean showed significant variation in FOS content ($P < 0.05$). Soybean had the highest GOS and FOS content followed by sesame. Sugar polyols were below the limit of quantification values for all grains and seeds under study.

Table 3.2: Characterisation of FODMAPs profiles of Millet, Sorghum, Soybean and Sesame

Crop type	Monosaccharides/Disaccharides (g/100g)				Polyols (g/100g)	Oligosaccharides (g/100g)	
	Glucose	Fructose	Excess fructose	Sucrose	Sorbitol/mannitol	Total GOS	Total FOS
Soybean	0.05 ^b ±0.03	0.08 ^b ±0.04	0.03 ^b ±0.06	3.37 ^a ±0.4	< LOQ	4.43 ^a ±0.85	3.7 ^a ±0.43
Millet	0.28 ^a ±0.15	0.37 ^a ±0.05	0.09 ^{ab} ±0.19	0.51 ^c ±0.17	< LOQ	< LOQ	0.57 ^b ±0.33
Sorghum	0.14 ^b ±0.03	0.34 ^a ±0.05	0.2 ^a ±0.08	0.41 ^c ±0.07	< LOQ	0.22 ^c ±0.1	0.63 ^b ±0.48
Sesame	0.07 ^b ±0.04	0.35 ^a ±0.09	0.28 ^a ±0.11	0.97 ^b ±0.09	< LOQ	1.93 ^b ±0.43	0.83 ^b ±0.23

Values show mean ± SD (n=6) for each crop type; values in the same column with different superscript are significantly different ($p < 0.05$); Excess Fructose=Fructose-Glucose; LOQ-Limit of Quantification (sorbitol LOQ = 3.56 µg/ml, mannitol LOQ = 5.1 µg/ml & total GOS LOQ = 27.1 µg/ml)

3.3.2 Effect of crop variety on the contents of Fermentable Oligo-Di-Monosaccharides and Polyols

Significant differences ($P < 0.05$) in FODMAPs content were observed between improved and indigenous varieties, with varying magnitudes (Table 3.3). The improved soybean (Maksoy 3N) and sorghum varieties, (Narosorg 2) had significantly lower ($P < 0.05$) excess

fructose content compared to the indigenous variety. The excess fructose content in Narosorg 2 was significantly lower by 0.12 and 0.17 g/100g compared to the indigenous and Narosorg 4 varieties, respectively.

Similarly, the improved varieties of soybean, sorghum and millet recorded significantly lower ($P < 0.05$) total oligosaccharide content except in Naromil 2 millet variety which recorded significantly higher ($P < 0.05$) values. Specifically, the indigenous variety of sorghum had significantly higher ($P < 0.05$) GOS (by 56%), and FOS (by 73.9%) compared to the improved varieties. Additionally, the percentage difference in GOS and FOS content between the indigenous soybean variety and the improved varieties ranged from 10.2% to 38.3%. Conversely, oligosaccharide content in sesame improved and indigenous varieties were not significantly different ($P > 0.05$). By implication therefore, varietal effect due to crop improvement technologies such as conventional breeding had a non-uniform effect on FODMAPs content within crop types.

Table 3.3: Influence of crop variety on Fermentable Oligo-Di-Monosaccharides and Polyols content in Millet, Sorghum, Soybean and Sesame

Crop	Variety	Monosaccharides (g/100g)			Disaccharides (g/100g)	Oligosaccharides (g/100g)	
		Glucose	Fructose	Excess fructose	Sucrose	Total GOS	Total FOS
Soybean	Maksoy 3N	0.06 ^a ±0.01	0.03 ^b ±0.01	0 ^b	3.78 ^a ±0.05	4.56 ^a ±0.09	3.52 ^a ±0.03
	Maksoy 6N	0.06 ^a ±0.02	0.09 ^b ±0.04	0.03 ^a ±0.02	3.31 ^b ±0.28	3.48 ^b ±0.16	3.80 ^a ±0.32
	Indigenous variety-soybean	0.08 ^a ±0.10	0.12 ^a ±0.01	0.04 ^a ±0.01	3.03 ^b ±0.07	5.13 ^a ±0.44	3.90 ^a ±0.58
Millet	Naromil 2	0.14 ^c ±0.03	0.40 ^a ±0.01	0.26 ^a ±0.02	0.7 ^a ±0.05	0.03±0.17	0.81 ^a ±0.19
	Seremi 2	0.27 ^b ±0.01	0.39 ^a ±0.01	0.12 ^a ±0.01	0.49 ^b ±0.01	< LOQ	0.4 ^b ±0.19
	Indigenous variety-millet	0.47 ^a ±0.07	0.31 ^a ±0.06	0 ^b	0.32 ^c ±0.09	< LOQ	0.58 ^b ±0.31
Sorghum	Narosorg 2	0.18 ^a ±0.01	0.26 ^b ±0.05	0.09 ^b ±0.05	0.31 ^b ±0.02	0.18 ^b ±0.02	0.52 ^b ±0.08
	Narosorg 4	0.12 ^b ±0.02	0.38 ^a ±0.01	0.26 ^a ±0.01	0.45 ^a ±0.05	0.17 ^b ±0.08	0.21 ^b ±0.03
	Indigenous variety-sorghum	0.13 ^b ±0.01	0.34 ^a ±0.04	0.21 ^a ±0.04	0.48 ^a ±0.02	0.32 ^a ±0.06	1.13 ^a ±0.29
Sesame	Sesim 2	0.10 ^a ±0.04	0.25 ^a ±0.14	0.16 ^a ±0.18	1.00 ^a ±0.08	2.30 ^a ±0.25	0.86 ^a ±0.07
	Sesim 3	0.06 ^a ±0.04	0.32 ^a ±0.10	0.26 ^a ±0.11	0.9 ^a ±0.05	1.86 ^{ab} ±0.04	0.73 ^a ±0.19
	Indigenous variety-sesame	0.05 ^a ±0.01	0.33 ^a ±0.1	0.27 ^a ±0.08	0.98 ^a ±0.07	1.5 ^b ±0.24	1.0 ^a ±0.37

Values show mean ± SD (n=6) for each grain variety; values in the same column with different superscript are significantly different ($P < 0.05$); Excess Fructose=Fructose-Glucose; LOQ; Limit of Quantification (Total GOS = 27.1 µg/ml).

3.3.3 Effect of processing on the contents of Fermentable Oligo-Di-Monosaccharides and Polyols

Table 3.4 shows the effect of processing techniques on FODMAPs contents in oil seeds, cereals, and legume grains investigated. The results indicate that the type of processing significantly ($P < 0.05$) influenced FODMAPs quantities, regardless of grain type albeit at varying magnitude. Malting had a greater potential to reduce FODMAPs in millet, sorghum, soybean and sesame compared to roasting. Malting significantly ($P < 0.05$) increased glucose levels across all grains, with the highest increase observed in sorghum (428.6%) and millet (250%). Conversely, malting significantly ($P < 0.05$) reduced fructose levels in millet, sorghum, and sesame, with sorghum showing the greatest reduction (79.4%), while soybean had an increase of over 100%. Additionally, malting significantly ($P < 0.05$) reduced galacto-oligosaccharides (GOS), especially in soybean (71.6%) and sesame (62.2%) whereas no significant reduction in GOS was observed in sorghum and millet. The effect of malting on fructo-oligosaccharides (FOS) was variable, increasing in cereals (11.1% in sorghum, 63.2% in millet) but significantly reducing in sesame and soybean by 14.5% and 68.9%, respectively.

In contrast, roasting did not significantly affect GOS and FOS irrespective of the crop type but resulted in a significant ($P < 0.05$) increase in fructose levels across all grains, ranging from 21.6% to 512.5%. Soybean and sesame showed the highest increase.

Table 3.4: Effect of processing on FODMAPs content in Millet, Sorghum, Soybean and Sesame

Crop	Type of processing	Monosaccharides/Disaccharides (g/100g)			Oligosaccharides (g/100g)		Total FODMAPs (g/100g)
		Glucose	Fructose	Excess fructose	Total GOS	Total FOS	
Soybean	Unprocessed	0.05 ^b ±0.03	0.08 ^c ±0.04	0.03 ^c ±0.06	4.43 ^a ±0.85	3.70 ^a ±0.43	8.16 ^a ±0.85
	Roasting	0.07 ^{ab} ±0.04	0.49 ^a ±0.03	0.41 ^a ±0.05	4.34 ^a ±0.95	3.59 ^a ±0.41	8.34 ^a ±1.28
	Malting	0.14 ^a ±0.06	0.28 ^b ±0.03	0.15 ^b ±0.05	1.26 ^b ±0.90	1.15 ^b ±0.73	2.56 ^b ±2.95
Millet	Unprocessed	0.28 ^b ±0.15	0.37 ^{ab} ±0.05	0.09 ^a ±0.19	< LOQ	0.57 ^a ±0.33	0.65 ^a ±0.41
	Roasting	0.3 ^b ±0.14	0.45 ^a ±0.1	0.17 ^a ±0.15	< LOQ	0.48 ^a ±0.44	0.65 ^a ±0.37
	Malting	0.98 ^a ±0.33	0.27 ^b ±0.15	0 ^b	< LOQ	0.93 ^a ±0.38	0.93 ^a ±0.38
Sorghum	Unprocessed	0.14 ^b ±0.14	0.34 ^b ±0.05	0.2 ^a ±0.08	0.22 ^a ±0.10	0.63 ^a ±0.48	1.05 ^a ±0.58
	Roasting	0.17 ^b ±0.17	0.42 ^a ±0.04	0.25 ^a ±0.09	0.30 ^a ±0.14	0.36 ^a ±0.13	0.91 ^a ±0.25
	Malting	0.74 ^a ±0.74	0.07 ^c ±0.03	0 ^b	0.20 ^a ±0.15	0.70 ^a ±0.86	0.90 ^a ±0.89
Sesame	Unprocessed	0.07 ^b ±0.04	0.35 ^{ab} ±0.09	0.28 ^a ±0.11	1.93 ^a ±0.43	0.83 ^a ±0.23	3.04 ^a ±0.36
	Roasting	0.04 ^b ±0.02	0.45 ^a ±0.09	0.41 ^a ±0.10	2.46 ^a ±0.41	0.49 ^a ±0.28	3.36 ^a ±0.64
	Malting	0.18 ^a ±0.03	0.26 ^b ±0.07	0.08 ^b ±0.08	0.73 ^b ±0.65	0.71 ^a ±0.54	1.52 ^b ±0.65

Values show mean ± SD (n=6) for each grain type. Means in the same column with different superscripts are significantly different ($P < 0.05$). Excess Fructose = Fructose - Glucose. Total FODMAPs = (Excess Fructose + total GOS + total FOS); LOQ; LOQ-Limit of Quantification (total GOS LOQ = 27.1 µg/ml)

3.3.4 The potential of roasting and malting in achieving low FODMAPs Food Products

Figures 3.1, 3.2 and 3.3 illustrate the potential of roasting and malting in reducing FODMAPs to the recommended threshold values for each FODMAPs category with the objective of obtaining a low FODMAPs food product. Malting effectively reduced excess fructose in all grain types to the recommended levels of $<0.15\text{g}/100\text{g}$, a reduction which was not achieved by roasting (Fig 3.1). The amount of excess fructose obtained after malting was not significantly different ($P > 0.05$) from the reference cut-off value for soybean but was significantly lower ($P < 0.05$) in sesame, millet and sorghum. After malting, millet and sorghum did not have any excess fructose content. Furthermore, malting had a greater effect on reducing the total oligosaccharides and total FODMAPs in soybean and sesame ($>50\%$ reduction) although this reduction did not result in attaining the recommended threshold of $0.3\text{g}/100\text{g}$ (Fig 3.2) and $0.5\text{g}/100\text{g}$ (Fig 3.3), respectively. Specifically, the reduction of both total oligosaccharides and total FODMAPs remained significantly higher ($P < 0.05$) in all crop types than the reference cut-off values.

Conversely, roasting significantly increased ($P < 0.05$) excess fructose, total oligosaccharide, and total FODMAPs content in soybean, sesame, and sorghum compared to the reference cut-off values. The percentage differences ranged from 50% to 185.4%, with sorghum registering the lowest and soybean the highest significant differences from the reference cut-off values.

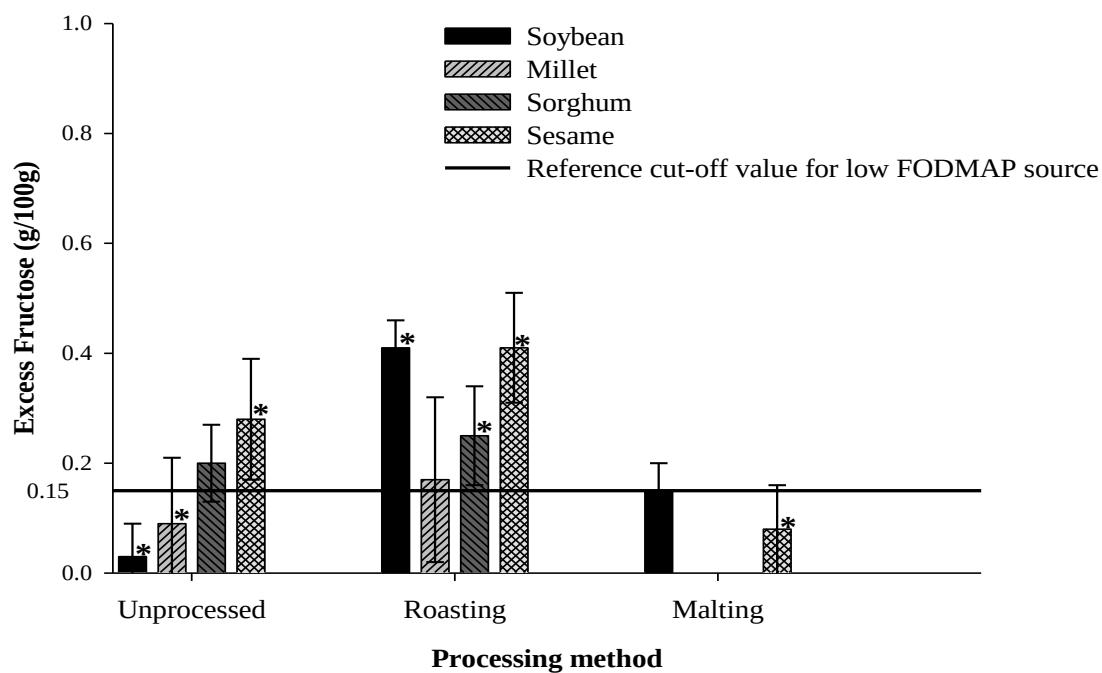


Figure 3.1: Effect of processing on excess fructose content ratings of millet, sorghum, soybean and sesame compared to the Monash University reference cut-off value for a low FODMAPs food (Varney et al., 2017). Values are means of three independent determinations. Error bars represent standard deviation. Graphs with asterisk are significantly different ($P < 0.05$) from the reference cut-off value.

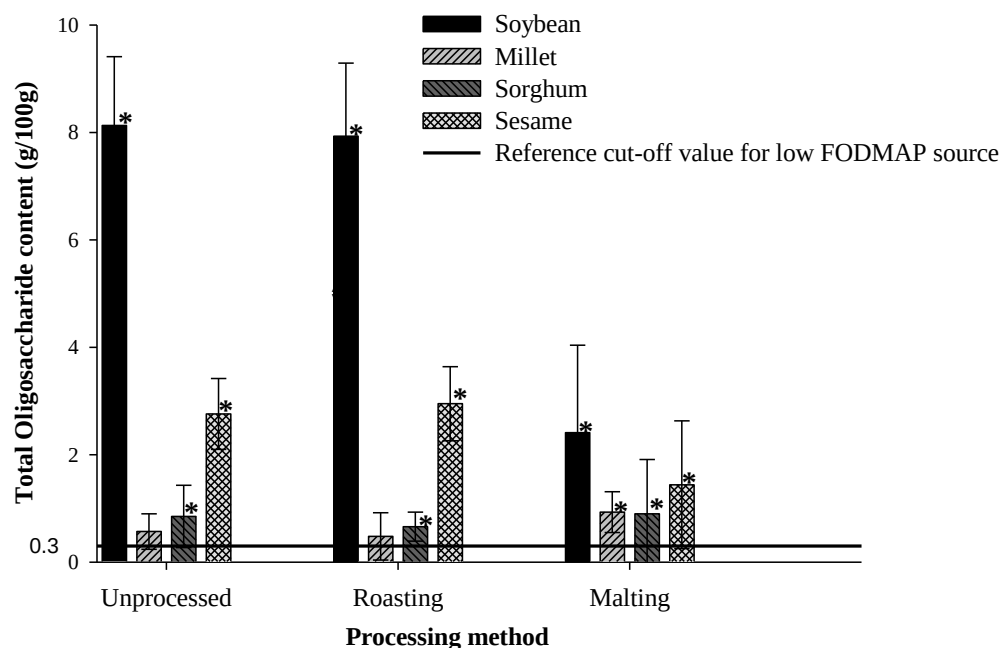


Figure 3.2: Effect of processing on total oligosaccharide content ratings of millet, sorghum, soybean and sesame compared to the Monash University reference cut-off value for a low FODMAPs food (Varney et al., 2017). Values are the means of three independent determinations. Error bars represent standard deviation. Graphs with asterisk are significantly different ($P < 0.05$) from the reference cut-off value.

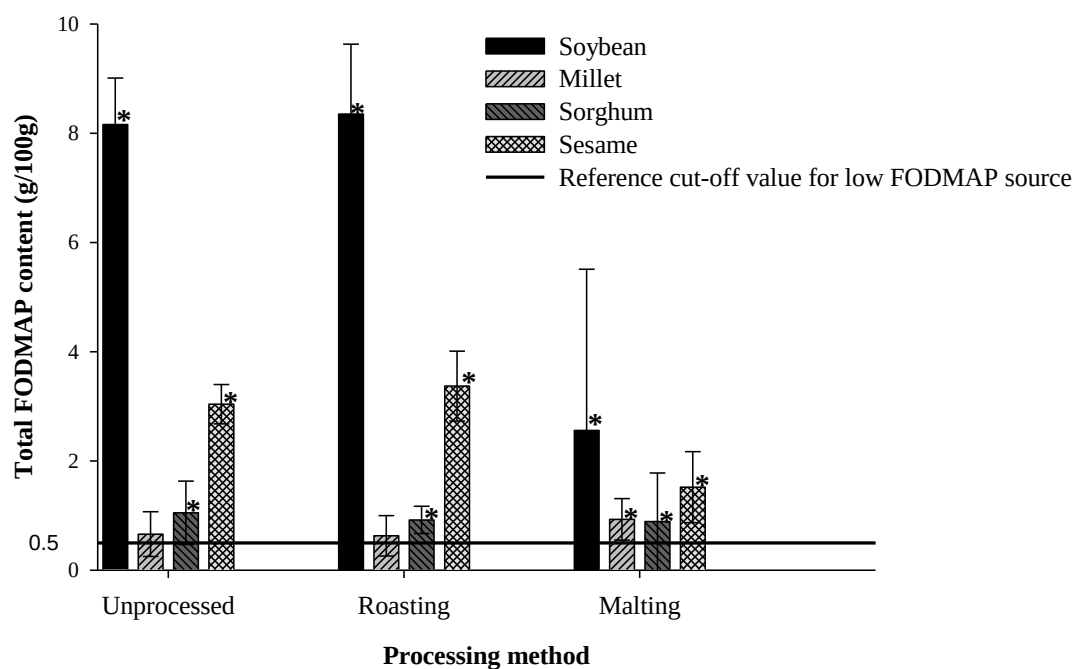


Figure 3.3: Effect of processing on total FODMAPs content ratings of millet, sorghum, soybean and sesame compared to the Monash University reference cut-off value for a low FODMAPs food (Varney et al., 2017). Values are the means of three independent determinations. Error bars represent standard deviation. Graphs with asterisk are significantly different ($P < 0.05$) from the reference cut-off value.

3.4 Discussion

3.4.1 Characterisation of Fermentable Oligo-Di-Monosaccharides and Polyols in selected grains and seeds

Understanding the characteristics of fermentable oligo-di-monosaccharides and polyols in cereal, legume and oil seeds provides an insight to allow for: (i) selection of nutritious and functional crop type for use in dietary modification therapies (Galgano et al., 2023), (ii) enhancing the content of bioactive compounds through food processing (Melilli et al., 2024), and (iii) updating the food composition tables (Liljebo et al., 2020). The characteristics of FODMAPs reported in this study delineated sesame, soybean, sorghum and millet as crops with varying quantities of FODMAPs (Table 3.2). They can be used as good candidates for modification through processing to develop products with lower FODMAPs content as dietary interventions for FGIDs. This information can also be used to update information in the current food composition tables for East and central Africa because these crop varieties are consumed in the two African regions (Mirriam et al., 2023).

The results obtained in the current study are comparable with those of Ispiryan et al. (2020) who reported legume such as peas and beans to contain substantial quantities of galacto-oligosaccharide (between 4.48 to 4.87 g/100g). Galacto-oligosaccharides and fructo-oligosaccharides are known prebiotic substrates that modulate gut microbiota and inhibit pathogen growth (Guarino et al., 2020). Conversely, the higher content of fructose (Table 3.2) in sorghum, millet and sesame may contribute to fructose-related gastrointestinal digestive disorders such as increased motility and flatulence in sensitive individuals. This is further emphasised by Larke et al. (2023) who reported increased gastrointestinal inflammation among adults who frequently consumed fructose rich diets.

These findings highlight the dual role of FODMAPs components in gastrointestinal health emphasising the necessity for personalised dietary recommendations tailored to individual needs. For instance, individuals predisposed to gastrointestinal disorders may benefit from a diet rich in oligosaccharides such as those in soybean (Maksoy 3N). Conversely, individuals sensitive to monosaccharides should limit their intake of foods like sorghum and sesame. This personalised approach could enhance overall gut health and reduce the risk of

digestive disorders contributing to the achievement of Sustainable Development Goal 3, which aims at achieving good health and well-being.

3.4.2 Effect of crop variety on the contents of Fermentable Oligo-Di-Monosaccharides and Polyols

FODMAPs content varied significantly between improved and indigenous varieties of millet, sorghum, soybean, and sesame (Table 3.3). These variations were comparable to those reported by Bhardwaj (2014) in S26 and S32 sesame varieties and Australian sweet Lupin legumes (Karnpanit et al., 2016). Moreover, Raja et al. (2016) reported variations in GOS content in different chickpea varieties while Huynh et al. (2007) reported variations in FOS content in different wheat and barley varieties.

These varietal differences in FODMAPs content could be attributed to crop improvement technologies that aim at improving agronomic characteristics potentially at the expense of total nutritional content and quality (Obua et al., 2020). Furthermore, genotypic differences and varying geographical environmental conditions (Varney et al., 2017) could also be a plausible explanation for the differences in FODMAPs content. The varying FODMAPs content reported in improved varieties has three major implications in the field of nutrition. Firstly, these varieties may increase the symptoms of functional gastrointestinal disorders in sensitive individuals especially those with high fructose (Killian et al., 2021). Secondly, those with higher total oligosaccharides (FOS & GOS) can provide ameliorating effect to FGIDs among vulnerable population such as children under 5 years of age. This is due to their proven prebiotic properties (Cao et al., 2023). Therefore, careful selection of crop varieties is essential to balance between nutritional benefits with potential digestive health implication. Thirdly, the high FODMAPs content presents an opportunity to apply food processing technologies to develop synbiotic food products which contain both prebiotic and probiotic properties (González-Herrera et al., 2021).

3.4.3 Effect of processing on the contents of Fermentable Oligo-Di-Monosaccharides and Polyols

The results of this study, which showed a reduction of more than 50% in FODMAPs content (Table 3.4) due to malting, are consistent with results from previous studies. Nyssölä et al. (2021) reported a 90% decrease in galacto-oligosaccharide (GOS) content in faba beans and yellow peas following enzymatic treatment. Similarly, Kaczmarek et al. (2017) reported a 44% reduction in total oligosaccharides in germinated soybean whereas Ispiryan et al. (2020) documented a 60-85% decrease in GOS content in malted chickpeas, barley, and oats. During malting, GOS are rapidly mobilised by endogenous catabolic α -galactosidases during the germination stage (Blöchl et al., 2007). This endogenous enzyme activation is crucial for energy metabolism to promote plant growth during the initial stages (Wang et al., 2023). This could be a plausible explanation for the reduction of GOS in malted plant-based foods such as soybean, sesame, millet, and sorghum.

The varying effect of malting on FOS content may be attributed to differences in grain type, inherent FOS quantity and type, and processing steps. Cereals such as wheat, rye and barley, millet and sorghum may contain varying quantities of Levan and inulin type fructo-oligosaccharides with varying glycosidic linkages of either β -2 $\rightarrow$ 1 or β -2 $\rightarrow$ 6 fructose-sucrose glycosidic linkages (Jackson et al., 2023). These linkages influence the degree of polymerisation which in turn influences their ability to be broken down by malting. Nevertheless, this study did not explore this perspective in detail.

Conversely, the effect of roasting was more visible at the total FODMAPs level rather than individual FODMAPs categories. This could be attributed to re-polymerisation and re-construction of new carbohydrate glycosidic linkages and moisture removal during roasting leading to increased concentration of FODMAPs (Martínez-Subirà et al., 2024; Yao et al., 2021). Overall, the study reveals that processing techniques significantly alter the content of FODMAPs content depending on the FODMAPs category and processing method. Malting is more effective than roasting in reducing FODMAPs content in cereals and legumes, making it a preferable method for producing low-FODMAPs foods. Malting is effective in

reducing FODMAPs categories such as excess fructose with adverse effects upon consumption by vulnerable population such as children under 5 years.

In most Ugandan households, millet, sesame, sorghum, and soybean are fundamental in the diet and nutrition. Millet and sorghum serve as staple cereals, frequently used to prepare porridge, non-alcoholic fermented beverages, bread, and other traditional dishes (Mukisa et al., 2010). Similarly, soybean and sesame are utilised either as single ingredients or in combination to develop snacks, composite flours, and pastes, contributing to the diversity and nutritional value of the diet (Alowo et al., 2018; Desire et al., 2021). Malting these food ingredients can lead to production of various food products with low FODMAPs content while preserving nutritional quality.

3.4.4 The potential of roasting and malting in achieving low FODMAPs Food Products

Functional gastro-intestinal disorders (FGIDs) incidences are increasing in low- and middle-income countries, and dietary management plays a crucial role in symptom control. Despite high FODMAPs foods being known triggers for FGID symptoms, they are also rich in nutrients (Killian et al., 2021). Therefore, elimination or significant reduction of daily intake of these foods may lead to nutrient deficiencies (Hill et al., 2017). Previous studies have documented that bioprocessing methods such as malting and fermentation can reduce FODMAPs content while maintaining nutritional quality (Nyyssölä et al., 2020). These methods also produce synbiotic food products with prebiotic and probiotic properties (Bamigbade et al., 2022).

The results of this study indicate that malting significantly reduced FODMAPs contents in cereal and legume grains commonly consumed in Uganda and other countries in eastern and central Africa. Nonetheless, reductions attained could not achieve the recommended cut-off values of below 0.3 g/100g (Fig. 3.2) and 0.5 g/100g (Fig. 3.3) for total oligosaccharides and total FODMAPs, respectively (Varney et al., 2017). Similar results were reported by Ispiryan et al. (2020), for chickpea (0.49 g/100g) and lentil malt (0.84 g/100g). Schmidt and Scieurba (2021) also found comparable FODMAPs content in fermented wheat flour varieties (0.53 g/100g). The failure to attain cut-off values as observed in the current study could be due to the initially high FODMAPs content in cereals and legumes investigated and ineffectiveness

of selected processing conditions. Therefore, optimisation of processing parameters for the methods investigated could be explored to improve their effectiveness in producing low FODMAPs food products. Optimising these processes is crucial for developing dietary interventions that minimise gastrointestinal discomfort for individuals sensitive to FODMAPs, particularly in regions where these grains are staple foods.

Based on the results of this study and others (Catassi et al., 2017; P. Hill et al., 2017), it is plausible to suggest that the 0.5 g/100g threshold for a low FODMAPs diet (Varney et al., 2017), may be excessively restrictive for high FODMAPs food resources such as cereals, legume grains, and oil seeds used in this current study. Given that FODMAPs confer health benefits primarily through prebiotic mechanism of action (Gibson et al., 2020), reducing them to such low levels might undermine these benefits. Notably, the FODMAPs levels achieved in this study through malting fall within the recommended prebiotic intake range of 5-8 g/day, which is believed to be adequate to elicit a prebiotic effect in the host (Kolida & Gibson, 2007). Nevertheless, the current study did not explore this aspect. Therefore, future studies should explore the prebiotic potential of FODMAPs at levels achieved through malting for the promising varieties of millet, soybean, sorghum and sesame identified in this study. This will provide a basis for striking a compromise between achieving the recommended FODMAPs threshold and the expected prebiotic benefit. These quantities maybe protective from functional gastrointestinal disorders rather than act as potential triggers.

3.5 Conclusion and Recommendation

This study successfully characterised the FODMAPs content in cereals, oil seeds, and legumes varieties commonly consumed in Uganda and showed the potential of malting to reduce FODMAPs content to recommended cut-off values. Specifically, the study revealed that; (i) Crop improvement led to varying changes in the total FODMAPs content of improved crop varieties. The improved varieties of soybean (Maksoy 3N), millet (Seremi 2) and sorghum (Narosorg 2) contain lower total FODMAPs content compared to indigenous varieties. (ii) malting was more effective than roasting at reducing total FODMAPs content, especially fructose which is known to have adverse digestive health effects when consumed

in high quantities. This study concluded that FODMAPs composition varied significantly among crop varieties and processing techniques. These findings demonstrate that targeted crop selection and processing can be used to manipulate dietary FODMAPs content, providing a food-based approach for developing low FODMAPs foods

Given that food processing has been shown to alter the FODMAPs composition of millet, sorghum, sesame, and soybean varieties, it is essential to investigate how these modifications influence the prebiotic functionality of the FODMAPs. Understanding the extent to which processing affects the fermentability, and selective utilisation of these FODMAPs by beneficial gut microbiota will provide critical insights into their potential to promote gastrointestinal health. While this Chapter presented important findings on the effect of crop varieties and processing methods on FODMAPs, it did not analyse the prebiotic properties of the FODMAPs. This gap was addressed in the following Chapter (four).

CHAPTER FOUR

Effect of crop variety on prebiotic potential of the Fermentable oligosaccharides extracted from millet, sorghum, soybean and sesame

This Chapter is adapted from:

Alowo, D., Olum, S., Mukisa, I. M., & Ongeng, D. (2025). Prebiotic potential of oligosaccharides extracted from improved Ugandan varieties of millet, sesame, soybean, and sorghum: enhancing probiotic growth and enteric pathogen inhibition. *BMC Microbiology*, 25(307), 1–17. <https://doi.org/10.1186/s12866-025-04028-x>

ABSTRACT

Functional gastrointestinal disorders like diarrhoea continue to affect children under five years in low- and middle-income countries. Incorporating health-enhancing bioactive compounds such as prebiotics in diet offers a promising solution. This study investigated prebiotic potential of oligosaccharides extracted from improved varieties of millet (Seremi 2, Naromil 2), sesame (Sesim 2, Sesim 3), soybean (Maksoy 3N, Maksoy 6N), and sorghum (Narosorg 2, Narosorg 4), commonly consumed in Uganda. These were compared to their respective indigenous variety. This study employed standardised methods for optical density measurement, culture preparation, and oligosaccharide extraction to evaluate prebiotic properties. The study investigated whether plant-based oligosaccharides could enhance the effectiveness of probiotics, specifically *Lactiplantibacillus plantarum* (ATCC 14917) and *Lacticaseibacillus rhamnosus* (ATCC 7469), in antagonising common enteric pathogens (*Salmonella enterica* subsp. *enterica* (ATCC 13076) and *Shigella flexneri* (ATCC 12022)). Approximately 5 and 8 log CFU/ml of each probiotic was incubated in 2% w/v oligosaccharide extracts at 37°C to evaluate the influence of the extracts on their growth (5 log CFU/ml of probiotic used), short-chain fatty acid (SCFA) production and antagonistic activity (8 log CFU/ml of probiotic was used). Maximum cell density, which exceeded the minimum recommended probiotic cell density (6 log CFU/ml), was achieved during 24-hour incubation period. The probiotics showed optimal growth in extracts of sesim 2, maksoy 3N, narosorg 2 and indigenous millet variety resulting in a 68-84% increase in cell densities. The concentration of SCFA concentration was significantly higher ($p < 0.05$) in soybean-based oligosaccharides. Both probiotics antagonised growth of *Salmonella* and *Shigella* by more than 40% when cultured on Sesim 2, Maksoy 3N, Narosorg 2 and indigenous millet variety, while maintaining the probiotic cell densities above the minimum recommended level. These varieties show great potential as functional ingredients for developing synbiotic-rich foods to promote gut and public health. However, to evaluate the oligosaccharides prebiotic efficacy, *in vitro* fermentation using faecal microbiota and *in vivo* studies are necessary to determine gut microbiota changes and interactions.

Key words: Fructo-oligosaccharide; Galacto-oligosaccharide; *Lactiplantibacillus plantarum*; *Lacticaseibacillus rhamnosus*; Crop variety; Enteric pathogens; Prebiotics, Probiotics.

4.1 Introduction

Functional gastrointestinal diseases including diarrhoea continue to be a substantial health issue for children under five years in low- and middle-income countries such as those in sub-Saharan Africa (SSA), contributing significantly to child mortality. Although diarrhoea only constitutes approximately 4% of child mortality worldwide, it is responsible for approximately 20% of deaths in low- and middle-income countries, highlighting a pressing health concern (Abu & Codjoe, 2018; Demissie et al., 2021). Particularly, in 2022, Uganda registered a 69% prevalence of diarrhoea among children under five years (Uganda Bureau of Statistics, 2022). The primary pathogens associated with diarrhoea within this demographic group include Enterotoxigenic *E. coli*, *Salmonella* and *Shigella* species (Kotloff et al., 2017; Liu et al., 2016). The prevalent route of exposure for these diarrhoea-inducing microorganisms is through ingestion of contaminated foods and beverages (Contreras et al., 2020).

Historically, there has been a strong association between diarrhoeal disease occurrence in SSA and the lack of sufficient water, sanitation, and hygiene (WASH) practices (Birhan et al., 2023; Nantege et al., 2022). Despite intensive efforts by governmental and non-governmental organisations to improve WASH and vaccination coverage, diarrhoea-related deaths still occur (Colding-Jørgensen et al., 2023). This situation is underscored by reports of over 80% adoption of improved WASH practices in the SSA region (Gizaw & Addisu, 2020). This suggests that sanitation and vaccination strategies are necessary but not sufficient to wipe out diarrhoeal disease. This situation emphasises the necessity for additional strategies to complement these essential interventions.

Adopting dietary interventions that extend beyond basic nutrition to incorporate health-promoting bioactive substances offers a feasible approach to alleviate functional gastrointestinal problems, such as diarrhoea, in children under 5 years. Prebiotics, among other bioactive ingredients, are essential for enhancing gastrointestinal health (Khan et al., 2019). They modulate the gut microbiota by homeostatic regulation of the diversity/abundance of health-promoting gut bacteria (probiotics), and reducing enteric pathogen (Berg et al., 2021; Guarino et al., 2020). Increased diversity and abundance of

probiotics such as *Lactocaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* species in the gut plays crucial roles by maintaining a healthy balance of gut bacteria; improving digestion and nutrient absorption and boosting the immune system (Papoff et al., 2012; Valero-Cases et al., 2020). Therefore, consumption of foods that have both prebiotic and probiotic potential provides synergistic effects on the overall well-being of an individual.

Prebiotics, primarily derived from minimally processed plant-based diets are selectively metabolised by probiotics in the lower gastrointestinal tract (Gibson et al., 2017). One of the primary beneficial metabolic products of this process are short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate (Yao et al., 2022). SCFAs lower the pH of the gastrointestinal tract creating an acidic environment that is unfavourable for the growth of pathogenic microorganisms (Demuth et al., 2021). Additionally, SCFAs, especially butyrate, serve as an energy source for intestinal epithelial cells, thereby enhancing the integrity of the intestinal barrier (Venegas et al., 2019). Butyrate is also important for the maintenance of specialised gut cells, such as the interstitial cells of Cajal, which play a role in mitigating inflammation and oxidative stress in the gastrointestinal tract (Carabotti et al., 2015).

Furthermore, acetate administration has been shown to significantly reduce inflammation in mice with gout (Vieira et al., 2017). Similarly, a dose-response study by Tayyeb et al. (2020), indicated increased inhibition in the production of pro-inflammatory mediators such as nuclear factor kappa B (NF- κ B) with increasing levels of propionate and butyrate, thereby strengthening the intestinal barrier function. Furthermore, SCFAs contribute to weight management and the prevention of non-communicable diseases by regulating appetite-regulating gut hormones such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (Alhabeeb et al., 2021). These studies highlight the comprehensive local and systemic benefits of SCFAs to the host produced by metabolism of dietary prebiotics.

Majorly recognised prebiotics are carbohydrate-based and are of plant-based origin, for instance, Fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS). These oligosaccharides have diverse applications in the food industry such as low-calorie sweeteners, texturisers, stabilisers, fat replacers, nutrition supplements and as prebiotic sources (Hanau et al., 2020; Kaur et al., 2021; Nikolić et al., 2024). They can either be

consumed as extracts/supplements or as part of whole foods. FOS and GOS can be obtained from plants such as millet, sorghum, soybean, and beans although, the amount of these prebiotic oligosaccharides in plants differs greatly (Kotha et al., 2020). For instance, cereals contain higher amounts of FOS whereas legumes largely contain GOS (Abdi & Joye, 2021). Furthermore, FOS and GOS differ in the structural composition and degree of polymerisation both within and between different food types (Sims & Tannock, 2020). Consequently, these variations influence the level at which the probiotic bacteria metabolise them. This in turn affects the type and quantity of metabolites such as SCFAs produced either *in vivo* or *in vitro* (Roupar et al., 2022).

An *in vitro* evaluation of the growth potential of 41 prospective *Lactocaseibacillus* probiotic strains on various carbohydrate substrates revealed a spectrum of carbohydrate utilisation, ranging from partial to complete (Nemska et al., 2022). Furthermore, Harris et al. (2020) observed an elevated production of butyrate and acetate by gut microbiota upon consumption of galacto-oligosaccharides, as compared to other carbohydrate sources such as polydextrose and rhamnose. This implies that different probiotic strains have varying abilities to utilise different carbohydrate types (Arapović et al., 2024). Consequently, this influences their growth and survival both in the gut and the food matrix. The type of carbohydrate consumed can also impact the metabolic activity of the probiotic, including the production of beneficial metabolites like SCFAs (Ai et al., 2022).

To develop food products that integrate both prebiotics and probiotics nutritionally termed as synbiotics, achieving the right combination of prebiotic compounds and probiotic strains is essential (González-Herrera et al., 2021). Synbiotic food products create a synergistic effect between prebiotics and probiotics that enhances host health. The prebiotics in the synbiotic food products not only increases gut microbiota but also increases the survival rate of probiotics as they pass through the harsh gastrointestinal tract (Pandey et al., 2015). Nonetheless, research in this area has largely focused on commercialised prebiotic oligosaccharides such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and inulin, paired with well-characterised probiotic strains from *Lactiplantibacillus plantarum*, *Lactocaseibacillus rhamnosus*, and *Bifidobacterium* (Cheon et al., 2023; Lafontaine et al., 2020; Yin et al., 2024). Studies investigating the prebiotic potential of

oligosaccharide extracts from various food sources have largely focused on wheat, seaweed, lotus seeds and human milk (Marotti et al., 2012; Okburan & Kızıler, 2023; Seong et al., 2019; Su et al., 2019). Nevertheless, there is limited information on prebiotic potential of oligosaccharides derived from foods including millet, sesame, soybean, and sorghum varieties commonly consumed in low-and middle-income countries such as Uganda.

Additionally, variations in oligosaccharide content of cereals, legumes and oilseeds resulting from differences in variety and processing methods have been documented (Abioye et al., 2022; Ispiryan, Kuktaite, et al., 2020; Qaim, 2020; Schmidt & Sciurba, 2021). However, the authors did not evaluate the potential of these oligosaccharides as prebiotics that can be combined with established probiotics to develop food products possessing synbiotic properties. Therefore, this study evaluated prebiotic potential of oligosaccharide extracts from millet, sorghum, sesame, and soybean varieties to determine the most appropriate oligosaccharide source that can be combined with probiotics to develop potential synbiotic food products using commonly consumed foods. The effect of these oligosaccharides on growth of probiotics, production of major SCFA (acetate, butyrate and propionate), and inhibition of common enteric pathogen was investigated since these aspects form the mechanism of action of prebiotics (Gibson et al., 2017). Evaluating prebiotic potential of these commonly consumed foods could create new opportunities for formulating innovative and diverse synbiotic food products, potentially enhancing accessibility, sustainability, and health benefits especially among individuals at risk of functional gastrointestinal disorders such as children under five years.

4.2 Materials and Methods

4.2.1 Prebiotic plant sources

Four crops were used: two cereals; finger millet (*Eleusine coracana*) and sorghum (*Sorghum bicolor*); one legume; soybean (*Glycine max*); and one oilseed; sesame (*Sesamum indicum*) were used as oligosaccharide sources. These were selected because they are commonly consumed in households in Uganda and other parts of Africa. Each crop consisted of two improved varieties and 1 indigenous variety (Table 4.1). The indigenous varieties were purchased from 10 different indigenous markets commonly used by most of the population

in Gulu City, northern Uganda. These were then pooled together to make 20 kg for each crop type. The improved varieties of millet, sorghum, and sesame were sourced from the National Semi-Arid Resources Research Institute (NASARRI) located in Serere district, Uganda, whereas the improved soybean varieties were acquired from the Zonal Agricultural Research and Development Institute in Ngetta, Lira, Uganda. These institutes lead plant breeding and crop improvement in the country and hence have pure lines of improved varieties. Plant breeding and crop improvement alters nutritional composition including prebiotic properties in addition to enhancing agronomic traits (de Albuquerque et al., 2020). Therefore, pure lines of highly adopted improved varieties were selected to determine the degree of alteration of prebiotic properties compared to indigenous varieties. Millet and sorghum were used for the study because they are the ingredients mostly used to produce fermented non-alcoholic beverages that can be consumed by children (Byakika et al., 2020). Sesame and soybean were used because they are the major plant protein sources used to enrich the nutritional quality of plant-based complementary foods (Alowo et al., 2018). Individual samples were sorted, cleaned, and sun-dried on a clean tarpaulin until a moisture content of 10-12% was achieved. Details on each crop used are shown in Table 4.1.

Table 4.1: Oligosaccharide plant sources used in this study segregated by crop type and variety

Name of Food/crop	Variety	Unique attributes
Sorghum	Indigenous variety	N/A
	Narosorg-2	Drought tolerant, minimal bird damage, used for food and yeast/brew
	Narosorg-4	Tolerant to striga weed, used for food
Finger Millet	Seremi 2	Good aroma & taste, early maturity, high marketability used for food & brewing
	Naromil 2	High yielding, early maturity
	Indigenous Variety	N/A
Soybean	Maksoy 3N	High yielding, large seed, high oil & protein content,
	Maksoy 6N	Early maturity, high yielding, high oil & protein content, resistant to rust disease
	Indigenous Variety	N/A
Sesame	Sesim 2	Drought tolerant, high yielding, high oil content, resistant to pests
	Sesim 3	High yielding
	Indigenous Variety	N/A

Source: (Hamba et al., 2024; Lubadde et al., 2019; Obua et al., 2021; Owere et al., 2014).
N/A- No relevant information on crop attributes available for the variety.

4.2.2 Microbial strains and growth conditions

Probiotic and pathogenic microbial strains were procured in lyophilised form from the American Type Culture Collection (ATCC) (Microbiologics, Minnesota, USA). To ensure their long-term viability, these strains were stored at -20°C. The probiotic strains used included *Lactocaseibacillus rhamnosus* (ATCC 7469) and *Lactiplantibacillus plantarum* (ATCC 14917). These specific strains were selected based on the international consensus criteria established by the FAO/WHO for probiotic evaluation, which require definitive taxonomic identification, proven safety, and demonstrable functional efficacy (FAO & WHO, 2006). Utilizing these stable, reference strains provided a validated, reproducible baseline to objectively benchmark and evaluate the probiotic attributes of the novel isolates investigated in this study.

Additionally, *Lpb. plantarum* is one of the most versatile lactic acid bacteria, commonly isolated from fermented cereals, and legume-based foods. It has a large, flexible genome with numerous carbohydrate-active enzymes, enabling it to metabolize diverse oligosaccharides (Carpi et al., 2022). This makes it a suitable reference to elucidate how crop-derived FODMAPs can support probiotic growth. On the other hand, *Lb. rhamnosus*, is among the most extensively characterized probiotics in clinical research. However, its metabolic repertoire for complex carbohydrates is relatively narrow (Suisa et al., 2023). Inclusion of *Lb. rhamnosus* in this study therefore provided a comparative benchmark, enabling assessment of substrate specificity. When FODMAPs extracts fail to sustain its growth, this outcome underscores the selective nature of prebiotic–probiotic interactions and highlights that not all probiotic strains respond uniformly to identical carbohydrate substrates.

The pathogenic organisms used for the study were *Salmonella enterica* subsp. *enterica* (ATCC 13076) and *Shigella flexneri* (ATCC 12022). *Lb. rhamnosus* and *Lpb. plantarum* were cultured anaerobically at 5% CO₂ in de Man, Rogosa, and Sharpe (MRS) agar (HiMedia, India) for 48 hours at 37°C. The enteric pathogenic microorganisms were cultured aerobically on a selective and differential Xylose Lysine Deoxycholate (XLD) agar (HiMedia, India) at 35°C for 24 to 48 hours. This medium selectively promotes the growth

of *Shigella* and *Salmonella* while inhibiting other enteric pathogens (Taylor & Schelhart, 1971).

4.2.3 Oligosaccharide extraction from selected crops

Oligosaccharide from malted millet, sesame, sorghum, and soybean varieties were extracted using ethanol absolute ($\geq 99.5\%$ purity) (Millipore, Darmstadt, Germany) extraction method described by Muir et al. (2009) and Pereira et al. (2018). Briefly, individual sun-dried crop varieties were ground using a laboratory grinder (Neo-Tech SA, Milmort, Belgium) and passed through a 40-mesh screen sieve with diameter size of 300 μm (HT/standard sieves). Subsequently, 1 g of the ground sample was accurately weighed into a Pyrex beaker to which 80 ml of 80% ethanol absolute solution was added and placed in a water bath shaker (MaxQ 7000, Thermo Fisher Scientific, UK) at 75 °C. The mixture was shaken at 200 rpm for 60 minutes to ensure maximum extraction of oligosaccharides without hydrolysis (Pereira et al., 2018). Following extraction, the mixture was centrifuged (VWR Micro star 17, 521-1646, Berlin, Germany) at 3000 x g for 10 minutes to obtain the supernatant.

The total oligosaccharide content in the extracts was quantified using Megazyme kits and determined by UV-VIS spectrophotometer (Jenway 6705, Milmort Belgium). K-RAFGK kit (last updated August 2023) (Megazyme, Bray Ireland) was used to determine galacto-oligosaccharide content whereas total fructo-oligosaccharide was determined by K-FRUC kit (last updated November 2022) (Megazyme, Bray Ireland) according to manufacturer's instructions. Subsequently, the oligosaccharide content of the extracts was used to standardise the oligosaccharide concentration to 2% (w/v) across all samples by dilution using distilled water or concentration by evaporation. Concentration was done using a rotary evaporator (Hei-vap precision, Heidolph, Germany) at 43°C, 75 mbars, and 125 rpm between 20 and 60 minutes depending on the degree of concentration required to achieve the standardised concentration. This was to ensure that the prebiotic effects observed were due to the oligosaccharides and not variations in extract concentration (Śliżewska & Chlebicz-Wójcik, 2020). The prepared solution was subsequently filter-sterilised using a 0.22 μm nylon syringe filter (AVF-100C-NY, Dubai, UAE) to eliminate any solid particles and bacteria and then stored at -14°C and analysed within 30 days.

4.2.4 Probiotic growth characteristics on oligosaccharides extracted from sorghum, millet, soybean, and sesame

The initial step involved preparation of overnight cultures of the probiotic strains *Lactiplantibacillus plantarum* (ATCC 14917) and *Lacticaseibacillus rhamnosus* (ATCC 7469). Cell pellets were harvested from the overnight cultures by centrifugation (VWR Micro Star 17, 521-1646, Berlin, Germany) at $8000 \times g$ for 10 minutes. The pellets were washed three times with phosphate-buffered saline (PBS) (Sigma-Aldrich, Chemie Taufkirchen, Germany), with centrifugation performed after each wash. Following the final wash, the pellets were re-suspended in 5 ml of PBS. Subsequently, 3 ml aliquots of the suspension were transferred into cuvettes, and the optical density (OD) at 600 nm was measured using a calibrated UV-VIS spectrophotometer (6705 Jenway, Milmort, Belgium). The OD readings were adjusted by diluting the suspension with PBS and standardised to a range of 0.8 to 1.0 at 600 nm, corresponding to approximately 1.0×10^8 CFU/ml (Karnjana et al., 2023) prior to evaluation. The microbial concentration was determined using culture media as described under section 4.2.2 microbial strains and growth conditions.

To evaluate their growth in the presence of different oligosaccharide sources, MRS broth (Millipore, Germany) composition was modified by adding 2% (w/v) of individual oligosaccharide extract (section 4.2.3) and the MRS broth without any modification was used as a control. Subsequently, a 5 ml aliquot of standardised probiotic culture was serially diluted with PBS and appropriate dilutions with about 5 log CFU/ml, determined by plate count method, was mixed with 50 ml of modified MRS broth and aliquoted for further analysis. A 10 ml aliquot of the mixture was incubated anaerobically in an anaerobic incubator (Forma Series 3 Water Jacketed CO₂ incubator, model 4111, Thermo Fisher Scientific, Marietta, USA) with 5% CO₂ at 37°C for 48 hours. A colony counter (Scan-100, Interscience, Paris, France) was used to enumerate microbial growth at 0, 24 and 48 hours of incubation on MRS agar (HiMedia, India). The growth rate of the probiotics was calculated using equation 4.1. Each treatment was performed in triplicates.

$$\text{Growth rate } \mu = 2.303 \times (\log_{10} N_t - \log_{10} N_0) \div (t - t_0) \dots \dots \text{Equation 4.1}$$

Where; N_t : number of bacteria at time point “t,” N_0 : number of bacteria at the initial time point (time point “0”), t : duration of observing time, and t_0 : initial time point (time point “0”)

4.2.5 Determination of short chain fatty acids from probiotic mixtures cultured in extracted oligosaccharides

Measurements were taken at 0, 24, and 48 hours to monitor SCFAs production over time. The SCFAs quantified were butyrate, Propionate and Acetate which were summed up to obtain total SCFA. SCFA concentration was quantified using High-Performance Liquid Chromatography (HPLC) with UV detector (LC-2050, Shimadzu, Kyoto, Japan) following the method described by Harris et al. (2020) with slight modifications. The HPLC analysis involved a derivatisation step using 2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br) (Sigma-Aldrich Co. LLC, USA) in acetone, following a procedure compatible with a SCFA analysis kit (Sigma-Aldrich Co. LLC, USA) (catalog number SBR00030).

The kit also provided the basis for constructing a calibration curve using a SCFA mix, with dilutions ranging from 0.0156 M to 0.5 M. This curve was used to determine the limit of detection (LOD) and limit of quantification (LOQ) of the standard using equation 4.2 and equation 3.1 in section 3.2.7, respectively. The LOD was 0.07 mmol and LOQ of SCFA mix was 0.22 mmol with an R^2 value of 0.994, and equation was $y=237.15x+18.483$. The derivatisation procedure of the standards and the sample mixtures were made according to the manufacturer’s instructions. Briefly, the derivatisation reaction mixtures were prepared by combining 100 μ L of SCFA standard or sample, 900 μ L of acetone and 3 μ L of PFB-Br. The mixtures were thoroughly homogenised by vortexing at maximum speed for 20 seconds. The mixtures were then placed in a water bath at 65°C for 24 hours to complete the derivatisation reaction. After derivatisation, the samples were filtered through 0.2 μ m nylon syringe filters into 1.5 ml HPLC vials.

A slight modification for the HPLC conditions was made as follows; HPLC was conducted using a C18 column (Luna^R 5 μ m C18 (2) 100 Å, LC Column 250 x 4.6mm) and UV detector at λ_{max} 245nm. Two mobile phases (80% Acetonitrile (gradient grade $\geq$ 99.9% purity, Sigma Aldrich, Chemie Taufkirchen, Germany) as solvent A and HPLC water + 0.1% Trifluoro acetic acid ($\geq$ 99.0% purity, Sigma Aldrich, Chemie Taufkirchen, Germany) as solvent B)

were used in gradient mode. The gradient conditions were set as; 0–15 min 20% solvent A, and 80% of solvent B, 15-18 minutes, 80% of solvent A and 20% of solvent B and 18-20 minutes, 20% of solvent A. The flow rate was set at 1ml/min, with 5 µL injection volume and a column temperature set at 30°C. The sample retention times were compared to the standard retention times and their concentration was determined by calculating the area under the corresponding chromatogram using Shimadzu Lab Solutions CS software. The analysis for each mixture was conducted in triplicates.

$$LOD = 3.3 \times (\text{standard deviation of intercept} \div \text{slope}) \dots\dots \text{Equation 4.2}$$

4.2.6 Potential of oligosaccharides extracted from different plant sources to enhance antagonistic activity of *Lpb. plantarum* and *Lb. rhamnosus* against *Shigella* and *Salmonella*

The antagonistic effects of probiotic strains with cell density of 8 log CFU/ml (*Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus*) cultured with 2% w/v extracted oligosaccharide against pathogenic bacteria (*Salmonella enterica* subsp. *enterica* and *Shigella flexneri*), was evaluated following the method of Śliżewska and Chlebicz-Wójcik (2020) with slight modifications. This current study used the total oligosaccharide content (Fructo-oligosaccharides and galacto-oligosaccharides) because these foods are consumed in their wholesome form rather than as isolated compounds. A 10 ml aliquot of the mixture prepared in section 4.2.4 was incubated anaerobically for 24 hours.

Overnight cultures of *Salmonella enterica* subsp. *enterica* and *Shigella flexneri* were prepared in Rappaport-Vassiliadis broth (Condalab, Madrid, Spain) and nutrient broth (Millipore, Germany), respectively at 37°C in a shaking incubator (AAH23433K, Lab companion, Oxfordshire, UK). The cell density for each pathogen was standardised as described earlier in section 4.2.4. Exactly 1 ml of each standardised pathogen culture was individually added to 1 ml of the 24-hour oligosaccharide extract-probiotic mixture. Individual probiotic and pathogen co-cultures without added oligosaccharide extract were used as a negative control. These samples were then incubated aerobically at 35°C for 24 hours. The growth of pathogenic bacteria was monitored at 6-hour intervals over a 24-hour period using the plate count method, whereby viable colonies were enumerated to assess

changes in population density over time. The pathogenic bacterial counts were enumerated using a Scan-100 colony counter (Interscience, Paris, France) and expressed as log CFU/ml. Each experiment was performed in triplicates.

4.2.7 Statistical analysis

One-way ANOVA was applied to assess statistical differences in the effects of oligosaccharides extracted from different crop varieties (millet, sorghum, sesame, and soybean) on probiotic growth, SCFA production, and antagonistic activity. Specifically, the analyses compared mean probiotic cell density, SCFA concentrations, and pathogen inhibition counts across the crop varieties. Post-hoc comparisons were conducted using Tukey's HSD test, with statistical significance set at $\alpha = 0.05$. Before running the ANOVA, homogeneity of variances was assessed using Levene's test ($F(5, 36) = 1.289$; $p = 0.290$), while normality was evaluated using the Shapiro-Wilk test ($W = 0.836$, $p = 0.203$). A Post hoc Tukey's test was performed to evaluate the significant differences between the means. Additionally, to visually represent the growth of the microbial cultures and SCFA production over time, Sigma Plot version 11.0 software was used to plot a graph of microbial counts and short chain fatty acid concentration against incubation time.

4.3 Results

4.3.1 Growth characteristics of *Lpb. plantarum* ATCC 14917 and *Lb. rhamnosus* ATCC 7469 on oligosaccharides extracted from sorghum, millet, soybean, and sesame

Figures 4.1 and 4.2 showed that oligosaccharide extracts from sorghum, millet, soybean, and sesame increased the cell density of both *Lpb. plantarum* ATCC 14917 and *Lacticaseibacillus rhamnosus* ATCC 7469 to levels between 8-9.3 log CFU/ml after 24 hours of incubation. The increase in cell density was observed to be within the range of 68% to 84%. In every case, the recorded cell counts (log CFU/ml) exceeded the minimum recommended threshold for probiotic classification (> 6 log CFU/ml) (Fig. 4.1 and Fig. 4.2). Specifically, the organism had a comparative growth advantage in oligosaccharide extracts from all sorghum and sesame varieties when contrasted with growth in millet (Fig 4.1-A) and soybean extracts (Fig 4.1-B), respectively. Among the sorghum extracts, growth of *Lpb. plantarum* was highest in Narosorg 2 sorghum variety with a count of 9.24 log CFU/ml,

followed by sesim 2 sesame extracts (9.04 log CFU/ml). After 48 hours, the cell density of *Lpb. plantarum* decreased in all crop extracts but only significantly ($p < 0.05$) in millet and sesame varieties with Naromil 2 and sesim 3 recording the highest decreases of 21% and 22%, respectively.

Figure 4.2 similarly presents a significant increase ($p < 0.05$) in growth of *Lacticaseibacillus rhamnosus* ATCC 7469 within the first 24-hour incubation period. However, a notably higher growth was observed in all sorghum and millet oligosaccharide extracts than growth observed in soybean and sesame extracts (Fig 4.2-B). Sorghum and millet oligosaccharide extracts increased *Lb. rhamnosus* cell density between 70 and 80% during the first 24-hour incubation period. Specifically, millet varieties recorded the maximum growth of *Lb. rhamnosus*, with the indigenous variety indicating the most substantial increase by 79.4%. Among sesame varieties, Sesim 3 displayed a maximum increase of 75.7% in the cell density. All crops recorded their maximum growth (> 8 log CFU/ml) of *Lb. rhamnosus* at 24 hours, followed by a stationary growth phase, as indicated by a plateau curve (Fig 4.2). Figures 4.1 and 4.2 showed that sorghum varieties produced the most pronounced and statistically significant ($P < 0.05$) increases relative to the control. The other crops also showed values higher than the control, though their increases were not significant ($P > 0.05$).

The data from Figures 4.1 and 4.2 indicates that both *Lactiplantibacillus plantarum* ATCC 14917 and *Lacticaseibacillus rhamnosus* ATCC 7469 reached maximum growth within the first 24-hour incubation period. This implies that 24-hour incubation time is the most favourable for achieving maximum growth of both *Lpb. plantarum* and *Lb. rhamnosus*. Consequently, the 24-hour incubation data were used to evaluate varietal differences among the different crop varieties concerning the growth characteristics of *Lpb. plantarum* ATCC 14917 and *Lb. rhamnosus* ATCC 7469, as indicated in Table 4.2.

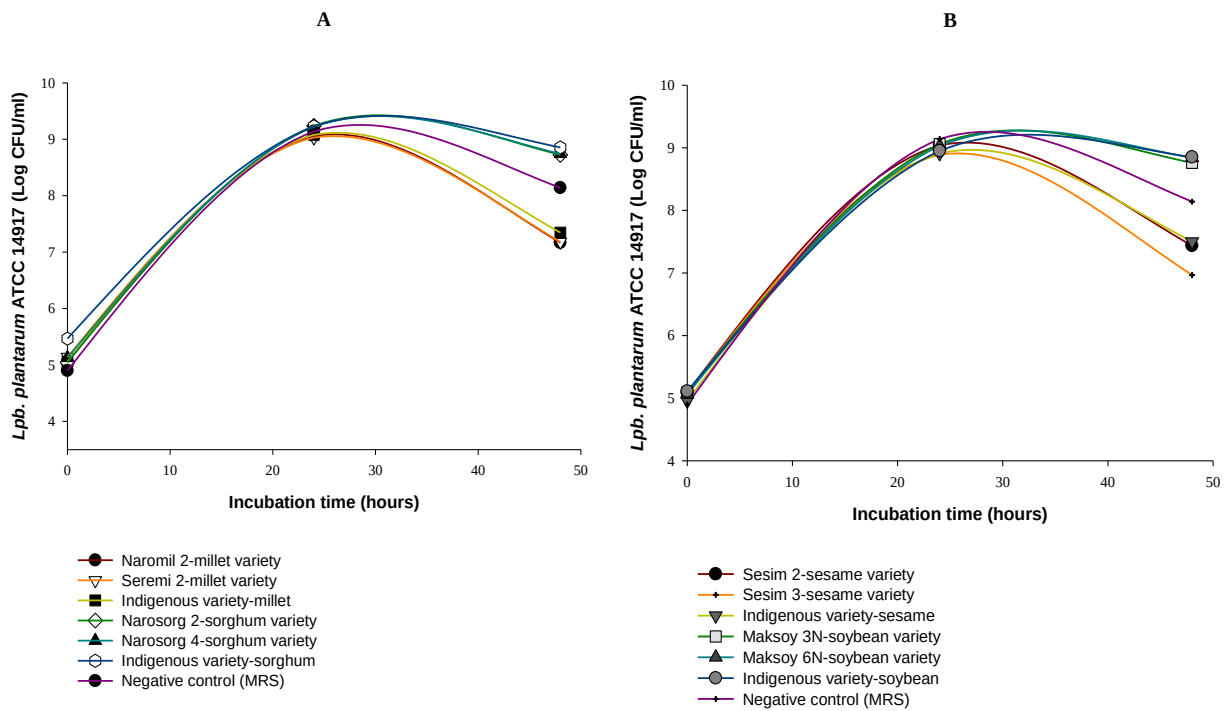


Figure 4.1: *Lactiplantibacillus plantarum* ATCC 14917 load during 48-hour incubation on different oligosaccharide plant source. A-*Lpb. plantarum* growth curve on millet and sorghum crop varieties, B-*Lpb. plantarum* growth curve on sesame and soybean crop varieties. Error bars are excluded for clarity.

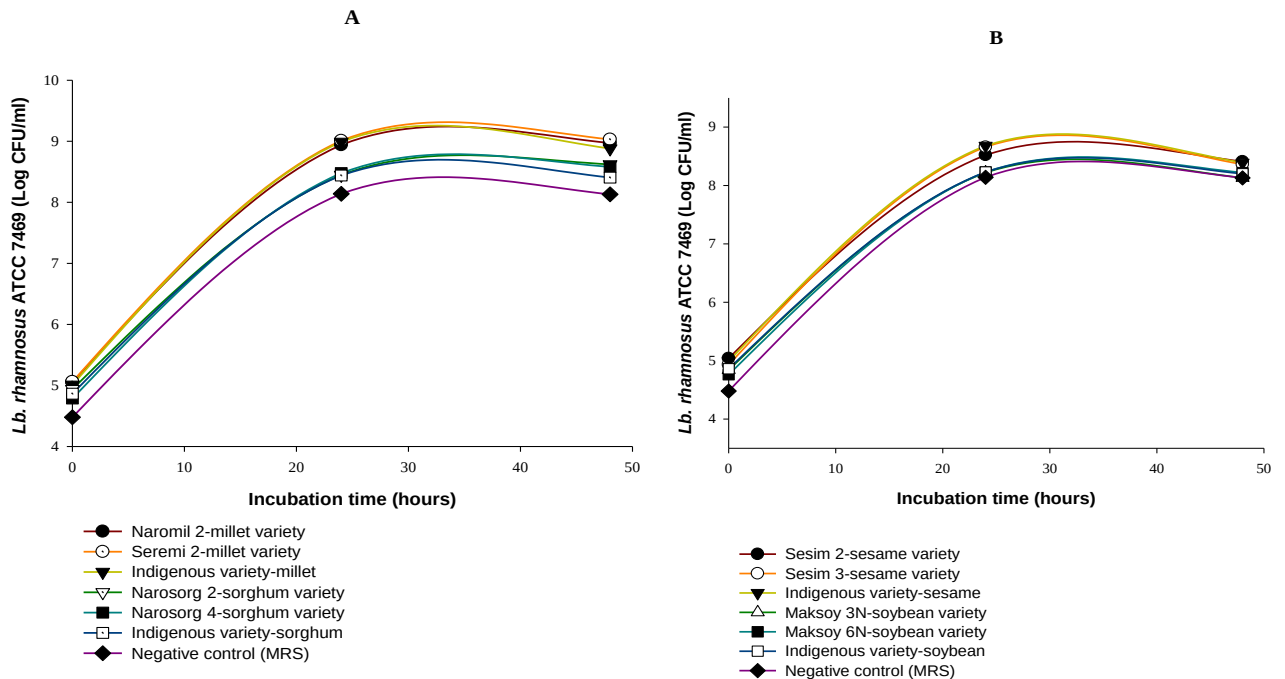


Figure 4.2: *Lactocaseibacillus rhamnosus* ATCC 7469 load during 48-hour incubation on different oligosaccharide plant source. A-*Lb. rhamnosus* growth curve on millet and sorghum crop varieties, B-*Lb. rhamnosus* growth curve on sesame and soybean crop varieties. Error bars are excluded for clarity.

Lpb. plantarum ATCC 14917 showed a comparative growth advantage over *Lb. rhamnosus* ATCC 7469 by more than one logarithmic cycle (Table 4.2) irrespective of oligosaccharide plant source. However, both bacteria had similar trend in the growth rate between 0.32 and 0.41 log CFU/ml/hour with Narosorg 2 recording the highest growth rate of 0.41 log CFU/ml/hour. Notably, the Narosorg 2 variety of sorghum significantly ($p < 0.05$) augmented the growth of *Lpb. plantarum*, with an average increase of more than 0.5 log units compared to other oligosaccharide plant extracts. Maksoy 3N soybean variety and Sesim 2 sesame variety extracts similarly facilitated growth of *Lpb. plantarum* comparable with sorghum varieties. When comparing improved and indigenous varieties of the extracts, it was observed that improved crop varieties significantly ($p < 0.05$) enhanced the growth of *Lpb. plantarum*, except for millet varieties. No significant difference ($p < 0.05$) was observed between the improved and indigenous varieties when *Lb. rhamnosus* was used as the probiotic bacteria. Therefore, oligosaccharide extracts from improved varieties (Maksoy 3N, Narosorg 2, and Sesim 2) and indigenous millet varieties were further evaluated for their potential in short chain fatty acid production and enteric pathogen inhibition.

Table 4.2: Growth characteristics of *Lpb. plantarum* ATCC 14917 and *Lb. rhamnosus* ATCC 7469 on different plant substrates (soybean, millet, sorghum, and sesame) selected for their oligosaccharide content

Crop type	Crop variety	Growth of <i>Lpb. plantarum</i> & <i>Lb. rhamnosus</i> (log CFU/ml) 0-hour incubation		Growth of <i>Lpb. plantarum</i> & <i>Lb. rhamnosus</i> (log CFU/ml) 24hour incubation		Growth rate (μ log CFU/ml/hour)	
		<i>Lpb. plantarum</i>	<i>Lb. rhamnosus</i>	<i>Lpb. plantarum</i>	<i>Lb. rhamnosus</i>	<i>Lpb. plantarum</i>	<i>Lb. rhamnosus</i>
Soybean	Maksoy 3N	5.09 ^a $\pm$ 0.03	4.84 ^{ab} $\pm$ 0.10	9.06 ^{bcd} $\pm$ 0.05	8.22 ^f $\pm$ 0.02	0.38 ^a $\pm$ 0.01	0.32 ^a $\pm$ 0.01
	Maksoy 6N	5.06 ^a $\pm$ 0.06	4.76 ^b $\pm$ 0.11	9.03 ^{bcd} $\pm$ 0.03	8.23 ^f $\pm$ 0.02	0.38 ^a $\pm$ 0.01	0.33 ^a $\pm$ 0.01
	Indigenous variety	5.11 ^a $\pm$ 0.12	4.86 ^a $\pm$ 0.07	8.95 ^{cde} $\pm$ 0.10	8.23 ^f $\pm$ 0.03	0.37 ^a $\pm$ 0.00	0.32 ^a $\pm$ 0.01
Millet	Naromil 2	5.05 ^a $\pm$ 0.08	5.05 ^a $\pm$ 0.31	9.06 ^{bcd} $\pm$ 0.05	8.94 ^a $\pm$ 0.08	0.39 ^a $\pm$ 0.01	0.37 ^a $\pm$ 0.03
	Seremi 2	5.14 ^a $\pm$ 0.18	5.06 ^a $\pm$ 0.29	9.03 ^{bcd} $\pm$ 0.02	9.01 ^a $\pm$ 0.03	0.37 ^a $\pm$ 0.02	0.38 ^a $\pm$ 0.03
	Indigenous variety	5.06 ^a $\pm$ 0.4	5.01 ^a $\pm$ 0.23	9.08 ^{abc} $\pm$ 0.03	8.99 ^a $\pm$ 0.05	0.38 ^a $\pm$ 0.04	0.38 ^a $\pm$ 0.02
Sorghum	Narosorg 2	5.04 ^a $\pm$ 0.14	4.94 ^a $\pm$ 0.17	9.24 ^a $\pm$ 0.01	8.45 ^d $\pm$ 0.08	0.4 ^a $\pm$ 0.01	0.34 ^a $\pm$ 0.01
	Narosorg 4	5.13 ^a $\pm$ 0.17	4.79 ^a $\pm$ 0.13	9.22 ^a $\pm$ 0.05	8.48 ^{cd} $\pm$ 0.04	0.40 ^a $\pm$ 0.02	0.35 ^a $\pm$ 0.02
	Indigenous variety	5.46 ^a $\pm$ 0.01	4.86 ^a $\pm$ 0.18	9.23 ^a $\pm$ 0.04	8.44 ^{de} $\pm$ 0.06	0.36 ^a $\pm$ 0.01	0.35 ^a $\pm$ 0.02
Sesame	Sesim 2	5.09 ^a $\pm$ 0.39	5.04 ^a $\pm$ 0.30	9.04 ^{bcd} $\pm$ 0.12	8.52 ^{bcd} $\pm$ 0.07	0.38 ^a $\pm$ 0.05	0.33 ^a $\pm$ 0.03
	Sesim 3	5.08 ^a $\pm$ 0.38	4.93 ^a $\pm$ 0.16	8.89 ^e $\pm$ 0.03	8.66 ^{bc} $\pm$ 0.13	0.37 ^a $\pm$ 0.04	0.36 ^a $\pm$ 0.01
	Indigenous variety	4.96 ^a $\pm$ 0.32	4.99 ^a $\pm$ 0.20	8.91 ^{de} $\pm$ 0.06	8.68 ^b $\pm$ 0.04	0.38 ^a $\pm$ 0.04	0.35 ^a $\pm$ 0.02
Control	negative control (MRS broth)	5.18 ^a $\pm$ 0.02	5.19 ^a $\pm$ 0.08	9.14 ^{ab} $\pm$ 0.04	8.14 ^f $\pm$ 0.01	0.38 ^a $\pm$ 0.01	0.28 ^b $\pm$ 0.01

Values indicate mean $\pm$ SD (n=3) for crop type under each probiotic. Means in the same column followed by different superscripts are significantly different from each other at 5% level of significance.

4.3.2 Production of short chain fatty acid from extracted oligosaccharide and probiotic mixtures

Figures 4.3 and 4.4 illustrate the production of SCFAs by different oligosaccharide and probiotic mixtures. The production of SCFAs (acetate, propionate, and butyrate) varied depending on the oligosaccharide source and probiotic combination during the incubation period. After incubation for 24 hours, a significant ($p < 0.05$) increase in acetate concentration (ranging between 4-and >50%) was observed in sesame, millet, and soybean oligosaccharide-*Lpb. plantarum* (Fig. 4.3). Acetate concentration after 24 hours of incubation in these oligosaccharide extract-*Lpb. plantarum* combinations ranged between 1.39 and 2.38 mmol, with the highest increase recorded in soybean oligosaccharide. However, a significant ($p < 0.05$) increase in propionate (0.65 mmol) and butyrate (0.5 mmol) concentration was recorded only in soybean. After 48 hours of incubation, all the SCFAs decreased albeit significantly ($p > 0.05$) except in sesame where butyrate and propionate increased significantly ($p < 0.05$).

In contrast, oligosaccharide-*Lb. rhamnosus* combinations (Fig. 4.4) recorded a significant decrease ($p < 0.05$) in butyrate concentration for the first 24 hours across all oligosaccharide combinations. Propionate concentrations remained largely unchanged except in millet and sesame oligosaccharides which showed a significant ($p < 0.05$) decrease in the first 24 hours followed by a significant ($p < 0.05$) increase after 48 hours of incubation. Acetate concentration significantly ($p < 0.05$) increased in all oligosaccharide combinations during the first 24 hours but decreased after 48 hours except in soybean oligosaccharides and the negative control.

These variations in individual SCFAs consequently influenced both the total and molar proportions of the SCFAs. For instance, soybean and sesame oligosaccharides recorded the highest net increase in total SCFAs after 24 hours and 48 hours of incubation, respectively. Furthermore, soybean oligosaccharide extract had (67:18:14-Acetate: propionate: butyrate) molar proportions of SCFAs comparable with recommended ranges (60:20:20- Acetate: propionate: butyrate) when *Lpb. plantarum* was used. However, the total SCFA concentration observed in oligosaccharide-*Lb. rhamnosus* combinations were significantly

lower than MRS broth except for soybean during the first 24 hours and sesame after 48 hours.

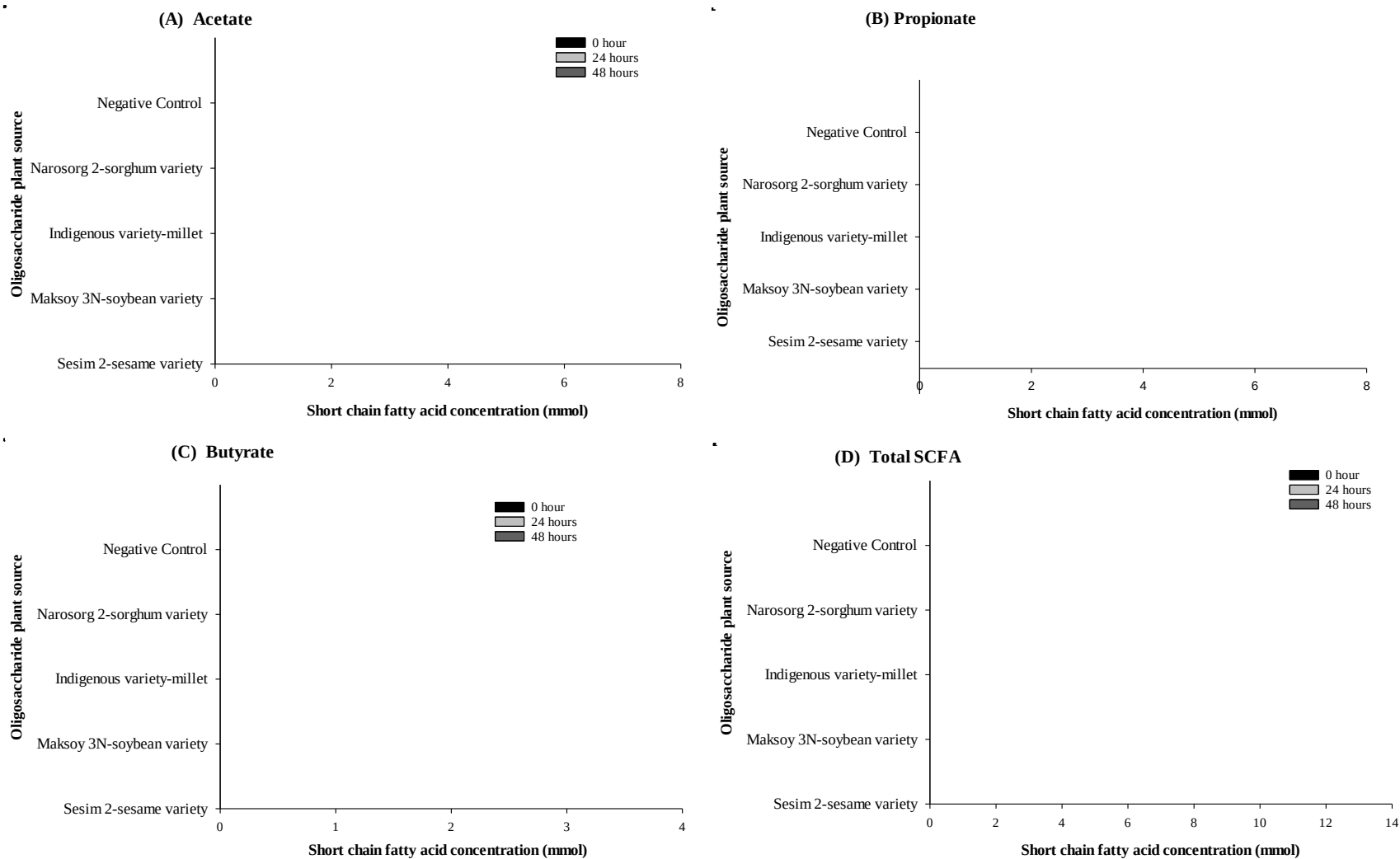


Figure 4.3: Production of short chain fatty acids (A: acetate, B: propionate, C: butyrate and D: total short chain fatty acids-total SCFA) in different oligosaccharide plant source fermented by *Lpb. plantarum* during 48-hour incubation.

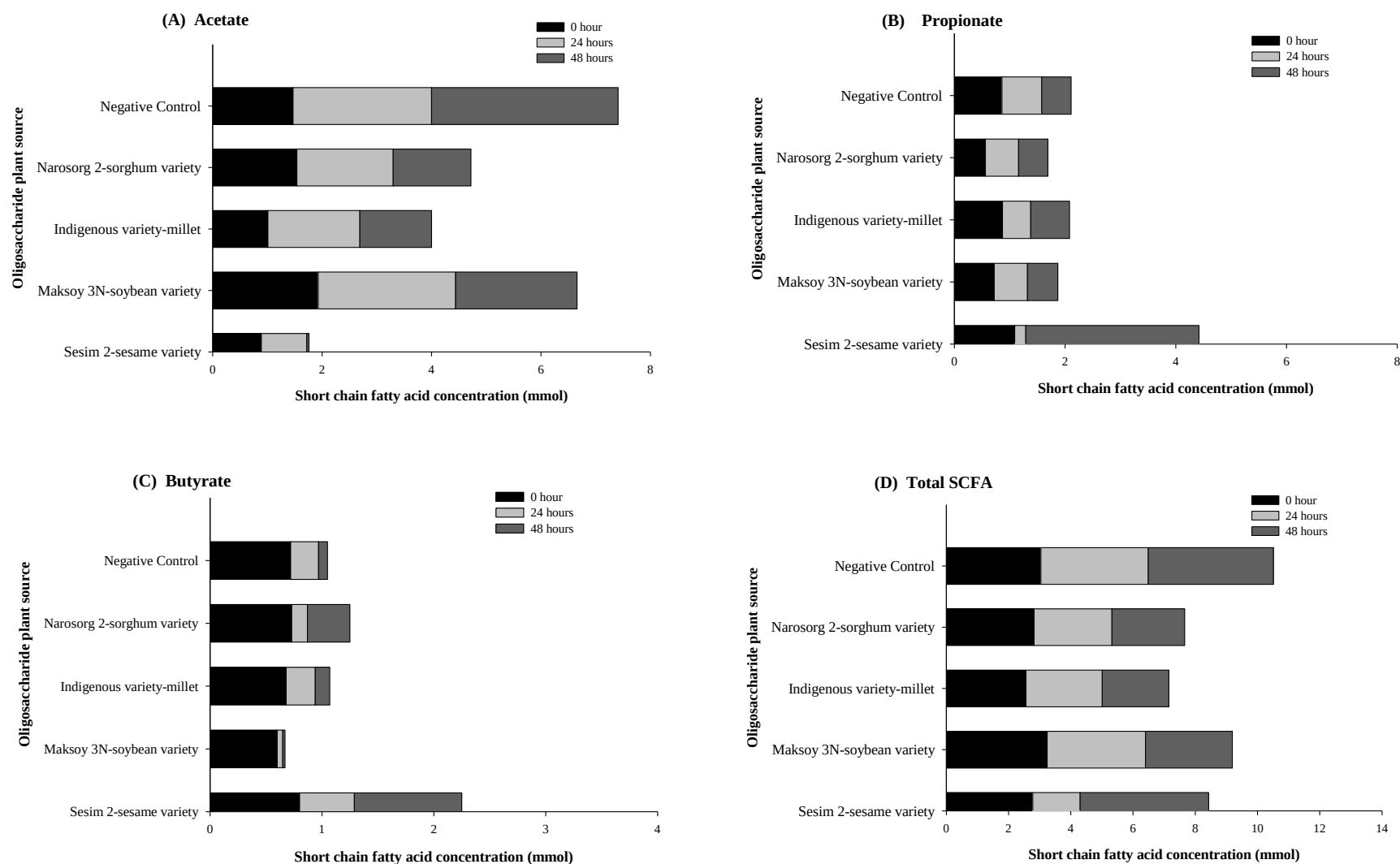


Figure 4.4: Production of short chain fatty acids (A: acetate, B: propionate, C: butyrate and D: total short chain fatty acids- total SCFA) in different oligosaccharide plant source fermented by *Lb. rhamnosus* during 48-hour incubation

4.3.3 Potential of oligosaccharide extracts from different plant sources to enhance antagonistic activity of *Lpb. plantarum* and *Lb. rhamnosus* against common enteric pathogens

The results of the present study establish that oligosaccharide extracts from all different plant sources studied enhanced the antagonistic potential of both single and mixed cultures of *Lpb. plantarum* and *Lb. rhamnosus* against *Salmonella enterica* ATCC 13076 and *Shigella flexneri* ATCC 12022. The antagonistic activity of both probiotics was enhanced by 30-80% when combined with various oligosaccharide sources, compared to the control. This enhancement was similar in pattern but varied in intensity following a 24-hour incubation period (as depicted in Figures 4.5 and 4.6). A consistent and statistically significant ($P < 0.05$) antagonistic activity against *Salmonella* was noted for both *Lpb. plantarum* and *Lb. rhamnosus* in both single and mixed cultures in the presence of all oligosaccharide plant sources (Fig. 4.5).

In contrast, the antagonistic activity of the oligosaccharide-probiotic mixtures on *Shigella* growth varied (Fig. 4.6). For example, sesame augmented *Shigella* growth when co-cultured with *Lpb. plantarum* and *Lb. rhamnosus* after a 24-hour incubation period, resulting in the bacterial count of 8.32 log CFU/ml. Conversely, millet oligosaccharide extracts did not enhance the antagonistic activity of *Lb. rhamnosus* against *Shigella* throughout the incubation period. The growth of *Shigella* ranged from 7.66 log CFU/ml to 8.13 log CFU/ml at 0- and 24-hour incubation, respectively. The inhibitory effect of *Lpb. plantarum* against *Shigella* was significantly enhanced by oligosaccharides from all plant sources, resulting in colony counts that were too few to enumerate (< 30 CFU) even at the lowest dilution factor, indicating a decrease in *Shigella* growth after 24 hours of incubation.

After 24 hours of antagonistic activity, the cell densities of both *Lactiplantibacillus plantarum* (*Lpb. plantarum*) and *Lacticaseibacillus rhamnosus* (*Lb. rhamnosus*) remained above the recommended threshold for probiotic products (6 log CFU/ml), regardless of the oligosaccharide source or the pathogen involved. Although a decline in microbial counts was observed over the incubation period, co-cultures of oligosaccharides with *Lpb. plantarum* consistently had significantly higher cell densities compared to those with *Lb. rhamnosus*, as presented in Table 4.3. The decrease in the microbial counts after 24 hours

ranged between 0.1 and 1.5 log CFU/ml with *rhamnosus* co-cultures showing the greatest decrease. Among the oligosaccharides evaluated, co-cultures with indigenous millet variety recorded the lowest microbial counts for both probiotics followed by sesame. These findings underscore the superior resilience of *Lpb. plantarum* in maintaining growth under antagonistic conditions and provide valuable insights for optimising oligosaccharide-probiotic-pathogen interactions for development of food products with synbiotic properties.

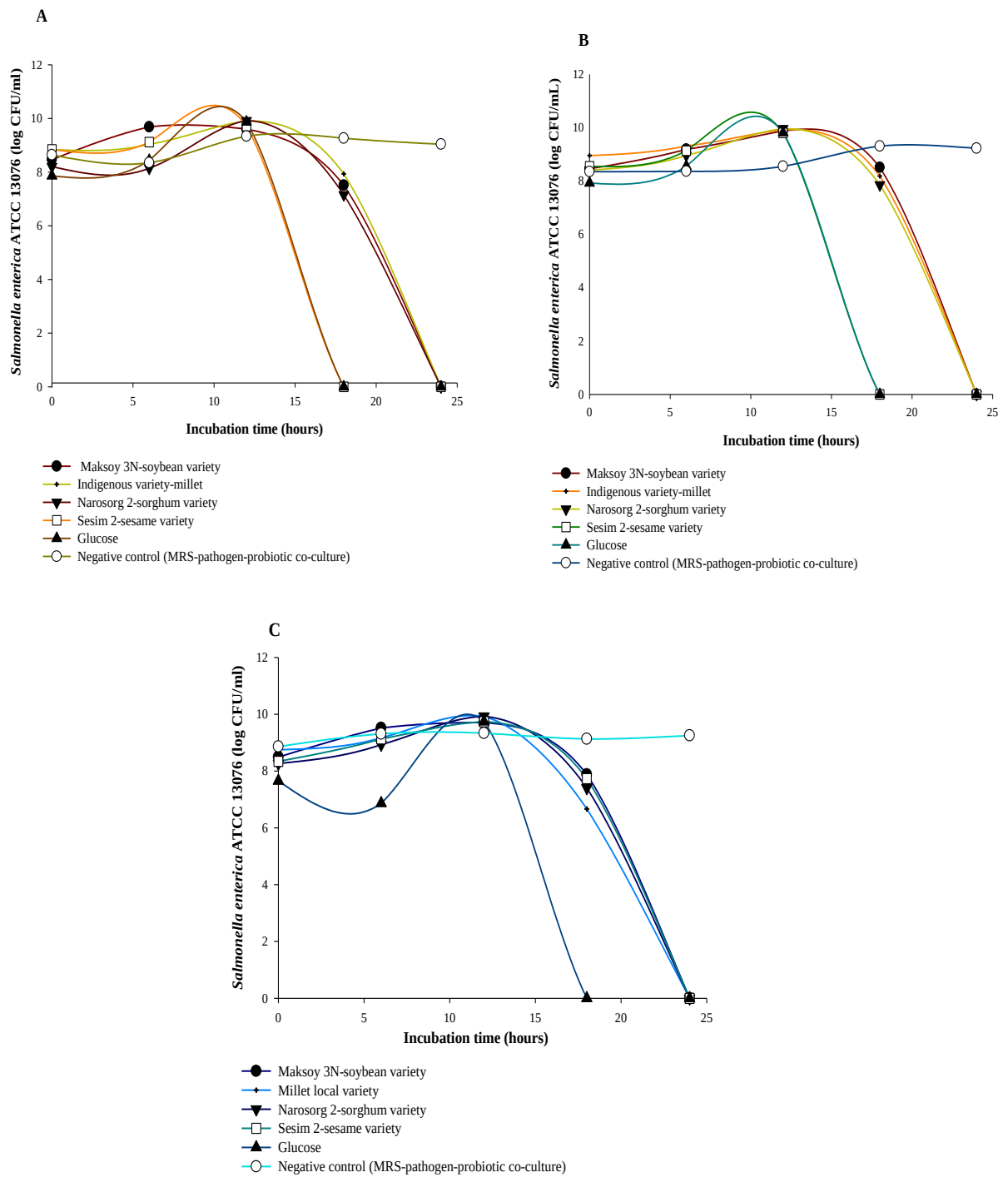


Figure 4.5: Effect of oligosaccharide plant source on antagonistic activity of monoculture and co-culture strains against *Salmonella enterica* ATCC 13024 over 24-hour incubation time: A-Antagonistic activity of *Lpb. plantarum* ATCC 14917 monoculture: B-antagonistic activity of *Lb. rhamnosus* ATCC 7469 monoculture: C-antagonistic activity of ATCC 14917 and ATCC 7469 co-culture. Error bars are excluded for clarity.

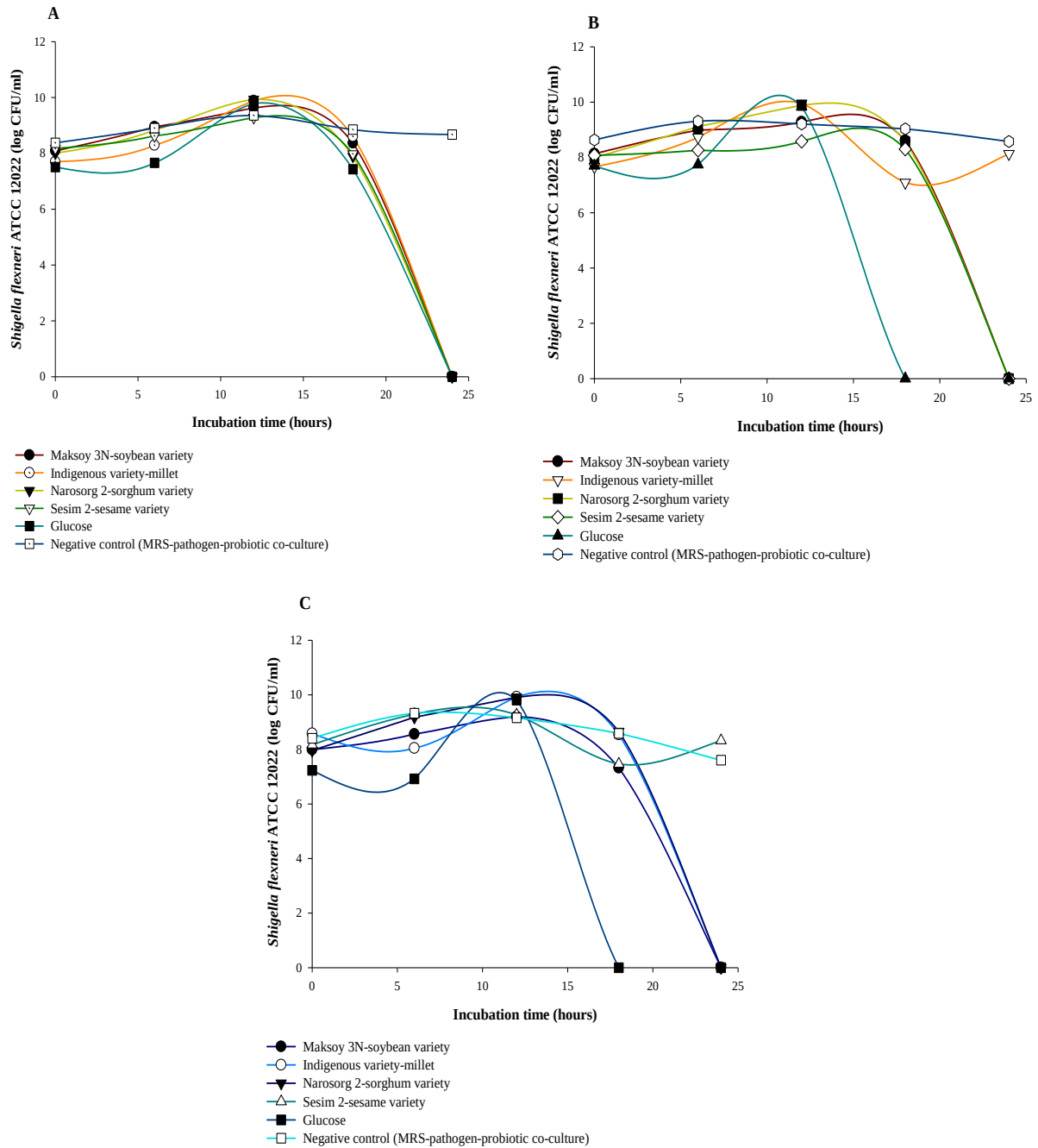


Figure 4.6: Effect of oligosaccharide plant source on antagonistic activity of monoculture and co-culture strains against *Shigella flexneri* ATCC 12022 over 24-hour incubation time: A-Antagonistic activity of *Lpb. plantarum* ATCC 14917 monoculture: B-antagonistic activity of *Lb. rhamnosus* ATCC 7469 monoculture: C-antagonistic activity of ATCC 14917 and ATCC 7469 co-culture. Error bars are excluded for clarity

Table 4.3: Probiotic microbial counts following pathogen inhibition across oligosaccharide-probiotic-pathogen co-cultures

Oligosaccharide source	probiotic count across oligosaccharide-probiotic-pathogen co-cultures after 24-hour incubation (log CFU/ml)							
	<i>Lpb. plantarum-salmonella</i>		<i>Lpb. plantarum-shigella</i>		<i>Lb. rhamnosus-salmonella</i>		<i>Lb. rhamnosus-shigella</i>	
	0 hour	24 hours	0 hour	24 hours	0 hour	24 hours	0 hour	24 hours
Maksoy 3N soybean	8.03 ^a ± 0.04	7.85 ^a ± 1.44	8.05 ^a ± 0.21	7.10 ^b ± 1.13	8.47 ^a ± 0.48	7.66 ^b ± 1.35	8.27 ^a ± 0.2	7.23 ^b ± 0.5
Indigenous variety-millet	8.05 ^a ± 0.21	7.48 ^b ± 2.22	7.96 ^a ± 0.08	6.79 ^b ± 0.16	8.34 ^a ± 0.96	6.95 ^b ± 0.13	7.84 ^a ± 0.25	6.81 ^b ± 0.21
Narosorg 2-sorghum	7.95 ^a ± 0.21	7.11 ^a ± 1.14	8.10 ^a ± 0.14	7.65 ^b ± 1.06	8.40 ^a ± 0.57	7.73 ^b ± 1.17	8.00 ^a ± 0.01	7.13 ^b ± 0.95
Sesim 2-sesame	8.10 ^a ± 0.28	7.75 ^a ± 0.64	8.25 ^a ± 0.21	7.65 ^b ± 1.78	7.94 ^a ± 0.2	6.55 ^b ± 1.06	8.09 ^a ± 0.01	6.90 ^b ± 0.57
Negative control	8.15 ^a ± 0.49	6.25 ^b ± 0.92	8.13 ^a ± 0.18	6.65 ^b ± 0.21	7.69 ^a ± 0.14	5.45 ^b ± 0.64	8.44 ^a ± 0.26	5.05 ^b ± 1.48

Values indicate mean ± SD (n=3) for oligosaccharide-probiotic-pathogen co-culture. Means in the same row under each co-culture followed by different superscripts are significantly different from each other at 5% level of significance.

4.4 Discussion

4.4.1 Growth characteristics of *Lpb. plantarum* and *Lb. rhamnosus* on oligosaccharides extracted from sorghum, millet, soybean, and sesame

To qualify as a prebiotic, an oligosaccharide must have the ability to enhance the growth of probiotics to a cell density ranging from 6 to 9 log CFU/ml or CFU/g (Anosike et al., 2022). Maximum proliferation (8-9 log CFU/ml) of six *Lactobacillus* strains on 3 different prebiotic carbohydrates were reported at 24-hour incubation (Hernandez-Hernandez et al., 2012). Similar results are reported in *Lb. plantarum* MNC 21 using different varieties of sorghum malt extracts as a growth media (Byakika et al., 2020). Moreover, Di Pede et al. (2024) reported significant growth of *LimosiLactobacillus reuteri*, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* GG when cultured on malto-oligosaccharide extracts similar to commercial FOS.

These results were comparable to the maximum proliferation of both *Lpb. plantarum* ATCC 14917 and *Lb. rhamnosus* ATCC 7469 recorded in the current study (Figure 4.1 and 4.2) for all oligosaccharide extracts after 24 hours of incubation. These results could be attributed to the additional energy sources provided by the oligosaccharides to the probiotics. Sorghum, millet, soybean and sesame extract majorly provide galacto-oligosaccharides and fructo-oligosaccharides which are good sources of energy for probiotic bacteria (Ispiryan et al., 2019; Schmidt & Sciurba, 2021). This implies that these plant-based oligosaccharides have a prebiotic property of enhancing growth of beneficial bacteria such as *Lpb. plantarum* and *Lb. rhamnosus*. When consumed, they could increase the gut microflora, which in turn boosts gut health, strengthens the immune system, and improves overall well-being.

The extent to which plant-based oligosaccharides are metabolised as energy sources by probiotic microorganisms depends on the degree of polymerisation and structural complexity of the plant source. The current study indicates that oligosaccharide from improved varieties of cereals, legumes and oil seed significantly enhance the growth of *Lpb. plantarum* and *Lb. rhamnosus* compared to indigenous varieties. These results concur with those of Marotti et al. (2012) who reported enhanced growth of *Lb. plantarum* L12 by improved wheat varieties compared to old varieties. Crop improvement through breeding

techniques may have caused a decrease in the degree of polymerisation of the oligosaccharides (Dwivedi et al., 2014).

A low degree of polymerisation increases the exposure of oligosaccharide structure to hydrolytic enzymes and probiotic micro-organisms that are responsible for their fermentation (Hallac & Ragauskas, 2011). This in turn influences the quantity of metabolites produced. Therefore, oligosaccharide extracts from improved varieties (Maksoy 3N, Narosorg 2, and Sesim 2) and indigenous millet varieties were further evaluated for their potential in short chain fatty acid production and antagonistic activity of the probiotics. This provides an opportunity for the development of fermented food products with both prebiotic and probiotic properties (synbiotic food products).

4.4.2 Production of short chain fatty acid from probiotic mixtures cultured in extracted oligosaccharides

The second key attribute of a substrate to be classified as a prebiotic is its capacity to produce bioactive metabolites that confer health benefits to the host (Gibson et al., 2017). In this study, sorghum, millet, soybean, and sesame oligosaccharides successfully produced significant amount of the three primary SCFAs (butyrate, acetate, and propionate) in the first 24 hours (Figure 4.3 and 4.4), which are well-documented for their health-promoting properties. The concentration of SCFAs observed in oligosaccharides in this study is consistent with the findings of Harris et al. (2020), who observed an elevated production of SCFAs by galacto-oligosaccharides, compared to polydextrose and rhamnose. Additionally, Roupar et al. (2022) reported similar results in *Lactobacillus*-FOS mixtures. This could be due to the quantity and structural composition of the available total oligosaccharides which have rapid and moderate fermentation rates by the probiotic bacteria.

The fermentation rate and metabolite production by bacteria are dependent on the structure of the carbon source, specifically the number of glycosidic linkages and branching levels (Davani-Davari et al., 2019). When these linkages and branches are moderately complex (with sugar residues between 3 and 10), they allow varying rates of enzymatic accessibility and utilisation by different bacterial species. This differential accessibility regulates the degree of fermentation, and the diversity of metabolites generated during the process (Di

Pede et al., 2024). Nemska et al. (2022) reported that 41 candidate *Lactocaseibacillus* probiotic strains had varying degree of fermenting different carbohydrate sources in an *in vitro* assessment study. Similarly, *Lpb. plantarum* ATCC BAA-793 metabolised Fructo-oligosaccharides and human milk oligosaccharides better than cranberry xyloglucan (Özcan et al., 2021).

The results of the current study indicated that plant oligosaccharide extracts when combined with *Lpb. plantarum* exhibits higher metabolite production compared to their combinations with *Lb. rhamnosus*. The results highlighted that oligosaccharide extract-*Lb. rhamnosus* combinations are not ideal choices as prebiotic-probiotic combinations for developing a synbiotic food product. This limitation can be attributed to strain-specific differences, as *Lb. rhamnosus* was unable to effectively utilise the available oligosaccharides (Uhegwu & Anumudu, 2025). *Lb. rhamnosus* has limited capacity to metabolize complex oligosaccharides with moderately higher degree of polymerisation such as GOS and FOS because it lacks key hydrolases like specific β -galactosidases and fructosidases required to cleave β -1,4 and β -1,6 glycosidic linkages (Arnold et al., 2018). Furthermore, even when partial hydrolysis occurs, *Lb. rhamnosus* often lacks the specialized transport machinery required for cellular uptake. Specifically, the absence of dedicated ATP-binding cassette (ABC) transporters or specific phosphotransferase systems (PTS) prevents the translocation of these larger oligosaccharide fragments across the bacterial cell membrane (Murtini et al., 2015).

On the other hand, *Lpb. plantarum* is known for its versatile metabolic pathways and ability to utilise a wide range of carbohydrates including oligosaccharides from different food types (Cui et al., 2021; Ming et al., 2018). This adaptability allows it to efficiently ferment various plant-based oligosaccharides, leading to enhanced metabolite production. Therefore, it is recommended to optimise oligosaccharide extract-*Lpb. plantarum* combinations for the formulation and advancement of synbiotic food products.

Variations in the individual concentrations of SCFAs affect the total SCFAs and their molar ratios produced. Only soybean oligosaccharide extract had (67:18:15-Acetate: propionate: butyrate) molar proportions of SCFAs comparable with recommended ranges (60:20:20-

Acetate: propionate: butyrate) when *Lpb. plantarum* was used (Besten et al., 2013). An optimal molar ratio of SCFAs is essential for maximising their local and systemic health benefits. For instance, the local effects include reduced gut pH, inhibition of gastrointestinal pathogens through decreased adherence of enteric pathogens to the epithelial cells of the gastrointestinal tract and improved gastrointestinal barrier (Al-Qadami et al., 2022; Yamamura et al., 2023). Systemically, SCFAs enhance the absorption of minerals like calcium and magnesium, and show immune-regulatory, cardio-protective, neuro-protective, anti-inflammatory, and anti-oxidant activities (Caetano & Castelucci, 2022; Kumari et al., 2024). The results of the current study indicate that the evaluated oligosaccharides particularly soybean oligosaccharides and *Lpb. plantarum* combinations have the potential to create prebiotic and probiotic product combinations that have the potential to synergistically improve host health when consumed. However, in vivo studies are necessary to determine the concentration of SCFAs after consumption of such food products to evaluate their associated beneficial attributes, particularly among vulnerable populations.

It also provides an opportunity to explore the potential of improving the molar proportions of total SCFA from other oligosaccharides extracts by making composite formulae. This processing technology has been used to increase the nutritional quality of plant-based foods in resource constrained settings (Abe-Inge et al., 2020; Agbor Asuk et al., 2020; Alowo et al., 2018). However, further studies should evaluate the effect of nutrient-nutrient interaction on the fermentability and quantity of SCFA produced by the composite products and their microbial diversity and abundance.

4.4.3 Potential of oligosaccharide extracts from different plant sources to enhance antagonistic activity of *Lpb. plantarum* and *Lb. rhamnosus* against common enteric pathogens

The enhanced antagonistic activity of the study probiotics against enteric pathogens concurs with those of Ślizewska and Chlebicz-Wójcik (2020) where *Lactobacillus* species cultured in inulin as a prebiotic source significantly inhibited *Salmonella* growth to non-detectable levels compared to β -glucan and maltodextrin. Furthermore, a combination of *Lb. rhamnosus* NCDC 298 and FOS reduced risk of entero-toxigenic *E. coli* infection (Anand et al., 2018). The enhancement of antagonistic activity is influenced by the specific probiotic

strain, the type of enteric pathogen, and the available prebiotic oligosaccharide source. Piatek et al. (2020) reported variation in the degree of pathogen inhibition of *Shigella* and *Salmonella* spp. by *Lb. rhamnosus* GG. This suggests that selecting the appropriate combination of probiotic and oligosaccharide source is crucial for achieving effective inhibition of enteric pathogens.

This study indicates that oligosaccharide extracts from sorghum, soybean and sesame selectively promoted the growth of probiotic bacteria (*Lpb. plantarum* and *Lb. rhamnosus*) (Figure 4.5 and 4.6) while inhibiting the growth of enteric pathogenic bacteria. This selective promotion is another crucial attribute for a substrate to be classified as a prebiotic (Gibson et al., 2017). *Shigella* and *Salmonella* infections are significant contributors to childhood functional gastrointestinal disorders such as diarrhoea in low- and middle-income countries (Kotloff et al., 2017). Traditionally, these infections have been treated with antibiotics such as azithromycin, and colistin (Moshynets et al., 2023). On the contrary, recent studies indicated a rise in antibiotic resistance in these regions, prompting the exploration of alternative therapeutic approaches (Yang et al., 2023). One such approach involves the use of prebiotic oligosaccharides and probiotics, either individually or in combination, to treat FGIDs (Ranjbar & Farahani, 2019).

The ability of oligosaccharide extract-probiotic combinations to reduce the bacterial pathogens depends on the extent of fermentation by the probiotic and the type of fermentation metabolites. SCFAs are the major health promoting metabolites produced which have anti-microbial, and anti-inflammatory properties (Hu et al., 2019; Liu et al., 2021). Additionally, SCFAs effectively inhibit enteric pathogens through a pH, concentration and complexity-dependent pathway (Xiong et al., 2022). When these SCFAs are ingested they enhance mucus production which increases intestinal barrier integrity (Deleu et al., 2021). However, other factors such as the initial concentration of host gut bacteria, and host responses influence the prebiotic mechanistic actions and hence the host's health outcome (Bussolo et al., 2019). Therefore, the efficacy and effectiveness of the oligosaccharides with prebiotic properties under physiological conditions need to be explored. Furthermore, to evaluate its potential as a functional food for children under the

age of five, it is essential to perform *in vitro* fermentation using faecal microbiota from this age group. This will allow for the observation of microbiota changes and interactions.

4.5 Conclusion and Recommendation

This study highlights the superior prebiotic properties of oligosaccharides extracted from improved cereal and legume varieties when fermented with selected probiotic strains. The oligosaccharide extracts promoted proliferation of the probiotic cell density exceeding the World Health Organisation's minimum recommended levels (6 log CFU/ml or log CFU/g) for probiotic products. Furthermore, the observed increase in the production of total short-chain fatty acids (SCFAs) when *Lpb. plantarum* was cultured in oligosaccharide substrates derived from Maksoy 3N soybean and Sesim 2 sesame varieties which may have contributed to enhanced antagonistic activity against pathogenic species. This positions *Lpb. plantarum* as a promising candidate for targeted applications in probiotic and synbiotic food production. Future research should focus on optimising combinations of plant oligosaccharide extracts with *Lpb. plantarum* and other potential probiotic micro-organisms to maximise their production of beneficial metabolites and probiotic functionality. This could include evaluating different oligosaccharide types, composite proportions, micro-organisms used, and fermentation conditions to enhance product yields.

Incorporating oligosaccharide plant sources with prebiotic potential, such as improved cereal and legume varieties, in the diet of populations at risk of functional gastrointestinal diseases may help reduce the incidence and severity of these conditions. These findings can also be used to develop targeted nutrition interventions such as the development of food products with both prebiotic and probiotic properties for vulnerable populations, such as children under five years old. However, it is crucial to investigate the efficacy and effectiveness of oligosaccharides with prebiotic properties under physiological conditions.

To evaluate the potential of prebiotic-probiotic combinations as effective functional foods for FGIDs, it is essential to perform comprehensive genomic analyses. Such investigations will enable the characterisation of the genotypic diversity of microbial strains present in these formulations, providing critical insights into their functional attributes, safety profiles, and adaptability as adjunct interventions for management of FGIDs. This approach will also

help identify strain-specific health-promoting properties and inform the development of targeted nutritional interventions for Gastrointestinal disorders among vulnerable populations.

This study showed that oligosaccharides from improved food resource varieties and *Lactiplantibacillus plantarum* ATCC 14917 combinations yielded better prebiotic properties compared to combinations with *Lactocaseibacillus rhamnosus* ATCC 7469. The finding highlights the critical role of selecting appropriate oligosaccharide food sources-probiotic combinations to optimise prebiotic effect. Moreover, the results provided a basis for recommending their use as raw materials in the development of single-line or composite foods and beverages with synbiotic characteristics. This recommendation informed the focus of the study in Chapter five.

CHAPTER FIVE

Influence of ingredient type and fermentation time on genotypic characteristics of lactic acid bacteria in fermented composite beverages

ABSTRACT

Functional gastrointestinal disorders (FGIDs) are increasingly associated with microbial dysbiosis and compromised gut physiology. Fermented plant-based foods present a promising avenue for probiotic intervention due to their bioactive potential. This study evaluated genotypic plasticity and orthogroup distribution of lactic acid bacteria (LAB) isolated from millet-sesame-soybean composite fermented beverages. Thirteen LAB isolates were subjected to whole-genome sequencing, and genome quality was assessed via N50, L50, and GC content metrics. Taxonomic classification was conducted using Average nucleotide identity, while multilocus sequence typing (MLST) evaluated strain-level differences. Orthogroup clustering via OrthoFinder enabled functional categorisation of core and accessory genes. The resulting genome assemblies were of high-quality with low L50 values and GC content between 42.29% and 46.71%, consistent with *Lactobacillaceae* reference genomes. Average Nucleotide Identity (ANI) hierarchical clustering revealed that ingredient composition had greater selective pressure on isolate clustering than fermentation time. Dominant species included *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus*. MLST revealed intra-species plasticity within composite beverages that registered potential novel strains irrespective of fermentation time. Twelve universally conserved core orthogroups (G50 = 12) were identified, alongside extensive accessory gene variability, underscoring the functional potential of composite substrates. These findings suggest that composite fermented beverages are valuable platforms for developing functional foods with multiple probiotic strains due to their genotypic plasticity. These could act as cost-effective vehicles for increasing consumption of beverages with positive bioactive properties that can improve gastrointestinal health. However, further *in vitro* and *in vivo* validation of probiotic traits is recommended.

Key words: Ready-to-use fermented composite beverages, Genotypic plasticity, Functional gastrointestinal disorders

5.1 Introduction

Fermentation is a traditional and widely practiced food processing method across cultures. It enhances food safety, shelf life, nutrition, and supports health through beneficial microbes and bioactive compounds (Sawant et al., 2025; Voidarou et al., 2021). In Sub-Saharan Africa, fermented foods are broadly categorised into non-alcoholic cereal-based products, fermented legumes, root crops, animal proteins, and alcoholic beverages (Bamigbade et al., 2022). The quality of these foods largely depends on the raw materials, microbial strains, and the hygiene of the processing conditions, equipment, and personnel.

Fermented foods are produced either through spontaneous fermentation or by inoculation with defined starter cultures. Spontaneous fermentation relies on native microbiota from raw materials and the environment, leading to dynamic microbial succession (Sooresh et al., 2023; Uzkuç et al., 2020). Common microbial groups in fermentation include lactic acid bacteria (LAB), acetic acid bacteria, yeasts, and *Enterobacteriaceae* (Diaz et al., 2019; Mukisa et al., 2012), with LAB genera such as *Lactobacillus*, *Lactococcus*, and *Weisella* being dominant (Wang et al., 2021). Products like *ogi* (Adisa & Enujiugha, 2020), *obushera* (Mukisa, et al., 2012), *mahewu* (Franz et al., 2014), and soy-based beverages (Mridula & Sharma, 2015) exemplify traditional spontaneous fermentations.

In contrast, industrial fermentations commonly use defined starter cultures to ensure product consistency, safety, and scalability (Bintsis, 2018; Kandasamy et al., 2018). These cultures may reduce microbial diversity and limit the spectrum of bioactive compounds, thereby diminishing the nutraceutical potential of the final product (Fitsum et al., 2025; Squillante et al., 2025). As a result, some industries now adopt modified spontaneous fermentation using native microbial communities (autochthonous microorganism) under controlled good manufacturing practices (GMPs) to retain complexity while improving safety (Praveen & Brogi, 2025; Sternes et al., 2017).

Spontaneously fermented foods produced under GMPs can harbour diverse microbial strains with potential technological, probiotic, and health-promoting attributes (Malongane & Berejena, 2024). While such foods have shown promise in managing gastrointestinal and cardio-metabolic conditions (Fitsum et al., 2025), detailed knowledge of the microbiomes

involved, especially in composite ingredients, is limited. Most microbial studies have focused on single-ingredient substrates like millet or soybean (Liang et al., 2018; Song et al., 2020). Cereal-legume composites remain underexplored despite their potential to enhance microbial diversity via increased genomic and metabolic plasticity driven by diverse available carbon substrates (Qiao et al., 2012). These diverse carbon substrates may facilitate microbial adaptation and substrate utilisation flexibility. Nevertheless, genotypic plasticity phenomenon of lactic acid bacteria within food composites remains poorly defined.

Additionally, fermentation duration is a critical determinant of substrate breakdown and metabolite yield (Gautam et al., 2025). The duration must be sufficient to generate beneficial metabolites such as short-chain fatty acids, exopolysaccharides, and bioactive peptides, while avoiding excessive fermentation that may lead to undesirable byproducts (e.g., biogenic amines, ethanol) (Siddiqui et al., 2023). Most probiotic-enriched fermented beverages are optimised within 24–48 hours of fermentation, a window that balances microbial growth, substrate utilisation, and metabolite production (Chaichana et al., 2026). However, there remains limited investigation into how fermentation time directly shapes the final lactic acid bacteria genotypic characteristics isolated from composite beverages. This study therefore evaluated the genotypic characteristics of LAB isolated from millet-sesame-soybean and sorghum-sesame-soybean composite fermented beverages subjected to varying fermentation durations of 24 and 48 hours.

5.2 Materials and methods

5.2.1 Food ingredients used to develop the ready-to-use fermented beverages

The following crop varieties identified in Chapter four of this dissertation for their superior prebiotic properties were selected as key raw materials for developing fermented beverages: finger millet (*Eleusine coracana*, indigenous variety), sorghum (*Sorghum bicolor*, variety Narosorg 2), sesame (*Sesamum indicum*, variety Sesim 2), and soybean (*Glycine max*, variety Maksoy 3N)

A total of eight fermented beverages (four ingredient types × two fermentation durations) were analysed to assess lactic acid bacteria diversity. Two widely consumed commercial

cereal-based fermented beverages within northern Uganda markets of Gulu and Lira were selected for comparative analysis of genotypic characteristics. These beverages did not undergo post-fermentation heat-treatment. For each treatment, 300 ml aliquots from four independent production batches were pooled and homogenised using a laboratory-grade blender to ensure sample uniformity (Varney et al., 2017). Formulations of the composite beverages were made as previously described by Alowo et al. (2018). Briefly, millet/sorghum, sesame and soybean were blended to obtain either millet or sorghum based composite flour. The blending ratios were 76% millet or sorghum, 4% sesame and 20% soybean. Spontaneous fermentation procedure was used as previously described by Mukisa et al. (2010) (Appendix 5). To ensure reliability, each fermentation experiment was conducted in duplicate.

In accordance with the Convention on Biological Diversity (CBD) and the Nagoya Protocol on Access and Benefit-Sharing, this study ensured the ethical and legal utilization of genetic resources (United Nations, 2014). Food samples containing the studied isolates were legally acquired from local suppliers in northern region. The isolates obtained were strictly for academic research and non-commercial purposes at this stage.

5.2.2 Enumeration and isolation of lactic acid bacteria (LAB)

Isolation of lactic acid bacteria (LAB) was carried out following the method described by Jin et al. (2018). Briefly, 1 ml of the supernatant was serially diluted in 9 ml of peptone water, and 200 µl of the selected dilution was spread-plated in duplicates on de Man, Rogosa, and Sharpe (MRS) agar (HiMedia, India). The inoculated plates were incubated anaerobically at 37°C for 48 hours. Colonies were enumerated and categorised based on morphological characteristics such as shape and size. Following incubation, approximately 120 presumptive lactic acid bacterial colonies (15 colonies per beverage) were randomly selected and purified by repeated streaking on MRS agar. All isolates were characterized based on colony morphology, Gram reaction, cell morphology and catalase activity. Isolates sharing similar phenotypic characteristics were clustered into phenotypic groups to minimise redundancy. Three representative isolates from the dominant or distinct phenotypic groups in each beverage were then selected for molecular characterization. This resulted in a total

of 24 isolates that were subjected to whole genome sequencing and comparative genomic analyses using *Lactiplantibacillus plantarum* ATCC 14917 as the reference strain. The isolates were suspended in 30% v/v glycerol and dispensed into sterile cryogenic vials at -80°C for subsequent analysis within six months.

5.2.3 Genomic DNA extraction

Genomic DNA from the isolates ($n = 24$) was extracted using a D6005 quick DNA Fungal/Bacterial MiniPrep kit following the manufacturer's (Zymo Research, Irvine, 17062 Murphy Ave, United States) instructions (Baer et al., 2024). Briefly, the isolates were washed, centrifuged, and a 50 ml of the concentrated solution was resuspended in 200 μL of PBS buffer. This was then transferred to ZR BashingBead™ Lysis Tube followed by addition of 750 μL BashingBead™ Buffer. The mixture was vortexed at maximum speed for 10 minutes to facilitate cell lysis to release DNA. The mixture was then centrifuged (VWR Micro star 17, 521-1646, Berlin, Germany) at $10000 \times g$ for 1 minute to obtain a supernatant. Exactly 400 μL of the supernatant was transferred into the Zymo-Spin™ III-F Filter in a collection Tube and centrifuged at $8,000 \times g$ for 1 minute. Subsequently, 1,200 μL of Genomic Lysis Buffer was added to the filtrate, and 800 μL of the mixture was passed through the Zymo-Spin™ IICR Column in a Collection Tube and centrifuged at $10,000 \times g$ for 1 minute. The DNA in the column was first washed with 200 μL of DNA pre-wash buffer and centrifuged at $10,000 \times g$ for 1 minute followed by a wash with 500 μL of g-DNA wash buffer and another centrifugation. Finally, 100 μL of DNA elution buffer was added to the column and centrifuged at $10,000 \times g$ for 30 seconds to elute the extracted genomic DNA.

The quality and concentration of the DNA was assessed using agarose gel electrophoresis (1% w/v agarose in 0.5X Tris-acetate-EDTA buffer) and spectroscopic analysis with a NanoDrop One spectrophotometer (Thermo Scientific, Saveen Werner ApS, Denmark), respectively. The final DNA concentration exceeded 100 ng/ μL , with a 260 nm/280 nm ratio ranging between 1.8 and 2.0. All extracted DNA samples were suspended in 30% v/v glycerol and dispensed into sterile cryogenic vials and stored at -20°C for subsequent analysis within 3 months.

5.2.4 Polymerase Chain Reaction (PCR) amplification of the extracted genomic DNA

The 16S rRNA gene for the 24 isolates was amplified using a thermal cycler, with the extracted genomic DNA serving as a template using the method described by Haarman and Knol (2006). Universal bacterial primers: Forward-eub 5'TCCTACGGGAGGCAGCAGT 3' and Reverse-eub 5' GGACTACCAGGGTATCTAATCCTGTT 3' (Macrogen, Inc., Europe BV) were used.

To select isolates of the *Lactobacillus* genus, genus-specific primers Forward_allact_IS (5'-TGGATGCCTTGGCACTAGGA-3') and Reverse_allact_IS (5'-AAATCTCCGGATCAAAGCTTACTTAT-3') were used (Macrogen, Inc., Europe BV) (Haarman & Knol, 2006). The rationale for focusing exclusively on *Lactobacillus* genus stems from extensive literature indicating their dominance in plant-based fermented beverages, where they play a pivotal role in acidification, flavour development, and probiotic functionality (Adimpong et al., 2012; Mukisa, et al., 2012). Studies have consistently shown that *Lactobacillus* genus have high species and strain-level diversity in various fermented products, making them ideal candidates for further genomic and functional characterisation (Xu et al., 2015; Yu et al., 2021; Zhang et al., 2021). By narrowing the scope to this genus, the study aims to capture the most ecologically and functionally relevant isolates for downstream analysis.

Each reaction mix contained 12.5 µL of 2X GoTaq® green master mix (Promega Corporation, Madison, USA), 9.5 µL of Nuclease free water, 0.5 µL of forward primers, 0.5 µL of reverse primer and 2 µL of DNA template (Horie et al., 2019). The reaction conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 seconds, annealing 53°C for 50 seconds for 16S rRNA and 49°C for 50 seconds for *Lactobacillus*, extension at 72°C for 30 seconds, 40 cycles and final extension 72°C for 10 minutes.

The PCR products were confirmed with 1% (w/v) agarose gel electrophoresis stained with Ethidium bromide and subsequently visualised on the gel documentation system with a 100 bp DNA ladder for reference. Distinct and strongly amplified bands were observed in 13 isolates following gel electrophoresis (Appendix 4). Inclusion of the reference strain

Lactiplantibacillus plantarum ATCC 14917 brought the total to 14 isolates selected for whole genome sequencing.

5.2.5 Whole genome sequencing of extracted genomic DNA

Whole genome sequencing of 14 isolates confirmed under sub-section 5.2.4 was done by Inqaba Biotec south Africa using PacBio Onso™ sequencing platform. The DNA libraries were prepared with the Pacific Biosciences Template Prep Kit 2.0 (Pacific Biosciences California, Inc) and were used for subsequent clustering and sequencing. Quality sequence assessment was conducted using FASTQC version (0.12.1) where low-quality sequences <100bp were removed. The sequences were then assembled following de Novo assembly using St. Petersburg genome Assembler (SPAdes) (v. 3.5.0; –sc –careful) (Sternes et al., 2017) and were analysed to determine contig metrics specifically, number of sequences, sequence size statistics (N50 and L50) and consensus contigs. The completeness of the assemblies was determined by Benchmarking Universal Single-Copy Orthologs (Busco) v. 5.8.3 (Tenea & Ortega, 2021).

5.2.6 Bioinformatic analysis

5.2.6.1 Gene prediction, annotation and genomic similarity assessment

Fourteen whole genome sequences of the isolates were annotated using Prokaryotic Genome annotation system (Prokka) v1.14.6 to determine samples genetic composition (De Angelis et al., 2020). Species taxonomic classification was evaluated using average nucleotide identity (ANI) with the following cut off value interpretation 95-96%-indicating genomes of the same species, 90-95% are closely related but distinct species, 85-90% indicates same genus but different species (Arahal, 2014; Chokesajjawatee et al., 2020). Pairwise genomic similarities across all sequenced isolates were determined by calculating ANI using Fast alignment free-computation of whole-genome Average Nucleotide Identity (FastANI) version 1.34. All-versus-all genomic comparisons were made utilising a reciprocal fragment mapping approach with a default fragment length of 3,000 base pairs. To satisfy the requirements of downstream distance-based clustering algorithms, the resulting asymmetric

ANI similarity matrix (S) was converted into a symmetric dissimilarity (distance) matrix (D) following equation 5.1.

$$D_{ij} = 1 - \left(\frac{ANI_{ij}}{100}\right) \dots \dots \dots \text{Equation 5.1}$$

Where; (D_{ij}) represents the genetic distance between genome (i) and genome (j), and (ANI_{ij}) represents the mean percentage of nucleotide identity shared between their homologous genomic segments.

The whole genome sequences of the isolates were aligned against six reference genomes using the Basic Local Alignment Search Tool (BLAST). The specific reference strains included *Lacticaseibacillus rhamnosus* (strains BBAH2, CP-1, and GG) and *Lactiplantibacillus plantarum* (strains RSCM103295, IDCC3501, and DSM20174). These reference genomes were selected based on preliminary alignment metrics showing >99% sequence identity and 100% query coverage with zero gaps in the local alignment.

5.2.6.2 Phylogenetic Reconstruction and Clustering via Neighbour-Joining

To account for potential variations in evolutionary mutation rates across different lineages without assuming a strict molecular clock, hierarchical clustering was performed using the Neighbour-Joining (NJ) method. The NJ algorithm was applied to the distance matrix to sequentially identify and pair operational taxonomic units (OTUs) that minimised the total sum of branch lengths at each clustering step (Fernández et al., 2023). This top-down decomposition resolved the phylogenetic topologies based on net divergence rather than raw average distances alone. All data parsing, matrix structural formatting, and neighbour-joining tree constructions were executed within the Python programming environment (v3.10) utilising the Biopython library (v1.81), specifically via the Bio.Phylo.TreeConstruction module (Cock et al., 2009). The final clustering topologies were evaluated against the standard 95-96% ANI threshold widely accepted for prokaryotic species delineation (Arahal, 2014).

5.2.6.3 MLST gene selection

Although whole genome sequencing data were generated for all isolates, *in silico* multilocus sequence typing (MLST) was additionally performed using the assembled genome sequences. Housekeeping gene alleles were identified from the WGS assemblies and compared with the corresponding reference MLST database to assign sequence types. The purpose of MLST was not to generate additional sequencing data, but to classify isolates using a globally standardized typing scheme that facilitates comparison with previously characterised strains and public databases. Whole genome sequencing provided comprehensive genomic information for functional annotation and comparative genomic analyses, whereas MLST served as a complementary tool for standardized strain identification and epidemiological comparison (Xu et al., 2015). To facilitate strain-level differentiation of LAB isolates, eight conserved housekeeping genes including *clpX*, *groEL*, *murC*, *murE*, *pheS*, *pyrG*, *recA*, and *uvrC* (Skotniczny & Satora, 2023), were used for MLST. These genes were chosen based on their chromosomal ubiquity and evolutionary conservation across LAB species, as they encode proteins essential for core cellular functions. The *clpX* gene encodes the ATP-dependent protease ATP-binding subunit *clpX*, which plays a role in protein quality control and stress response. The *GroEL* gene encodes the chaperonin *GroEL*, a molecular chaperone critical for proper protein folding. Both *murC* and *murE* are involved in peptidoglycan biosynthesis, encoding UDP-N-acetylmuramyl tripeptide synthase and UDP-N-acetylmuramoyl-alanyl-D-glutamate-2,6-diaminopimelate ligase, respectively. The *pheS* gene encodes the alpha subunit of phenylalanyl-tRNA synthetase, which is essential for accurate translation (Huang et al., 2020; Xu et al., 2015).

For each gene, distinct nucleotide sequences defined individual alleles. The combination of alleles across all eight loci constituted an isolate's unique allelic profile, which was used to assign a specific sequence type (ST). Isolates sharing identical allelic profiles were grouped under the same ST, enabling robust strain-level classification. To establish a reliable MLST framework, whole-genome sequences from the NCBI database exhibiting >98% similarity to the sample genomes were selected as references. Ten well-annotated genomes from *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* species were used to

construct the allele database (Table 5.1). These included CP039121.1

Lactiplantibacillus plantarum strain DSM 20174, CP096853.1: *Lactiplantibacillus plantarum* strain LPgoji, CP104753.1: *Lactiplantibacillus plantarum* strain MGEL20154, CP005942.2: *Lactobacillus plantarum* subsp. *plantarum* P-8, NZ_CP028221.1: *Lactiplantibacillus plantarum* strain SRCM100442, NZ_CP040780.1: *Lacticaseibacillus rhamnosus* strain 1.0320, CP184536.1: *Lacticaseibacillus rhamnosus* strain BBAH2, CP099396.1: *Lacticaseibacillus rhamnosus* strain P118, CP092033.1: *Lacticaseibacillus rhamnosus* strain VHProbi Y39, CP073711.1: *Lacticaseibacillus rhamnosus* strain X253.

To infer orthologous relationships and phylogenetic structure among the isolates, OrthoFinder v3.1.0 was employed (Emms & Kelly, 2019). This enabled comparative genomic analysis and evolutionary insights across the sampled LAB strains.

Table 5.1: Multi locus sequence typing generate database with eight *Lactobacillus* housekeeping genes

Representative strains with highest % identity per cluster	Housekeeping genes & respective clusters								assigned sequence type (ST)
	<i>MurE</i>	<i>MurC</i>	<i>GroEL</i>	<i>Phes</i>	<i>recA</i>	<i>UvrC</i>	<i>pyrG</i>	<i>Clpx</i>	
<i>Lactiplantibacillus plantarum</i> strain DSM 20174	1	1	1	1	1	1	1	1	ST1
<i>Lacticaseibacillus rhamnosus</i> strain 1.0320	2	3	2	2	2	4	2	2	ST2
<i>Lacticaseibacillus rhamnosus</i> strain BBAH2	3	2	3	3	3	5	3	2	ST3
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> P-8	1	1	1	1	1	2	1	1	ST4
<i>Lactiplantibacillus plantarum</i> strain SRCM100442	1	1	1	1	1	3	1	1	ST5

5.2.7 Statistical Analyses

To evaluate differences in gene family sizes among the individual isolates, statistical analysis was performed on the orthogroup gene count matrix, treating each identified orthogroup as an independent genomic feature. Because the distribution of gene counts per orthogroup was heavily skewed and non-normal, non-parametric methods were applied to compare the distributions. For descriptive reporting, mean gene copy numbers per orthogroup were calculated for each strain to assess global tendencies toward gene duplication or contraction.

The mean, rather than the median, was selected for this purpose because the median for almost every single strain in the OrthoFinder matrix was exactly 1. Kruskal–Wallis’s test, followed by Dunn’s post hoc comparisons with Bonferroni correction to control for Type I error. Statistical analysis was performed in python anaconda3 distribution with statistical significance set at $p < 0.05$.

5.3 Results

5.3.1 Genotypic characterisation of lactic acid bacteria from millet- and sorghum-based fermented beverages: Influence of ingredients and fermentation time

5.3.1.2 Hierarchical genomic clustering of microbial Isolates

A total of seven phylogenetic clusters were resolved (Fig. 5.1). The clustering patterns were driven more strongly by the type of ingredients used in producing the fermented beverages than by the duration of fermentation. For example, isolates derived from single-ingredient fermentations such as AK-S10D, AK-S11D, AK-S12D, AK-S8D, and AK-S13D formed distinct clusters separate from those obtained from composite fermentations, regardless of fermentation time. Within the primary cluster, the topology revealed a highly structured sub-lineage originating from a common ancestral node (branch length: 0.018), corresponding to 98.2% ANI similarity with *Lpb. plantarum*. From this node, two distinct evolutionary paths emerged: one leading to a moderately divergent lineage terminating in isolate AK-S6D (distance: 0.035; 96.5% ANI), and the other forming a highly conserved sister branch (distance: 0.013; 98.7% ANI). This conserved branch further bifurcated into two sub-clusters. The first displayed intermediate divergence (branch length: 0.032; 96.8% ANI), splitting into two closely related terminal strains *Lpb. plantarum* SRCM103295 and isolate AK-S1D each with equal branch lengths of 0.023 (97.7% ANI).

In contrast, the second sub-cluster displayed asymmetric evolution: one nearly invariant terminal branch (distance: 0.005; 99.5% ANI) encompassing isolates AK-S5D, the positive control AK-S14D, and the reference strain *Lpb. plantarum* DSM20174; and a more divergent companion lineage (distance: 0.032; 96.8% ANI) comprising isolate AK-S4D and the *Lpb. plantarum* IDCC3501 reference strain. Despite micro-evolutionary variations at the

strain level, all members of this nested sub-tree consistently exceeded the 95% ANI threshold, confirming their classification within *Lpb. plantarum*.

In contrast, isolates AK-S7D through AK-S13D clustered with *Lb. rhamnosus* species. Comparative ANI analysis against reference genomes revealed strong species-level identity that is; AK-S7D and AK-S9D shared 99.1% ANI with *Lb. rhamnosus* CP-1; AK-S8D and AK-S13D showed 99.2% ANI with *Lb. rhamnosus* GG; while AK-S10D, AK-S11D, and AK-S12D recorded 99.2% ANI with *Lb. rhamnosus* BBAH2. Notably, three isolates AK-S2D, AK-S3D, and AK-S6D formed a distinct cluster, underscoring their unique phylogenetic positioning relative to both *Lpb. plantarum* and *Lb. rhamnosus* lineages.

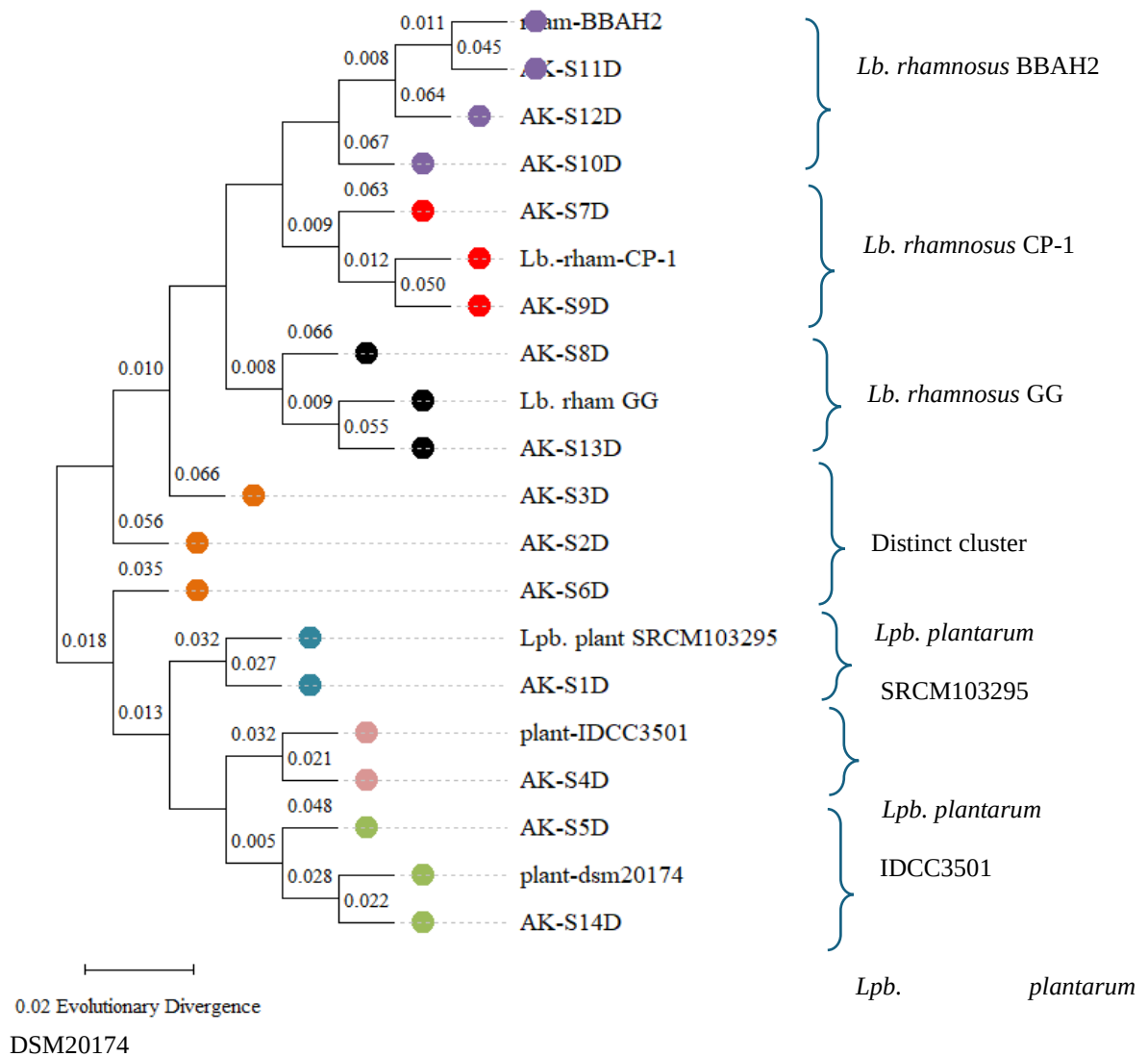


Figure 5.1: A dendrogram representation of isolated genome clusters based on average nucleotide identity. AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917, AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1.

5.3.1.3 Quality of the genome assembly and genetic diversity of the isolates

The genome assemblies of the isolates had lower L50 values and higher N50 values, indicating high contiguity. GC content ranged between 44% and 47% across most isolates,

except for AK-S5D, which showed a lower GC content of 42.32%. Multi-locus sequence typing revealed that the isolates had highest sequence identity exceeding 96% to *Lacticaseibacillus rhamnosus* strains BBAH2, CP-1, GG and *Lactiplantibacillus plantarum* strains DSM20174, IDCC350, and SRCM103295.

Except for strains AK-S2D and AK-S3D which exhibited unique gene loci combinations that may represent novel strains (Table 5.2), *Lactiplantibacillus plantarum* was detected in all composite beverage samples, regardless of fermentation duration. Specifically, AK-S2D varied in the *clpX* gene, AK-S3D varied in both *murC* and *clpX* gene alleles.

Table 5.2: Genome quality assembly metrics, species, and strain identification

Isolate ID	Isolate source	Fermentation time (hrs)	MLST strain classification with >96% identity	N50	L50	E-size	Genome size	GC (%)
AK-S9D	Millet	24	<i>Lacticaseibacillus rhamnosus</i> CP-1	11456	72	16776.7	2975865	46.46
AK-S1D	Millet-sesame-soybean	24	<i>Lactiplantibacillus plantarum</i> SRCM103295	3373057	1	3011798.2	3777746	45.58
AK-S2D	Millet-sesame-soybean	24	<i>Novel strain</i>	3305809	1	1732159.9	6316859	45.31
AK-S5D	Sorghum-sesame-soybean	24	<i>Lactiplantibacillus plantarum</i> DSM20174	3257043	1	2143265.1	4951888	42.32
AK-S6D	Sorghum	24	<i>Novel strain</i>	3982089	1	2308879.6	6892986	44.95
AK-S10D	Millet	48	<i>Lacticaseibacillus rhamnosus</i> BBAH2	3597750	1	1969266.1	6580060	45.54
AK-S11D	Millet	48	<i>Lacticaseibacillus rhamnosus</i> BBAH2	17139	26	655917.7	4692966	45.97
AK-S12D	Commercial <i>bushera</i>	n/a	<i>Lacticaseibacillus rhamnosus</i> BBAH2	3736613	1	2054390.5	6810535	45.55
AK-S13D	Commercial <i>bushera</i>	n/a	<i>Lacticaseibacillus rhamnosus</i> GG	14884	56	22977.5	3051326	46.66
AK-S14D	<i>Lactiplantibacillus plantarum</i> ATCC14917	n/a	<i>Lactiplantibacillus plantarum</i> DSM20174	15092	4	1777617.8	7280049	44.48
AK-S4D	Millet-sesame-soybean	48	<i>Lactiplantibacillus plantarum</i> IDCC3501	3274307	1	3177716.8	3373859	45.44
AK-S3D	Millet-sesame-soybean	48	<i>Novel strain</i>	4063001	1	2194984.1	7531409	44
AK-S7D	Sorghum-sesame-soybean	48	<i>Lacticaseibacillus rhamnosus</i> CP-1	3982089	1	2308879.6	6892986	44.88
AK-S8D	Sorghum	48	<i>Lacticaseibacillus rhamnosus</i> GG	12769	92	15679	6055738	45.6

n/a- not applicable

5.3.1.4: Genotypic comparative analysis using OrthoFinder

Table 5.3 presents the gene distribution patterns across the 14 isolates. A total of 69,371 genes were identified across the 14 isolates, with gene counts varying per isolate. AK-S13D recorded the lowest number of genes, totalling to 2,749. These genes were clustered into 6,186 orthogroups, of which 653 were classified as core orthogroups present in all isolates. Twenty-six orthogroups were unique to specific isolates and comprised 58 genes. Unique species orthogroups in 6 samples isolates (AK-S12D, AK-S1D, AK-S5D, AK-S6D, AK-S7D, and AK-S8D) were significantly different ($p < 0.05$) from those of other isolates. Out of these samples, 3 of the isolates were derived from composite beverages. Half of the assigned genes were in orthogroups containing 12 or more genes (G50), and 50% of all assigned genes were distributed among the largest 1,952 orthogroups (O50 = 1,952). Isolates AK-S13D and AK-S9D had the lowest orthogroup representation of less than 45%.

Table 5.3: Genomic Distribution Patterns of Gene Orthologues Across Bacterial Isolates

ortholog metrics									
Isolate ID	Number of genes	Number of genes in orthogroups	Number of unassigned genes	Number of orthogroups containing species	Number of species-specific orthogroups	Number of genes in species-specific orthogroups	Core orthogroups	G50	O50
AK-S10D	5882	5771 (98.1% ^a)	111 (1.9% ^a)	4810(77.8% ^a)	0	0			
AK-S11D	3353	3296 (98.3% ^a)	57 (1.7% ^a)	2976 (48.1% ^b)	0	0			
AK-S12D	5755	5636 (97.9% ^a)	119 (2.1% ^a)	4612 (74.6% ^a)	1	7 (0.1% ^a)			
AK-S13D	2749	2743 (99.8% ^a)	6 (0.2% ^a)	2570 (41.5% ^b)	0	0			
AK-S14D	3038	3027 (99.6% ^a)	11 (0.4% ^a)	2989 (48.3% ^b)	1	3 (0.1% ^a)			
AK-S1D	5066	4853 (95.8% ^b)	213 (4.2% ^b)	3987 (64.5% ^a)	3	6 (0.1% ^a)			
AK-S2D	5872	5824 (99.2% ^a)	48 (0.8% ^a)	4837 (78.2% ^a)	0	0			
AK-S3D	7212	6980 (96.8% ^b)	232 (3.2% ^b)	5363 (86.7% ^a)	0	0			
AK-S4D	3559	3503 (98.4% ^a)	56 (1.6% ^a)	3344 (54.1% ^b)	0	0			
AK-S5D	4911	4755 (96.8% ^b)	156 (3.2% ^b)	4621 (74.7% ^a)	3	6 (0.1% ^a)			
AK-S6D	6093	5976 (98.1% ^a)	117 (1.9% ^a)	4864 (78.6% ^a)	3	6 (0.1% ^a)			
AK-S7D	7471	7138 (95.5% ^b)	333 (4.5% ^b)	5259 (85% ^a)	9	18 (0.2% ^a)			
AK-S8D	5622	5352 (95.2% ^b)	270 (4.8% ^b)	4232 (68.4% ^{ab})	6	12 (0.2% ^a)			
AK-S9D	2788	2778 (99.6% ^a)	10 (0.4% ^a)	2577 (41.7% ^b)	0	0			
Total	69371	67632 (97.5%)	1739 (2.5%)	6186	26	58 (0.1%)	653	12	1952

G50: orthogroup size at which 50% of all genes are found in orthogroups of that size or larger. O50: minimum number of orthogroups needed to account for 50% of the genes. AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917, AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, Ak-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1. Means in the same column with different superscripts are significantly different from each other at alpha value of 0.05%

Figures 5.2 and 5.3 depict the distribution of orthogroups (OGs) across the genome samples using binary heatmaps. Figure 5.2 presents the total orthogroups, including both core and accessory OGs identified in each genome, while Figure 5.3 specifically highlights accessory orthogroups that are not universally conserved. A set of eight core orthogroups (OG0000000, OG0000141, OG0000282, OG0000423, OG0000564, OG0000705, OG0000846, and OG0000987) were consistently present across all isolates. In contrast, orthogroups OG0005781, OG0005922, and OG0006063 were predominantly found in isolates AK-S3D, AK-S5D, and AK-S7D. Figure 5.3 reveals pronounced variability in the distribution of accessory orthogroups across genome samples, underscoring their contribution to strain-specific diversity. Isolates AK-S11D, AK-S13D, and AK-S9D lacked a suite of seven accessory orthogroups including OG0002357, OG0002570, OG0005765, OG0005978, OG0005339, OG0005126, and OG0005552 compared to other samples. These orthogroups encode a range of gene products, including hypothetical proteins, tRNA (Met) cytidine acetyltransferase, major cardiolipin synthase ClsA, arsenate reductase, and the HTH-type transcriptional regulator CueR, suggesting potential differences in stress response mechanisms and transcriptional regulation among the isolates.

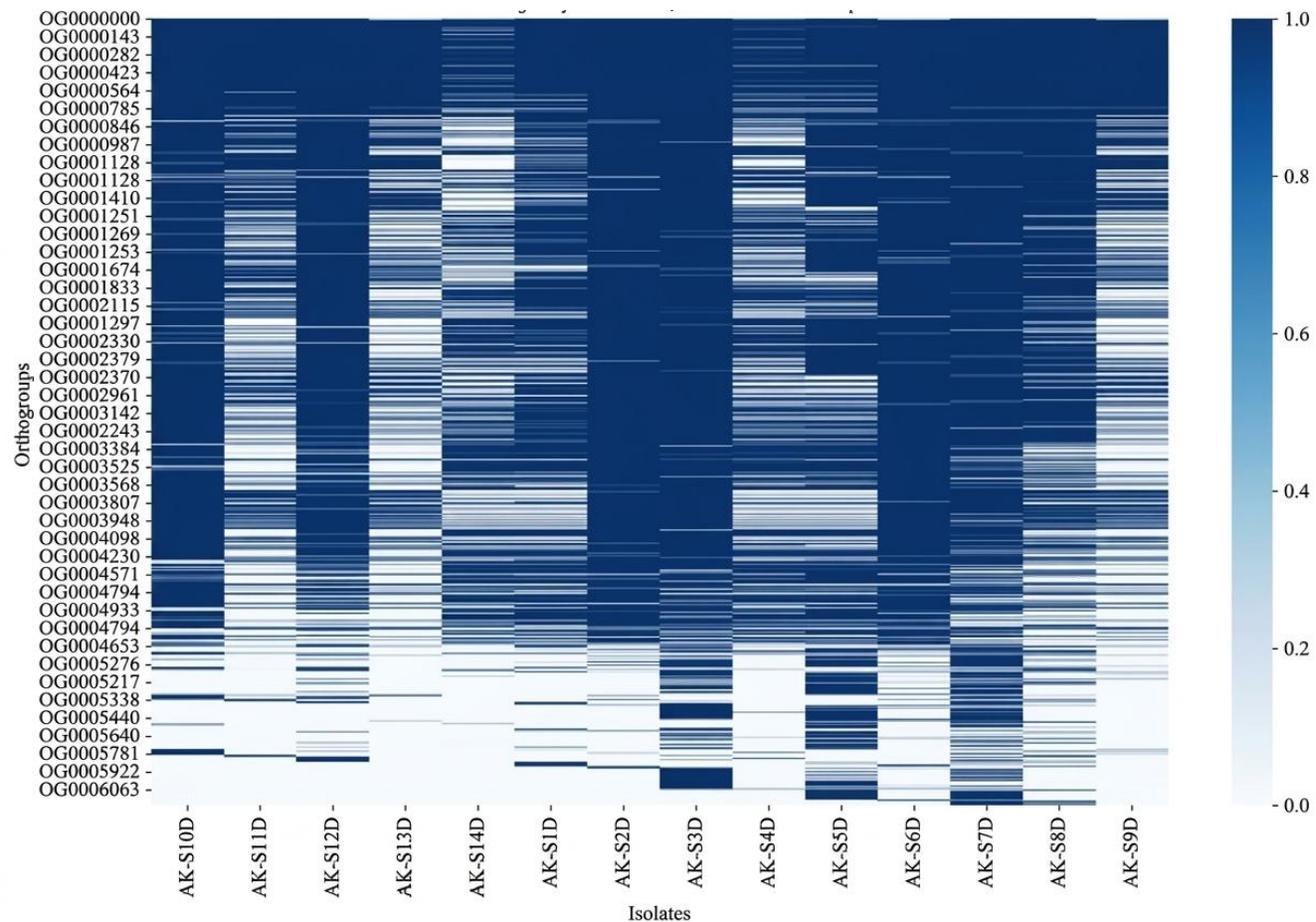


Figure 5.2: A heatmap showing the presence of orthogroups (core and accessory) across different genome species AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917, AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, Ak-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1. The darker coloured cells indicate presence, and the light-coloured cells indicate absence of orthogroups

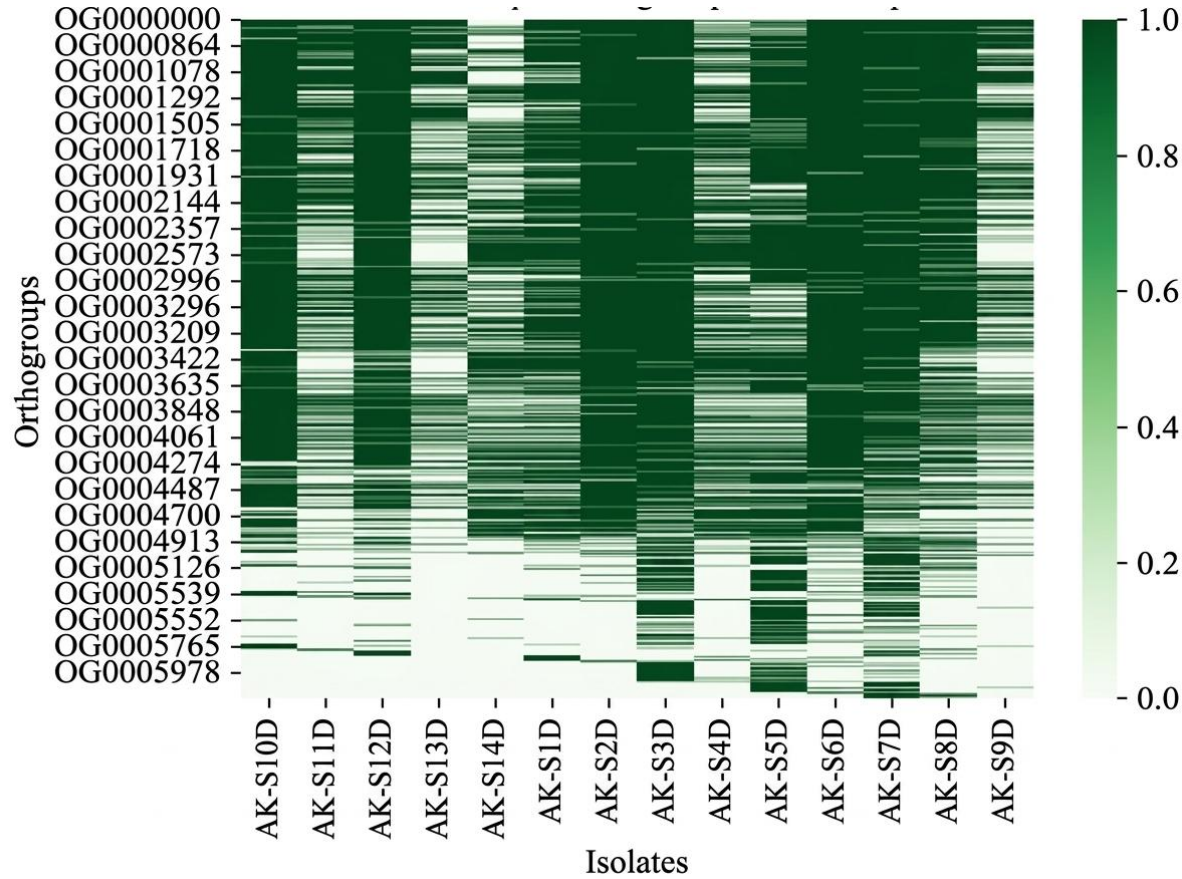


Figure 5.3: A heatmap showing the presence of accessory orthogroups across different genome species AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: positive control, AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D: sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1. The darker coloured cells indicate presence, and the light-coloured cells indicate absence of accessory orthogroups.

5.4 Discussion

The bacterial isolates from single and composite fermented beverages showed robust genome assembly quality, as evidenced by low L50 values. Given that L50 denotes the smallest number of contigs required to span 50% of the genome, lower values reflect greater assembly contiguity which is a prerequisite for reliable gene prediction and orthogroup delineation (Nunvar et al., 2010). High-quality assemblies, therefore, enhance the resolution of comparative genomic analyses. The GC content of these isolates ranged from 42.29% to 46.71%, aligning with the established genomic signatures of the *Lactobacillaceae* family (Stefanovic & McAuliffe, 2018). Notably, GC content variability may hold functional implications for microbial performance under fermentation stress. Isolates with GC content exceeding 45%, particularly within stress-related genes are postulated to have superior stress tolerance during processing conditions (Ilyin et al., 2023). This may stem from enhanced transcriptional stability, stronger transcription factor binding, and prolonged mRNA half-life under acidic and osmotic stress conditions (Mann & Chen, 2010).

Genome isolates that cluster closely with a reference genome typically represent high sequence identity and conserved genomic architecture, suggesting a close evolutionary relationship and adaptation to similar ecological niches (Fig 5.1). This is supported by the observed average nucleotide identity (ANI) values ranging above 95% across all clusters, which fall within the species-level threshold proposed by Richter and Rossello (2009). Palleja et al. (2018) further emphasised that the 95% value is stringent enough to identify closely related genes. Similar ANI ranges have been reported for *Lactobacillus* strains (Chokesajjawatee et al., 2020).

In contrast, isolates forming a distinct cluster may represent a subspecies or divergent lineage, potentially shaped by ecological or functional pressures. Such divergence is often accompanied by unique gene repertoires that reflect adaptation to specific environmental substrates. In this context, Chapter four of the dissertation showed that oligosaccharides extracted from improved Ugandan varieties of millet, sorghum, sesame, and soybean selectively enhanced growth and SCFA production in *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus*, suggesting substrate-specific genomic adaptations. This is

further supported by comparative genomic studies from Zhang et al. (2025) who reported that host-associated microbial clusters were enriched in carbohydrate-active enzymes and adhesion factors, indicating niche-specific genomic plasticity.

The dendrogram clusters were further confirmed by Multilocus sequence typing (MLST) that revealed dominance *Lactiplantibacillus plantarum* from composite beverages and *Lactocaseibacillus rhamnosus* single-ingredient beverages, with sequence identity values above 98%. The potential novel strain registered in the distinct cluster could be due to functional divergence and genomic plasticity. Recent comparative genomic studies confirm that *Lpb. plantarum* harbours extensive repertoires of carbohydrate-active enzymes (CAZymes), stress tolerance genes, and multiple *plantaricin* bacteriocin operons, enabling survival in diverse niches (Chaichana et al., 2026). Furthermore, the detection of highly conserved branches alongside moderately divergent lineages in the study (Fig. 5.1) aligns with reports that *Lpb. plantarum* strains balance genomic conservation with niche-specific adaptations, such as in composite fermented food environments (Tenea et al., 2025). Genomic plasticity enables genomes to undergo structural and functional changes in response to alternative available fermentable substrate source like in composite beverages (Tsushima et al., 2019).

Additionally, *Lpb. plantarum* has an open pan-genome with excessive accessory gene variability (Carpi et al., 2022). This genomic architecture enhances their ecological versatility, functional adaptability, and evolutionary plasticity especially within the context of fermented food systems and probiotic formulations (You et al., 2023). An open pan-genome implies that sequencing additional strains continually reveals novel genetic features, rather than converging on a core set of conserved genes. Such ongoing discovery, as highlighted by Bhattacharya et al. (2025), emphasises the genetic fluidity and horizontal gene acquisition potential of *Lpb. plantarum* and *Lb. rhamnosus*. This trait enhances their capacity to thrive across diverse niches and respond dynamically to environmental alterations. Moreover, the detection of potential novel strains differing by only one or two genes from reference genomes further supports the presence of micro-variations and functional divergence such as short-chain fatty acid (SCFA) synthesis, mucosal adhesion ability, and bile salt hydrolase activity that contribute to host interactions and probiotic

efficacy (You et al., 2023). Nonetheless, this study did not further relate the observed plasticity with functional insights.

Whole genome sequencing provides the highest genomic resolution and encompasses all information contained within conventional MLST schemes (Pham et al., 2025). Nevertheless, MLST remains valuable because it offers a universally accepted framework for strain nomenclature and enables direct comparison with isolates reported in international databases and previous studies. Accordingly, the present study used WGS as the primary analytical platform, with MLST profiles derived in silico from the genome assemblies to provide standardized strain classification and facilitate comparison with existing *Lactobacillus* sequence types.

The identification of universally conserved core orthogroups (Fig 5.2) in this study corresponds closely with findings by Emms and Kelly (2019) who emphasised that core orthogroups are integral to fundamental cellular functions with high conservation across taxa. These conserved gene families often encode proteins crucial to cellular integrity, environmental resilience, and microbial fitness which traits are particularly pertinent to probiotic organisms (Yu et al., 2018). One such example is the cold shock protein (CSP) gene family, which plays a pivotal role in stabilising mRNA transcripts under low-temperature stress (Giuliodori et al., 2023). These proteins enhance microbial survivability and stress tolerance, a defining probiotic characteristic that supports colonisation and functionality in diverse environments (Horn et al., 2007). Moreover, central glycolytic gene regulators were also conserved across genomes. These genes modulate the expression of glycolytic enzymes, governing energy production via glucose metabolism which is a critical process in both fermentation dynamics and microbial growth (Zhang et al., 2021). These gene attributes influence the ability of *Lpb. plantarum* and *Lb. rhamnosus* to exert their probiotic characteristics when consumed. However, this Chapter did not investigate their probiotic characteristics.

5.5 Conclusion and Recommendation

This study showed that substrate composition exerts a more pronounced influence on genotypic plasticity among microbial isolates than fermentation duration. The predominance

of *Lactiplantibacillus plantarum* in composite samples underscores its potential as a lead candidate for development of a probiotic composite beverage. Such formulations offer promising avenues for industrial-scale development of functional food products tailored to alleviating symptoms among populations susceptible to functional gastrointestinal disorders (FGIDs). However, further *in vitro* and *in vivo* validation of probiotic traits is recommended. The probiotic potential of the isolates was evaluated using *in vitro* methods under Chapter six of the dissertation.

Although the study focused on characterisation of *Lactobacillus* genus only, which provided valuable insights into genotypic characteristics, it restricted broader understanding of the microbial community and succession patterns that occur during fermentation. This limitation was compounded by the relatively small sample size, which reduced statistical power and constrained the generalizability of the findings. Additionally, the early screening of isolates to eliminate non-target species may have excluded other potentially relevant bacterial strains whose functional attributes could also contribute to probiotic potential, thereby limiting the scope of conclusions that could be drawn from the analysis.

Despite the limitations stated, this Chapter established a baseline information on *Lactobacillus* genus in locally developed composite substrates, which remain underexplored in Uganda and similar contexts. Furthermore, even with a relatively small sample size, the study provided preliminary scientific evidence that composite food substrates can support the growth of probiotic LAB, thereby laying the groundwork for functional food development tailored to regional nutritional needs. Future investigations should therefore, expand beyond *Lactobacillus* to include the broader microbial community and succession dynamics during fermentation, using metagenomic or multi-omics approaches to capture diversity and functional interactions.

CHAPTER SIX

***In vitro* evaluation of the probiotic potential of *Lactobacillus* strains isolated from ready-to-use millet-sesame-soybean and sorghum-sesame-soybean fermented composite beverages**

ABSTRACT

Various strains isolated from different indigenous fermented foods and beverages have shown promising probiotic properties. These studies have largely focused on foods derived from single-ingredient substrates including cereals, legumes, or vegetables with limited investigation into probiotics sourced from fermented products formulated using composite ingredients. Composite food matrices, often designed to enhance nutritional density in resource-constrained settings, remain underexplored in probiotic research, particularly when fermented into beverages. Lactic acid bacteria (LAB) isolated from spontaneously fermented composite beverages was evaluated for key probiotic properties (acid and bile tolerance, gastrointestinal tolerance, adhesion, antimicrobial activity, microbial safety and coaggregation). Comparative evaluations were conducted across isolates using standardised *in vitro* methods. The probiotic properties of the isolates were evaluated using one way analysis of variance (ANOVA) to determine significant differences among them at 5% level of significance. Strains isolated from composite beverages displayed superior ($p < 0.05$) probiotic functionality compared to those from sorghum-based beverages. Three composite isolates registered survival rates above 50% when subjected to harsh environment and gastrointestinal conditions. Sample AK-S3D-miseso-48hr-isolate recorded highest ($p < 0.05$) survival ability in low pH (99%) and bile salts (98%). All isolates were resistant to at least more than 5 commonly used antibiotics in Uganda and did not show any haemolytic or DNase activity. The inhibition of enteric pathogens was primarily attributed to short-chain fatty acid production, which significantly lowered pH. Among the formulations evaluated, millet-sesame-soybean-based composites recorded generally superior performance across these probiotic benchmarks compared to their sorghum-based counterparts. Fermented composite beverage formulations can be optimised to enhance probiotic functionality and support gut health. Their integration into public health strategies and dietary guidelines may offer accessible interventions for gastrointestinal and nutrition-related disorders. To ensure safe application, future research should include comprehensive genomic safety profiling and long-term ecological assessments of host–microbiome interactions.

Key words: Probiotic attributes, Fermented composite beverages, Enteric pathogens.

6.1 Introduction

Microorganisms that inhabit the gastrointestinal tract (GIT) collectively known as gut microbiota are approximately 1×10^{14} cells (Thursby & Juge, 2017). These microorganisms play an essential role in maintaining intestinal homeostasis, supporting development, and providing protection against pathogens (Marques et al., 2023). The gut microbiota is predominantly composed of Bacteroidetes and Firmicutes phyla, which together account for over 80% of the total microbiota (Magne et al., 2020). At genus level *Lactobacillus* (now subdivided into 25 new genera) (Zheng et al., 2020) and *Bifidobacteria* are among the most prevalent (Rastogi & Singh, 2022).

The abundance of these microorganisms is maintained by continuous intake of either new live microorganisms (probiotics) from fermented foods, supplements or intake of food substrates (prebiotics) that selectively promote their growth and proliferation (Amenyogbe et al., 2024; Zeng et al., 2025). The composition of the gut microbiota relatively remains stable due to its plasticity which allows it to return to its original state (Thriene & Michels, 2023). However, continuous exposure to stressors such as highly processed and high fat foods, antibiotics as evidenced by current nutrition transition in low- and middle-income countries can chronically alter the gut microbiota (Francino, 2016; Popkin & Ng, 2022). Consequently, the quality of diet consumed plays a dual role on either improving gut microbiota integrity or causing dysbiosis.

Recent scientific evidence has shown that diets predominantly consisting of minimally processed plant-based and fermented foods and beverages promote gut health and overall well-being. These diets have been found to be cardioprotective, immune-protective due to antiatherogenic, anti-inflammatory, and antioxidative (García-Montero et al., 2021). Fermented foods and beverages, including *Boza* a cereal-based drink and traditionally produced *tempeh*, have recorded beneficial effects on metabolic and gastrointestinal health, such as anti-diabetic properties and enhanced gut function (Kumari et al., 2024; Taylor et al., 2020; Wicaksono et al., 2024). These health benefits are largely attributed to the diverse live microorganisms they contain, which metabolise available nutrients like prebiotic

oligosaccharides into bioactive compounds that support microbial balance and intestinal integrity (Hakim et al., 2023; Omak & Yilmaz-Ersan, 2022).

Furthermore, because most fermented foods and beverages are not subjected to post-fermentation processing, they retain high microbial loads of good gut bacteria exceeding the minimum threshold of 10^6 CFU/g or ml required for probiotic functionality (Leeuwendaal et al., 2022). Consequently, a substantial number of the viable microorganisms may withstand the acidic and enzymatic conditions of the upper gastrointestinal tract (GIT) and reach the lower GIT, where they can potentially integrate into the resident microbiome (Rezac et al., 2018). This integration can promote a shift toward beneficial microbial populations, enhancing colonisation resistance and suppressing pathogenic species through competitive exclusion (Plaza-Diaz et al., 2019).

However, the functional efficacy of these good gut bacteria (probiotics) is inherently species and strain specific (McFarland et al., 2018), underscoring the necessity of characterising individual strains isolated from newly innovated fermented food products such as millet-sesame-soybean fermented composite beverage. Such evaluations facilitate the identification of strain-specific interactions that can inform the development of tailored probiotic formulations with diverse health-promoting effects. To exert beneficial outcomes in the host, probiotic strains must have a suite of essential attributes, including tolerance to processing and gastrointestinal stressors, adhesion to intestinal epithelial surfaces, antimicrobial activity, non-pathogenicity, and established safety profiles (Kwofie et al., 2020; Rastogi et al., 2021).

Common Lactic acid producing bacterial species evaluated for probiotic capacity include *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Carnobacterium*, and *Weissella* (Amenu & Bacha, 2023; Zielinska & Kolozyn-Krajewska, 2018). Among these, *Lactobacillus* genus is the most widely utilised probiotic in the food industry for the production of fermented and other functional foods (Khushboo et al., 2023). These bacteria are either used as starter cultures or as autochthonous agents that facilitate the fermentation process (Sooresh et al., 2023).

Majority of these bacterial strains have been isolated from plant-based fermented food products. Notable examples include *Lactobacillus paracasei* MRS59 isolated from Brazilian *kefir* grains (Leite et al., 2015); *Lactobacillus plantarum* RYPR1 from fermented cereal-based Indian *Rabaadi* (Yadav et al., 2016); *Lactobacillus plantarum* MNC21 from *obushera*, a Ugandan cereal-based fermented beverage (Byakika et al., 2020); and 24 *Lactobacillus* strains isolated from fermented vegetables (Liu et al., 2022). These studies have largely focused on foods derived from single-ingredient substrates including cereals, legumes, or vegetables with limited investigation into probiotics sourced from fermented products formulated using composite ingredients. Chapter five of this dissertation revealed that millet-sesame-soybean and sorghum-sesame-soybean composite fermented beverages could act as viable vehicles for the enrichment and delivery of novel probiotic strains, owing to their diverse carbohydrate profiles and fermentable energy sources that enhanced genotypic plasticity. However, systematic evaluation of their probiotic properties and efficacy has not been previously conducted.

To address this gap, this Chapter (six) therefore aimed to characterise the probiotic properties of these isolated strains using standardised *in vitro* assays. Evaluation criteria were based on established benchmarks for probiotic classification, including tolerance to gastrointestinal conditions (e.g., acid and bile resistance), adhesion to intestinal epithelial cells, antimicrobial activity, safety status (e.g., GRAS designation), and capacity to confer health benefits to the host (Hill et al., 2014; Sarita et al., 2024). This approach enables a targeted assessment of whether the genetically diverse strains from composite beverages fulfil the functional standards required for probiotic designation.

6.2 Materials and Methods

6.2.1 Micro-organisms and growth conditions

The probiotic reference strain *Lactiplantibacillus plantarum* (ATCC 14917), and pathogenic microbes *Salmonella enterica* subsp. *enterica* (ATCC 13076), *Escherichia coli* (ATCC 25922) and *Shigella flexneri* (ATCC 12022) were purchased from American Type Culture Collection (ATCC) through Sigma-Aldrich Co. (Microbiology, Minnesota, USA) in lyophilised form. Lactic acid bacteria species used in this study were isolated from millet

and sorghum based fermented composite beverages (millet-sesame-soybean and sorghum-sesame-soybean composites). *Lactiplantibacillus plantarum* (ATCC 14917) was used as a positive control. Microbial stock cultures were stored at -80°C in 30% (v/v) glycerol. The stocks were resuscitated overnight at 37 °C and then 0.1 ml of fresh culture was prepared in 10 ml of appropriate broth for each culture.

To obtain fresh cultures, the isolates and reference probiotic strains were incubated anaerobically in de Man, Rogosa, and Sharpe (MRS) broth at 37 °C for 24 hours. Conversely, the enteric pathogens *Salmonella enterica* subsp. *enterica* (ATCC 13076), *Shigella flexneri* (ATCC 12022), and *Escherichia coli* (ATCC 25922) were cultured aerobically at 37 °C for 24 h. Nutrient broth (Millipore, Berlin, Germany) was used for *S. enterica* and *S. flexneri*, while *E. coli* was grown in tryptic soy broth. The cells were harvested by centrifugation (VWR Micro Star 17, 521-1646, Berlin, Germany) at 8000 × g for 10 minutes after being washed three times with phosphate-buffered saline (PBS) (Sigma-Aldrich Chemie, Taufkirchen, Germany). Subsequently, the cells were re-suspended in PBS for further assays.

6.2.2 Evaluation of probiotic properties of the microbial isolates

6.2.2.1 Resistance to low pH

Ability of the isolates to resist low pH conditions was determined according to the method described by Amenu and Bacha (2023). LAB isolates and the reference strain were cultured in acidified MRS broth (pH=3) using concentrated HCL. Viable cells were enumerated on MRS agar (HiMedia, India) at 0- and 3 hours of anaerobic incubation at 37 °C to simulate physiological body temperature. The colonies were counted using colony counter (Scan-100, Interscience, Paris, France) and the results were used to determine the percentage survival rate (SR) of the LAB isolates using equation 6.1 as applied in an earlier (Rastogi et al., 2021).

$$SR (\%) = \left(\frac{\text{Log number of viable cells at time } t=3 \text{ hours}}{\text{Log number of viable cells at } t=0 \text{ hours}} \right) \times 100 \dots\dots\dots \text{Equation 6.1}$$

6.2.2.2 Tolerance to bile salts and quantitative bile salt deconjugation activity

Bile salt tolerance of the *Lactobacillus* isolates was evaluated according to Amenu and Bacha (2023). Fresh *Lactobacillus* isolates were cultured overnight in MRS broth

anaerobically at 37 °C. Their optical density OD was measured at 600 nm using UV-VIS spectrophotometer (6705 Jenway, Milmort, Belgium) and adjusted to 0.1 corresponding to 1×10^8 cells/ml using PBS. After adjusting the cell density, 1 ml of each isolate was inoculated in 10 ml of MRS broth containing 1% w/v bile salts (cholic acid sodium salt, 50%; tauro-deoxycholic acid sodium salt, 50%) (Sigma-Aldrich, Chemie, Taufkirchen, Germany). The mixture was then incubated anaerobically at 37°C for 24 hours to determine bile salt tolerance. The OD of the isolates was taken at 0- and 24 hours incubation time which was used to calculate the percentage survival rate (SR) of isolates to bile salts using Equation 6.2.

$$SR (\%) = \left(\frac{OD_{600nm} \text{ after 24 hour incubation}}{OD_{600nm} \text{ at 0 hour}} \right) \times 100 \dots\dots\dots \text{Equation 6.2}$$

To determine bile salt deconjugation activity, LAB isolates were cultured in duplicates on modified MRS agar containing 0.5% (w/v) Tauro deoxycholic acid (Sigma-Aldrich, Chemie, Taufkirchen, Germany) and 0.037% (w/v) calcium chloride to aid precipitation of deconjugated bile acids and aid visualisation on the agar plates. The agar plates were incubated anaerobically at 37 °C for 48 hours. The diameter of the precipitation zones around the colonies were measured in mm and classified as follows; 0, no precipitation zone, low activity (class 1) with a precipitation zone not exceeding the colony edge (1-10 mm); medium activity (class 2) with a precipitation zone of 11-15 mm and, high activity (class 3) with a precipitation zone between 16-20 mm (Mathara et al., 2008). MRS agar plate without bile salts was used as control.

Low pH and bile salt tolerances are distinct physiological parameters representing the gastric and intestinal barriers, respectively. The purpose of conducting both assays was to comprehensively map the sequential survival profile of the isolates as they transit through the gastrointestinal tract.

6.2.2.3 Invitro assay on gastrointestinal environment tolerance by LAB isolates

6.2.2.3.1 Preparation of gastric and pancreatic Juices

Gastric juice used to simulate digestion in the upper gastrointestinal tract was prepared by dissolving 3 g/L of porcine pepsin (P-7000) (Sigma-Aldrich, St. Louis, USA) in 0.5% (w/v)

sterile saline and the pH of the solution was adjusted to 2 with 2M HCL using a benchtop pH meter (C6010, Consort, Belgium). Pancreatic juice used to simulate digestion in the lower gastrointestinal tract was prepared by dissolving 1 g/L of porcine pancreatin (P-3292) (Sigma-Aldrich, St. Louis, USA) in 0.5% (w/v) sterile saline. The pH of the solution was then adjusted to 8 with 0.1M NaOH.

6.2.2.3.2 Preparation of washed cell suspensions

Overnight cell culture suspensions were centrifuged at 10000 x g for 10 minutes to obtain cell pellets. The recovered cell pellets were washed twice with 10 ml of PBS solution and later resuspended in PBS after which the cell density was standardised to absorbance of 0.8-1 at 600 nm which approximately corresponds to 7-8 log CFU/ml.

6.2.2.3.3 Determination of gastrointestinal transit tolerance

The survival ability of LAB isolates in the upper and lower gastrointestinal tract was assessed using the methods described by Charteris et al. (1998b) and Amenu and Bacha (2023). Initial cell density for all the samples was standardised to 6-7 log CFU/ml by either addition of PBS or washed cell pellets. A 0.2 ml aliquot of the standardised cell suspension from each LAB isolate was mixed with 1 ml of either gastric (pH=2) or pancreatic juices (pH=8) and vortexed for 10 seconds. The mixture was then incubated anaerobically at 37°C for 3 hours. Cell viability was evaluated at 1.5 and 3 hours after incubation by measuring optical cell density at 600 nm. To assess overall tolerance during gastric transit, the isolates incubated with gastric juices for 3 hours were harvested and washed with PBS solution and centrifuged to obtain concentrated cells. The concentrated cells were then resuspended in 1 ml of pancreatic juices and incubated for an additional 3 hours. The optical density (OD 600 nm) of the cultures was determined at 1.5- and 3-hours post-incubation. The percentage survivability of the isolates was calculated using equation 6.3.

Survivability (%) =

$$\left(\frac{OD \text{ at } 600nm \text{ at } 0 \text{ hours} - OD \text{ at } 600nm \text{ at time } t=1.5 \text{ or } 3 \text{ hours}}{OD \text{ at } 600nm \text{ at } 0 \text{ hours}} \right) \times 100 \dots\dots \text{Equation 6.3}$$

6.2.2.4 Adhesion assay

6.2.2.4.1 Cell surface Hydrophobicity and Auto-aggregation capacity

The cell surface hydrophobicity of LAB isolates was assessed using a mixture of hydrocarbons and ethylbenzenes (Sigma-Aldrich Chemie, Taufkirchen, Germany) as previously described by Rastogi et al. (2021). Overnight cell suspensions were centrifuged at 10,000 x g for 10 minutes. The resulting cell pellets were washed twice with 10 ml PBS solution and then resuspended in PBS to achieve an absorbance of 0.8-1 at 600 nm. A 3 ml aliquot was taken and mixed with 1 ml of hydrocarbon, then homogenised by vigorously vortexing on a vortexer for 2 minutes. The mixture was incubated at 25°C for 20 minutes, after which the aqueous layer was removed and the absorbance measured at 600 nm using a UV-Vis spectrophotometer (6705 Jenway, Milmort, Belgium).

Auto-aggregation was determined according to the procedure described by Kos et al. (2003). Exactly 4 ml overnight cell culture suspension with cell density of 8 log CFU/ml was vortexed for 10 s and incubated at 37°C for 24 hr. Auto-aggregation capacity was then evaluated at 5 hr and 24 hr incubation where 0.1 ml of the upper suspension was withdrawn and transferred into a tube containing 3.9 ml of phosphate-buffered saline (PBS). The absorbance was read at 600 nm following the same procedure used for assessing cell surface hydrophobicity. The 5 hr incubation period aligns well with typical gastrointestinal transit time, representing the period which ingested probiotics or food pathogens pass through the stomach and the small intestine. In contrast, the 24 hr period enables evaluation of long-term persistence of the isolates under conditions simulating prolonged residence within the gastrointestinal tract (Trunk et al., 2018). Both percentage hydrophobicity or auto-aggregation was calculated using equation 6.4.

$$\text{hydrophobicity or auto - aggregation (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \dots\dots\dots \text{Equation 6.4}$$

Where; A0 is the absorbance at 0 hour and A1 is the absorbance at 5 and 24 hours.

6.2.2.5 Safety assay

6.2.2.5.1 Haemolytic and DNase activity

Haemolytic and DNase activity assay were conducted according to the method described by Wang et al. (2018). Briefly, overnight cultures of LAB isolates were streaked on blood agar plates (Condalab, Madrid, Spain) containing 5% (w/v) sheep blood and incubated at 37°C for 48 hours. The plates were evaluated for β -haemolysis (clear zones), α -haemolysis (green zones), and non-haemolytic activities (γ -haemolysis-no zones around the colonies).

DNase activity was determined following the method followed by Shuhadha et al. (2017). The isolates were enumerated on DNase agar (Condalab, Madrid, Spain) using the spread plate method and incubated at 37°C for 48 hours to determine the production of DNase enzyme. The plates were flooded with 3% v/v HCL after which halo zones were examined after 8 minutes. Presence of halo zones indicated positive DNase activity.

6.2.2.5.2 Antibiotic susceptibility test

The antibiotic susceptibility test was conducted in duplicates using Kirby-Bauer disc diffusion method according to the method described by Charteris et al. (1998a). Sterile cotton swabs were used to swab standardised (8 log CFU/ml) overnight *Lactobacillus* isolates on MRS agar (HiMedia, India). After 1-hour, antibiotic discs were placed on the surface of the inoculated MRS agar and incubated anaerobically at 37°C for 48 hours. After incubation, the diameter of inhibition zones of 13 antibiotics from various classes commonly used to treat antibiotic related infections (Table 6.1) was measured using a clear millimetre ruler placed against the back of the Petri dish under a good light source against a dark background. The isolates were classified as resistant, moderately susceptible, or susceptible to each antibiotic, following the criteria established by Charteris et al. (1998a).

Table 6.1: Description of commonly used antibiotic drugs to treat bacterial infections

Antibiotic groups (mechanism of action)	Antibiotic disc	Concentration (µg/disc)	Abbreviation	Interpretation of diameter mm		
				R	MS	S
Group 1-inhibitors of cell wall synthesis	Ampicillin	10	AMP	≤12	13-15	≥16
	Vancomycin	30	VA	≤14	15-16	≥17
	Cephalexin*	30	CL	≤14	15-17	≥18
	Ceftriaxone	30	CRO	≤13	14-20	≥21
Group 2-protein synthesis inhibitors	Clindamycin	2	DA	≤8	9-11	≥12
	Erythromycin	15	E	≤13	14-17	≥18
	Gentamicin	10	CN	≤12	-	≥13
	Tetracycline	30	TE	≤14	15-18	≥19
	Azithromycin*	15	AZM	≤13	14-17	≥18
	Kanamycin	30	K	≤13	14-17	≥18
Group 3-inhibitors of nucleic acid synthesis	Metronidazole	5	MTZ	≤14	15-17	≥18
	Ciprofloxacin	5	CIP	≤13	14-18	≥19
Group 4- inhibitors of cytoplasmic membrane function	Colistin	10	CT	≤8	9-10	≥11

Source: (Charteris et al., 1998a) *Interpretation based on other antibiotics within the same class. R-Resistant, MS-Moderately Susceptible, and S-Susceptible.

6.2.2.6 Pathogen inhibition capacity of the *Lactobacillus* isolates

6.2.2.6.1 Co-aggregation assay

Co-aggregation of *Lactobacillus* isolates was assessed according to the method described by Jayaram et al. (2025). Specifically, 2 ml of overnight cultures of *Lactobacillus* isolates and 2 ml of individual pathogen (*S. enterica* subsp. *enterica* (ATCC 13076), *E.coli* (ATCC 25922), and *Shigella flexneri* (ATCC 12022)) were mixed and homogenised by vortexing at low speed for 10 s. The homogenised suspensions were incubated at 35 °C for 5 hours. Controls consisted of 4 ml of individual pathogens or *Lactobacillus* isolates. Absorbance of the samples was measured at 600 nm (OD₆₀₀) at 0- and 5-hours post-incubation. The percentage co-aggregation was calculated according to equation 6.5.

$$\text{Co-aggregation (\%)} = \frac{\frac{AODx+AODy}{2} - A(ODx+ODy)}{\frac{AODx+AODy}{2}} \times 100 \dots\dots\dots \text{Equation 6.5}$$

Where AODx=absorbance of LAB alone and AODy= absorbance of specific pathogen alone and A (ODx + ODy) = absorbance of LAB + pathogen

6.2.2.6.2 Production of anti-microbial compounds

Antimicrobial activity was evaluated using the agar well diffusion method (Vinderola et al., 2002). Briefly, overnight LAB culture isolates were centrifuged at 10000 x g for 10 minutes to obtain cell free culture supernatant (CFCS). Residual cells were removed by filtration using a 0.22 µm syringe filter (AVF-100C-NY, Dubai, UAE). PCA plates were swabbed with standardised (8 log CFU/ml) fresh pathogenic cultures of (*S. enterica* subsp. *enterica* (ATCC 13076), *E.coli* (ATCC 25922), and *Shigella flexneri* (ATCC 12022). Subsequently, a 4 mm diameter well was created in the inoculated plates where 100 µL of CFCS was pipetted and plates were incubated aerobically at 37°C for 24 h. The inhibition zone diameter was measured in mm.

To evaluate whether the antimicrobial activity was due to organic acids, pH of the CFCS was modified to 6.5 using 1M NaOH. The heat stability of the antimicrobial compound was assessed by heating the CFCS at 100°C for 30 minutes. To confirm production of bacteriocins, 1 mg/ml of Proteinase K (Qiagen Sciences Inc, Germantown, USA) was added to the CFCS, followed by incubating the mixture at 37°C for 3 hours as previously applied by Byakika et al. (2020). All tests were performed in duplicate.

6.2.3 Statistical analysis

The probiotic properties of the isolates were evaluated using one-way analysis of variance (ANOVA) to determine whether significant differences existed among them at 5% level of significance. Before running the ANOVA, homogeneity of variances was assessed using Levene's test ($F(13, 196) = 2.138; p = 0.199$), while normality was evaluated using the Shapiro-Wilk test ($W = 0.763, p = 0.18$). Tukey's HSD Post Hoc test was used to identify the significant mean differences. Analysis was performed using SPSS software version 25.0 (SPSS Inc., Chicago, IL)

6.3 Results

6.3.1 Acid tolerance

All isolates recorded a high survival rate when exposed to a pH of 3, with each isolate showing more than 90% survival (Table 6.2). After 3 hours of incubation, most isolates maintained the minimum cell density required for a probiotic culture, (6 log CFU/ml). Although, 5 isolates including AK-S1D, AK-S2D, AK-S5D, AK-S6D, AK-S8D and the reference strain, showed a decline in cell density ranging from 5.8 to 5.97 log CFU/ml. This was significantly lower ($p < 0.05$) than the rest of the isolates. AK-S3D recorded the highest ($p < 0.05$) survival rate of 99%.

Table 6.2: Acid tolerance capacity of lactic acid bacteria isolated from Ugandan millet and sorghum based fermented beverages

Ingredient source of isolate	Acid Tolerance (pH=3)	
	3 hr (log CFU/ml)	Survivability (%)
AK-S1D	5.87 ^{def} ±0.04	90.56 ^d ±1.4
AK-S2D	5.97 ^{de} ±0.1	93.98 ^{bcd} ±3.33
AK-S3D	6.61 ^a ±0.03	99.16 ^a ±0.59
AK-S4D	6.4 ^{ab} ±0.16	96 ^{abc} ±0.89
AK-S5D	5.95 ^{de} ±0.06	91.52 ^{cd} ±1.02
AK-S6D	5.83 ^{ef} ±0.05	93.74 ^{bcd} ±1.57
AK-S7D	6.22 ^{bc} ±0.03	95.98 ^{abc} ±2.32
AK-S8D	5.7 ^f ±0.05	95.69 ^{abc} ±0.41
AK-S9D	6.59 ^a ±0.02	96.58 ^{ab} ±0.13
AK-S10D	6.24 ^{bc} ±0.04	92.51 ^{bcd} ±0.67
AK-S11D	6.22 ^{bc} ±0.1	96.35 ^{ab} ±0.98
AK-S12D	6.06 ^{cd} ±0.08	95.08 ^{abcd} ±1.71
AK-S13D	6.05 ^{cde} ±0.09	96.64 ^{ab} ±1.85
AK-S14D	5.85 ^{def} ±0.07	95.83 ^{abc} ±1.79

Mean values ± SD (n=2) with different superscripts in the same column are significantly different ($p < 0.05$) corresponding to the attributes displayed. AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.3.2 Resistance to bile salts and bile salts deconjugation activity

Table 6.3 summarises the survival rates of isolates exposed to 1% bile salt and their bile salt deconjugation activity. After 24 hours of incubation, all isolates registered a decline in cell

counts, ranging between 6.0 and 7.0 log CFU/ml. Despite this reduction, viability remained within the acceptable threshold for probiotic functionality. Statistical analysis indicated no significant differences ($p > 0.05$) in survival rates or cell densities between the test isolates and the reference strain except for AK-S3D that recorded a significantly higher survivability of 98%. In contrast, bile salt deconjugation activity was uniformly lower among all isolates (1–10 mm inhibition zones) when compared to the reference strain.

Table 6.3: Bile salt tolerance and bile salt deconjugation activity of lactic acid bacteria isolated from Ugandan millet and sorghum based fermented beverages

Isolates	Bile salt tolerance		Bile salt deconjugation
	24 hr	% survivability	
AK-S1D	6.85 ^{ab} ±0.3	93.59 ^b ±5.01	+
AK-S2D	6.92 ^{ab} ±0.24	94.54 ^b ±2.78	+
AK-S3D	7.31 ^a ±0.03	98.21 ^a ±0.65	+
AK-S4D	7.10 ^{ab} ±0.16	95.49 ^b ±2.12	+
AK-S5D	6.64 ^b ±0.06	91.43 ^b ±2.29	+
AK-S6D	6.87 ^{ab} ±0.27	94.49 ^b ±3.68	+
AK-S7D	6.91 ^{ab} ±0.03	93.73 ^b ±0.55	+
AK-S8D	6.62 ^b ±0.21	92.46 ^b ±2.97	+
AK-S9D	7.09 ^{ab} ±0.15	94.32 ^b ±2.33	+
AK-S10D	7.06 ^{ab} ±0.17	94.79 ^b ±2.13	+
AK-S11D	6.94 ^{ab} ±0.11	96.95 ^b ±1.02	+
AK-S12D	6.86 ^{ab} ±0.14	96.97 ^b ±2.4	+
AK-S13D	6.85 ^{ab} ±0.12	95.51 ^b ±3.62	+
AK-S14D	6.71 ^b ±0.21	91.74 ^b ±2.03	++

Mean values ± SD (n=2) with different superscripts in the same column are significantly different ($p < 0.05$) corresponding to the attributes displayed. + 1- 10 mm low activity, ++ 11-15 mm medium activity, >16 mm high bile salt deconjugation activity (Mathara et al., 2008). AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.3.3 *In vitro* tolerance to gastrointestinal conditions

Table 6.4 presents the survivability of isolates following a 3-hour simulated digestion, structured in three phases: upper gastrointestinal tract, lower gastrointestinal tract, and overall digestion. When compared to the reference strain, all isolates had superior viability in the upper gastrointestinal tract, except for AK-S6D, which showed reduced performance.

This pattern was consistent across the lower gastrointestinal phase and overall digestion outcomes.

After 1.5 hours of exposure to simulated gastric juices, most isolates retained higher viability than the reference strain, apart from AK-S6D. Among the isolates derived from composite beverages, survivability ranged from 30% to 63%, with AK-S4D recording the highest value. In contrast, isolates from single-ingredient beverages had greater acid tolerance, notably AK-S11D, which achieved a maximum survival rate of 75%. Probiotic candidate strains from millet-based formulations registered superior resilience to gastric stress compared to those sourced from sorghum-based counterparts, regardless of fermentation type (composite or single-ingredient)

After 3 hours of gastric incubation, AK-S6D recorded the lowest tolerance, with a survival rate of 8% significantly ($p < 0.05$) lower than that of the reference strain. In contrast, all remaining isolates performed comparably to or better than the reference strain, with several showing statistically significant improvements ($p < 0.05$) in survivability. Notable examples include AK-S12D, AK-S13D, AK-S9D, AK-S10D, AK-S11D, AK-S5D, and AK-S3D, each achieving survival rates ranging from 12% to 99%.

After 1.5 hours of pancreatic incubation, the survival rates of isolates ranged from 60% to 97%. Notably, AK-S7D, the reference strain, AK-S1D, and AK-S4D showed significantly ($p < 0.05$) lower survival rates compared to the other isolates ($p < 0.05$). Among composite beverage isolates, the AK-S4D had the highest survival advantage, exhibiting a significant percentage difference of 18% ($p < 0.05$) over the reference strain, followed by the AK-S5D with a 14% increase. In single-ingredient beverages, the AK-S9D achieved the highest pancreatic tolerance, surpassing the reference strain by 20%. A similar pattern of enhanced survivability was observed after 3 hours of incubation.

After 3 hours of simulated gastrointestinal digestion, isolates from single-ingredient fermentations had significantly higher ($P < 0.05$) survival rates exceeding 50% compared to composite beverage isolates, except AK-S5D, AK-S3D, and AK-S1D, which also maintained relatively strong viability. Despite generally lower survivability among

composite isolates, most strains still had significantly higher survival ($p < 0.05$) than the reference strain (38%).

Table 6.4: In vitro gastro-intestinal tolerance of LAB isolated from Ugandan fermented millet and sorghum-based beverages

Isolates	Gastric stress tolerance (% survivability)		Pancreatic stress tolerance (% survivability)		Overall digestion stress tolerance(% survivability)	
	1.5 hrs	3 hrs	1.5 hrs	3 hrs	1.5 hrs	3 hrs
AK-S1D	32.28 ^f ±1.78	29.63 ^{fg} ±0.75	77.69 ^c ±5.71	71.72 ^{bcd} ±3.33	60.94 ^d ±2.91	53.69 ^d ±1.43
AK-S2D	37.44 ^e ±1.27	32.93 ^{efg} ±1.44	86.04 ^{abc} ±3.96	75.87 ^{abc} ±0.45	50.34 ^e ±1.78	45.99 ^{ed} ±1.03
AK-S3D	63.05 ^c ±1.81	60.25 ^b ±0.78	95.61 ^a ±1.23	89.74 ^{ab} ±3.94	80.83 ^b ±0.45	77.48 ^b ±0.90
AK-S4D	38.37 ^e ±2.13	35.54 ^{def} ±0.95	60.64 ^d ±7.13	52.9 ^d ±3.35	30.71 ^f ±1.09	26.34 ^f ±0.61
AK-S5D	61.57 ^c ±2.80	56.15 ^b ±4.18	91.67 ^{bca} ±4.86	86.94 ^{abc} ±5.79	89.27 ^a ±1.14	85.87 ^a ±1.19
AK-S6D	9.75 ^g ±0.98	7.55 ⁱ ±0.55	86.91 ^{abc} ±6.91	51.81 ^d ±11.8	15.46 ^g ±4.15	12.53 ^g ±3.61
AK-S7D	30.16 ^f ±2.26	26.53 ^{gh} ±1.05	81.21 ^{bc} ±9.58	71.03 ^{bcd} ±16.11	49.96 ^e ±2.67	40.64 ^e ±1.68
AK-S8D	67.96 ^b ±1.37	63.27 ^{ab} ±3.86	96.35 ^a ±1.69	93.62 ^a ±0.68	91.08 ^a ±2.21	87.18 ^a ±1.62
AK-S9D	62.03 ^c ±1.55	44.65 ^c ±2.51	97.36 ^a ±0.47	92.44 ^a ±3.99	85.82 ^b ±2.24	82.41 ^{ba} ±1.32
AK-S10D	59.45 ^d ±2.30	40.31 ^{cd} ±2.50	89.19 ^{abc} ±0.76	86.02 ^{abc} ±1.41	70.40 ^c ±1.52	66.72 ^c ±2.41
AK-S11D	74.78 ^a ±2.04	69.46 ^a ±4.88	92.68 ^{ab} ±4.95	87.31 ^{ab} ±7.12	90.76 ^a ±1.24	90.12 ^a ±0.49
AK-S12D	49.32 ^d ±1.42	46.04 ^c ±0.56	88.87 ^{abc} ±2.34	79.71 ^{abc} ±7.6	90.04 ^a ±2.08	85.86 ^a ±0.62
AK-S13D	44.98 ^d ±0.8	39.80 ^{cde} ±2.35	96.39 ^a ±0.25	89.95 ^{ab} ±4.5	90.86 ^a ±3.82	83.74 ^{ba} ±3.17
AK-S14D	28.14 ^f ±1.45	23.55 ^h ±1.39	80.05 ^{bc} ±1.83	67.16 ^{cd} ±3.8	49.74 ^f ±3.39	38.28 ^e ±5.99

Mean values ± SD (n=2) with different superscripts in the same column are significantly different ($p < 0.05$) corresponding to the attributes displayed. Gastric stress tolerance (pH=2 pepsin, 0.3% w/v pepsin) Pancreatic stress tolerance (pH= 8, 0.5% w/v pancreatin) Overall digestion stress tolerance (pH=2 pepsin, 0.3%w/v pepsin pH=8 0.5% w/v pancreatin). AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.3.4 Adhesion assay

6.3.4.1 Cell surface Hydrophobicity and auto-aggregation

Table 6.5 shows the cell surface hydrophobicity and auto-aggregation properties of the evaluated isolates. Significant variation in hydrophobicity was observed among the strains. Hydrophobicity of isolates from single-ingredient sorghum-based beverages were significantly higher ($p < 0.05$) than those from composite beverages. Among the isolates, AK-6SD and AK-S11D displayed the highest hydrophobicity levels, exceeding 44% ($p < 0.05$). Conversely, millet-based composite beverages demonstrated significantly greater ($p < 0.05$) hydrophobicity compared to single-ingredient fermented products of similar composition. In the millet-based composite category, AK-S1D and AK-S4D recorded the highest hydrophobicity values at 19% and 21%, respectively. The lowest hydrophobicity was noted in sorghum composite isolate AK-S5D, with a value of 6%. Despite differences in hydrophobic properties, all isolates showed strong auto-aggregation capacity. Aggregation rates exceeded 95% and 96% at 5 and 24 hours of incubation, respectively, and showed no statistically significant variation ($p > 0.05$) compared to the reference strain.

Table 6.5: Cell surface hydrophobicity and auto-aggregation capacity of LAB isolated from Uganda fermented millet and sorghum-based beverages

Isolates	% Hydrophobicity	% Auto-aggregation	
		5 hours	24 hours
AK-S1D	19.25 ^d ±2.57	96.5 ^a ±0.40	99.38 ^a ±0.24
AK-S2D	13.8 ^{ef} ±0.68	98.05 ^a ±0.18	99.52 ^a ±0.34
AK-S3D	11.58 ^{gf} ±1.69	97.32 ^a ±1.13	99.45 ^a ±0.16
AK-S4D	20.82 ^d ±1.34	98.18 ^a ±0.43	99.47 ^a ±0.55
AK-S5D	5.98 ^h ±0.25	97.93 ^a ±0.43	99.39 ^a ±0.6
AK-S6D	44.24 ^a ±1.79	95.82 ^a ±5.24	98.69 ^a ±1.82
AK-S7D	13.47 ^{ef} ±0.94	95.84 ^a ±4.08	99.54 ^a ±0.3
AK-S8D	35.10 ^b ±3.3	95.18 ^a ±5.04	96.31 ^a ±5.1
AK-S9D	6.21 ^h ±0.99	98.26 ^a ±0.07	99.61 ^a ±0.37
AK-S10D	18.58 ^{ed} ±0.81	97.12 ^a ±1.86	99.6 ^a ±0.53
AK-S11D	7.28 ^{gh} ±0.55	95.77 ^a ±3.53	99.52 ^a ±0.54
AK-S12D	44.42 ^a ±3.80	97.12 ^a ±1.81	99.26 ^a ±0.21
AK-S13D	28.10 ^c ±0.42	97.86 ^a ±0.77	98.12 ^a ±2.74
AK-S14D	43.53 ^a ±0.19	97.76 ^a ±0.59	98.79 ^a ±0.63

Mean values ± SD (n=2) with different superscripts in the same column are significantly different ($p < 0.05$) corresponding to the attributes displayed. AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.3.5 Evaluation of safety of *Lactobacillus* isolates

6.3.5.1 Antibiotic susceptibility.

Table 6.6 summarises the antibiotic susceptibility profiles of lactic acid bacteria (LAB) isolates obtained from millet-, sorghum-based fermented beverages. Most isolates, including the reference strain, had resistance to at least 5 out of 13 antibiotics. Notably, all isolates were resistant to vancomycin (Va-30 µg, glycopeptide), kanamycin (K-30 µg, aminoglycoside), metronidazole (MTZ-5 µg, nitroimidazole), ciprofloxacin (CIP-5 µg, fluoroquinolone), and colistin (CT-10 µg, polymyxin), except AK-S12D, which showed susceptibility to vancomycin. On the contrary, most isolates (11) were susceptible to ceftriaxone (CRO-30 µg, β-lactam), clindamycin (DA-2 µg, lincosamide), and ampicillin (AMP-10 µg, β-lactam). Exceptions included AK-S9D and AK-S5D, which recorded moderate susceptibility and resistance to ceftriaxone, respectively.

Overall, all isolates were resistant to five or more antibiotics, except AK-S12D. Specific resistance profiles included: i) Resistance to 5 antibiotics: AK-S3D, AK-S7D, AK-S11D, and AK-S13D; ii) Resistance to 6 antibiotics: AK-S1D, AK-S2D, AK-S4D, and AK-S8D; iii) Resistance to 7 antibiotics: AK-S5D and AK-S9D. Millet-based isolates generally recorded greater resistance across antibiotic classes than their sorghum-based counterparts. Furthermore, isolates from millet-sesame-soybean composite beverages showed more robust antibiotic resistance profiles than those derived from single-ingredient fermentations, suggesting that composite substrates may influence strain-level antibiotic tolerance.

Table 6.6: Antibiotic susceptibility of LAB isolated from Ugandan fermented millet and sorghum-based beverages

Isolates	Anti-biotic discs/concentration											
	AMP- 10 µg	VA-30 µg	CRO-30 µg	DA-2 µg	E-15 µg	CN-10 µg	TE-30 µg	AZM- µg	K-30 µg	MTZ-5 µg	CIP-5 µg	CT-10 µg
AK-S1D	S	R	S	S	S	S	R	S	R	R	R	R
AK-S2D	S	R	S	S	S	S	S	R	R	R	R	R
AK-S3D	S	R	S	S	S	S	S	MS	R	R	R	R
AK-S4D	S	R	S	S	S	R	S	MS	R	R	R	R
AK-S5D	S	R	R	S	MS	R	MS	MS	R	R	R	R
AK-S6D	S	R	S	S	R	R	S	R	R	R	R	R
AK-S7D	S	R	S	S	MS	S	S	MS	R	R	R	R
AK-S8D	S	R	S	S	S	R	S	S	R	R	R	R
AK-S9D	S	R	MS	S	R	S	R	S	R	R	R	R
AK-S10D	S	R	S	S	R	R	S	R	R	R	R	R
AK-S11D	S	R	S	S	S	S	S	S	R	R	R	R
AK-S12D	S	S	S	S	S	S	S	MS	R	R	R	R
AK-S13D	S	R	S	S	S	S	S	S	R	R	R	R
AK-S14D	S	R	S	S	S	S	S	S	R	R	R	R

R: resistant; MS: Moderately Susceptible; S: Susceptible. AMP-Ampicillin; VA- Vancomycin; CRO-Ceftriaxone; DA- Clindamycin; E-Erythromycin; CN-Gentamycin; TE-Tetracycline; AZM-Azithromycin; K-Kanamycin; MTZ-Metronidazole; CIP-Ciprofloxacin; CT-Colistin. AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.3.5.2 Haemolytic and DNase activity

All isolates did not have haemolytic and DNase activity.

6.3.6 Pathogen inhibition capacity of *Lactobacillus* isolates

6.3.6.1 Co-aggregation

The coaggregation capacity of isolates with the pathogens showed variability and lacked a specific pattern (Table 6.7). AK-S12D, AK-S13D, demonstrated the highest coaggregation, which was significantly different ($p < 0.05$) from the other isolates when tested with all three pathogens. AK-S12D showed the highest coaggregation with *Salmonella enterica* subsp. *enterica* (ATCC 13076) at 55%, while AK-S13D registered the highest coaggregation with *Shigella flexneri* (ATCC 12022) at 62%. Additionally, AK-S9D recorded coaggregation values exceeding 50% for all tested pathogens, indicating a strong interactive capacity. In contrast, coaggregation among the composite-based isolates ranged between 20–35% for most pathogens, except AK-S7D, which showed a markedly lower coaggregation with *Salmonella* (13%) and *Shigella* (18%). Overall, coaggregation capacity was generally higher with *Escherichia coli* and *Shigella flexneri* compared to *Salmonella enterica* subsp. *enterica*, suggesting differential affinity patterns among the isolates for specific pathogenic targets.

Table 6.7: Co-aggregation capacity of LAB isolated from Ugandan fermented millet and sorghum-based beverages

Isolates	% co-aggregation of pathogenic bacteria		
	<i>Salmonella enterica</i> subsp. <i>enterica</i> (ATCC 13076)	<i>Shigella flexneri</i> (ATCC 12022)	<i>Escherichia coli</i> (ATCC 25922)
AK-S1D	31.43 ^b ±6.25	27.21 ^{cd} ±0.66	31.98 ^{cd} ±0.63
AK-S2D	24.52 ^{cd} ±0.66	25.17 ^{cd} ±2.67	34.52 ^c ±1.03
AK-S3D	27.82 ^c ±2.08	34.39 ^b ±5.81	30.81 ^{def} ±0.61
AK-S4D	26.61 ^c ±0.63	28.14 ^{bcd} ±0.24	32.89 ^{cde} ±0.35
AK-S5D	22.16 ^d ±2.44	24.03 ^{cde} ±1.65	31.56 ^{cdefg} ±0.01
AK-S6D	22.05 ^d ±0.67	19.37 ^{fe} ±3.46	28.19 ^{fg} ±0.6
AK-S7D	13.27 ^e ±1.01	17.89 ^f ±1.92	29.82 ^{efg} ±2.48
AK-S8D	24.47 ^{cd} ±3.62	21.78 ^{def} ±1.18	28.09 ^g ±2.28
AK-S9D	50.44 ^a ±3.47	53.61 ^b ±0.85	52.54 ^b ±1.96
AK-S10D	22.14 ^c ±0.24	24.53 ^{cdef} ±1.29	28.91 ^{fg} ±1.05
AK-S11D	20.26 ^c ±0.69	22.21 ^{def} ±0.61	28.33 ^{fg} ±0.22
AK-S12D	55.2 ^a ±4.30	55.07 ^{ba} ±2.88	54.56 ^{ab} ±0.93
AK-S13D	49.84 ^a ±2.38	62.05 ^a ±2.08	57.25 ^a ±1.19
AK-S14D	31.19 ^b ±4.14	30.38 ^{db} ±0.67	34.45 ^{cd} ±0.31

Mean values ± SD (n=2) with different superscripts in the same column are significantly different ($p < 0.05$) corresponding to the attributes displayed. AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.3.6.2 Production of antimicrobial compounds

The production of antimicrobial compounds and its effect on enteric pathogens is summarised in Table 6.8 and 6.9. The untreated cell free culture supernatants of the isolates inhibited *Salmonella enterica* subsp. *enterica* (ATCC 13076), *Shigella flexneri* (ATCC 12022), *Escherichia coli* (ATCC 25922) at varying magnitude with *Shigella* being inhibited more than *Salmonella* and *E. coli*. After heat treatment, the isolates retained their antimicrobial activity which was not the case when pH was altered. Addition of proteinase K did not significantly inhibit antimicrobial activity of all the isolates against *Salmonella* and *E. coli* (6.9).

Table 6.8: Antimicrobial production potential of LAB isolates from millet-and sorghum-based fermented beverages

Isolates	AMT-no treatment		
	<i>Salmonella enterica</i> subsp. <i>enterica</i> (ATCC 13076)	<i>Shigella flexneri</i> (ATCC 12022)	<i>Escherichia coli</i> (ATCC 25922)
AK-S1D	++	+	++
AK-S2D	++	++	++
AK-S3D	++	+++	++
AK-S4D	++	+++	++
AK-S5D	+++	+++	++
AK-S6D	++	++	++
AK-S7D	+	+++	++
AK-S8D	+++	++++	++
AK-S9D	++	++	++
AK-S10D	+	+++	++
AK-S11D	+	+++	++
AK-S12D	++	+++	++
AK-S13D	++	+++	++
AK-S14D	+	++	++
Isolates	AMT-heat 100°C for 20 minutes		
	<i>Salmonella enterica</i> subsp. <i>enterica</i> (ATCC 13076)	<i>Shigella flexneri</i> (ATCC 12022)	<i>Escherichia coli</i> (ATCC 25922)
AK-S1D	++	+	++
AK-S2D	++	+++	-
AK-S3D	+++	+++	++
AK-S4D	++	++	++
AK-S5D	-	+++	++
AK-S6D	+	+++	++
AK-S7D	+++	+++	++
AK-S8D	+++	++++	++
AK-S9D	+++	-	++
AK-S10D	+++	++++	+++
AK-S11D	+++	++++	+++
AK-S12D	++	++++	-
AK-S13D	++	++++	++
AK-S14D	+	+	++

cell-free culture supernatant treatment-no inhibition/ +(7-9mm)-weak inhibition/ ++ (10-13mm) intermediate/ +++(14-16mm) strong inhibition and +++++ >17mm very strong inhibition. AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

Table 6.9: Antimicrobial production potential of LAB isolates from millet-and sorghum-based fermented beverages

Isolates	AMT-pH		
	<i>Salmonella enterica</i> subsp. <i>enterica</i> (ATCC 13076)	<i>Shigella flexneri</i> (ATCC 12022)	<i>Escherichia coli</i> (ATCC 25922)
AK-S1D	-	-	-
AK-S2D	-	-	-
AK-S3D	-	-	-
AK-S4D	-	-	-
AK-S5D	-	-	-
AK-S6D	-	-	-
AK-S7D	-	-	-
AK-S8D	-	-	-
AK-S9D	-	-	-
AK-S10D	-	-	-
AK-S11D	-	-	-
AK-S12D	-	-	-
AK-S13D	-	-	-
AK-S14D	-	-	-

Isolates	AMT-Proteinase K		
	<i>Salmonella enterica</i> subsp. <i>enterica</i> (ATCC 13076)	<i>Shigella flexneri</i> (ATCC 12022)	<i>Escherichia coli</i> (ATCC 25922)
AK-S1D	++	+	+
AK-S2D	++++	+	+
AK-S3D	++	+	+
AK-S4D	+	+	+
AK-S5D	+++	+	+
AK-S6D	++	+	+
AK-S7D	++	+	+
AK-S8D	+++	+	+++
AK-S9D	++	+	+
AK-S10D	+++	+	++
AK-S11D	+++	+	++++
AK-S12D	+++	+	++
AK-S13D	++	+	++
AK-S14D	++++	+	++

cell-free culture supernatant treatment-no inhibition/ +(7-9mm)-weak inhibition/ ++ (10-13mm) intermediate/ +++(14-16mm) strong inhibition and ++++ >17mm very strong inhibition. AKS-1D: millet-sesame-soybean 24hr-isolate 1, AK-S2D: millet-sesame-soybean 24hr-isolate 2, AK-S3D: millet-sesame-soybean 48hr isolate 1, AK-S4D: millet-sesame-soybean 48hr isolate 2, AK-S5D: sorghum-sesame-soybean 24hr isolate 1, AK-S6D: sorghum 24hr isolate 1, AK-S7D: sorghum-sesame-soybean 48hr isolate 1, AK-S8D, sorghum 48hr isolate, AK-S9D: millet 24hr isolate 1, AK-S10D: millet 48hr isolate 1, AK-S11D: millet 48hr isolate 2, AK-S12D: commercial *bushera* isolate 1, AK-S13D: commercial *bushera* isolate 2, AK-S14D: *Lactiplantibacillus plantarum* ATCC 14917.

6.4. Discussion

6.4.1 Tolerance to harsh environmental and gastrointestinal conditions

This study demonstrated that *Lactobacillus* isolates derived from millet- and sorghum-based fermented beverages displayed strong resilience under simulated gastrointestinal conditions (Table 6.4). Several strains surpassed the reference strain in acid, bile, and pancreatic tolerance, indicating promising probiotic potential. Notably, these isolates were tolerant to 1% bile salt, which exceeds the physiological concentration of 0.3% (w/v) typically found in the human small intestine with a residence time of 3–4 hours (Amenu & Bacha, 2023). Both gastric and pancreatic juices act as first-line non-specific physiological barriers to microorganisms in the gastrointestinal tract (Zhang et al., 2022). Therefore, beneficial micro-organisms should be able to possess properties that enable them bypass this barrier to reach the lower GIT where they exact their benefits (Sanders et al., 2019).

Comparable acid and bile tolerance (>80% survival) has been reported in 34 lactic acid bacteria strains isolated from Ethiopian fermented foods by Mulaw et al. (2019). Such results reinforce the concept of strain-specific resilience, as emphasised by Wendel (2022), who noted that the survivability and functionality of probiotics are largely dependent on both the microbial species and the fermentation substrate. In this context, millet-based composite beverages may provide a selective advantage. These oligosaccharides create structural barriers that neutralizes the gastric and pancreatic juices thus providing physical barrier protection to the isolates (Ji et al., 2025). Moreover, the diverse oligosaccharide content could offer metabolic flexibility to probiotic strains, enabling them to utilise alternative energy sources when preferred substrates are limited thereby enhancing stress tolerance as elaborated in Chapter five of the dissertation where isolates from composite beverages had higher number of accessory genes.

Genomic analysis in Chapter five revealed that dominant *Lpb. plantarum* and *Lb. rhamnosus* strains contained elevated numbers of accessory orthologous genes, that would enhance genomic plasticity to be able to utilise the diverse carbohydrate options in composite beverages. These findings are supported by Treven et al. (2024), who demonstrated that gruel-based substrates such as porridge enhanced probiotic survival more effectively than

juice or water, likely due to buffering effects provided by prebiotic oligosaccharides. It is important to note that, the protective capacity of a food matrix is strain-dependent, emphasizing the need for precise matching between substrate composition and microbial strains (Jiang et al., 2026).

The low bile salt activity in the isolates could be due to down regulation of bile salt hydrolase (BSH) genes. Expression levels BSH genes may vary due to regulatory mechanisms or mutations that reduce enzyme functionality especially in environments without bile salts (Begley et al., 2006). By implication therefore, continued exposure of these isolates to bile salts exerts a strong selective pressure, driving genomic rearrangements that can lead to the stable integration, duplication, or altered expression of bile salt hydrolase (*bsh*) genes, which are frequently carried on mobile genetic elements (Foley et al., 2019; Zhang et al., 2013). Nevertheless, this Chapter did not explore this further.

6.4.2 Adhesion potential of LAB isolates from millet-and sorghum-based beverages

The current study reported significant variability in cell surface hydrophobicity (CSH) among isolates derived from fermented millet- and sorghum-based beverages. Notably, isolates from single-ingredient sorghum and composite millet beverages registered the highest CSH values, ranging from 19% to 44% (Table 6.5). Despite this variability, all isolates had a uniformly high auto-aggregation capacity exceeding 95% at 24 hours. CSH is a non-specific physicochemical property driven by thermodynamic forces, allowing hydrophobic cells to coalesce and minimize their surface energy within aqueous environments like the gastrointestinal tract (GIT) (Krausova et al., 2019). This initial, non-specific interaction serves as a crucial preliminary phase that facilitates adhesion by overcoming electrostatic repulsion and bringing microbial cells into close proximity with the host epithelial lining (Bennett et al., 2014). However, long-term colonization and stable tissue attachment depend strictly on highly specialized surface components rather than general hydrophobicity (Krausova et al., 2019).

In this context, auto-aggregation serves as a more reliable indicator of stable colonization potential. Unlike CSH, auto-aggregation relies on specific macromolecular components referred to as auto-agglutinins which facilitate the self-recognition and clumping of

genetically identical cells (Trunk et al., 2018). This process is typically mediated by surface protein adhesins that operate through specific structural complementation, creating a physical bond far more resilient than non-specific hydrophobic associations (Nwoko & Okeke, 2021). Furthermore, many *Lactobacillus* species possess crystalline surface layer (S-layer) proteins (Angelescu et al., 2024). These S-layers directly mediate strong, specific self-association and cellular interlocking independently of the baseline CSH of the cell wall (Wang et al., 2024). This distinct molecular mechanism provides a plausible explanation why isolates in this study achieved near-complete auto-aggregation despite possessing low-to-moderate surface hydrophobicity.

These differences may reflect underlying genomic variation, particularly in accessory orthologous genes encoding surface-associated proteins that mediate hydrophobic interactions. The observed hydrophobicity levels were higher than those reported by Byakika et al. (2020) in *Lpb. plantarum* MNC21 isolated from sorghum-millet beverages. Auto-aggregation capacity was uniformly high across all isolates, exceeding 95% at 24 hours, and aligning with the findings of Amenu and Bacha (2023), who reported aggregation rates between 45% and 120%. These values were substantially higher than those reported by Ramos et al. (2013) for *Lactobacillus* strains from Brazilian fermented foods, which ranged from 12% to 21%.

Cell surface hydrophobicity and auto-aggregation are widely recognised as proxy indicators of probiotic adhesion potential. Hydrophobic interactions facilitate the initial non-specific attachment of probiotic cells to intestinal mucosal surfaces (Monteagudo-Mera et al., 2019), which may be followed by specific ligand-receptor binding events that enable colonisation and host interaction (Krausova et al., 2019). Auto-aggregation contributes to microcolony formation and biofilm development, enhancing persistence and competitive exclusion of pathogens (Salas-Jara et al., 2016). The high auto-aggregation capacity observed in isolates from sorghum single-ingredient and millet composite beverages show promising adhesion potential which is an essential attribute for probiotic viability and functionality. However, given that these are indirect measures, further *in vitro* and *in vivo* adhesion assays are necessary to confirm the ability of these strains to adhere to intestinal epithelial cells and exert beneficial effects.

6.4.3 Microbial safety of the LAB isolates

The present study identified diverse antibiotic resistance patterns among *Lactiplantibacillus plantarum* isolates recovered from millet-, and sorghum-based fermented beverages (Table 6.6). Majority of the isolates registered resistance to multiple antibiotic classes, notably glycopeptides (vancomycin), aminoglycosides (kanamycin), nitroimidazoles (metronidazole), fluoroquinolones (ciprofloxacin), and polymyxins (colistin). Such resistance phenotypes have been previously reported in *Lactobacillus* strains from traditional fermented foods in Ethiopia (Amenu & Bacha, 2023) and Uganda (Byakika et al., 2020). However, several isolates remained susceptible to β -lactam antibiotics, including ceftriaxone and ampicillin. This susceptibility aligns with observations in strains such as *Lactocaseibacillus paracasei* K56 and ET-22, and *Bifidobacterium lactis* BL-99 (Lu et al., 2021). Furthermore, Mulaw et al. (2019) also reported susceptibility of *Lactobacilli* strains isolated from Ethiopian traditional fermented food and beverages to β -lactam antibiotics.

Previous studies have reported that *Lactobacilli* species have intrinsic antibiotic resistance, which is naturally occurring and non-transferable (Meleh et al., 2020). As a result, these species are generally regarded as safe (GRAS) (Amenu & Bacha, 2023). This intrinsic resistance is beneficial in restoring gut microbiota following antibiotic treatment (Gueimonde et al., 2013). Gueimonde further emphasised that intrinsic resistance mechanisms in *Lactobacillus* species such as vancomycin resistance due to the absence of D-Ala-D-lactate targets are not considered safety concerns unless they are associated with mobile genetic elements. The resistance observed in the study isolates to vancomycin and kanamycin is likely attributable to these intrinsic mechanisms and therefore may not pose a safety risk.

The relative resistance to Kanamycin could be intrinsically linked to the anaerobic and fermentative metabolism of *Lactobacillus* species. These bacteria lack the cytochrome-mediated electron transport systems required to generate the proton motive force needed for the active cellular uptake of these drugs (Zafar & Saier, 2020). Similarly, the intrinsic resistance to ciprofloxacin could be due to the restricted permeability of the bacterial cell envelope to hydrophobic compounds, coupled with subtle structural variations in the target

enzymes DNA gyrase and topoisomerase IV that diminish drug binding affinity (Hooper & Jacoby, 2017; Redgrave et al., 2014)

Nevertheless, Ezeanya-Bakpa and Omorinbola (2024) cautions that probiotics carrying transferable antibiotic resistance genes (ARGs) may facilitate horizontal gene transfer to gut pathogens, such as *Salmonella* and *Shigella* species. Although the isolates examined in the present study demonstrated resistance to multiple antibiotics, the lack of evaluation for mobile genetic elements leaves the question of transferability unresolved. This could be an important aspect to address in future safety assessments.

6.4.4 Pathogen inhibition of *Lactobacillus* strains isolated from millet-and sorghum-based beverages

The coaggregation capacity (Table 6.7) observed among isolates derived from commercial *bushera* and millet-based fermentations offers compelling insights into their probiotic efficacy, especially regarding pathogen exclusion and colonisation potential in the gastrointestinal tract. Coaggregation is a vital mechanism through which probiotics interact with other microbial species via specific surface adhesins and receptors, facilitating the formation of mixed-species clusters or biofilms (Zymovets et al., 2025). This interaction plays a crucial defensive role by entrapping pathogenic bacteria within microbial aggregates, thereby preventing their adhesion to epithelial cells and reducing infection risk (Golletz et al., 2025). In the current study, *Lactobacillus* isolates had higher coaggregation with *Salmonella enterica* and *Escherichia coli* compared to the 11–17% range reported by Byakika et al. (2020). However, the current results were lower than those (50-116%) reported by Amenu and Bacha (2023) but comparable (15-55%) to those reported by Abdulla et al. (2014). These variations may reflect inherent differences in strain-specific surface characteristics, including molecular composition of adhesins, hydrophobicity, and biofilm-forming capacity, as well as differences in fermentation substrates and conditions used (Ji et al., 2025; Jiang et al., 2026; Yin et al., 2024).

The antimicrobial activity observed across all isolates (Table 6.8 and 6.9) was predominantly attributed to the production of non-proteinaceous compounds, including organic acids such as lactic acid and short-chain fatty acids. Multiple studies have demonstrated that

Lactobacillus spp. synthesise a diverse array of antimicrobial metabolites. For instance, Hussein et al. (2025) reported that the inhibitory activity of cell-free culture supernatants (CFCS) remained unaffected following thermal and protease treatments. Similarly, Mariam et al. (2014) revealed that CFCS retained antimicrobial potency after exposure to heat and Proteinase K. These findings strongly imply that pathogen inhibition in both studies and in the present study is primarily mediated by heat- and protease-stable metabolites, with organic acids playing a central role.

Organic acid functions by lowering the extracellular pH and compromising microbial membrane integrity, thereby collapsing the proton motive force across the membrane (Nkosi et al., 2021). This impairs core physiological processes and increases microbial sensitivity to other antimicrobial compounds (Sun & O’Riordan, 2013). The demonstrated stability of these metabolites in protease-rich environments, such as the gastrointestinal tract, supports the suitability of these isolates for food preservation and therapeutic applications. Furthermore, their robust and consistent antimicrobial performance highlights their value in probiotic product development, particularly in formulations requiring extended shelf-life and functional resilience.

6.5 Conclusion and Recommendation

The *Lactobacillus* isolates demonstrated a wide spectrum of probiotic properties, including tolerance to acidic and bile environments, adhesion to intestinal surfaces, safety-related characteristics, and pathogen inhibition capacity. Among the formulations evaluated, millet-sesame-soybean-based composites had superior performance across these probiotic benchmarks compared to single-component beverages.

Future assessments of probiotic strains should incorporate comprehensive genomic screening for mobile genetic elements. This will enable differentiation between intrinsic antibiotic resistance and transferable resistance mechanisms which is an essential step in ensuring microbial safety. Additionally, *in vitro* and *in vivo* evaluations must also be complemented with targeted risk assessments, particularly for strains with multi-drug resistance, to evaluate their potential for horizontal gene transfer. To facilitate wider adoption and therapeutic utilisation, fermented foods and beverages with validated probiotic

properties should be integrated into national food composition databases and dietary guidelines. This strategy may promote their recognition and uptake as adjunct nutritional interventions, especially for the management of functional gastrointestinal disorders and other diet-related conditions affecting vulnerable populations.

CHAPTER SEVEN

GENERAL DISCUSSIONS, CONCLUSIONS, RECOMMENDATIONS AND STUDY LIMITATIONS

7.1 General Discussions

7.1.1 Effect of varietal differences and processing methods on the content of Fermentable Oligo-Di-Monosaccharides, and polyols (FODMAPs)

The composition of FODMAPs in plant-derived foods are shaped by both genetic and processing techniques (Tuck et al., 2018; Varney et al., 2017). Therefore, investigating crop varietal differences is crucial because FODMAPs profiles are highly cultivar-specific, governed by distinct genetic expressions of biosynthetic enzymes such as fructan sucrose and galactinol synthase (Huynh et al., 2007; Martins et al., 2019). Identifying naturally low-FODMAPs germplasms provides an agronomic strategy to select and cultivate crops tailored for individuals with irritable bowel syndrome (IBS) and other functional gastrointestinal disorders.

Concurrently, evaluating the impact of distinct processing methods is essential, as these matrices are rarely consumed raw. Thermal treatments, mechanical disruptions, hydration, and fermentations alter the food matrix, driving the reduction of osmotic short-chain carbohydrates such as FODMAPs through two primary mechanisms (Adebiyi et al., 2018; Al Loman & Ju, 2017; Thirunathan & Manickavasagan, 2019). These mechanisms include thermal/enzymatic activation of endogenous hydrolases that cleave complex oligosaccharides into simpler monomers (Ispiryan, et al., 2020; Yao et al., 2021), and the physical leaching of water-soluble FODMAPs fractions (Njoumi et al., 2019). Documenting these shifts across commonly consumed various crop varieties establishes an empirical framework for the food industry to optimise commercial processing parameters while preserving the nutritional and structural integrity of the final food product.

To comprehensively map the diverse FODMAPs categories contributing to the total FODMAPs pool in the different crop varieties in the present study, a complementary, dual-platform analytical approach was deployed. High-Performance Liquid Chemistry (HPLC)

was selected for the precise isolation and quantification of low-molecular-weight free monosaccharides and sugar alcohols (polyols). This chromatographic approach offers high resolution and structural specificity, preventing co-elution and misidentification within chemically dense matrices (Jalaludin & Kim, 2021).

Conversely, directly quantifying FOS and GOS, standard HPLC systems can be technically challenging due to co-elution (Austin et al., 2014). Therefore, highly specific enzymatic assay kits (K-FRUC and K-RAFGL, Megazyme, Ireland) were used to quantify the respective oligosaccharide fractions. These Megazyme kits deploy targeted enzymatic cascades using a fructanase mixture (comprising recombinant endo- and exo-inulinases and endo-levanase) and an α -galactosidase/invertase complex to selectively isolate and hydrolyse specific glycosidic linkages without disturbing baseline free monomer pools (Mccleary et al., 2019).

Cross-method analytical overlap and the subsequent inflation of the total FODMAPs sum were prevented through internal chemical controls built into the assay protocols. In the K-FRUC workflow, a preliminary alkaline borohydride reduction step rendered all free background reducing sugars (fructose and glucose, independently quantified by HPLC) chemically inert, ensuring that the downstream spectrophotometric absorbance readings originated exclusively from hydrolysed fructan polymers. In the K-RAFGL workflow, the baseline content of free D-glucose was determined and mathematically subtracted from the post-hydrolysis glucose pool during the final calculation matrix, effectively isolating the true GOS values and preventing double-counting with HPLC monomer data.

The findings from this work (Chapter three) showed that crop type, genetic variety, and processing technique significantly modulate the concentration and composition of fermentable oligosaccharides. Previously, crop breeding programs have prioritised traits such as environmental resilience, yield optimisation, and micronutrient enrichment (Beebe, 2020; Obua et al., 2020; Tukamuhabwa et al., 2012). This study provided empirical evidence that improved varieties of sorghum, soybean, and sesame have lower oligosaccharide levels compared to their indigenous counterparts, ranging from 0.38 to 8.03 g/100 g. However, these values for instance 8.03g/100g in soybean were still relatively high especially among

individuals with functional gastrointestinal disorders (FGIDs). Daily intake of more than a serving may exacerbate symptoms such as bloating, flatulence, and mucosal hypersensitivity. This therefore implies that although breeding techniques such as conventional breeding may serve as a potential strategy for development of low-FODMAPs crop varieties, it requires optimisation. Currently, the variability in oligosaccharide reduction underscores the limitations of relying solely on varietal selection to achieve low-FODMAPs dietary profiles.

To address this gap, the study further investigated the impact of traditional processing techniques specifically roasting and malting on FODMAPs reduction in millet, sorghum, soybean, and sesame. The results of this study showed that malting consistently achieved significant reductions across all FODMAPs categories, lowering concentrations to between 0.93 and 2.56 g/100 g. These levels fall within the recommended daily intake range of 5–8 g required to elicit a prebiotic effect. This proposes that targeted processing can optimise oligosaccharide profiles without compromising functional potential (Kolida & Gibson, 2007). The modulation of FODMAPs profiles through targeted processing techniques such as fermentation, malting, soaking, and thermal treatment enables the development of plant-based foods with optimised prebiotic-properties.

It is important to emphasize that this study assessed varietal and processing strategies independently in order to generate foundational baseline data, as their specific effects had not been previously documented. As a result, potential synergistic interactions between crop variety and processing methods were not examined. The findings demonstrated that improved varieties had lower FODMAPs content, while malting significantly reduced FODMAPs levels. Therefore, applying malting to varieties that are already genetically low in FODMAPs content could further enhance the effectiveness of reduction. This represents a promising avenue for future investigation.

7.1.2 Effect of crop type and variety on prebiotic potential of Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAPs) extracted from millet, sorghum, soybean and sesame varieties

While compositional profiling of FODMAPs provides valuable baseline data, it remains incomplete without assessing their prebiotic potential, which is the primary mechanism through which they influence gut health. Accordingly, Chapter Four of this dissertation evaluated the prebiotic attributes of FODMAPs extracted from millet, sorghum, soybean, and sesame varieties. Given the observed varietal differences across FODMAPs categories, it was critical to determine how these compositional variations translate into functional outcomes. Relying solely on chemical quantification is insufficient, as structural isomerism and carbohydrate complexity dictate microbial fermentability (Lee et al., 2024).

Therefore, *in vitro* anaerobic fermentation assays were conducted using well-characterized probiotic strains (*Lpb. plantarum* ATCC 14917, *Lb. rhamnosus* ATCC 7469). This approach directly measured microbial growth using plate count method and metabolic activity via SCFA production, thereby providing functional validation of the extracts that chemical analysis alone cannot achieve. These findings underscore the importance of integrating compositional profiling with biological assays to establish the nutritional and therapeutic relevance of FODMAPs in Ugandan crop varieties.

The major findings revealed that improved crop varieties had better prebiotic properties (selectively promoted growth of well characterised probiotics, enhanced production of total SCFA and antagonistic activity against common enteric pathogens) than indigenous varieties. These outcomes may be attributed to a more accessible cellular architecture in improved varieties, potentially involving reduced concentrations of tightly bound matrix cross-linkages such as lignified fibre fractions (Verspreet et al., 2015). This structural shift enhances the extractability and bio-accessibility of the internal oligosaccharides, that readily support the growth of beneficial lactic acid bacteria during fermentation assays (Karakoula et al., 2026; Tariq et al., 2025). Furthermore, crop breeding programs designed to optimize agronomic performance frequently alter source-sink relationships within the plant (Slewinski, 2012). This selection pressure can upregulate enzymes involved in the synthesis of specialized storage carbohydrates, such as FOS and GOS.

7.1.3 Genotypic characteristics of lactic acid bacteria isolated from millet-sesame-soybean and sorghum-sesame-soybean fermented composite beverages

The findings from this study (Chapter five) underscore the critical role of fermentation substrates in shaping microbial genotypic plasticity, particularly in response to composite carbohydrate sources compared to fermentation time. The use of multi-ingredient matrices such as millet-sesame-soybean composites appears to stimulate genomic and phenotypic diversification, potentially driving the emergence of novel *Lactiplantibacillus plantarum* probiotic strains with enhanced functional attributes. These findings diverge from previous reports on single ingredient cereal-based fermentation microbiota. For example, Mukisa et al. (2012) documented *Streptococcus gallolyticus*, *S. infantarius subsp. infantarius*, *Lactobacillus fermentum*, *Lb. delbrueckii*, and *Weissella confusa* as the dominant strains in traditional Ugandan millet and sorghum fermentations, locally referred to as *bushera*. Similarly, Qin et al. (2016) identified *Lactobacillus fermentum* as the predominant species in Chinese fermented cereal gruel.

Several factors may account for these variations especially at species and strain level. First, the ingredient matrix plays a critical role in microbial selection (Śliżewska & Chlebicz-Wójcik, 2020). Single-component substrates typically offer limited carbon sources, whereas composite formulations such as those used in the present study provide a broader spectrum of fermentable oligosaccharides, shaped further by crop variety and regional cultivation practices. These differences can influence microbial competitiveness and metabolic functionality (Kumoro et al., 2025).

Furthermore, methodological variations in microbial profiling significantly affect strain diversity estimates. The current study employed MLST and whole genome sequencing technologies with PacBio SMRT, enabling fine-scale resolution of closely related strains. Nevertheless, previous studies largely relied on Denaturing Gradient Gel Electrophoresis (DGGE) and short-read 16S rRNA sequencing (Mukisa et al., 2012; Qin et al., 2016). DGGE, while useful for community fingerprinting, suffers from low taxonomic resolution due to overlapping melting domains and indistinct band profiles, leading to potential misidentification or redundancy (Bhadury & Ghosh, 2022). Similarly, sequencing partial regions of the 16S rRNA gene often limits discrimination among phylogenetically proximate

taxa, particularly at the strain level (Buetas et al., 2024) and omits other variable regions that are crucial for accurate classification (Johnson et al., 2019). This highlights the need for methodological standardization and ingredient contextualization in future comparative studies of microbial fermentation ecology.

The presence of high number of accessory gene orthogroups confers adaptive advantages, such as enhanced antimicrobial production or niche-specific metabolic capabilities. Comparative genomic analyses have reported that accessory genes often encode functions critical for environmental adaptation, such as bacteriocin synthesis, stress response, and specialised carbohydrate metabolism (Evanovich et al., 2019; Rajput et al., 2023). Adaptive modulation of accessory genes in microbial strains is largely driven by genomic alterations, encompassing differential gene expression, regulatory network evolution, and epigenetic remodelling (Handy et al., 2011; Weiner & Katz, 2021). When exposed to nutrient-diverse substrates such as millet-sesame-soybean composite beverages, these strains undergo transcriptional reprogramming that reflects the biochemical complexity of their environment (Tomar et al., 2025; Weiner & Katz, 2021). Such selective pressures can induce lineage-specific shifts in gene expression, fostering niche adaptation and functional diversification (Huber & Chávez, 2025; Sultan, 2017).

In *Lactobacillus plantarum*, for instance, pan-genomic studies revealed that strain-specific accessory elements contribute to functional diversity (Carpi et al., 2022). Similarly, *Lactobacillus reuteri* isolates possess accessory genes encoding reuterin and other antimicrobial compounds that facilitate competitive exclusion and host colonisation (Lee et al., 2017). These genomic features underpin strain-level specialisation and niche adaptation. While this study primarily aimed to assess potential shifts in LAB genotypic characteristics arising from exposure to millet-sesame-soybean composite substrates and fermentation time, it did not extend into the mechanistic underpinnings driving these changes. Specifically, the investigation did not dissect the regulatory or epigenetic pathways responsible for transcriptional reprogramming, nor did it explore the functional consequences of accessory gene modulation across lineages. Therefore, future studies should incorporate integrative multi-omics approaches to unravel the regulatory, epigenetic, and transcriptional

mechanisms underpinning genotypic plastic shifts of strains in composite fermented beverages.

7.1.4 Probiotic potential of lactic acid bacteria strains isolated from ready-to-use millet- and sorghum-based fermented composite beverages

Isolates derived from composite fermented beverages had better performance across eight core probiotic *in vitro* evaluation criteria including acid and bile tolerance, cell hydrophobicity and auto-aggregation adhesion assays, disc and agar well diffusion for antimicrobial activity, and antibiotic resistance profiling (Chapter six) compared to the reference strain. The Kirby–Bauer disk diffusion assay evaluated antimicrobial susceptibility by measuring inhibition zones formed as antibiotics diffuse radially from impregnated paper disks into agar seeded with the target *Lactobacillus* strain (Hudzicki, 2016). A concentration gradient develops, and growth is suppressed where drug levels exceed the minimal inhibitory concentration (MIC). Diffusion dynamics depend on molecular weight, agar depth, and viscosity. This implies that zone diameters do not always directly reflect clinical efficacy as larger molecules often diffuse more slowly, potentially underestimating susceptibility (CLSI, 2020; Flanagan & Steck, 2017). For instance Vancomycin and Colistin diffuse poorly, producing smaller inhibition zones that can underestimate susceptibility whereas Metronidazole, Ciprofloxacin diffuse rapidly, yielding larger zones that more closely reflect MIC values (Peláez et al., 2008; Wong et al., 1999) as observed in this study.

Despite these limitations, disk diffusion remains a cost-effective, standardized screening tool that provides a preliminary profile of resistance just as observed in this study where all isolates were resistant to Kanamycin, Vancomycin, Metronidazole, Colistin and Ciprofloxacin. However, for definitive determination of MIC against the isolates, complementary quantitative methods such as broth microdilution or E-test strips are recommended (CLSI, 2020). Borderline or resistant phenotypes should be further validated using genomic approaches to exclude mobile genetic elements (MGEs) that could disseminate resistance (Kerek et al., 2025).

The adhesive potential of the isolates to the lower GIT mucosa was evaluated using auto-aggregation and cell surface hydrophobicity assays. These biophysical metrics act as highly

correlated, reliable screening proxies that offer valuable preliminary quantitative data regarding a strain's capacity to colonize the host intestine (Bennett et al., 2014). Although testing direct adherence to human cell lines like Caco-2 or HT-29 represents the gold standard, it requires sophisticated tissue culture facilities, and high operational costs (Fedi et al., 2021). Utilizing these *in vitro* surface property assays allowed for an efficient, cost-effective evaluation of the isolates' competitive exclusion and colonization potential.

In vitro transit assay featuring simulated gastric and pancreatic juices to mimic the dynamic and hostile physiological conditions characteristics of the upper gastrointestinal tract (GIT) was conducted. To effectively colonize the human gut and subsequently confer systemic health benefits, candidate probiotic strains must possess the metabolic resilience to survive severe biological barriers, specifically low gastric pH and enzymatic degradation (Amenu & Bacha, 2023). Consequently, simulated *in vitro* digestion models serve as highly reproducible, ethically viable, and reliable initial screening approaches to rigorously select robust strains. This screening method proved instrumental in isolating and validating the viability of the LAB strains derived from composite beverages, as detailed in Chapter Six. Having showed *in vitro* gastrointestinal tolerance, these promising strains can be advanced to necessary *in vivo* and functional efficacy trials. Such clinical validation must be established prior to large-scale commercial manufacturing and formal consumer product recommendation (Hill et al., 2014).

However, translating these functional formulations into scalable products often involves advanced processing techniques, which can substantially increase production costs. This raises a pertinent question about consumers' willingness to pay for the product. This economic-health trade-off underscores the need for targeted market segmentation, frugal innovation strategies, and supportive policy mechanisms to enhance affordability and uptake especially in low-resource settings where diet-related disease burdens remain disproportionately high amidst economic constraints (Kuijken et al., 2017).

Although both households and food industries in Uganda commonly produce fermented food products using single-ingredient substrates and traditional processing methods (Mukisa et al., 2010; Namugumya & Muyanja, 2009), the concept of using cereal-legume composites

as raw materials remains a new and novel approach. This study contributes to advancing local food systems by demonstrating the functional potential of composite substrates to enhance probiotic properties and nutritional value of ready-to-use fermented composite beverages. This approach supports the development of culturally resonant, health-promoting fermented products that align with local dietary patterns and public health priorities, particularly in the context of functional gastrointestinal disorders and nutrition-related non-communicable diseases. Although consumer acceptability is a critical determinant for the successful adoption of any new formulated product (Tao et al., 2020), this study did not evaluate sensory perception or user preferences. Future research should therefore incorporate consumer behaviour and acceptability studies to ensure that health-enhancing composite beverages align with cultural expectations and palatability standards across target populations.

7.2 General Conclusions

This study demonstrated that use of improved crop varieties and application of common processing methods particularly malting are viable strategies for reducing FODMAPs content in plant-based foods. The extracts obtained from improved crop varieties with low FODMAPs content showed better capacity as prebiotic substrates for *Lactiplantibacillus plantarum* ATCC 14917 and *Lacticaseibacillus rhamnosus* ATCC 7469 probiotic strains than the indigenous varieties. The magnitude of growth, short-chain fatty acid production and antagonistic activity was consistently greater when *Lpb. plantarum* strain was incubated in the extracts from improved crop varieties. This highlights the importance of strain-specific substrate utilization, where a metabolically versatile strain such as *Lpb. plantarum* ATCC 14917 derives greater benefit from varietal improvements compared to a more restricted strain like *Lb. rhamnosus* ATCC 7469.

Lactic acid bacteria isolated from composite fermented beverages formulated with improved crop varieties exhibited enhanced genotypic plasticity and better probiotic potential irrespective of fermentation time. This implies that ingredient type such as millet-sesame-soybean and sorghum-sesame-soybean composite substrates, serve as the primary driver of microbial adaptation, exerting stronger selective pressure through its biochemical

composition. Collectively, these findings provide critical insights into how crop improvement, processing, and ingredient composition interact to shape the nutritional and functional potential of plant-based foods. They also establish an *in vitro* foundation for developing locally relevant functional food formulations that leverage Ugandan crop diversity to improve gut health outcomes.

7.3 General Recommendations

7.3.1 Recommendations for future research

While crop improvement and malting demonstrate potential for reducing FODMAPs content in plant-based foods, these strategies require further optimization to achieve the internationally recognized threshold for classification as low-FODMAPs (≤ 0.5 g/100 g). Future research should therefore focus on refining varietal selection and advancing processing technologies, to ensure that reductions are both nutritionally meaningful and clinically effective for individuals with functional gastrointestinal disorders.

Given the limited diversity of food types evaluated in this study, broader conclusions regarding the generalizability of these prebiotic outcomes across other foods consumed within the country remain tentative. Therefore, extensive research could be conducted on a wider variety of nationally available foods such as fruits, vegetables, cereals, legumes, pulses, and oily seeds, consumed to document their FODMAPs profiles and to provide a more comprehensive understanding of their prebiotic benefits. This information could be used to update already existing food composition tables that can be used by nutritionists to implement effective dietary interventions for FGIDs.

Static *in vitro* models cannot fully replicate the dynamic, dose-dependent conditions of the gastrointestinal tract. This phase of the study was designed to provide a controlled baseline screening rather than simulate *in vivo* efficacy. By standardizing doses, incubation times, and conditions, direct comparison of prebiotic potential across crop varieties was achieved. Consistent with established *in vitro* methodologies, these findings warrant validation through future *in vivo* studies. While this study establishes a foundational baseline regarding the genotypic characteristics and probiotic potential of isolated *Lactobacillus* strains, broader ecological and temporal mechanics within the fermenting ecosystem remain

uncharacterized. Therefore, subsequent investigations of bacterial communities by mapping metagenomic successional paths over time is recommended.

It is important to note that this study did not investigate genomic or functional analyses that could elucidate the molecular basis linking observed genotypic plasticity in isolates from composite beverages to their phenotypic traits. Therefore, to uncover the molecular mechanisms underlying substrate-driven microbial adaptation, it is recommended that future studies integrate genomic, transcriptomic, and metabolomic approaches. These analyses will provide deeper insight into strain-level functional responses to dietary substrates. Furthermore, for successful adoption of health-enhancing products such as fermented composite beverages, it is essential to conduct, clinical trials, consumer studies assessing acceptability, sensory preferences, and willingness to pay.

7.3.2 Recommendation for practice

Household could incorporate malted food resources rich in oligosaccharides with prebiotic potential such as Maksoy 3N (soybean), Sesim 2 (sesame), indigenous millet, and Narosorg 2 (sorghum) in their daily diets. Moreover, these resources could be utilised in the formulation of composite dietary products. One such example is a millet-sesame-soybean composite flour, which may be prepared as either a gruel or a solid food. This composite flour can further act as a raw material for development of probiotic composite beverages with potential gut health benefits.

For individuals with irritable bowel syndrome, choosing crop varieties with low FODMAPs content and applying malting as a processing method could significantly reduce the fermentable sugars such as excess fructose that trigger bloating and discomfort. This implies that locally available grains and legumes, when carefully selected and malted, could be transformed into more gut-friendly foods and beverages, offering practical dietary options that ease FGIDs symptoms.

7.3.3 Recommendation for policy

Prioritize investment in the commercialization of fermented composite blends specifically millet–sesame–soybean and sorghum–sesame–soybean as a strategic, low-cost bioactive

food solution. Scaling these formulations into affordable products offers a dual impact: tackling malnutrition through enhanced nutrient density while simultaneously promoting gut health via prebiotic and probiotic activity.

7.4 Study Limitations

This PhD study has provided a range of useful findings and results with respect to FODMAPs. Despite the useful findings, this study is not devoid of limitations. Presenting these limitations provides opportunities for future research.

Firstly, although compositional profiling and *in vitro* assays provided valuable functional insights, extensive research across a wider range of commonly consumed foods is needed to document their FODMAPs profiles and to conduct clinical trials that establish tolerable intake levels for vulnerable individuals.

Secondly, the exclusive focus on isolates with positive bands for *Lactobacillus* genus restricted insight into the broader microbial community and succession dynamics during fermentation, while the early narrowing of isolates may have excluded other potentially relevant bacterial strains capable of contributing to probiotic potential. This relatively small sample size reduced statistical power and limited the generalizability of the findings.

The third limitation of this study concerns the interpretation of genomic diversity. Although the detection of potential novel strains differing by only one or two genes from reference genomes supports the presence of micro-variations and functional divergence, the analysis did not extend to linking this observed genomic plasticity with functional outcomes. As a result, the study could not fully establish how subtle genetic differences translate into probiotic efficacy and host interactions.

Lastly, this study did not incorporate comprehensive genomic screening for mobile genetic elements, which is essential to differentiate between intrinsic antibiotic resistance and transferable resistance determinants. The absence of such screening limits the ability to fully assess microbial safety and the risk of resistance dissemination.

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APPENDICES

Appendix 1: Ethical clearance letter by Gulu University Research Ethics Committee



11/10/2024

To: Docus Alowo

Gulu University
0778766419

Type: Annual Renewal

Re: Prebiotic and probiotic capacity of ready-to-use composite foods developed from non-starch polysaccharides plant-based sources

I am pleased to inform you that at the **118th** convened meeting on **19/09/2024**, the **Gulu University REC** reviewed the progress report to the above study reference number **GUREC-2022-242** and found it satisfactory. In this respect, annual renewal of the study is granted. The study was initially approved on **21/07/2023** and expired on **21/07/2024**.

The Approval of the research is for the period of **11/10/2024** to **11/10/2025**.

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC **eight weeks** prior to the expiration date of **11/10/2025** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to notify the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.
8. **All approved study documents should be stamped by the REC before commencement of data collection.**

The following is the list of all documents reviewed in this application by **Gulu University REC**:

No.	Document Title	Version	Date
1	Protocol-version 2		2022-07-29
2	Translated informed consent		2022-08-16
3	COVID-19 Risk Management plan		2022-07-29
4	Informed Consent-version 3		2022-08-16
5	Data Collection tool version 2		2022-07-29

Yours Sincerely

Julaina A. Obika (PhD)
For: Gulu University REC

Appendix 2. Ethical clearance letter by Uganda National Council for Science and Technology



Uganda National Council for Science and Technology (Established by Act of Parliament of the Republic of Uganda)

Our Ref: A226ES

6 September 2022

Docus Alowo
Gulu University
Gulu

Re: Research Approval: Prebiotic and probiotic capacity of ready-to-use composite foods developed from non-starch polysaccharides plant-based sources

I am pleased to inform you that on **06/09/2022**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **06/09/2022** to **06/09/2025**.

Your research registration number with the UNCST is **A226ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project. As the Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. Keeping all co-investigators informed of the status of the research.
2. Submitting all changes, amendments, and addenda to the research protocol or the consent form (where applicable) to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority and a notification to the UNCST.
4. Unanticipated problems involving risks to research participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST notification after review by the REC.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

Please note that this approval includes all study related tools submitted as part of the application as shown below:

No.	Document Title	Language	Version Number	Version Date
1	Informed Consent forms	English	3	16 August 2022
2	Data collection tools	English	2	29 July 2022
3	Translated informed consent form	Luo-Acholi	3	16 August 2022
4	COVID 19 RISK MANAGEMENT PLAN	English	2	29 July 2022
5	Project Proposal	English	2	
6	Approval Letter	English		
7	Administrative Clearance	English		

Yours sincerely,

Hellen Opolot
For: Executive Secretary

UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Appendix 3: Anti-plagiarism Certificate

GULU
P.O. Box 166
Gulu, Uganda



UNIVERSITY

Tel: +256-471-32924
Fax: +256 471343095
Emails: ul@gu.ac.ug
URL: www.gu.ac.ug

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PLAGIARISM CHECK

Declaration for Originality Form

This form must be completed and signed for all scholarly work produced.

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Personal File No.(staff) Reg. (student). REG. No. 20/U/3085/PHD/PS

Faculty/Institute: AGRICULTURE AND ENVIRONMENT, GULU UNIVERSITY

Department: FOOD SCIENCE AND POSTHARVEST TECHNOLOGY

Title and bibliographic description of the work

**IN VITRO EVALUATION OF THE BIOACTIVE POTENTIAL OF
PLANT-BASED OLIGOSACCHARIDES AND READY-TO-USE FERMENTED
COMPOSITE BEVERAGES**

Declaration.

1. I understand what plagiarism is and I am aware of the University policy on this regard,
2. I declare that this ... Dissertation ... (Thesis,, Proposal, essay, assignment, paper, report etc.) is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work or my work has been used, this has been properly acknowledged and referenced in accordance with Gulu University requirements,
3. I have not sought or used the services of any professional agency to produce this work,
4. I have not allowed, and shall not allow anyone to copy my work with the intention of passing it off as his/her own,
5. I understand that any false claim of this work shall result in disciplinary action in accordance with University Policy on Plagiarism.

Signature 

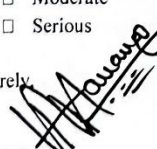
Date 27/8/2025

Originality Report

The article/thesis/document titled above have been subjected to plagiarism check and the level was found to be.....**18%** (percent) this is classified as:

- ☒ Minor
☐ Moderate
☐ Serious

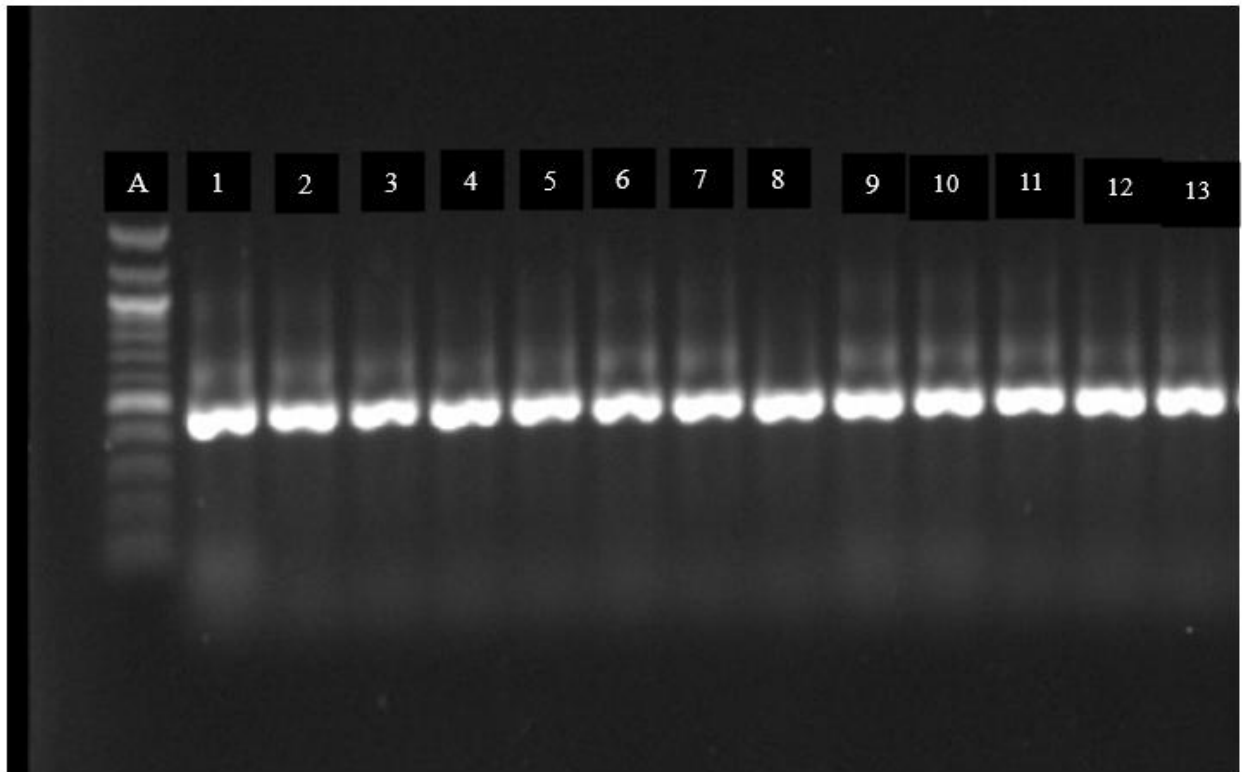
Yours sincerely,


Manana N Herbert
Ag. University Librarian

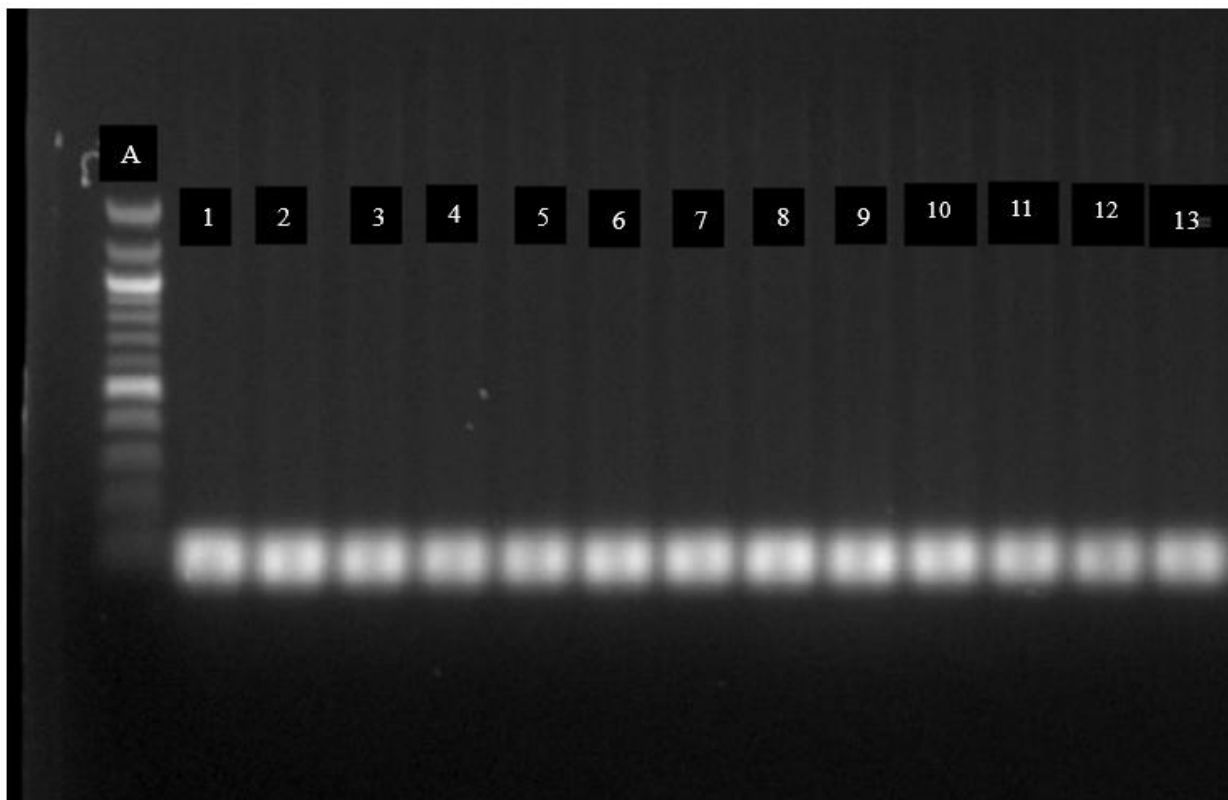


(This similarity test is exclusive of published articles by the student from the Dissertation)

Appendix 4: Agarose gel electrophoresis image of PCR amplicon



Agarose gel electrophoresis image of PCR amplicon length of 466 bp of the 16S rRNA gene from selected LAB isolates. Lane A: 100 bp DNA ladder; Lanes 1–13: LAB Isolates from fermented beverage samples



Agarose gel electrophoresis image of PCR amplicon length of 92 bp of the *Lactobacillus* rRNA gene from selected LAB isolates. Lane A: 100 bp DNA ladder; Lanes 1–13: LAB Isolates from fermented beverage samples

Appendix 5: Flow chart for production of spontaneous fermented beverage

