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PROBIOTIC LACTIC ACID BACTERIA ASSOCIATED WITH FERMENTED MILLET-BASED MILK BEVERAGE '*BRUKINA*' AND EFFECTS ON THE GUT MICROBIOME

BY

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THIS THESIS IS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE AWARD OF DEGREE OF MASTER OF PHILOSOPHY IN MOLECULAR, CELL BIOLOGY OF INFECTIOUS DISEASES

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DECLARATION

I, Bless Hodasi, affirm that this thesis is the result of my independent research conducted at the Department of Biochemistry, Cell, and Molecular Biology, University of Ghana, Legon-Accra. I was supervised by Dr. Elmer Ametefe and Prof. Winfred Seth K. Gbewonyo. I confirm that this thesis does not contain any previously approved or published material from any other source. All references cited in the text have been appropriately acknowledged.

…… **13/12/2023** ……

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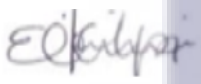

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ABSTRACT

Our diet significantly influences our gut microbiome. Fermented foods offer various nutritional advantages and may contain beneficial microorganisms known as probiotics. *Brukina*, a fermented beverage originating from Burkina Faso, but widely consumed in Ghana is produced from cooked millet and fermented cow milk. For probiotics to be effective, they must survive the acidity levels and bile salt concentration in the gastrointestinal tract. Their adhesion to gastrointestinal epithelial cells is also crucial for colonization and persistence in the gut. Probiotic strains should have limited resistance to antibiotics. This study sought to determine the lactic acid bacteria (LAB) present in *Brukina* and their impact on the gut microbiome of consumers.

FDA approved *Brukina* sample was obtained from supermarkets in Accra, Ghana. LAB load was determined. 16S rRNA amplicons from cultured LAB strains and DNA extracts from *Brukina* was sequenced. Acid and bile tolerance, adhesion capacity, antimicrobial activity and antibiotic susceptibility profile of the isolated lactic acid bacteria were determined. Human participants and animal models were given *Brukina* samples for two weeks. Fecal samples were collected at different time points, DNA was extracted and subjected to 16S PCR amplification. Next generation sequencing was carried out and the sequence data was analyzed using Shannon indices and metagenomics parameters.

LAB load ranged from 10^4 CFU/ml to 10^6 CFU/ml. 16S rRNA sequencing of genomic DNA identified the cultured LAB strains as *Limosilactobacillus fermentum*, *Enterobacter hormaechei*, *Alishewanella agri, Neobacillus fumarioli, Bacillus safensis* and *Faecalibaculum rodentia*. The direct extraction from *Brukina* showed the presence of *Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus johnsonii, Lactobacillus prophage,* and *Lactobacillus taiwanensis.* The strains exhibited antimicrobial activity against four pathogens (*Escherichia coli NCTC 11954 TEM*

1, K. pneumoniae NTC 13368, Salmonella typhi ACT 1, and Staphylococcus aureus). There was a notable increase in the abundance of LAB particularly, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* in the gastrointestinal tracts of human participants and rat models after two weeks of consistent consumption of *Brukina*. Most strains that adhered very well to Intestinal Epithelial Cells (IECs) also exhibited a higher tolerance to acid and bile coupled with low resistance to orally administered antibiotics.

The abundance of lactic acid bacteria in the GIT after regular consumption of *Brukina*, helps in the competitive elimination of enteric pathogens. The presence of a diverse range of microbes in the sample underscores the complexity of the microbial community in fermented millet-based milk beverage (*Brukina). Brukina* is therefore, a potential functional food with a significant LAB load. It also impacts the gut microbiome in a matter of three days of consistent consumption. The LAB strains have good probiotic potential and can be used to formulate a cocktail of starter cultures that would be used in the preparation of yoghurts, fermented foods and other probiotic products.

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DEDICATION

This dissertation is dedicated to my family particularly my mother, Emilia Fasemkye.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
LAB	Lactic acid bacteria
CKD	Chronic Kidney Disease
SCFAs	Short Chain Fatty acids
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
ATP	Adenosine triphosphate
EPS	Exopolysaccharide
GABA	Gamma-aminobutyric acid
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
FDA	Food and Drug Administration
CREs	Carbapenem-Resistant Enterobacteriaceae
DMEM	Dulbecco's Modified Eagle Medium
MRS	De Man Rogosa Sharpe agar
CFS	Cell-free supernatant
TLR	Toll-Like Receptors
NCTC	National Collection of Type Cultures
ATCC	The American Type Culture Collection
PyrE	Orotate phosphoribosyl transferase
IEC	Intestinal Epithelial Cell

PBS	Phosphate buffered saline
TAE	Tris Acetate EDTA
MHA	Muller Hinton Agar

CHAPTER ONE

1.0 Introduction

1.1 Background

Brukina is a traditional fermented millet-based milk beverage consumed in Ghana, made by combining milled and steam-cooked millet with fermented cow milk and sugar (Kortei et al., 2022). The milk used for the production is normally allowed to undergo fermentation spontaneously or through back slopping with leftover fermented milk (Nduko et al., 2016). The name "*Brukina*" comes from Burkina Faso, its country of origin; however it is called "dèguè" in the Northern part of Ghana (Kortei et al., 2022). Researchers have highlighted the nutritional, medicinal, economic, and social benefits of *Brukina*, emphasizing its role as a cost-effective and nutrient-rich source, especially in African regions facing malnutrition and hunger (Falade et al., 2022). *Brukina* provides essential nutrients and beneficial microorganisms, supporting growth and development in areas where these necessities are crucial (Franz et al., 2014). It contributes significantly to hunger alleviation and offers numerous health benefits through its rich nutritional profile and fermentation-enhanced bioavailability. Made from millet and milk, it is dense in complex carbohydrates, protein, and dietary fiber, promoting satiety by slowing digestion and maintaining stable blood glucose levels (Maurya et al., 2023). Fermentation improves the absorption of essential micronutrients such as iron, zinc, and calcium by breaking down antinutrients, while also increasing the levels of B-vitamins critical for energy metabolism and cognitive function (Emmanuel et al., 2024). The presence of probiotics from the fermentation process supports gut health, strengthens the immune system, and improves digestion (Dahiya & Nigam, 2022b). As a nutrient-dense, affordable, and shelf-stable beverage, *Brukina* serves as a sustainable food source in low-resource settings, especially beneficial for children's growth,

hydration, and development (Bazzano et al., 2017). Beyond nutrition, its local production empowers women and supports food security by utilizing readily available agricultural resources, making *Brukina* a functional food with broad socio-economic and health impacts (MONDO, 2024).

The human microbiome encompasses a variety of microorganisms, including bacteria, viruses archaea, and eukaryotic microorganisms, residing both inside and outside the body (Ogunrinola et al., 2020). These microorganisms have significant capacity to influence the overall well-being, impacting our physiology in diseased and healthy conditions (Makki et al., 2018). The intestinal tract of mammals houses a lot of unique microbes (Savage, 1977). These microorganisms are referred to as intestinal microbiota (Nishida et al., 2018). Microbiome can be used synonymously with microbiota (Serban, 2014). The genes or combined genetic compositions of microorganisms present in microbial communities is termed microbiome (Singh et al., 2017). One crucial counterpart that is considered to have evolved with the human genome is the human microbiome (Grice & Segre, 2012). This concept accentuates the diversity and dynamic interactions of facultative symbionts within a specified host (Guégan et al., 2018). Probiotics are live microorganisms that when consumed in sufficient amounts confer a health benefit on the host (Sanders et al., 2007). These group of microorganisms positively impact the host by deploying diverse mechanisms (Sánchez et al., 2017). These include, intrusion with potential harmful microbes, amelioration of barrier function, immune system modulation and synthesis of neurotransmitters (Ghosh et al., 2021).

Probiotics have gained significant attention for promoting intestinal health in humans (Angelin & Kavitha, 2020). As a result, the general probiotics market has experienced rapid growth, with probiotics transitioning from being just supplements to a part of our food (Klein et al., 2010). This

unique group of microorganisms has been linked to improvements in health and immune function (Pickard et al., 2017). Furthermore, while understanding the genomic and microbiological properties of each probiotic strain is crucial, it is equally important to explore their impact on the human gut microbiome when consumed (Sánchez et al., 2017).

Gut dysbiosis can lead to an increase in steatosis through metabolic alterations (Kim et al., 2018). When indigestible carbohydrates and proteins are fermented by bacteria that are situated in the intestinal tract, they synthesize fatty acids containing a small number of carbon atoms which are very relevant to energy metabolism and serve as both energy sources and signaling molecules in mammalian host (Sánchez-Tapia et al., 2019). These short chain fatty acids (SCFAs) specifically butyric acid, propionic acid, and acetic acid, contribute to around one third of the energy derived from the foods consumed (Ziętek et al., 2021). Among these, acetic acid, predominantly synthesized by Bacteroidetes, promotes obesity by stimulating lipid and cholesterol biosynthesis in adipose tissue as well as the liver (Tang et al., 2021). In contrast, butyric acid is believed to have anti-obesity effects, as it enhances the sensitivity to insulin, improves gut absorptivity, and increases the levels of leptin(Tan et al., 2018). During gut dysbiosis, the alteration in the Bacteroidetes to Firmicutes increases resulting in the overall increase in acetic acid production (Magne et al., 2020).

Probiotic bacteria, often introduced through food, face a tough journey from the mouth to the lower intestines (Castro-López et al., 2022). They must withstand challenges such as oral enzymes like lysozyme, survive stomach and intestinal digestion, and cope with the presence of bile (Kailasapathy & Chin, 2000). These bacteria spend approximately 90 minutes in the stomach before progressing further (Kailasapathy & Chin, 2000). However, the digestive processes in the intestines are more time-consuming (Wojtunik-Kulesza et al., 2020). Thus, bacteria must endure

the harsh conditions of the stomach and upper intestines, which includes exposure to bile, initiating cellular stress (Mills et al., 2011). The stomach has an extremely low pH of about 1.5, while the upper intestine secretes bile, with varying concentrations that are difficult to predict (Chou & Weimer, 1999). Once they navigate through this demanding environment, the bacteria settle in the lower intestine's epithelium (Alesa et al., 2019). To be effective as probiotics, specific strains must be able to endure 90 minutes of exposure to acidic conditions, tolerate bile acids, adhere to the epithelium, and thrive in the lower intestines to provide health benefits (Ayyash et al., 2021).

The application of probiotics in clinical practice is associated with a vast range of merits (McFarland, 2009). Notable among them are improvement of antibiotics and enteropathogens-related diarrhea and respiratory diseases (Omar, 2015). Probiotics used in cancer patients restores the functionality and populations of commensal bacteria, that decrease significantly after treatment regimens (Reid et al., 2011). Besides, the administration of probiotics in numerous clinical trials has been shown to restore healthy gastrointestinal microbes and to diminish diarrhea and mucositis (Miknevicius et al., 2021). Consistently, *Lactobacillus* containing probiotic supplements and other products protect individuals from the development of diarrhea and mucositis especially those who have undergone chemotherapy/radiotherapy for pelvic malignancy (Alam et al., 2022).

Lactic acid bacteria (LAB) have been an area of extensive research for quite a long time (Adams, 1999). They demonstrate very unique roles in diverse biological processes and ecosystems, mainly in the context of foods that are capable of undergoing fermentation (Korcz et al., 2018). Fermentation has been a focus of study for a long period of time, and the practice of transforming raw foods into edible products (Motarjemi, 2002).

In industrial fermentation processes, carefully selected cultures of LAB are used as starters or adjuncts (Leroy & De Vuyst, 2004). However, artisanal or traditional fermentation procedures

typically do not involve the utilization of predefined starter cultures (Russo et al., 2017). Instead, *Lactobacillus* originally present in probiotic materials or obtained from previous batches guide the fermentation process (Wu et al., 2012). They find themselves in competition with a multitude of other microbial species in the gut before they can confer their beneficial properties (Plaza-Diaz et al., 2019). LAB are considered a part of temporal gut environment, originating from natural community, with diet as a fundamental residence (Walter, 2008). This community mingles each day with the more established inhabitants of the gut microbiome (Dethlefsen & Relman, 2011). However, despite this general understanding, it remains unclear how components of the food microbiome transit into becoming part of the gut microbiome and the specific roles played in this intricate community (Milani et al., 2017).

In recent times, there has been growing speculation among researchers regarding the possible role of harmless microbes as pools of antibiotic resistance genes, which is analogous to that seen in pathogenic microbes (Papadimitriou et al., 2015). This concept is of significant importance in the comprehension of the mechanism of persistence and dissemination of these antibiotic resistance genes within bacterial environments (Barbosa & Levy, 2000). The primary concern lies in the ability of these bacteria to transfer resistance genes to harmful bacteria (Sharma et al., 2014). It is possible that such intricate organisms may be present in different foods and products that have vast concentrations of probiotic bacteria due to their natural production processes (LeBlanc et al., 2013). In this context, the food chain emerges as the primary means of transmitting antibiotic-resistant bacteria between animals and humans, with a particular emphasis on fermented dairy and meat products that are not subjected to heat treatment before consumption (Ruiz & Alvarez-Ordóñez, 2017). This creates a straightforward relationship between the resident microflora of animals and microbes in the gut (Canny & McCormick, 2008). Bacteria, known for their

adaptability, have responded to the significant change in their environment by developing various well-established mechanisms of antibiotic resistance (Capita & Alonso-Calleja, 2013). Both clinical and non-clinical bacterial populations are becoming increasingly resistant to conventional antibiotics, and this trend is seen the most in Gram-negative bacteria compared to Gram-positive microorganisms (Brunel & Guery, 2017). Moreover, the horizontal transfer and promiscuous exchange of antibiotic resistance genes have facilitated the widespread dissemination of resistant strains (Crits-Christoph et al., 2022). Consequently, the emergence of antibiotic-resistant strains is frequently observed shortly after the introduction of new antibiotics (Capita & Alonso-Calleja, 2013). Conversely, discontinuing certain antibiotics has contributed to a notable reduction in relevant resistance to antibiotics (Davies, 2007).

Utilizing lactic acid bacteria (LAB) that is well vested with antimicrobial property in the production of probiotic products offers an additional processing advantage (Oluwajoba et al., 2013). These additives can enhance the safety of probiotic products, serving as extra safeguard against the risk of foodborne illnesses (Mostafidi et al., 2020). Bacteriocins are naturally occurring bioactive peptides synthesized by bacteria, known for their antimicrobial properties against either closely related or unrelated bacteria (Heilbronner et al., 2021). They have gained significant attention for potential use in the food industry. Bacteriocins are categorized into two major classes based on modifications to their precursor peptides (Zacharof & Lovitt, 2012). Class I bacteriocins undergo modifications after translation and this introduce ether amino acids like methyllanthionine (Sahl et al., 1995). Recently, new bacteriocins having non-standard modifications have been discovered (Kumariya et al., 2019). Class II bacteriocins consist of peptides that are not modified and are further subdivided into four groups: IIa (one-peptide pediocin-like bacteriocins), IIb (two-peptide bacteriocins), IIc (cyclic bacteriocins), and IId (linear non-pediocin-like one-peptide

bacteriocins) (Rea et al., 2011). Bacteriocins are effective against Gram-positive harmful bacteria like *Listeria monocytogenes* and *Staphylococcus aureus* (Etayash et al., 2014). It can also be useful against Gram-negative bacteria if the outer covering is not stabilized (Helander et al., 1997). Incorporating strains that produce antimicrobial peptides as starter cultures in the production of fermented probiotic products can enhance product safety and the organoleptic properties of the product (Behera et al., 2018). For example, the first antibacterial peptide that was discovered in *lactobacillus* is nisin and this is synthesized by a unique strain known as *Lactococcus lactis* (Perin & Nero, 2014). Nisin is employed in biological preservation, and commercial formulations of these antimicrobial compounds are used (Field et al., 2015). The utilization of starter cultures that synthesize bacteriocins typically doesn't need approval from a regulatory body and is often seen as an attractive strategy for incorporating bacteriocins into foods (Yi et al., 2022). Additionally, combining bacteriocins with other therapies can increase their effectiveness, especially in targeting Gram-negative pathogens, as demonstrated by synergistic antimicrobial effects (Prudêncio et al., 2015).

Gastric cancers are induced by certain factors such as *H. pylori* mediated inflammation (Lamb & Chen, 2013). Some probiotics have a unique potential of controlling some of these carcinogenic or pathogenic microorganisms (Saber et al., 2017). *Lactobacillus plantarum* is one of the microbes that aids in triggering a robust hindering effect on the growth rate of *Helicobacter pylori* (Chua et al., 2017).

Advancements in technology have significantly advanced the study of the intestinal microbiome (Arnold et al., 2016). Many studies now employ culture-independent techniques for analysis (Fouhy et al., 2012). Typically, the bacterial components within a community of diverse microorganisms are picked out through 16S rRNA gene sequencing and comparing the results to

established reference sequences in the databases (Langille et al., 2013). Metagenomic analysis, involving the sequencing of a collection of DNA within a complicated environment, offers an added pros of assessing the genetic capabilities of the group of microbes (Creer et al., 2016). Additionally, other methodologies, like analyzing the microbial proteome, transcriptome and metabolome, provide further insights into various aspects of microbial physiology (de Graaf & Venema, 2007). Culture-independent techniques, such as metagenomics and high-throughput sequencing, offer significant advantages over traditional culture-based methods (Ercolini, 2013). They allow for the comprehensive analysis of microbial communities, including the detection of non-culturable, rare, or slow-growing organisms that may be missed using conventional methods. These approaches provide deeper insights into microbial diversity, functional potential, and community dynamics without the bias introduced by selective culturing conditions (Widder et al., 2016).

However, there are also challenges associated with culture-independent techniques. These include high costs, the need for specialized equipment and expertise, potential biases in DNA extraction and amplification, and difficulties in distinguishing between live and dead cells (Fittipaldi et al., 2012). Additionally, the vast amount of data generated requires robust bioinformatics tools and databases, which may not always be complete or fully accurate. Despite these limitations, culture-independent methods remain powerful tools for advancing microbial ecology and diagnostics when appropriately applied (Su et al., 2012).

1.2 Problem Statement

Despite the widespread consumption and cultural significance of *Brukina*, a traditional fermented millet-based milk beverage, there is limited scientific evidence characterizing its nutritional completeness, functional food properties, and antimicrobial potential. While anecdotal and preliminary reports suggest that *Brukina* offers notable nutritional, medicinal, and socio-economic benefits, including bacteriostatic and bactericidal activity against enteric pathogens, systematic studies validating these claims are lacking. Furthermore, its potential role in hunger alleviation, particularly in nutritionally vulnerable regions of West Africa, remains underexplored. This gap in scientific understanding hinders the optimization, standardization, and policy integration of *Brukina* as a sustainable, health-promoting dietary intervention.

1.3 Rationale for the study

Brukina, a widely consumed indigenous fermented beverage in Ghana and other parts of West Africa, stands out not only for its cultural significance but also for its remarkable nutritional, medicinal, economic, and social value. As a fermented cereal-dairy product, Brukina possesses characteristics consistent with functional foods, including documented bacteriostatic and bactericidal effects against enteric pathogens. Its rich nutrient profile, coupled with its low cost and widespread availability, positions it as a promising dietary tool in addressing malnutrition and food insecurity across resource-limited settings. Given its potential to improve both gastrointestinal health and nutritional outcomes, *Brukina* merits rigorous scientific exploration to validate and potentially harness its probiotic properties for broader public health impact.

1.4 Significance of the Study

This study will provide scientific evidence on the nutritional value and functional properties of *Brukina*, highlighting its potential as a health-promoting, affordable food. By exploring its

antimicrobial activity and role in hunger alleviation, the research aims to support the use of traditional fermented foods like *Brukina* in combating malnutrition and informing public health and nutrition policies in resource-limited settings.

1.5 Aim

To evaluate the effects of probiotic lactic acid bacteria (LAB) isolated from the indigenous fermented beverage *Brukina* on the gut microbiome.

1.6 Specific Objectives

1. To screen fermented millet-based milk beverage '*Brukina'* for probiotic strains of lactic acid bacteria.
2. To assess the antimicrobial activity of the probiotic strains against enteric pathogens.
3. To determine the percentage difference in specific gut microbial populations resulting from regular *Brukina* consumption.

CHAPTER TWO

2.0 Literature Review

2.1 Functional foods, Fermented foods and Probiotics products

Functional foods particularly organic items and products with reduced fat, salt, and sugar content face competition in the market (Villaño et al., 2022). The growing concern over the food supply chain and various issues related to food has elevated the demand for "pure" organic food substances, possibly posing a challenge to functional foods purported to be "adulterated" (Sanders & Huis in't Veld, 1999). To succeed, functional foods need to meet specific criteria: they must offer health benefits appealing to a broad audience, clearly communicate these benefits through comprehensive health claims or well-known active ingredients, compete effectively on various aspects including taste, convenience, and pricing, and not solely rely on their health benefits. While functional foods can offer higher profit margins, success isn't guaranteed (Wansink, 2005). Factors like brand loyalty, advertising, promotion, quality control, competition, and economic conditions also play significant roles (Granato et al., 2010).

Functional foods contain live microorganisms (probiotics) which serves as a source of added benefit (Peng et al., 2020). For a probiotic bacterium to demonstrate its positive effects, certain properties are anticipated (Gotcheva et al., 2002). These comprise acid and bile resistance, vital for oral intake, attachment to mucosal and epithelial surfaces to facilitate effective immune regulation, exclusion of pathogens through competition, prevention of pathogen adhesion and establishment, and antimicrobial action against detrimental bacteria (Servin, 2004). Additionally, bile salt hydrolase activity holds significance in this context (Yao et al., 2018). However, the significance of these parameters is debated due to *in vivo* and *in vitro* disparities and scarcity of standardized measures. Determining a strain's properties should involve specific studies based on

the target population and intended physiological function (Cencic & Chingwaru, 2010). In terms of the ultimate probiotic product, probiotic dose has to be determined depending on effective levels in studies involving humans, and the colony forming units of the product is a crucial criterion (Kechagia et al., 2013). Fermented foods are gaining recognition for their numerous health advantages, categorizing them as 'functional foods' that offer extra health benefits beyond basic nutrition (Roberfroid, 2002).

Fermentation, the ancient preservation method for fruits and vegetables, is widely used due to the spoilage susceptibility of untreated produce (Juodeikiene et al., 2012). Fermented items like olives, sauerkraut, kimchi, and pickled cucumbers are essential in global nutrition (Steinkraus, 1997). The process, primarily lactic acid fermentation, occurs naturally with lactic acid bacteria (LAB), being the dominant microorganisms involved (Ashaolu & Reale, 2020). LAB fermentation converts carbohydrates into carbon dioxide, alcohol, and organic acids, inhibiting harmful microorganisms (Bangar et al., 2022). Fermented fruits and vegetables containing probiotics derived from LAB can prevent diseases like cirrhosis and diarrhea (Swain et al., 2014). Additionally, the antioxidants in these foods combat harmful free radicals, contributing to the prevention of degenerative diseases (Pham-Huy et al., 2008).

The positive impacts of fermentation are supported by various hypotheses, some of which are highlighted in Figure 1. One explanation suggests that fermentation alters the chemical composition, enhancing the food's activity and availability (Leonard et al., 2021). Research indicates that fermentation enriches bioactive peptides and produces phytochemicals, enhancing neuroprotective effects (Kim et al., 2016). Furthermore, modified components through fermentation can enhance bioavailability, facilitating the absorption and utilization of nutrients in the digestive system (Schneeman, 2002).

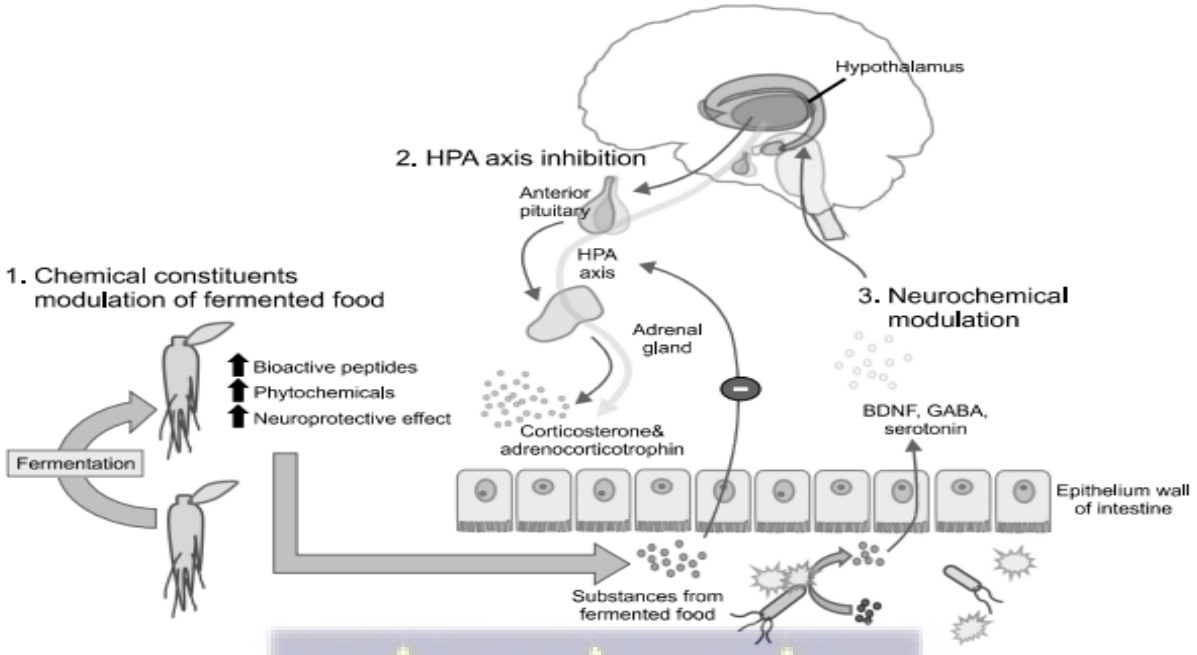


Figure 1.0: Potential ways fermented foods enhance brain and cognitive function.

The figure above highlights three hypotheses: modulation of chemical constituents, inhibition of the hypothalamic-pituitary-adrenal (HPA) axis, and modulation of neurochemicals. These mechanisms impact brain health by altering specific chemicals and pathways, such as brain-derived neurotrophic factor (BDNF) and gamma-aminobutyric acid (GABA) (Sun-Waterhouse et al., 2014).

2.1.1 Probiotics and Prebiotics

Intestinal microbiota is involved in nutritional, immunologic, and physiological functions. They therefore play an essential role in the maintenance of host health (Hooper & Gordon, 2001). Certain chronic debilitating diseases such as obesity, inflammatory bowel disease, cancer, and autism can be caused by dysbiosis of the intestinal microbiota (Zhang et al., 2015). Several strains of *Saccharomyces cerevisiae, Aspergillus niger*, and *Bacillus* have been investigated. However,

Lactobacillus and *Bifidobacterium* are the most widely investigated ones. Probiotics have been used as natural therapeutic agents with health benefits (Lee et al., 2019).

The adherence of beneficial microorganisms is largely related to stability, such as survival of strains that have been exposed to the GI tract, autoaggregation, and hydrophobicity (Ambalam et al., 2016). The commercial *Bacillus* probiotics such as *B. cereus, B. clausii*, and *B. pumilus* that could persist in mouse GI tract for up to 16 days were revealed through fecal analysis (Duc le et al., 2004). Probiotics contain antimicrobial substances, including bacteriocin, short chain fatty acids, and organic acids, and they modulate GI disorders by antimicrobial and antiadhesion effect against pathogenic strains (Vitetta et al., 2014).

Some unique properties or characteristics of certain strains of probiotics include, maintaining gut epithelium barrier function, competing with pathogenic bacteria for nutrients, regulating host immune response, improvement of host metabolism, mitigation of uremic intoxication, reduction of pro-inflammatory markers, and delay in the progression of renal dysfunction (Koppe et al., 2015).

Living microorganisms that, when ingested in adequate amounts, can ameliorate the intestinal microbiota profile by multiplying beneficial bacteria are termed Probiotics (Zheng et al., 2021). Prebiotics on the other hand are known as nonliving indigestible fibers that may enhance the growth of beneficial microorganisms in the gut (Gibson et al., 2017). Probiotics play a major role in the management of chronic kidney diseases (CKD). They play a vibrant role in the modulation of relevant processes in CKD by mitigating the production of colon-derived uremic solutes (Felizardo et al., 2019). Also, they are largely implicated in the increment of production of short-chain fatty acids (SCFAs). This unique biomolecule ameliorates the regulation of the incretin axis thereby contributing to the reduction of inflammation (Huang et al., 2022).

Products that contain probiotics are available for aquaculture, human nutrition and for animal feed supplements (Pineda-Quiroga et al., 2019). Probiotics are administered as prophylactic agents in certain countries to prevent childhood diarrhea for instance. However, in southeast Asia they are also largely used as therapeutic agents (Preidis & Versalovic, 2009).

Products containing endospores of members of the genus *Bacillus* (in single doses of up to 10^9 spores/g or 10^9 spores/ml) are used commercially as probiotics. The unique advantage offered by *Bacillus* products over the more common *Lactobacillus* products is the fact that, they can be stored indefinitely in a desiccated form (Kim et al., 2022). Initially, numerous commercial products were sold as products that carry *Bacillus subtilis* spores, but recent studies have shown that most products are mislabeled and carry other *Bacillus* species, including *Bacillus clausii*, *Bacillus pumilus*, and a variety of *Bacillus cereus* strains (Sorokulova et al., 1997).

2.2 Probiotics and Gut Health

Probiotics can be found in various products, such as fermented foods, dietary supplements, and cosmetics (Jiang et al., 2021). They can affect the make-up and role of the gastrointestinal microbiome, which refers to collection of predominantly bacteria and fungi that live in the digestive tract (Penders et al., 2007). The gut microbiome influences many aspects of human health, such as digestion, metabolism, immunity, and mood (Menni et al., 2017)

Some common gastrointestinal disorders are associated with an imbalance or dysbiosis of the gut microbiome (Passos & Moraes-Filho, 2017). Examples of these disorders are inflammatory bowel disease (IBD), diarrhea, constipation and irritable bowel syndrome (IBS) (Lee et al., 2017). Probiotics may help restore or maintain a healthy balance of the gut microbiome and alleviate some of the symptoms of these disorders (Dahiya & Nigam, 2022a). For example, probiotics may improve stool frequency and consistency in constipated patients, reduce the duration and severity

of diarrhea, modulate the immune system and inflammation in IBS and IBD patients, and enhance the intestinal barrier function (Currò et al., 2017). Probiotic foods provide other nutrients and health benefits, such as vitamins, minerals, antioxidants, and enzymes (Arias et al., 2022). Probiotics can also be taken as food supplements that take the form of tablets, capsules, powders, and liquids (Varzakas et al., 2018). Supplements may offer higher doses and more specific strains of probiotics than foods (Fenster et al., 2019). However, they may also have lower quality and stability than foods, and their safety and efficacy may not be well-regulated or tested (Socas-Rodríguez et al., 2021).

Lactobacillus and *Bifidobacterium* are two probiotic strains that have been extensively researched and widely acknowledged for their gut health benefits (Picard et al., 2005). These beneficial microbes affect the gut in various ways (Hirt, 2020). For instance, they can create a lower pH level in the colon, which favors themselves and other helpful gut residents (Wexler & Goodman, 2017). Moreover, they can enhance the absorption of essential proteins and nutrients, which helps the body to optimize its nutritional intake (Jäger et al., 2019). Additionally, these probiotic agents have shown their ability to reduce the occurrence of antibiotic-related diarrhea, a frequent adverse effect of antibiotic treatment (Nista et al., 2004).

Probiotics generally have a good safety profile, but they may also cause some side effects or interact with certain medications (Sanders et al., 2019). Therefore, it is wise to consult a healthcare professional before adding probiotics to your daily routine (Morrison et al., 2010) . This step can help to identify and address any possible interactions or contraindications promptly (Claes et al., 2011). Furthermore, following the recommended dosage and storage instructions is crucial to ensure the effectiveness of probiotics and protect their viability (Tripathi & Giri, 2014).

2.2.1 Probiotics as starter cultures

Fermented foods have been part of human diets for centuries, offering unique flavors, textures, and extended shelf life (Ray & Joshi, 2014). Probiotics, predominantly lactic acid bacteria (LAB) and *Bifidobacteria,* are natural components of fermented foods and are recognized for their potential health benefits (Mokoena et al., 2016). Incorporating probiotics as starter cultures in food fermentation not only improves the organoleptic properties of the products but also adds functional attributes (Mohammadi et al., 2012). Probiotic microorganisms can be added to fermented milk in two ways: either as non-fermenting microbes aimed at gastrointestinal benefits (non-starter probiotics) or as part of starter cultures used in milk fermentation (starter probiotics) (Schutte, 2013). Commonly, probiotics are combined with traditional yogurt bacteria like *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, as probiotics alone struggle to ferment milk adequately (Gao et al., 2021). When used as a starter culture, probiotics' safety, milk acidification ability, fermentation time, viability in the product, flavor/texture production, and cost-effectiveness are crucial factors (Mani-López et al., 2014).

2.2.2 Multiplicity versus prevalence of probiotics in dairy products

Several different kinds of probiotic bacteria are found in dairy products (Vijaya Kumar et al., 2015). Several studies have established the presence of different species of lactic acid bacteria in dairy products (Albesharat et al., 2011). They are responsible for the fermentation of carbohydrates in milk, which leads to the production of lactic acid (Harper et al., 2022). The diversity of LAB in dairy products varies based on the type of milk and the fermentation procedure employed (Macori & Cotter, 2018). Some of the common genera of LAB found in fermented dairy products include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Bacillus*, *Weissella*, and *Enterococcus* (Macori & Cotter, 2018). LAB have a significant impact on the flavor and texture of dairy products

due to their fermentation process (Hati et al., 2013). The use of LAB in dairy starter cultures is important for the production of high-quality dairy products (Høier et al., 2010).

2.3 Types and Classification of Lactic Acid Bacteria (LAB)

According to carbohydrate fermentation, lactic acid bacteria (LAB) refer to a class of Gram-positive bacteria that synthesize lactic acid as the major product (Axelsson, 2004). LAB have various applications in food and feed fermentation, probiotics, and health (Giraffa et al., 2010). Different genera of LAB can be identified based on their morphology, metabolism, physiology, and phylogeny (Axelsson, 2004). The most common genera are *Lactobacillus, Lactococcus, Pediococcus, Streptococcus, Enterococcus, Weissella*, and others (Muñoz-Atienza et al., 2013). Each genus contains many species and strains with different characteristics and applications.

LAB can be placed into two categories particularly based on their mode of hexose fermentation: homofermentative and heterofermentative (Buron-Moles et al., 2019). Homofermentative LAB convert glucose into two molecules of lactic acid, while heterofermentative LAB produces lactic acid, carbon dioxide, and ethanol or acetic acid (Florou-Paneri et al., 2013). For instance, *Lactobacillus casei* and *Lactococcus lactis* are homofermentative, while *Leuconostoc mesenteroides* and *Lactobacillus brevis* are heterofermentative (Xiong et al., 2012). Another way to classify LAB is by their optimal growth temperature: thermophilic, mesophilic, and psychrotrophic (Mun et al., 2021). Thermophilic LAB grow best at high temperatures (above 45°C), mesophilic LAB grow very well at average temperatures (15-45°C), and psychrotrophic LAB grow very well at reduced temperatures (below 15°C) (Carminati et al., 2014). For example, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* are thermophilic, *Lactococcus lactis* and *Enterococcus faecalis* are mesophilic, and *Leuconostoc lactis* and *Carnobacterium piscicola* are psychrotrophic (Mater et al., 2005).

Other criteria for classifying LAB include the configuration of lactic acid (D- or L-) they produce, their ability to grow in high salt concentrations (halotolerant or halophilic), their tolerance to acid or alkaline conditions (acidophilic or alkaliphilic), their fatty acid composition, their cell wall constituents, and their phylogenetic relationships based on molecular methods (Matti et al., 2019). These criteria help to distinguish between different species and strains of LAB that have different properties and functions (Schaer-Zammaretti & Ubbink, 2003).

2.3.1 Metabolic capacity of Lactobacilli

Lactic acid bacteria (LAB) have various essential metabolic potentials that make them useful in various applications (LeBlanc et al., 2011). Additionally, some LAB can use mixed or alternative bioenergetic strategies, such as extracellular electron transfer, for energy conservation (Yao et al., 2022) . Lactic acid bacteria (LAB) produce ATP through carbohydrate fermentation linked to substrate-level phosphorylation (Von Wright & Axelsson, 2019). The metabolic profiles of LAB are species- and strain-dependent (Harlé et al., 2020). Recent studies have explored the genome-associated metabolic landscapes of LAB, revealing strain-specific probiotic idiosyncrasies (Karlsen et al., 2023). These unique metabolic strategies may have interesting biotechnological applications. Overall, LAB have a diverse metabolic capacity that makes them useful in various fields, including food science, biotechnology, and medicine (Pedersen et al., 2012) .

In the fermentation industry, some lactic acid bacteria are able so convert certain non-digestible carbohydrates or sugars known as prebiotics and other detrimental inhabitants in food (Hugenholtz & Smid, 2002). Transformation of undesirable compounds in the context of the food and fermentation production ventures, lactic acid bacteria possess the ability not only to break down major nutritional macromolecules like polysaccharides, peptides and proteins but also to degrade various substances (Balla et al., 2021). Notably, these bacteria can serve as a means to prevent the

accumulation of mycotoxins in stored cereal products (Magan & Aldred, 2007). For instance, studies have shown that, the introduction of *Lentilactobacillus kefiri* FR7 into artificially contaminated almonds has demonstrated a significant reduction in the buildup of aflatoxins (Bangar et al., 2021). *Lactobacilli* acts to breakdown dangerous byproducts which will arise during heterofermentative and homofermentative processes (Dastmalchi et al., 2016). Extensive research has demonstrated the ability of *lactobacilli* to make riboflavin, cobalamin, folic acid, and pyridoxal (Gu & Li, 2016). Vitamins produced during lactic acid bacteria fermentation in the industry can be utilized for nutritional enrichment in foods (LeBlanc et al., 2011). This enrichment widens the scope of using lactobacillus to craft foods that undergo fermentation with elevated vitamin content tailored for specific populations or dietary needs (Zhu et al., 2020).

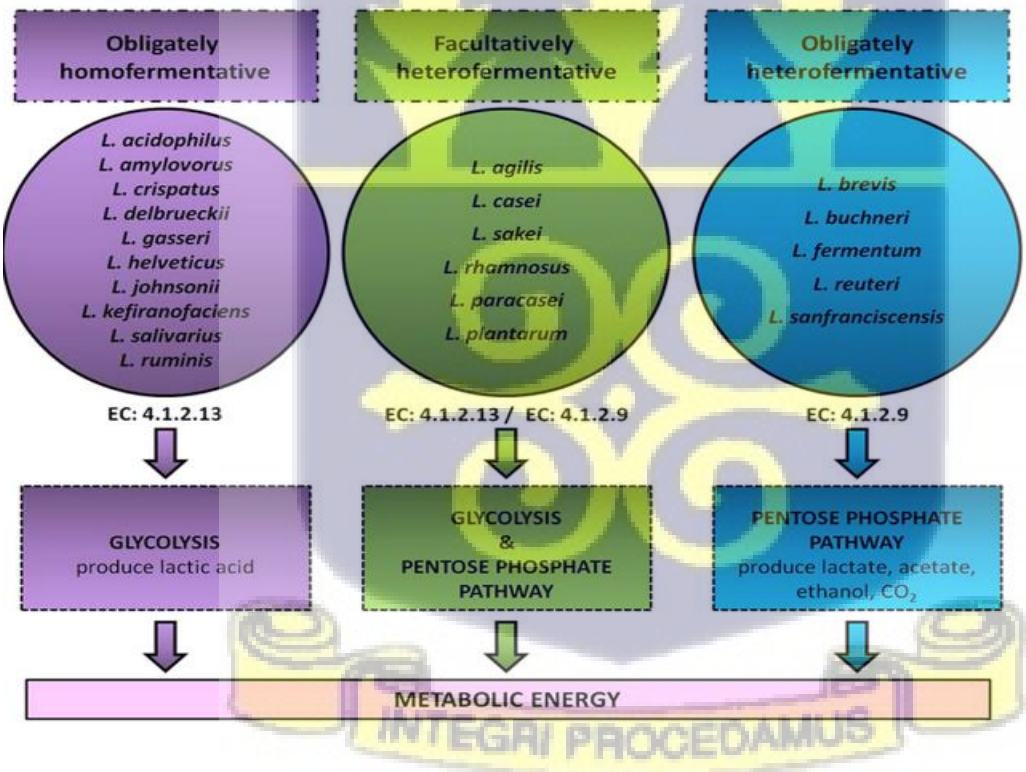


Figure 2.0: Metabolic classification of *Lactobacilli (Pot & Tsakalidou, 2009)*

Lactobacilli are versatile bacteria that can operate with or without oxygen, possessing numerous enzymes related to carbohydrate metabolism. They are categorized based on two metabolic pathways for glucose and xylose assimilation. *Lactobacillus* species in the obligate heterofermentative group produce lactate, acetate, ethanol, and CO_2 by fermenting hexoses and pentoses via the pentose phosphate pathway. These bacteria lack a crucial enzyme, fructose-1,6-diphosphate aldolase (EC: 4.1.2.13) (Von Wright & Axelsson, 2019).

2.4 Microbial Growth Suppression by LAB

Lactobacilli were found to possess antibacterial potential against a range of pathogens such as *Escherichia coli*, *Klebsiella spp*., *Shigella*, *Listeria innocua*, *Listeria monocytogenes and Salmonella enterica* (Darbandi et al., 2022). This antimicrobial activity comes about through different mechanisms like the organic acid biosynthesis which reduce pH as found in *Leuconostoc mesenteroides* where lactic acid secreted effectively kills pathogens with the production of hydrogen peroxide (Timothy et al., 2021). Certain lactic acid bacteria produce bacteriocins which are antimicrobial polypeptides that can inhibit the growth of other bacteria, they may have broad and narrow inhibitory spectrum respectively (Nes & Holo, 2000). Bacteriocins vary and effect their antimicrobial activity in different ways (Sidhu & Nehra, 2019).

Two classes of bacteriocins have been recognized: Category I encompasses antibiotics characterized by the presence of lanthionine, including examples like epidermin and streptin (Nes et al., 2007). Nisin, synthesized by *Lactococci*, was the pioneer among category I bacteriocins to be discovered (O'Connor et al., 2020). Its mechanism involves inducing the formation of minute pores of the bacteria's plasma membrane, leading to cellular breakdown (Andrews et al., 2014). The next category encompasses proteinaceous compounds known as bacteriocins (Snyder &

Worobo, 2014). Certain bacteriocins interact with the cell surface, inhibiting cell wall production, nucleic acid synthesis, and protein synthesis (Kumariya et al., 2019)

Lactobacillus acidophilus has also been shown to produce bacteriocins primarily active against Gram-positive bacteria (Oh et al., 2000). Antimicrobial peptide synthesized by *Lactobacillus rhamnosus* after purification and study showed its antimicrobial activity occurs due to the generation of apertures on the outer surface of the cell membrane which increases permeability, reduces transmembrane potential and pH gradient whilst compromising membrane integrity leading to loss of cell content and cell death (Krishnamoorthi et al., 2022). Many other classes of antimicrobial proteins act by the same mechanism of dissipating proton motive force as observed in LAB source bacteriocins (Montville & Bruno, 1994).

Enzyme deactivation is another well documented mechanism of antimicrobial activity of lactic acid producing bacteria (Carrillo et al., 2014). LAB possess the ability to deactivate key enzymes in pathogenic bacteria resulting in the modification of their catalytic activity and ultimately leading to their inactivation (Ibrahim et al., 2021). This antimicrobial property of LAB has made them useful in various applications like the food production of preservatives or bacteriostatic agents and as probiotics (Šušković et al., 2010).

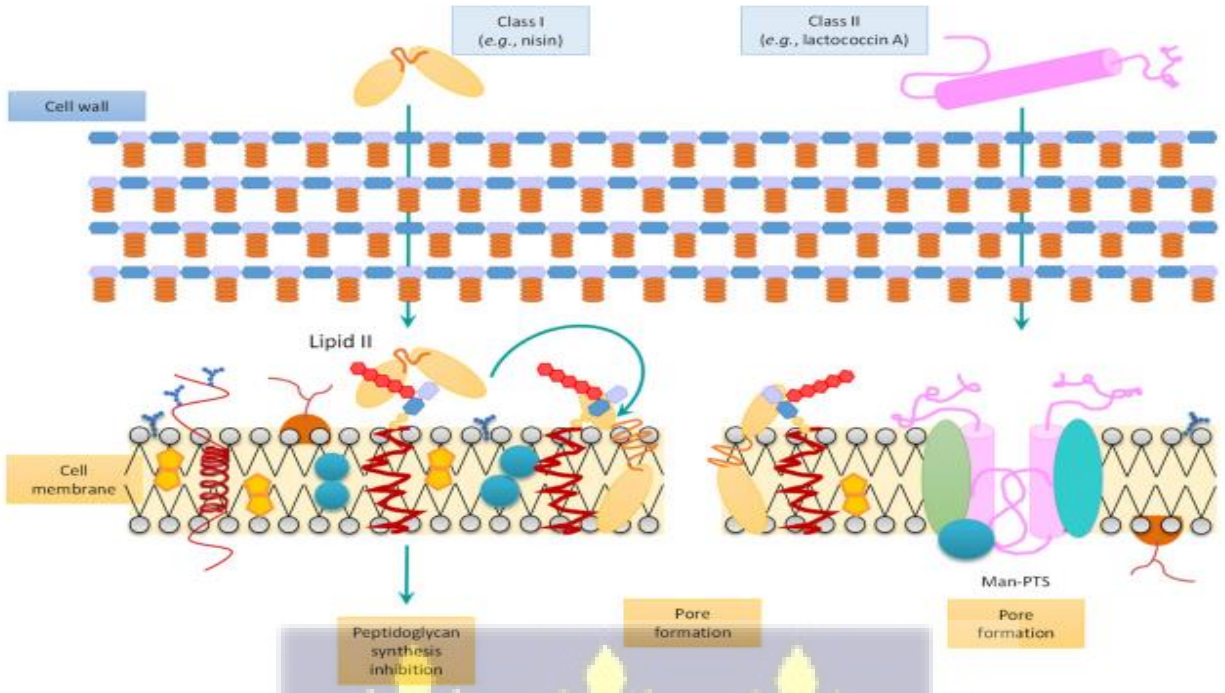


Figure 3: Mechanism of action of bacteriocins on Gram-positive bacteria (Héchard & Sahl, 2002).

The effectiveness of bacteriocins in inhibiting bacterial growth (bacteriostatic activity) relies on factors such as dosage, level of purification, growth stage, pH, temperature, and the presence of other antimicrobial substances that can impact cell wall integrity (Parada et al., 2007). Nisin and pediocin are widely recognized bacteriocins known for their potent antimicrobial properties, capable of inhibiting both spoilage and pathogenic bacteria (Jeevaratnam et al., 2005).

Bacteria that produce bacteriocins have mechanisms to defend against their own toxins, creating resistance (Beis & Rebuffat, 2019). However, this resistance against their own harmful substances is achieved through different methods. Various types of bacteriocins work by interacting with crucial components like lipid II in bacterial cell walls, specific enzymes (such as permease mannose phosphotransferase and zinc-dependent membrane-bound proteases), and transporters like the maltose ABC transporter (Bhattacharya et al., 2022). Initially, these bacteriocins target

membrane receptors, which act as docking sites (Ríos Colombo et al., 2018). For instance, the maltose ABC transporter complex is essential for Garvicin ML as receptors; its absence can result in resistance development within the *Enterococcaceae* family (Bhattacharya et al., 2022).

2.5 Probiotics and the immune system

Probiotics are essential for ensuring a healthy equilibrium between essential and superfluous immune responses, comprising both inborn and acquired defenses (Georgieva et al., 2015). Probiotics interact with the immune system in various ways, such as directly engaging with intestinal cells, being ingested by M cells and interacting with dendritic cells and epithelial cells in the gut (Forsythe & Bienenstock, 2010). The lining of the intestine acts as a natural blockade against harmful microbes and the gut content (Li et al., 2020). Recent studies have explored how probiotics influence intestinal epithelial cells (McLoughlin & Mills, 2011). These effects are a result of probiotics activating specific signaling pathways within the epithelial cells (Vanderpool et al., 2008). Research with *Lactobacillus johnsonii* N6.2 on human Caco-2 cell monolayers demonstrated that this probiotic upregulates TLR7 and TLR9 expression, leading to increased levels of IFN type 1, Stat1, and IRF7 (Kingma et al., 2011). This indicates that probiotics like *L. johnsonii* N6.2 enhance the immunological alertness of intestinal epithelial cells, potentially serving as a method for preventing and treating diseases (Yan & Polk, 2011) .

Probiotics also influence the immune response by modifying cytokine production in the intestinal immune system (Begum et al., 2021). For example, probiotic administration in humans elevated the production of cytokines like IFN-γ and TNF-α by T cells in the lamina propria (Pathmakanthan et al., 2004). In a recent animal studies, probiotic supplementation down-regulated certain inflammatory markers in the intestinal mucosa while upregulating others, indicating their ability to modulate immune function in intestinal epithelial cells (Plaza-Díaz et al., 2017). Thus, *Bacillus*

amyloliquefaciens SC06 activated the Toll-like receptor (TLR) signaling pathway, demonstrating its ability to influence the immune function of intestinal epithelial cells (Du et al., 2018). In laying hen, these findings underscore the diverse and significant impact of probiotics on the immune system within the gut environment (Dung, 2020).

Metagenomic analysis has enhanced our knowledge about probiotic genes that regulate the host immune responses (Shin et al., 2019). By comparing strain-specific cytokine responses and genome profiles, six genes associated with immune modulation were pinpointed (Van Hemert et al., 2010). It was established that, deleting these genes in *L. plantarum* WCFS1 eliminated its cytokine-stimulating capacity (Rook et al., 2014).

2.6 Adhesion activity of LAB

Microorganisms must adhere to the gastrointestinal epithelial cells in order to be characterized as a probiotic (Nishiyama et al., 2016). The adhesive activity of lactic acid bacteria plays a crucial role in their probiotic potential and their ability to promote gut health (Adikari et al., 2020). Adhesion is mediated by mutual influences of adjacent surfaces; these include weak molecular attractions and static electric attractions (Alizadeh Behbahani et al., 2019). Interactions between microorganism adhesive proteins and binding sites on epithelial cells then come into play facilitating proper adhesion (Sidira et al., 2015). The course of adhesion of LAB to intestinal epithelial cells is also influenced by factors, such as bacterial cellular barrier components, gastrointestinal mucosal layer, or external factors (Zawistowska-Rojek et al., 2022). Lactic acid bacteria possess various surface proteins, glycocalyx component, cell envelope acids which facilitate adhesion and the biofilm formation (Oloketuyi & Khan, 2017). The gastrointestinal tract also possesses structures such as mucin and extracellular matrix adhesion proteins which allow the binding of probiotic microbes to the intestinal epithelium (Sengupta et al., 2013).

The benefits of adhesion include the inhibition of colonization of the gut by exogenous bacteria, some of which may be pathogenic (Del Piano et al., 2006). This phenomenon has been described as “competitive exclusion” and it describes a situation where one species of bacteria is more adapted to colonizing a particular habitat than other bacteria hence have an advantage in the competition for resources vital for growth and survival (Rohmer et al., 2011). The rate of infections thus decrease due to probiotic colonization of the sites that pathogens may have been attached to in the host (Lindow & Brandl, 2003). This makes probiotics a more effective prevention mechanism than treatment for diseases caused by GIT pathogens (Friedland & Chapman, 2017). LAB strains have been shown to inhibit this kind of colonization which helps maintain a healthy balance and reduce the presence of pathogenic microbes (Duar et al., 2020).

2.7 Bile tolerance mechanisms of LAB

Bile tolerance determines the ability of microbes to survive in the gastrointestinal tract and is an essential characteristic of probiotic bacteria (Gonzalez-Rodriguez et al., 2013). Bile salts are strong surfactants and can act as antimicrobial molecules thus they influence the intestinal microflora necessitating the evaluation of capability of probiotic bacteria to survive bile salts when considering the use of LABs as probiotic products (Sarao & Arora, 2017). Conjugated bile acids have been found to inhibit the development of certain variants of lactobacilli by compromising the stability of membrane, reducing the electric gradient across the cell membrane and lowering ATP levels (Hofmann & Mysels, 1992). Probiotic lactic bacteria must have ways of surviving in the gastrointestinal tract despite the presence of these compounds (Tewari et al., 2019).

Lactic acid bacteria survive bile salt-induced stress by varying means, these include; the use of special enzymes referred to as bile salt hydrolases, through bile salt efflux, and alterations in the composition of bacterial membranes (Chen et al., 2022). Some strains of Lactobacilli have been

found to have the ability to remove cholesterol. This is related to their bile salt hydrolase activity (Kumar et al., 2012). The bile acid hydrolytic enzyme, (BSH, EC3.5.1.24) is the main bile salt defense for lactic acid bacteria (Morinaga et al., 2022). This enzyme deconjugates glycine and taurine linked bile salts thereby detoxifying the bile salts and making the intestinal environment more favorable to LAB variants which possess the enzyme and the presence of this enzyme has thus been described as a probiotic biomarker (Enright et al., 2018). *Lactobacilli* and *bifidobacteria* have been observed to possess functional copies of bile salt hydrolase genes in their genomes, functioning in bile salt deconjugation (Begley et al., 2006). Notably, enzyme specificity of bile salt hydrolase is based on the distinct amino acid therefore, the availability of this enzyme has also been hypothesized to grant a nutritional edge to the bacteria engaged in production, since they make use of the amino acids liberated from their conjugated bile salts (Jia et al., 2020).

Alterations in the bacterial cell membranes by the external synthesis of exopolysaccharide (EPS) layers is a characteristic of some gastrointestinal microbes (Angelin & Kavitha, 2020). The exopolymers function as a defensive layer and protect bacteria in the face of external factors (Skorupska et al., 2006). Consistently, bile is known to stimulate EPS biosynthesis in some variants of *B. animalis*, possibly as defense against bile (Ruiz et al., 2013). Relationship between EPS synthesis and resilience to bile exposure in various bifidobacterial species have also been established however, the impact of bile salts on EPS synthesis in *Limosilactobacilli* remains less evident (Averina et al., 2021).

Bile salts induce oxidative stress in bacteria by generating deleterious oxygen or nitrogen components (Bhattacharyya et al., 2014). Deconjugation of bile salts also leads to a release of protons, causing a decrease in pH (Thanassi et al., 1997). When challenged with bile, bacteria respond in a manner akin to reactions against stressors like acids and highly reactive oxygen

species, as demonstrated through microarray studies in various species including *L. reuteri, and L. rhamnosus* (Lebeer et al., 2008). This response is an attempt to mitigate the adverse impacts of bile, involving perturbations to cell membrane integrity, oxidative pressure, DNA damage, protein structural alteration, and acidic conditions within the cell (García et al., 2014). Notably, contact of bacteria to bile exhibit an upregulation of diverse proteins to curb these detriments. Protein degradation is addressed using a responsive mechanism involving chaperones and proteases, facilitating the rapid regeneration of impaired polypeptides and the appropriate activity enhancement of newly synthesized peptides (Guerriero & Brodsky, 2012). In *bifidobacteria*, both bile-induced and adapted states have been associated with an increased production of an array of proteases and chaperones (Ruiz et al., 2013).

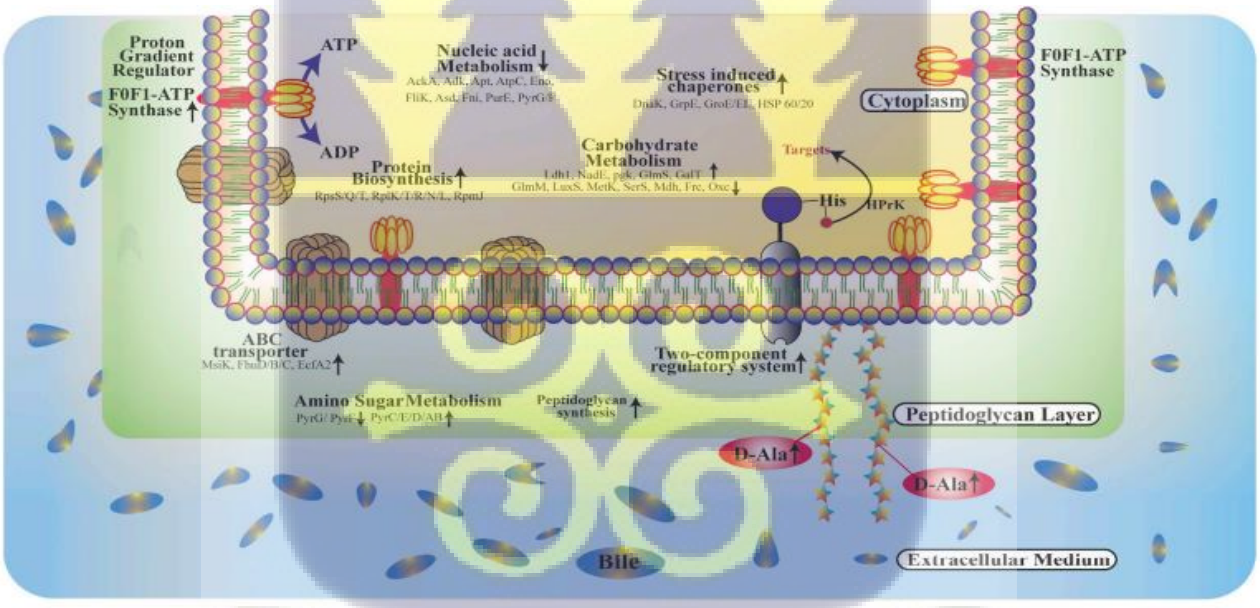


Figure 4.0: Proposed model for response mechanisms of *L. fermentum* NCDC 605 to bile stress (Ali et al., 2020).

The gene expression changes, both induced and repressed, are indicated by up and down arrows respectively in the list of genes provided and their corresponding abbreviations. This list

encompasses a variety of genes involved in different biological processes, such as enzymatic activities, transport systems, and chaperone proteins, among others namely; Acetate kinase (AckA), Adenylate kinase (Adk), Adenine phosphoribosyl transferase (Apt), ATP synthase epsilon chain (AptC), Enolase (Eno), Flagellar hook-length control protein FliK (FliK), 50S ribosomal protein L11 (RplK), 50S ribosomal protein L20 (RplT), 50S ribosomal protein L18 (RplR), 50S ribosomal protein L14 (RplN), 50S ribosomal protein L7/L12 (RplL), 50S ribosomal protein L36 (RpmJ), L-lactate dehydrogenase (Ldh1), NH(3)-dependent NAD(+) synthetase (nADe), Phosphoglycerate kinase (pgk), Glutamine-fructose-6-phosphate aminotransferase (glmS), Galactose-1-phosphate uridylyltransferase (GalT), Glutamine-fructose-6-phosphate aminotransferase (GlmS), S-ribosyl homocysteinelyase (LuxA, S-adenosyl methionine synthase (MetK), Serine-tRNA ligase (SerS), Malate dehydrogenase (NAD) (Mdh), Formyl-CoA transferase (frc), Oxalyl-CoA decarboxylase (oxc), Chaperone protein DnaK (DnaK), 60 kDa chaperonin; Hsp60 (GroEL), Cellobiose/maltose ABC transporter (Msik);, Iron(3+)-hydroxamatebinding protein (FhuD), Iron(3+)-hydroxamate import system permease protein (FhuB), Iron(3+)-hydroxamate import ATP-binding protein (FhuC), Energy-coupling factor transporter ATP-binding protein (EcfA2), Dihydroorotase (PyrC), Orotate phosphoribosyl transferase (PyrE), Dihydroorotate dehydrogenase (PyrD), Carbamoylphosphate synthase pyrimidine-specific large chain (PyrAB), HPr kinase/phosphorylase (HrpK) (Ali et al., 2020).

2.8 Gamma-Aminobutyric Acid (GABA) Production

Gamma-aminobutyric acid (GABA), a non-proteinaceous substance found predominantly in nature and, hold significant importance as a neurotransmitter (Jain & Ghodke, 2021). This is located in the central nervous system and has associations with various diseases based on its amount in the brain of humans (Meyerhoff, 2001). The key mechanism for intracellular GABA

synthesis involves the decarboxylation of L-glutamate through the action of glutamate decarboxylase (GAD) in conjunction with pyridoxal-5'-phosphate (Shi et al., 2014). *Levilactobacillus brevis* D17, characterized by high GABA production, exhibits a potential transcriptional regulator gene gadR controlling both GABA biosynthesis and strain resistance to acids (Lee et al., 2021). *Levilactobacillus brevis* in the industry is most often used alone or together with other strains, stands out for GABA production (Banerjee et al., 2021). Immobilization techniques also contribute significantly to optimizing the biosynthesis of GABA (Pannerchelvan et al., 2023).

Microbes known for safe GABA production can be harnessed to craft functional health food components in food sectors (Abedin et al., 2023). Fermented cereals can experience enhanced GABA content due to certain lactic acid bacteria (Zareian et al., 2012). Additionally, dairy items like cheese, yogurt, and cultured milk created using lactic acid bacterial fermentation can be transformed into products fortified with elevated GABA content (Yogeswara et al., 2020). Ancient Chinese food product, like Sufu, can benefit from the combined inoculation of *Levilactobacillus brevis* and *Bacillus subtilis* to elevate GABA concentrations and diminish the amounts of detrimental compounds like histamine (Wang et al., 2021).

2.8.1 General Antimicrobial Activity of Probiotics

Probiotics can produce substances that can kill or stop the growth of harmful bacteria or prevent them from forming biofilms (Dufour et al., 2010). These substances are found in the liquid part of the probiotic culture, called the cell-free supernatant (CFS), which is obtained by spinning or filtering the culture (Moradi et al., 2021). The CFS contains different types of substances, such as organic acids, hydrogen peroxide, biosurfactants, bacteriocins, and antimicrobial peptides (Nataraj

et al., 2020). Some of these substances can be carried by extracellular vesicles, which are like tiny bubbles that can transfer information between cells (Shen et al., 2022).

The CFS of probiotics has antibacterial and antibiofilm effects against various harmful bacteria, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Helicobacter pylori* (El-Mokhtar et al., 2020). The CFS of probiotics can also affect the immune system and strengthen the intestinal barrier, which can stop harmful bacteria from entering and spreading in the body (Cristofori et al., 2021). The CFS of probiotics could be a good alternative to regular antibiotics because it can avoid the problem of antibiotic resistance and reduce the negative effects on the good bacteria in the body (Algburi et al., 2021). However, more research is needed to understand how the CFS of probiotics works and how to best use it (Ozma et al., 2023).

2.8.2 Bactericidal Activity of Probiotics

The ability of probiotic microorganisms to kill or inhibit growth of harmful bacteria is called bactericidal activity of probiotics (Chuayana et al., 2003). To measure the smallest concentration of an antibacterial compound that can kill or eliminate bacteria over a specific time under certain conditions, the minimum bactericidal concentration (MBC) test can be used (Prabhurajeshwar & Chandrakanth, 2019). The MBC test can evaluate the antimicrobial activity of probiotic supernatants, which are the liquid fractions obtained after centrifuging probiotic cultures (Abouloifa et al., 2022). The MBC test can also compare the bactericidal activity of different probiotic strains or formulations against various pathogens, such as carbapenem-resistant *Enterobacteriaceae* (CREs), which are a major cause of healthcare-associated infections (Franca et al., 2014).

The minimum bactericidal activity of probiotics is an important factor to consider when developing new products containing probiotics, such as foods and dietary supplements (Binda et al., 2020).

Probiotics must be completely characterized, safe, supported by at least one positive human clinical trial, and alive in the product at an effectual dose throughout the shelf life (Binda et al., 2020). The minimum bactericidal activity of probiotics can help demonstrate the health benefits of probiotics on the host, such as preventing or treating infections, modulating immune responses, or improving intestinal barrier function (Ohland & MacNaughton, 2010).

Lowest inhibitory concentration (MIC) is the minimum concentration of an antimicrobial agent that can prevent the visible growth of a microorganism (Wiegand et al., 2008). MIC is an important parameter for evaluating the antimicrobial activity of probiotics (Neu & Ellner, 1983). Probiotics exert antimicrobial effects against various pathogens by producing organic acids, hydrogen peroxide, bacteriocins, and other substances that lower the pH, create oxidative stress, or disorganize the cell surface membrane of the target microorganisms (Monika et al., 2021). MIC for probiotics can vary depending on the strain, the target pathogen, the growth medium, the incubation time and temperature, and other factors (Tejero-Sariñena et al., 2012).

Several methods have been used to determine the MIC of probiotics, such as broth dilution, agar dilution, disk diffusion, and E-test (Charteris et al., 2001). Among these methods, broth dilution is considered the most accurate and reproducible, as it allows for a precise measurement of the antimicrobial concentration and a direct observation of the microbial growth (Smith & Kirby, 2016). Broth dilution can be performed in microtiter plates or test tubes, using either liquid or solidified broth as the growth medium (Hsieh et al., 2012). The procedure involves inoculating a standardized amount of the target microorganism into wells or tubes containing serial dilutions of the probiotic product or its supernatant (Greppi et al., 2017). After incubating for a suitable period, usually 18 to 24 hours, the wells or tubes are examined for turbidity or colony formation (Wiegand et al., 2008). Probiotics with low MIC values against specific pathogens can be considered as

promising alternatives or adjuncts to conventional antibiotics, especially in the context of increasing antibiotic resistance and adverse effects (Silva et al., 2020). Moreover, knowing the MIC of probiotics can help to determine the optimal amount and frequency of administration that can achieve effective antimicrobial concentrations in the target site without causing undesirable side effects or interactions with other substances (Leekha et al., 2011). Furthermore, the comparison of MIC of probiotics with their minimum bactericidal concentration (MBC), which is the minimum concentration that kills a certain percentage of the microorganisms, can indicate whether the probiotics have bacteriostatic or bactericidal effects against the target pathogens (LeBel et al., 2013). MIC is therefore, a valuable parameter for evaluating the antimicrobial activity of probiotics against various microorganisms (LeBel et al., 2013). MIC can be determined by different methods, but broth dilution is preferred for its accuracy and reproducibility (Waites et al., 2012);(Grumet et al., 2020).

CHAPTER THREE

3.0 Materials and Methods

3.1 Chemicals and Reagents

All solvents used were of analytical grade. Sodium chloride (Park Scientific Limited, Northampton, UK), methanol (Chem-Lab, France), Muller Hinton Agar (Sigma-Aldrich, St. Louis, USA), Muller Hinton Broth (Sigma-Aldrich, St. Louis, USA), de Man Rogosa and Sharp (MRS) Agar (Sigma-Aldrich, St. Louis, USA), de Man Rogosa and Sharp (MRS) Broth (Sigma-Aldrich, St. Louis, USA), Agar Bacteriological (Sigma-Aldrich, St. Louis, USA), McConkey Agar (Sigma-Aldrich, St. Louis, USA), McConkey broth (Sigma-Aldrich, St. Louis, USA), Agarose powder (ThermoFisher Scientific, Waltham, MA, USA), Ethidium bromide (Sigma-Aldrich, St. Louis, USA), TAE buffer (ThermoFisher Scientific, Waltham, MA, USA), Crystal violet, Grams iodine solution (ThermoFisher Scientific, Waltham, MA, USA), Ethanol (ThermoFisher Scientific, Waltham, MA, USA), 0.3% bile salt solution (ThermoFisher Scientific, Waltham, MA, USA), Penicillin-Streptomycin (Hyclone Laboratories, Inc., USA), Non-essential amino acid (Hyclone Laboratories, Inc., USA), Trypsin (Hyclone Laboratories, Inc., USA), absolute ethanol (Park Scientific Limited, Northampton, UK), chloroform (Park Scientific Limited, Northampton, UK), Peptone (Park Scientific Limited, Northampton, UK), Glycerol (Park Scientific Limited, Northampton, UK), HCl (Park Scientific Limited, Northampton, UK), Sodium hydroxide (Park Scientific Limited, Northampton, UK), Picric acid (ThermoFisher Scientific, Waltham, MA, USA), Nuclease free water (ThermoFisher Scientific, Waltham, MA, USA), Proteinase K (Sigma-Aldrich, St. Louis, USA), Dulbecco's modified Eagle's minimal essential medium (DMEM; Hyclone Laboratories, Inc., USA) supplement with 20% (v/v) inactivated fetal bovine serum

(Hyclone Laboratories, Inc., USA), 1% streptomycin (100 mg/mL) and 1% penicillin (100 mg/mL), One Taq 2X Master mix (ThermoFisher Scientific, Waltham, MA, USA).

3.1.1 Microorganisms

Pathogenic strains of bacteria used in this study were sourced from the National Collection of Type Cultures (NCTC) and the American Type Culture Collection (ATCC). The identity of these strains (*Escherichia coli NCTC 11954 TEM 1, K. pneumoniae NTC 13368, Salmonella typhi ACT 1, and Staphylococcus aureus*) were confirmed using MALDI-TOF. The non-pathogenic strains were isolated directly from the fermented beverage, except for the control group (*Lactobacillus casei*), which was previously identified through MALDI-TOF analysis.

3.2 Sample collection and description

3.2.1 Study samples

This study was approved by the Ethics committee for Basic and Applied Sciences (ECBAS) in University of Ghana (reference number: **ECBAS003/22-23**) and The Institutional Animal Care and Use Committee (IACUC) at Noguchi Memorial Institute for Medical Research (NMIMR) (reference number: **UG-IACUC 023/21-22**). Informed consent was obtained from all recruited participants prior to participation through completion of detailed questionnaires. All experiments and procedures involving animal models were conducted in accordance with the IACUC principles. Samples consisting of fermented millet-based milk beverage called "*Brukina*" as well as fecal samples obtained from humans and animals at different time points following *Brukina* sample consumption were used for this study. The primary reason for selecting the fermented millet-based milk beverage (*Brukina*) was its potential as a reservoir for probiotic strains, nutritional value, medicinal purposes and widespread consumption

across the country. Fecal samples were also carefully chosen to gain insights into how the consumption of fermented millet-based milk beverage (*Brukina*) affects the microorganisms associated with the gastrointestinal system.

3.2.2 Sampling locations

Samples were procured from open markets located in the Greater Accra Region of Ghana. They were obtained from distinct supermarkets, as detailed in Table 1.

Table 1: Sampling locations

Region	**Market**	**District**
Greater Accra Region	Dansoman Market	Ayawaso East
	Madina Market	La-Nkwantanang-Madina
	Accra Market	Korley-Klottey
	Ashaiman Supermarket	Ashaiman Municipal
	Kasoa supermarket	Awutu Senya East District
	Dodowa supermarket	Shai Osudoku District

Figure 5.0. Geographic distribution of sample collection sites within the Greater Accra Region of Ghana for microbiota study.

The map shows six urban and peri-urban locations: Dansoman, Madina, Accra, Ashaiman, Kasoa, and Dodowa, indicated by directional arrows. These sites represent diverse socio-environmental contexts from which *Brukina* product samples were obtained for microbiological and sequencing analyses. Regional placement highlights sampling coverage across coastal and inland zones, which are relevant to dietary exposure assessment.

3.2.3 Survey and sample collection

3.2.3.1 Survey

The study employed purposive sampling to choose supermarkets that were recognized for selling the research samples (*Brukina*) in large quantities. These were Food and Drugs Board (FDA) approved samples. Data encompassing various aspects of the samples (*Brukina*) were collected, including storage conditions (such as packaging materials, storage structures, and duration), hygiene practices to prevent the growth of harmful microorganisms, and potential mycotoxin buildup.

3.3 Bacterial strains and culturing conditions.

3.3.1 Biochemical Characterization of Probiotics

Fifteen (15) different FDA-approved '*Brukina*' samples were sourced from different supermarkets in Accra. Following the assessment of microbial load, 6 brands with counts exceeding 10^7 CFU/mL were selected for subsequent animal and human trials. These selected samples were collected in multiples and administered to both human and animal models over a two-week period. The volume of each sample was 150 milliliters. The '*Brukina*' samples were serially diluted and pour plated on MRS agar plates under aseptic conditions and incubated anaerobically for 48 hours. Colonies were counted and recorded in CFUs/ml for each of the samples. With colony morphology as the criterion, a total of thirty-nine (39) isolates were selected and sub-cultured for further biochemical and molecular analysis. Crystal violet was added to a heat-fixed bacterial column. The crystal violet was washed off followed by the addition of iodine to trap the crystal violet. To quickly decolorize it, ethanol was added, followed by washing off of the ethanol and the addition of safranin as counterstain. A light microscope was used to examine the bacteria colonies. The isolates were also subjected to catalase and oxidase testing. Out of the thirty-nine isolates that were

obtained from the fermented millet-based milk beverage (*Brukina)*, twenty-two were tentative *Lactobacilli* isolates and seventeen yeast strains.

3.3.2 Tolerance to acidic environments

Tentative *Lactobacillus* strains were cultured in MRS broth for 48 hours at 37°C. A volume of 0.1 mL from each active culture were added to MRS broth adjusted to pH 3.0 and 2.0 using 5 N HCl. These were then incubated at 37°C for 3 hours. Samples were collected every hour over the 3-hour period, bacterial growth was tracked by measuring absorbance at 600 nm using a plate reader from ThermoFisher Scientific, USA. Each assay was repeated twice.

3.3.3 Bile Tolerance

The *Lactobacillus* strains were grown in MRS broth at 37 °C for 48 hours. A saturated bile solution was prepared by dissolving 3g of powdered bile extract (Oxoid) in 100 mL MRS broth with a final concentration of 0.3% (w/v) bile salts. The bile solution was sterilized using a 4-micron filter. The bile containing media was inoculated with the *Lactobacillus* strains and incubated at 37 °C for 3 hours. Bacterial growth was monitored by measuring absorbance at 600 nm using a plate reader from ThermoFisher Scientific, USA. This entire process was duplicated for each assay.

3.3.4 Caco-2 cell culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 20% inactivated faetal bovine serum, 1% streptomycin (100 mg/mL), 1% penicillin (100 mg/mL), and non-essential amino acids. The cells were maintained at a temperature of 37°C in a controlled environment containing 5% CO_2 and 95% air. Passage of cells was facilitated using 0.25% trypsin. The cell culture medium was refreshed every 2 days, and adhesion assays were carried out after the cells had undergone six passages.

3.3.5 Adhesion assays on Caco-2 cells

Bacterial adhesion assessments were performed on Caco-2 cell monolayers using 6-well tissue culture plates sized at 22 x 22 mm. Pre-cultured Caco-2 cells were introduced to each well at a density of twenty thousand cells per milliliter. The cells were incubated in 5% CO_2 at 37°C until a densely packed cell layer formed. After washing the Caco-2 cell monolayers twice with phosphate buffer saline (PBS) of pH 7.2, individual wells received a bacterial suspension of 1 milliliter, and 1 milliliter of DMEM culture. These were then incubated for 60 minutes. Subsequently, any non-adherent cells were eliminated by washing with PBS buffer, and the cells that adhered to the monolayer were fixed using methanol for 20 minutes and then subjected to Gram staining. The adherent bacterial cells were counted by examining twenty randomly selected microscope fields. The count of bacteria adhered to 100 cells was used to determine the bacterial adherence value.

3.3.6 Antibiotic susceptibility test

Isolated Lactic acid bacteria were grown for 48 hours, normalized to a 0.5 McFarland, and streaked on Muller Hinton agar. Commercial antibiotic discs (Neomycin (30 µg), Metronidazole (5 µg), Vancomycin (30 µg), Streptomycin (10 µg), Kanamycin (30 µg), Ampicillin (10 µg), Gentamycin (10 µg), Ciprofloxacin (10 µg), Erythromycin (15 µg), Clindamycin (10 µg)) were placed on the agar plates with the strains and incubated for 48 hours at 37°C and the zones of inhibition measured.

3.3.7 Antimicrobial assessment

The inhibitory effect of *Lactobacillus* strains on specific enteropathogenic reference strains was determined using the well-diffusion procedure. For the agar well diffusion assay, overnight cultures of enteric pathogenic strains (*Escherichia coli NCTC 11954 TEM 1, K. pneumoniae NTC 13368, Salmonella typhi ACT 1, and Staphylococcus aureus*) were adjusted to a 0.5 McFarland

standard. These pathogenic strains were applied to Muller Hinton (MH) agar growth media. Wells with a diameter of 5mm were created on the agar plates. In each of these wells, 100 µL of *Lactobacillus* culture was placed. After a 48-hour incubation period at 37 °C, the zones of inhibition produced by *Lactobacillus* were observed and examined.

3.3.7.1 Cell free Supernatant for Bacteriocins Assay

Bacteriocin production by tentative probiotic *Lactobacillus* strains was determined. The *Lactobacillus* strains were cultured in MRS broth at 37°C for 48 hours. Following this, cell suspensions were centrifuged at 5000 rpm for 15 minutes. The pH of the resulting cell-free supernatant was then adjusted to 7.0 using 1N NaOH. To assess the antagonistic properties of the bacteriocins, a well diffusion method was employed. A 0.5 McFarland standard of enteric pathogenic strains (*Escherichia coli NCTC 11954 TEM 1, K. pneumoniae NTC 13368, Salmonella typhi ACT 1, and Staphylococcus aureus*) was spread plated on Muller Hinton (MH) agar growth media. Wells with a diameter of 5mm were created on the agar plates. In each of these wells, 100 µL of *Lactobacillus* cell free supernatant was placed. After a 48-hour incubation period at 37 °C, the zones of inhibition produced by the cell free supernatant were determined.

3.3.7.2 Determination of the growth patterns of *Lactobacillus* strains

Lactobacillus strains that demonstrated antimicrobial activity were cultured in 20 milliliters of MRS broth and incubated at 37°C for 24 hours. The optical density was taken hourly using the plate reader for a period of 24 hours. The growth pattern was determined using the Graph pad prism. The generation time was then calculated.

3.4 The effect of regular *Brukina* consumption on the Gut Microbiome profile of consumers.

3.4.1 Animal Study

A total of 210 samples were used, and the administered quantity was determined based on the weight of each animal. A total of 35 animals (Sprague Dawley Rats) were obtained and placed in groups of five. The animals were weighed prior to the commencement of sample administration using the weighing balance. Six groups were given different brands of *Brukina* sample in addition to their normal feed and the last group which was the control group was given only distilled water in addition to their usual feed for a period of 28 days. The homogenized sample (Brukina) was then administered to the animals using the oral gavage mode of administration daily. Fecal samples were collected after the administration at different time points. The fecal samples were collected directly with 1.5 ml Eppendorf tubes on day 0,3,7,14 and 28. DNA was directly extracted from the fecal sample using the Qiagen Kit for fecal samples according to the manufacturer's instructions. Fecal samples were collected from rats in accordance with approved ethical guidelines and institutional protocols. Freshly voided pellets were obtained by placing individual animals in sterile, clean cages without bedding. Using sterile forceps, fecal samples were collected immediately after defecation to prevent contamination and transferred into pre-labeled sterile microcentrifuge tubes. To preserve microbial and nucleic acid integrity, samples were either processed immediately. All tools and surfaces were sterilized with 70% ethanol between samples, and biosafety precautions were strictly followed throughout the procedure. Relevant metadata, including animal ID, feeding group, and sampling time, were documented for each specimen. Fragments of the 16SrRNA gene from the direct DNA extractions was amplified and sequenced at the Next Generation Sequencing (N.G.S.) Laboratory at West African Centre for Cell Biology

of Infectious Pathogens (WACCBIP) to determine the microbial profile of the experimental rats fed with *Brukina.*

3.4.2 Human study

Participant selection was guided by specific inclusion and exclusion criteria. Individuals who were on antibiotics, lactose intolerant, or consuming other probiotic products were excluded from the study. The final sample size for the human study comprised 420 *Brukina* samples, with each participant receiving 300 milliliters daily over a 14-day period. A total of thirty human participants were recruited and placed in groups of five. Every individual in each group was given a particular brand of 300 ml *Brukina* sample daily for a period of 14 days. Fecal samples were collected after the consumption at different time points on day 0,3,7 and 14. Human fecal samples were collected following ethical approval and informed consent from all participants. Fresh stool samples were self-collected by participants using sterile, labeled collection containers provided with clear instructions. Samples were transported to the laboratory within two hours of collection, stored on ice during transit, and immediately processed upon arrival. For molecular analysis, a portion of each sample was transferred into sterile microcentrifuge tubes containing DNA/RNA Shield solution and stored at −80°C to preserve microbial composition and nucleic acid integrity. All handling was performed under sterile conditions using appropriate biosafety procedures, and relevant metadata, including participant ID, dietary habits, and sampling time, were recorded for traceability and analysis. DNA was directly extracted from the fecal sample using the Qiagen Kit for fecal samples according to the manufacturer's instructions.

3.5 Molecular Characterization and Assessment of the Relative Abundance and Diversity of Lactobacillus species of Bacteria

3.5.1 Genomic DNA Extraction.

Bacterial isolates were grown on MRS medium and incubated at 37°C for 48 hours. Specifically, 22 *Lactobacilli* isolates were chosen based on their morphological and adhesion traits. The DNA of these pure LAB isolates were extracted and purified using a Qiagen Spin DNA Extraction kit, following the manufacturer's instructions.

3.5.2 Amplification of 16S rRNA of Bacterial Isolates.

The fragments of the 16S rRNA genes from each bacterial isolates were amplified using universal primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3'). These universal primers were used as forward and reverse primers respectively to amplify the 16S rRNA regions in a 50 µL reaction mixture (Wambui et al., 2014). A thermocycler (PCR Max) was used for the amplification process with respective PCR reaction (tables 2 and 3). Following PCR, 10 µL of the PCR products were subjected to agarose gel electrophoresis consisting of 1.5 % agarose (agarose, 1.5 g; 100 mL TAE buffer, ethidium bromide, 2 µL). The PCR products were loaded alongside a positive control, a negative control, and a standard molecular marker 100-bp DNA ladder. The electrophoresis setup was run at 90 V until the dye travelled about 80% down the gel. The gel was then visualized under UV light and photographed (Amersham Imager 600). The amplified products were then sequenced. The Shannon indices was then used to determine the percentage abundance and diversity of microorganisms, particularly Lactobacillus species in the samples.

Table 2: PCR Master mix

Reaction Mixture (50µL)	
Components	**Volume (µL)**
2X Master Mix	25
Nuclease free water	13
27F	1
1492R	1
Template DNA	10

Table 3: PCR Reaction conditions

Reaction Conditions (40 cycles)		
Step	**Temperature (℃)**	**Duration**
Initiation	95	1 min
Denaturation	95	60 sec
Annealing	51	30 sec
Extension	72	60 sec
Final Extension	72	1 min
Pause	4°C	Indefinite

3.5.3 Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.0), Microsoft Excel (Microsoft Office 2021), and R Studio - 2025. Data obtained from experimental replicates were presented as mean ± standard deviation (SD). Prior to analysis, data normality was assessed using the Shapiro-Wilk test to determine the appropriate statistical tests to apply.

For comparisons between multiple groups, one or two-way analysis of variance (ANOVA) was employed, followed by post hoc Tukey's multiple comparison test to identify significant differences between means. For pairwise comparisons, t-test was used when the data met parametric assumptions.

Categorical data, such as antimicrobial susceptibility profiles, were analyzed using chi-square (χ^2) tests to evaluate differences in frequency distributions. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant across all tests. Graphical representations were generated using GraphPad Prism and Microsoft Excel to visually support the statistical findings.

CHAPTER FOUR

4.0 Results

4.1 Survey on Consumption of Milk, Millet, Millet-based Milk Beverage (*Brukina*) and the Perceived Knowledge of Probiotics.

4.1.1 Demographics of participants

Table 4 shows the demographics of the participants. Thirty-one female participants participated in the survey as compared to twenty-four male participants. All the respondents were within the 20-to-26-year age range, accounting for 100% total. Additionally, all participants lived within the University of Ghana community at the time of survey.

Table 4: Demographics of Participants

	Description	Frequency	Percentage
Gender	Male	24	43.63
	Female	31	56.36
Age	20 – 26 years	55	100
Education	Tertiary	55	100

4.1.2 Consumption of *Brukina*, millet, and fresh milk

Table 5, shows that, participants accounting for 63.36% and 60% of the total, confirmed their consumption of millet and milk respectively. Similarly, 36 participants, making up 64.45%, acknowledged consuming of the millet-based milk beverage (*Brukina*).

Table 5: Consumption of *Brukina*, millet and milk by participants

Samples	Response				Frequency of *Brukina*			
	Yes		**No**		**3-5 times weekly**		**Once a month**	
	PP	% PP	PP	% PP	PP	% PP	PP	% PP
Brukina	36	65.45	19	34.54	39	70.90	16	29.09
Milk	33	60.00	22	40	41	74.54	14	25.45
Millet	35	63.63	20	36.36	37	67.27	18	32.72

Only participants (PP) who responded "Yes" to the consumption of *Brukina* were further involved in the study. Individuals who affirmed consuming millet-based milk beverage (*Brukina*), milk, and millet, 70.90%, 74.54%, and 67.27%, respectively, revealed consuming them three to five times weekly. On the other hand, 29.09%, 25.45%, and 32.72% indicated the consumption of these items monthly. Among various derivatives, *Brukina* was the most frequently consumed by most of the participants.

4.1.3 Knowledge on *Brukina*

In this section, data regarding participants' familiarity with *Brukina*, including their ability to recognize the beverage and whether they have consumed it, was collected (as presented in Table 6). 55 participants were interviewed. Findings from the survey revealed that a small proportion, specifically 9.09%, of participants were not familiar *Brukina*. However, around 90.90% of these individuals were still able to correctly identify fermented millet-based milk beverage (*Brukina*) in

its various forms. About half (49.09%) of respondents acknowledged being aware of any adverse effects associated with *Brukina,* while a significant majority (81.81%) were knowledgeable about the positive impacts it offers. Furthermore, the majority (81.81%) of participants were aware that consuming *Brukina* could have positive implications on their health.

Table 6: Knowledge on *Brukina*

Questions	Responses			Percent responses		
	Yes	No	Maybe	Yes	No	Maybe
Do you know *Brukina*?	50	5	0	90.90	9.09	0
Do you consume?	36	19	0	65.45	34.54	0
Do you know any health benefit of *Brukina* *Brukina*?	45	8	2	81.81	17.77	4.44
Do you know any detrimental impact of *Brukina*?	27	14	4	49.09	31.11	8.88

4.2 Knowledge of probiotics

The survey also aimed to determine participants' knowledge of probiotics (as shown in Table 7). Of the total of 55 participants, 28 individuals (50.90%) indicated that they were familiar with the concept of probiotics. Also 26 individuals (47.27%) were aware that probiotics are employed in the production of yoghurts, whilst only 23 (41.81%) understood that these beneficial bacteria can lose their effectiveness when exposed to heat. Moreover, a substantial number of the participants, specifically 72.73% out of 100%, possessed knowledge about certain health conditions that can be addressed through probiotics, while the remaining 27.27% did not possess this awareness.

Table 7: Knowledge on Probiotics

Questions	Responses					
	Frequency			Percent responses		
	Yes	No	Maybe	Yes	No	Maybe
Do you know what probiotics are?	28	27	0	50.90	49.09	0
Do you know probiotics are used in manufacturing yoghurts?	26	29	0	47.27	52.72	0
Do you think probiotics can be killed by heat?	23	21	11	41.81	38.18	20
Do you know any health condition managed with probiotics	40	15	0	72.73	27.27	0

4.3 Microbial Load Determination

The bacterial load (Log_{10} CFU/ml) in fifteen FDA-approved *Brukina* samples, measured in duplicate and expressed as mean ± SD is presented in Figures 5.0a and 5.0b. LAB counts ranged from 10^4 to 10^6 CFU/ml, meeting levels generally associated with probiotic potential. In samples A–G (Figure 5.0a), bacterial concentrations varied significantly, with sample G consistently recording the highest load and showing the most precise levels of statistical separation from other samples (***$p < 0.001$, ****$p < 0.0001$), corresponding to ≤0.1% and ≤0.01% probabilities, respectively. These differences were due to random variation. Lesser but still meaningful differences occurred at $p < 0.01$ (≤1% probability) and $p < 0.05$ (≤5% probability). In samples H–O (Figure 5.0b), sample L exhibited significantly higher counts than M, N, and O (**$p < 0.01$) as well as H (*$p < 0.01$) and I, J, and K ($p < 0.05$), placing it at the ultimate level within the group. Biochemically, higher LAB loads such as those seen in samples G and L may reflect more favorable fermentation conditions, including optimal carbohydrate availability from millet starch hydrolysis, higher protein content supporting microbial growth, and pH levels conducive to LAB metabolism and acid tolerance. Conversely, lower bacterial loads in samples such as N and E may result from suboptimal substrate composition, accumulation of inhibitory organic acids or bacteriocins during fermentation, or differences in microbial starter cultures. The prevalence of low p-values across multiple comparisons shows that, these differences are both statistically robust and biologically meaningful, pointing to consistent compositional and process-related factors rather than random experimental variation.

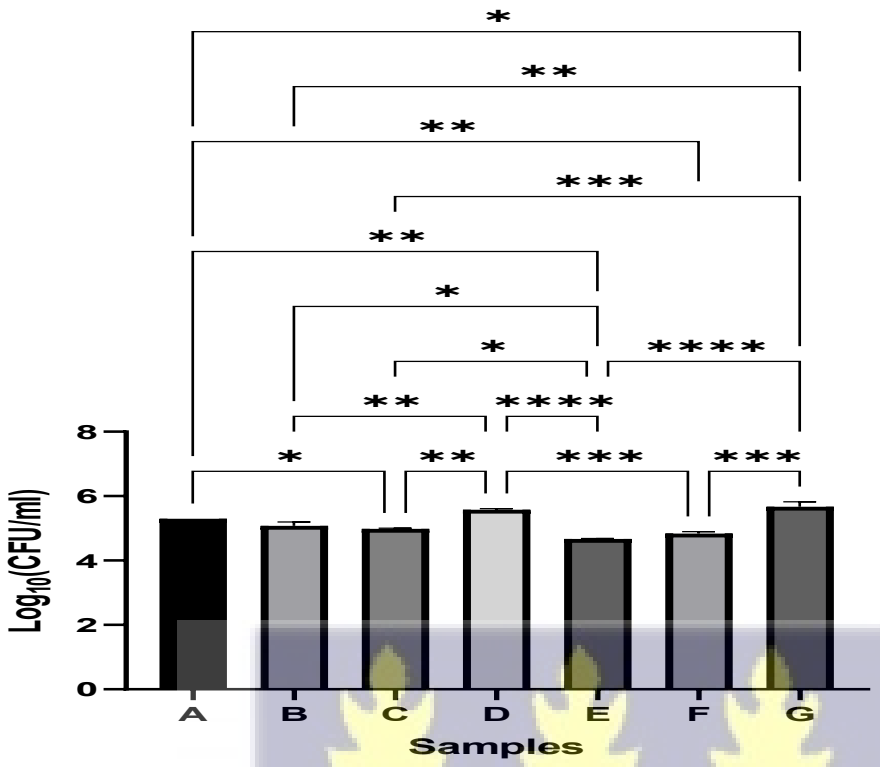


Figure 5.0a: Quantitative analysis of bacterial load (Log_{10} CFU/ml) across different *Brukina* samples.

Each bar represents the mean ± SEM of Log_{10} CFU/ml from two independent replicates. Statistical significance was evaluated using one-way ANOVA followed by Bonferroni's multiple comparison test. Asterisks indicate significant differences relative to group G or between designated groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Group G showed the highest bacterial load, whereas groups A through F exhibited sequential declines, indicating differential antimicrobial activity.

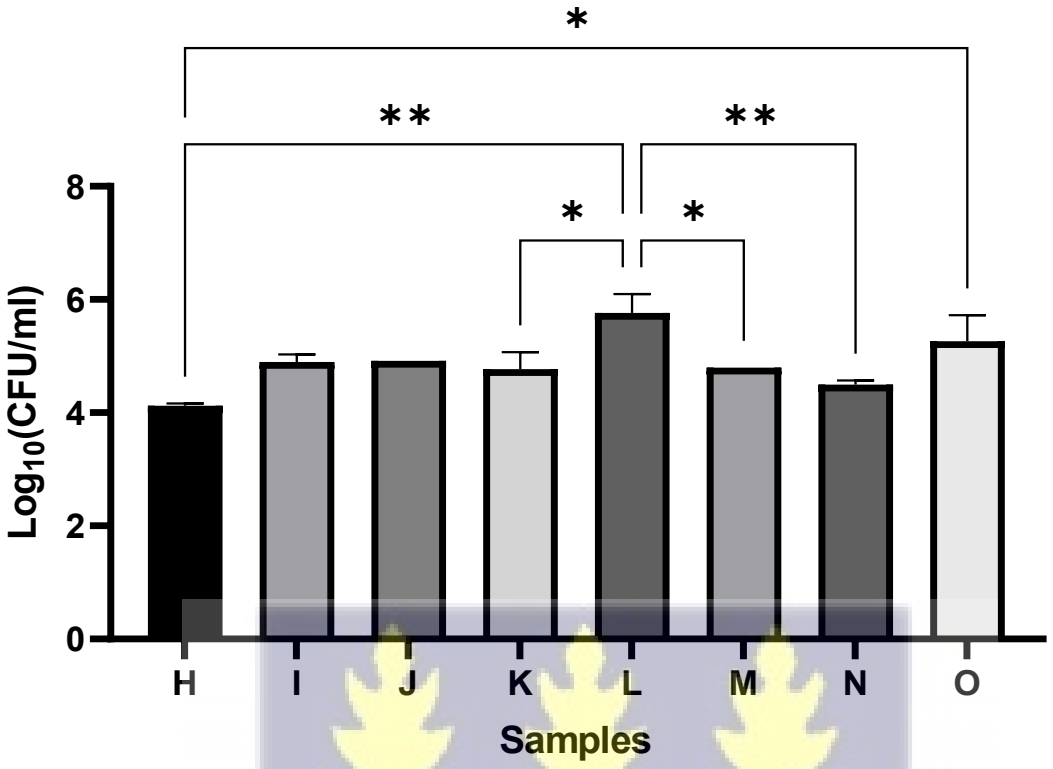


Figure 5.0b: Quantitative analysis of bacterial load (Log_{10} CFU/ml) across different *Brukina* samples.

The bar graph represents the mean Log10(CFU/ml) ± standard error for each sample (H-O). Statistical comparisons were conducted using one-way ANOVA followed by appropriate post-hoc testing. Significant reductions in bacterial load were observed when comparing sample L to samples H ($\mathbf{p} < 0.01$), I, J, and K ($p < 0.05$), as well as significant increases relative to samples M, N, and O ($\mathbf{p} < 0.01$). Asterisks indicate levels of statistical significance ($p < 0.05$, $\mathbf{p} < 0.01$). These results highlight the markedly elevated bacterial CFU in sample L, suggesting potential biological or environmental factors influencing microbial proliferation under those conditions.

4.4 Acid Tolerance at pH 2.0, 3.0 & 7.0

4.4.1 Acid Tolerance at pH 2.0.

Figure 5.1a and Fig 5.1b show the acid tolerance capabilities of 22 *Lactobacillus* strains isolated from a millet-based milk beverage (*Brukina*) following exposure to pH 2.0 over a 3-hour period. Most strains demonstrated substantial resilience, maintaining relatively high OD_{600} values throughout the time course, indicative of strong acid tolerance and potential for survival in gastric-like conditions. However, strains A12III, A2II, A4II, A13III, A8II, A8III, and A1I exhibited only short-term survival, with OD_{600} values declining sharply after 1 hour and remaining low at 2 and 3 hours. This trend suggests limited acid resistance, likely due to insufficient stress-response mechanisms or structural vulnerabilities under low pH.

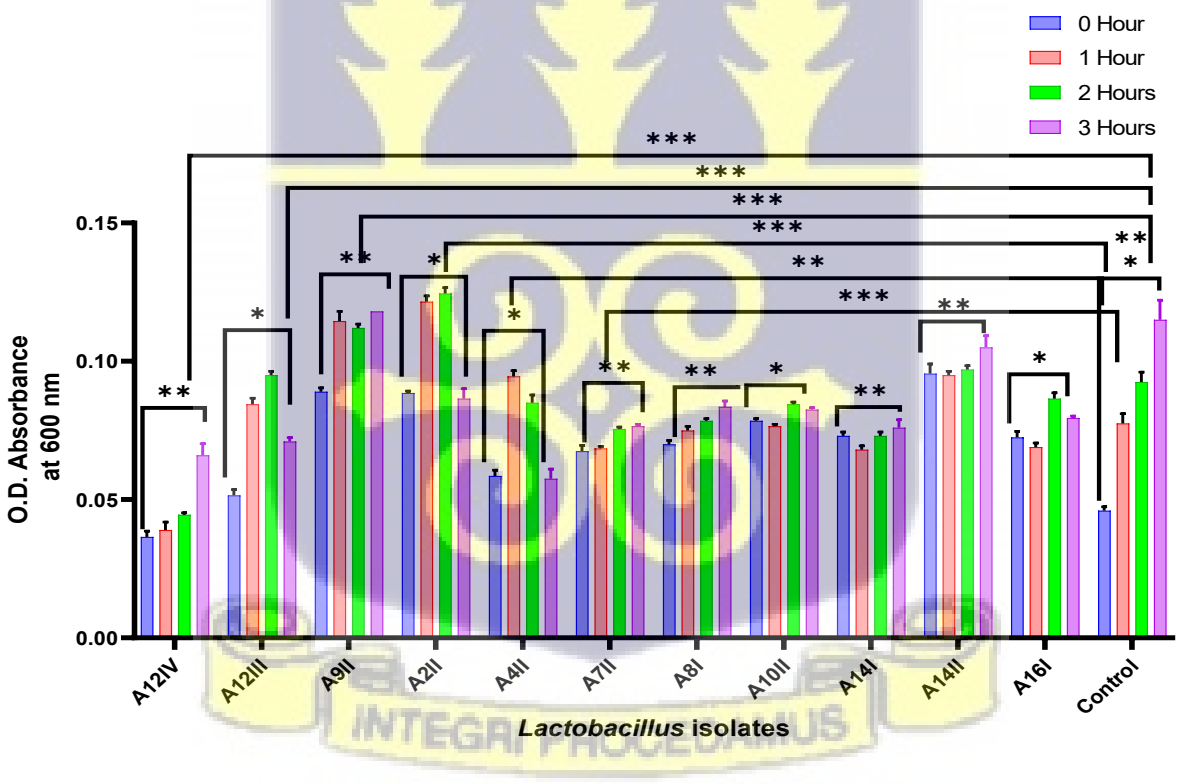


Figure 5.1a: Acid tolerance of *Lactobacillus* isolates at pH 2.0 over time (3 h).

Bar graph depicts the optical density (OD_{600}) of *Lactobacillus* isolates after exposure to acidic conditions (pH 2.0) for 0 h (blue), 1 h (red), 2 h (green), and 3 h (purple). Each isolate was compared to a control using two-way ANOVA followed by Dunnett's multiple comparisons test. Significant differences in acid tolerance were observed ($p < 0.0001$). Data represent mean OD_{600} values from two independent replicates, with color-coded bars denoting time-dependent survivability under low pH stress. Asterisks indicate statistically significant differences between the test and control isolates, as determined by Dunnett's post hoc test groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

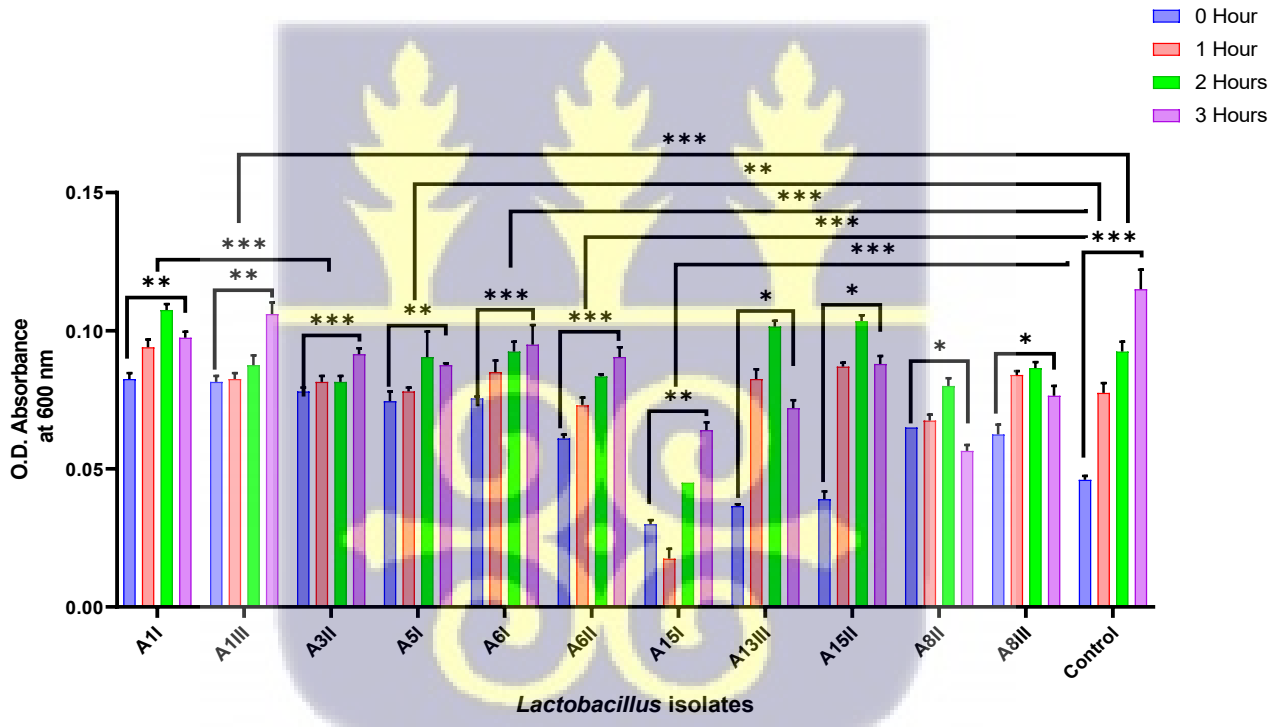


Figure 5.1b: Low pH survival of *Lactobacillus* isolates over a 3-hour period at pH 2.0.

Bar graph displays the optical density at 600 nm (OD_{600}) for eleven *Lactobacillus* isolates (A1I to A8III) following exposure to acidic conditions at pH 2.0 for 0 h (blue), 1 h (red), 2 h (green), and 3 h (purple). A two-way ANOVA followed by Dunnett's multiple comparisons test was performed

to compare each isolate against the control microbe (*Lactobacillus casei*). Significant increases in OD_{600} values were observed for all isolates ($\mathbf{p} < 0.0001$), indicating robust acid tolerance across isolates. Data represent mean values from two independent experiments; error bars reflect standard deviation. Asterisks indicate statistically significant differences between the test and control isolates, as determined by Dunnett's post hoc test groups (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

4.4.2 Acid Tolerance at pH 3.0

Figures 6.1a and 6.1b present the acid tolerance profiles of 22 *Lactobacillus* strains isolated from the millet-based milk beverage, *Brukina*. The majority of isolates exhibited a strong capacity to survive in a moderately acidic environment (pH 3.0), consistent with their potential resilience during gastrointestinal transit. In contrast, a small subset specifically strains A10II, A15II, A8II, A3II, and A15I demonstrated markedly reduced survival, indicating they could only withstand acidic exposure for a limited duration. Among all strains, A2II recorded the highest optical density after three hours, reflecting superior persistence under acidic stress, whereas A12III showed the lowest initial optical density prior to acid exposure, suggestive of slower baseline growth. These differences likely reflect inherent variations in growth kinetics and acid tolerance mechanisms among the *Lactobacillus* isolates.

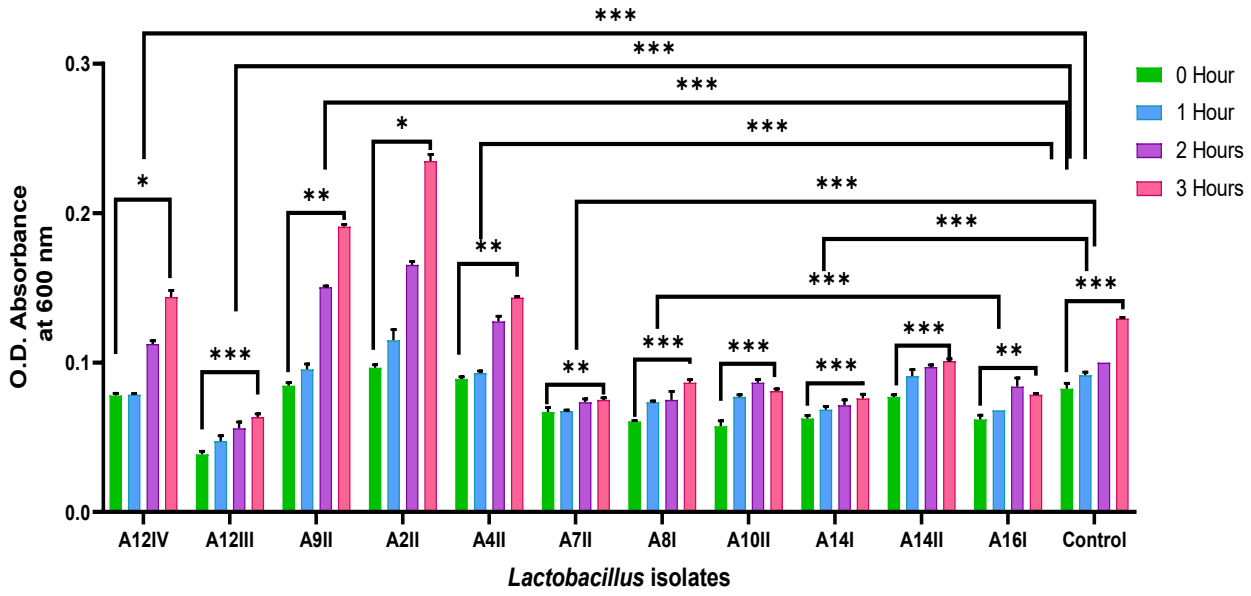


Figure 6.1a: Survival of *Lactobacillus* isolates under acidic conditions (pH 3.0) over time.

The bar graph illustrates the optical density (OD_{600}) of thirteen *Lactobacillus* isolates A12IV to A16I and a control strain (*Lactobacillus casei*) following exposure to pH 3.0 for 0 h (green), 1 h (blue), 2 h (purple), and 3 h (pink). Two-way ANOVA followed by Dunnett's multiple comparisons test was employed to assess significant differences between each isolate and the control. All isolates demonstrated statistically significant acid tolerance at pH 3.0 ($\mathbf{p} < 0.0001$), with survivability varying across time points. The data represent mean OD_{600} values from two biological replicates; error bars denote standard deviation. Asterisks indicate statistically significant differences between the test and control isolates, as determined by Dunnett's post hoc test groups (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

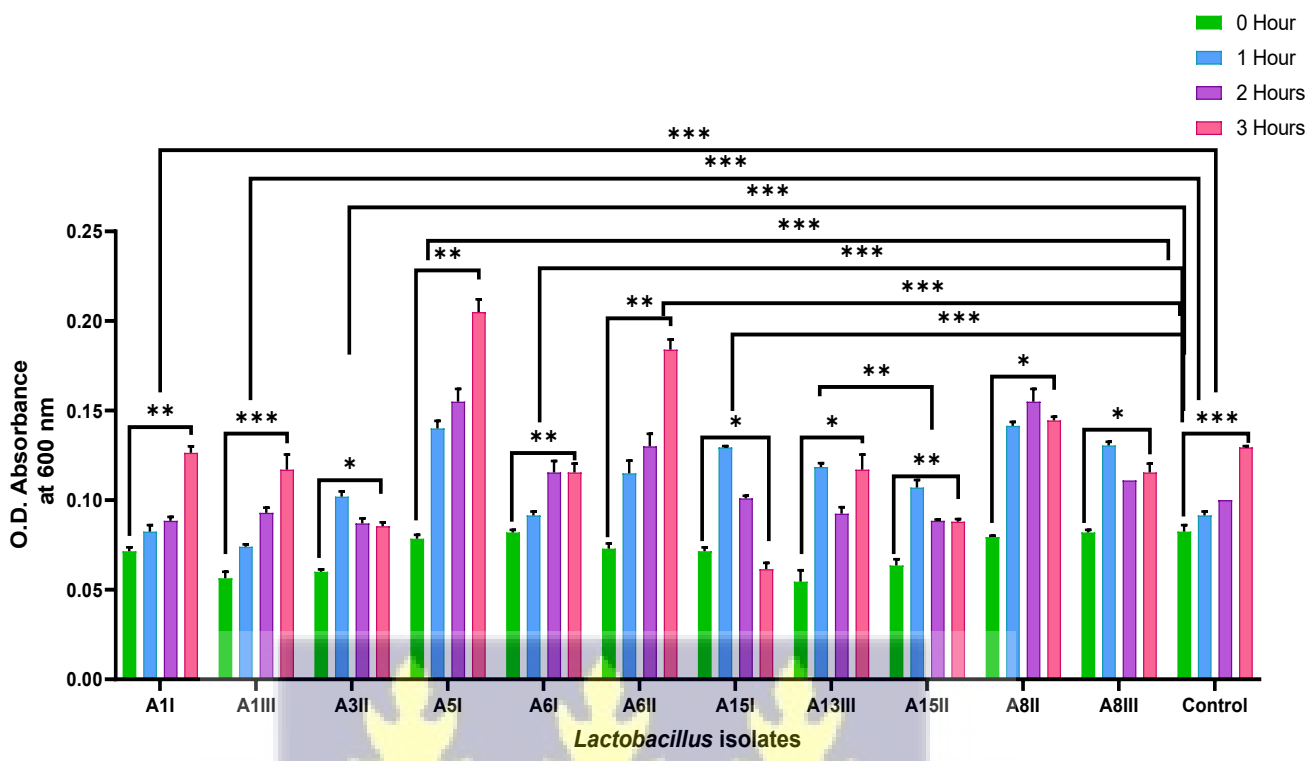


Figure 6.1b: Growth dynamics of *Lactobacillus* isolates assessed via optical density measurements at 600 nm over a 3-hour incubation period.

Bars represent mean O.D. values at four designated time points: 0 hours (green), 1 hour (blue), 2 hours (purple), and 3 hours (pink) for isolates A1I, A1III, A3II, A5I, A6I, A6II, A15I, A13III, A15II, A8II, A8III, and a control (*Lactobacillus casei*). Each measurement reflects the mean of duplicate samples (n = 2). Error bars indicate standard deviations. Statistical analysis was performed using repeated measures ANOVA followed by Tukey's post-hoc test. Significant differences were observed across time points and among isolates ($p < 0.0001$), indicating distinct growth profiles and metabolic activity under standardized conditions. Asterisks indicate statistically significant differences between the test and control isolates, as determined by Dunnett's post hoc test groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

4.4.3 Acid Tolerance at pH 7.0

The temporal growth trends of *Lactobacillus* isolate over a 3-hour period, as shown in Figure 7.1a, reveal several distinct patterns. Most isolates, including A12IV, A12III, A9II, A2III, and A4II, exhibited a gradual increase in OD_{600} from 0 to 3 hours, indicating progressive growth. Isolates A7II, A8I, and A14I showed moderate increases in absorbance over time. In contrast, isolates A10II, A14I, and A16I displayed minimal changes in OD_{600}, suggesting limited growth. Isolate A4II demonstrated a significant reduction in optical density as time increased. The control group consistently recorded the highest OD_{600} values at each time point, particularly at 3 hours. Statistical significance markers (*, **, ***) highlight notable differences in growth performance between isolates and across time intervals.

Over the 3-hour incubation period, all *Lactobacillus* isolates demonstrated time-dependent increases in optical density (OD_{600}), indicative of active growth (Figure 7.1b). Isolates A1I, A1III, A3II, A5I, and A6I exhibited the most pronounced growth, with statistically significant differences observed between time points (** to ****). Moderate increases were noted for A6II, A15I, and A13III, while A8II showed comparatively lower OD_{600} values, suggesting slower growth kinetics. The control consistently recorded the highest OD_{600} values across all time points, serving as a benchmark for growth performance. These trends clearly show the variability in growth dynamics among the isolates.

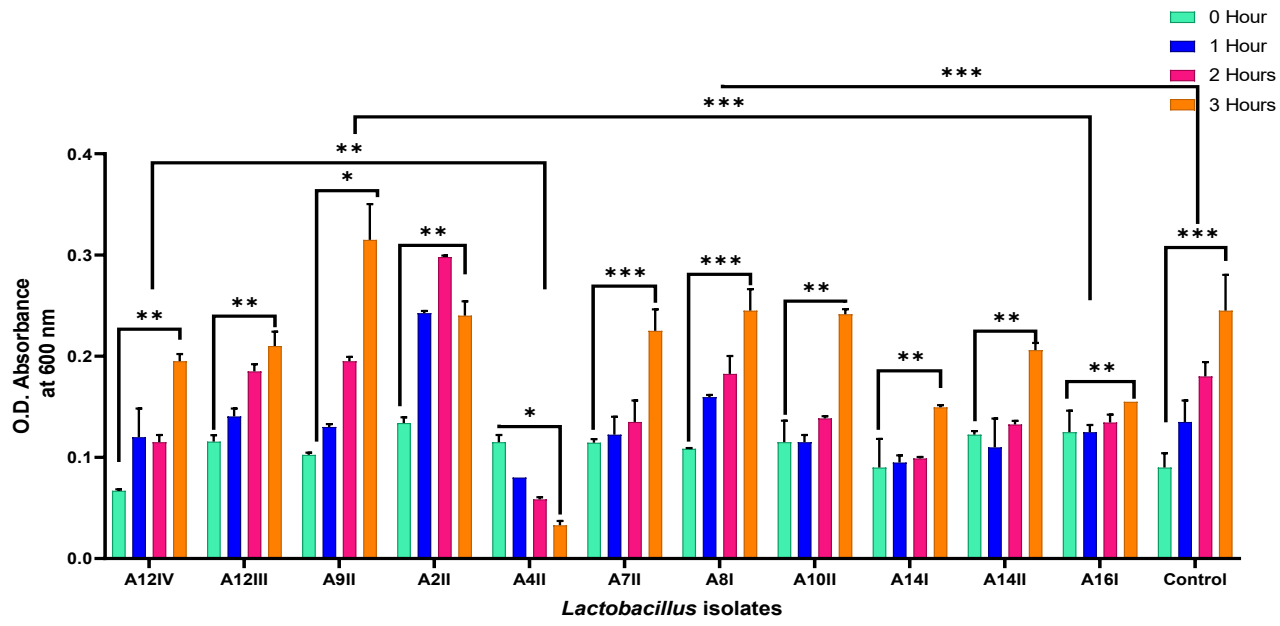


Figure 7.1a**:** Temporal growth trends of *Lactobacillus* isolates assessed by optical density (O.D.) measurements at 600 nm over 3 hours.

Bars represent mean O.D. values for isolates A12IV, A12III, A9II, A2II, A4II, A7II, A8I, A10II, A14I, A14II, A16I, and a control sample (*Lactobacillus casei*), recorded at 0 hours (green), 1 hour (blue), 2 hours (purple), and 3 hours (pink). Each data point reflects the average of duplicate measurements. Error bars indicate standard deviations. Differences in growth over time were analyzed using repeated measures ANOVA, followed by Tukey's post-hoc test to determine pairwise significance. The figure captures distinct growth trajectories among isolates, with progressive increases in absorbance suggesting differential metabolic activity and proliferation rates. Asterisks indicate statistically significant differences between the test and control isolates, as determined by Dunnett's post hoc test groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

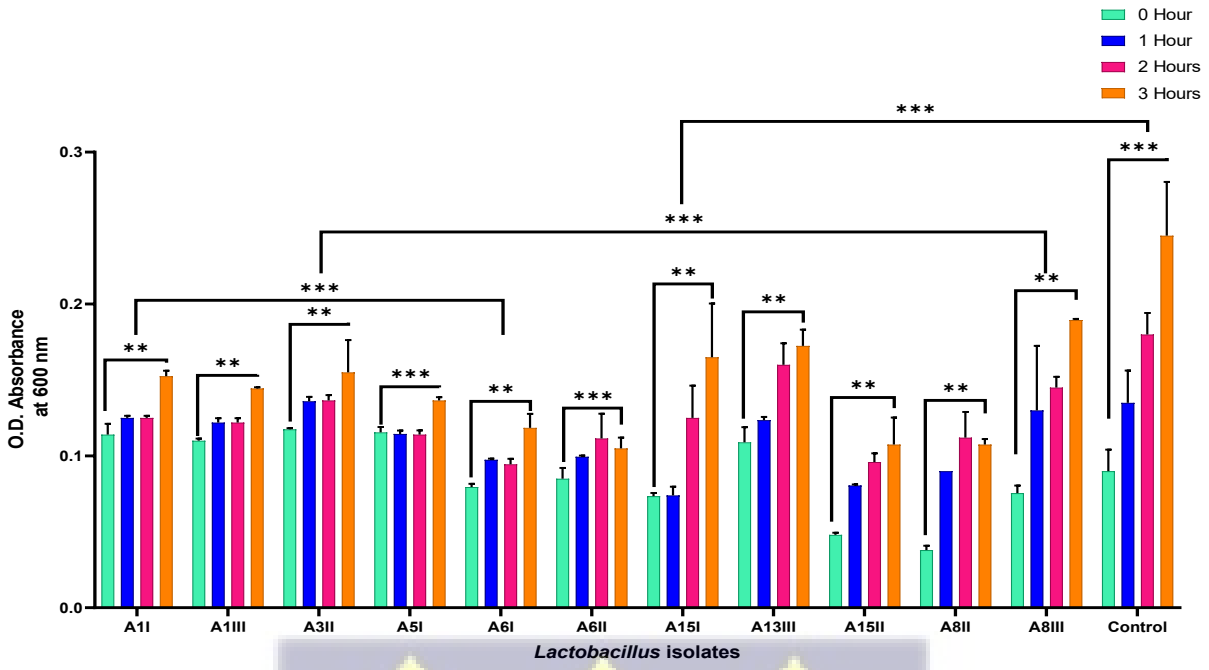


Figure 7.1b: Temporal changes in optical density (O.D.) at 600 nm for *Lactobacillus* isolates monitored over a 3-hour incubation period.

Bars represent mean O.D. values for isolates A1I, A1III, A3II, A5I, A6I, A6II, A15I, A13III, A15III, A8II, A8III, and a control sample, recorded at 0 hours (green), 1 hour (blue), 2 hours (purple), and 3 hours (pink). Each data point reflects the average of duplicate measurements. Error bars indicate standard deviations. Growth trends were statistically evaluated using repeated measures ANOVA, followed by Tukey's post-hoc test for pairwise comparisons. The figure illustrates differential growth kinetics among isolates, with progressive increases in absorbance indicating metabolic activity and bacterial proliferation. Asterisks indicate statistically significant differences between the test and control isolates, as determined by Dunnett's post hoc test groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

4.5 Summary of Acid Tolerance at pH 2.0, 3.0 & 7.0

As shown in Figure 8.0, vast majority of *Lactobacillus* isolates (77%) exhibited tolerance across all tested pH levels (2.0, 3.0, and 7.0), indicating strong acid resilience and potential for survival in harsh gastrointestinal conditions. In contrast, 23% of the isolates were intolerant to all tested pH levels. This distribution illustrates the predominance of acid-tolerant strains within the food matrix and showcase their suitability for probiotic applications.

Figure 8.0: Distribution of acid tolerance among *Lactobacillus* isolates assessed at pH 2.0, 3.0, and 7.0.

The pie chart depicts the proportion of isolates tolerant to all tested pH levels (blue, 77%) versus that intolerant to all pH levels (red, 23%). Acid tolerance was evaluated using duplicate measurements across 22 isolates. Statistical significance between tolerant and intolerant groups

was determined using a chi-square test for independence. The figure highlights robust survivability in acidic environments for the majority of isolates, underscoring their potential for probiotic applications and gastrointestinal resilience.

4.5.1 Tolerance of *Lactobacillus* strains to Bile Salts

All *Lactobacillus* isolates demonstrated varying degrees of tolerance to 0.3% bile salts over the 3-hour incubation period, as evidenced by progressive increases in OD_{600} values (Figure 9.1a). Isolates A12IV, A12III, A9III, A2III, and A4III showed marked growth, with OD_{600} values rising steadily from 0 to 3 hours, indicating strong bile salt tolerance. Moderate growth trends were observed for A14I, A8I, and A10II, while isolates A7II and A14II exhibited relatively lower OD_{600} values. The control consistently recorded the highest OD_{600} values across all time points.

In figure 9.1b, it was observed that, isolates A1I, A1III, A3II and A5I demonstrated the highest growth trends, with OD_{600} values increasing consistently over the 3-hour periods. Moderate tolerance was observed in isolates A6II, A15II, A13III, and A15I, while A8II, A8III, and A16II showed comparatively lower OD_{600} values, suggesting reduced bile salt tolerance. Isolate A6I demonstrated a significant decrease in optical density width respect to time. The control maintained the highest OD_{600} readings across all time points.

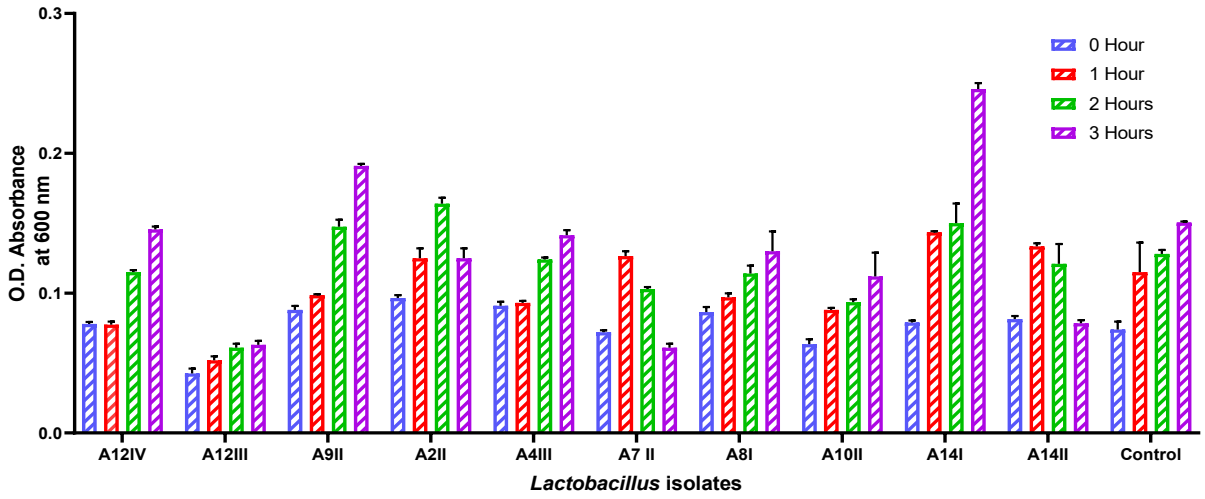


Figure 9.1a: Tolerance of *Lactobacillus* isolates to 0.3% bile salts assessed via optical density (O.D.) measurements at 600 nm over a 3-hour incubation period.

Bars represent mean O.D. values for isolates A12IV, A12III, A9II, A2II, A4III, A7II, A8I, A10II, A14I, A14II, and a control sample, recorded at 0 hours (blue), 1 hour (red), 2 hours (green), and 3 hours (purple). Each data point reflects the average of duplicate measurements. Error bars denote standard deviations. Statistical differences across time points and isolates were analyzed using repeated measures ANOVA followed by Tukey's post-hoc test. The figure highlights differential bile salt tolerance among isolates, with varying growth kinetics indicative of potential probiotic resilience in gastrointestinal environments.

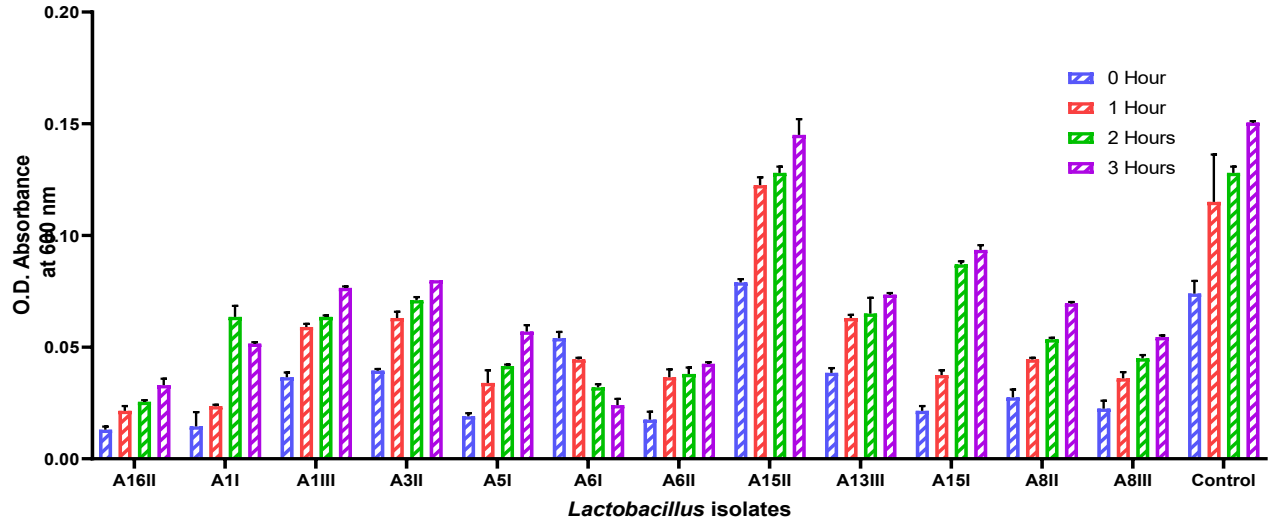


Figure 9.1b: Assessment of *Lactobacillus* isolate tolerance to 0.3% bile salts via optical density (O.D.) measurements at 600 nm over a 3-hour incubation period.

Bars depict mean O.D. values for isolates A16II, A1I, A1III, A3II, A5I, A6I, A6II, A15II, A13III, A15I, A8II, A8III, and a control sample, measured at 0 hours (blue), 1 hour (red), 2 hours (green), and 3 hours (purple). Each data point represents the average of duplicate measurements, and error bars indicate standard deviations. Statistical analysis was performed using repeated measures ANOVA to evaluate time-dependent differences in growth, followed by Tukey's post-hoc test for pairwise comparisons. The figure illustrates varying bile salt tolerance across isolates, with progressive increases in absorbance reflecting differential growth kinetics and survivability under bile salt stress conditions.

4.6 Antibiotic Susceptibility Profile of *Lactobacillus* strains

As illustrated in Figure 10.0, the antibiotic susceptibility profiles of the isolated *Lactobacillus* strains show distinct patterns of resistance and sensitivity across the tested antibiotics. Most isolates exhibited susceptibility to erythromycin, clindamycin, and ampicillin, indicating potential compatibility with commonly used therapeutic agents. Resistance was frequently observed against vancomycin, streptomycin, and gentamycin, with several isolates including A5I, A16I, A6I, and A10I showing consistent resistance across multiple antibiotics. Intermediate responses were noted for neomycin and kanamycin in a subset of strains, such as A12IV and A13I. Ciprofloxacin elicited variable responses, with some isolates demonstrating susceptibility while others were resistant or intermediate.

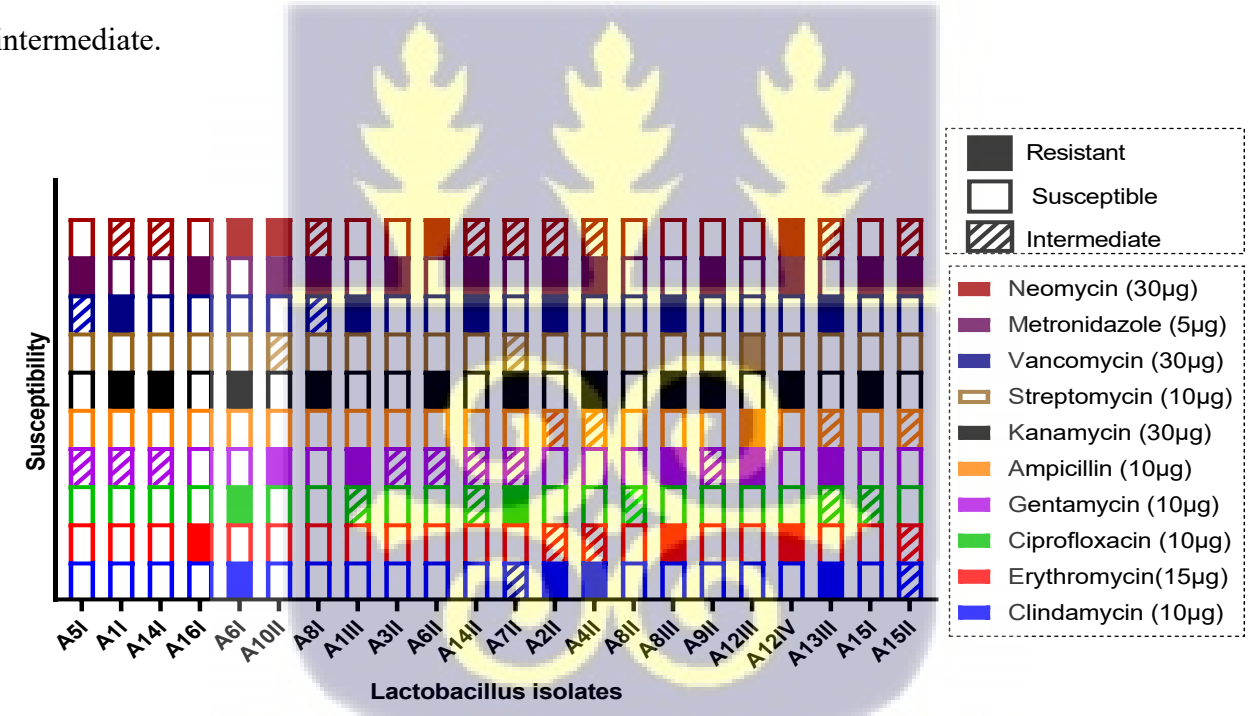


Fig 10.0; Antibiotic susceptibility profile of isolated *Lactobacillus* strains.

The figure presents the antibiotic susceptibility patterns of nine *Lactobacillus* strains (A51–I51) against ten antibiotics commonly evaluated for Gram-positive bacterial resistance: Neomycin (30 µg), Metronidazole (5 µg), Vancomycin (30 µg), Streptomycin (10 µg), Kanamycin (30 µg),

Ampicillin (10 µg), Gentamycin (10 µg), Ciprofloxacin (10 µg), Erythromycin (15 µg), and Clindamycin (10 µg). Each antibiotic is uniquely color-coded, with resistance categories indicated using distinct shading: resistant (solid black), intermediate (diagonal stripes), and susceptible (white). Duplicate measurements were performed for each strain-antibiotic combination to ensure reproducibility and data integrity. The bar heights represent the mean values from these replicates. A significance threshold of $p < 0.05$ was applied. Patterns observed suggest strain-dependent variability in resistance, with notable multidrug resistance in some isolates. These findings inform the potential clinical implications of *Lactobacillus* antibiotic resistance and guide therapeutic strategy development for probiotic applications.

4.7.1 Antimicrobial Activity of Lactic acid bacteria

Vast majority of selected isolates, apart from two (A7II and A10II) showed effective antibacterial properties against two or more enteric pathogens (Figs 12.1a, 12.1b). Strains A4II, A8II, A8III and A2II displayed bacteriostatic activity against four different harmful microorganisms (*Escherichia coli NCTC 11954 TEM 1, K. pneumoniae NTC 13368, Salmonella typhi ACT 1, and Staphylococcus aureus*). A13III and A15I inhibited the growth of all pathogens tested. Strain A10II specifically inhibited the growth of *Staphylococcus aureus*, while the remaining strains exhibited bacteriostatic effects against two or more pathogenic microorganisms (*Escherichia coli NCTC 11954 TEM 1, K. pneumoniae NTC 13368, Salmonella typhi ACT 1, and Staphylococcus aureus*).

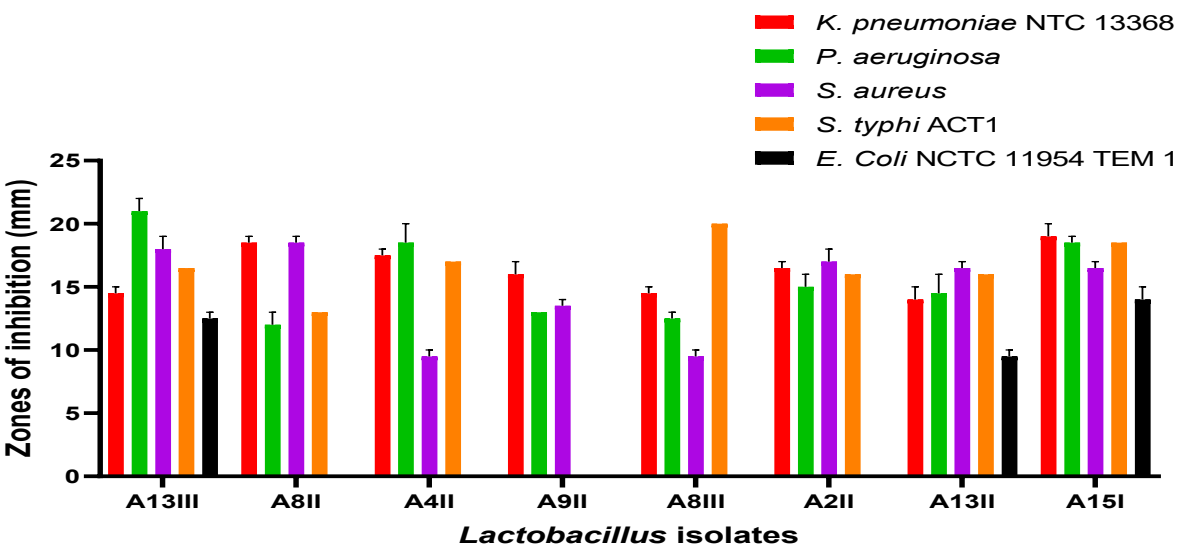


Figure 12.1a: Antimicrobial activity of isolated *Lactobacillus* strains.

The figure illustrates the mean zones of inhibition (in millimeters) produced by eight *Lactobacillus* isolates (A13III, A8II, A4III, A9II, A8III, A2II, A13II, A15I) against five clinically relevant pathogenic strains: *Klebsiella pneumoniae* NCTC 13368 (red), *Pseudomonas aeruginosa* (green), *Staphylococcus aureus* (purple), *Salmonella Typhi* ACT1 (orange), and *Escherichia coli* NCTC 11954 TEM-1 (black). Each isolate-pathogen interaction was assessed in duplicate, and the bars represent the mean zone diameter, with error bars indicating standard deviation (SD). Statistical analysis was conducted using two-way ANOVA to evaluate differences in inhibitory activity across isolates and pathogen types. Tukey's post-hoc test was performed to identify statistically significant pairwise differences between groups, with a significance threshold of $p < 0.05$. Distinct color coding was used to differentiate pathogens, enabling visual comparison of strain-specific antimicrobial profiles.

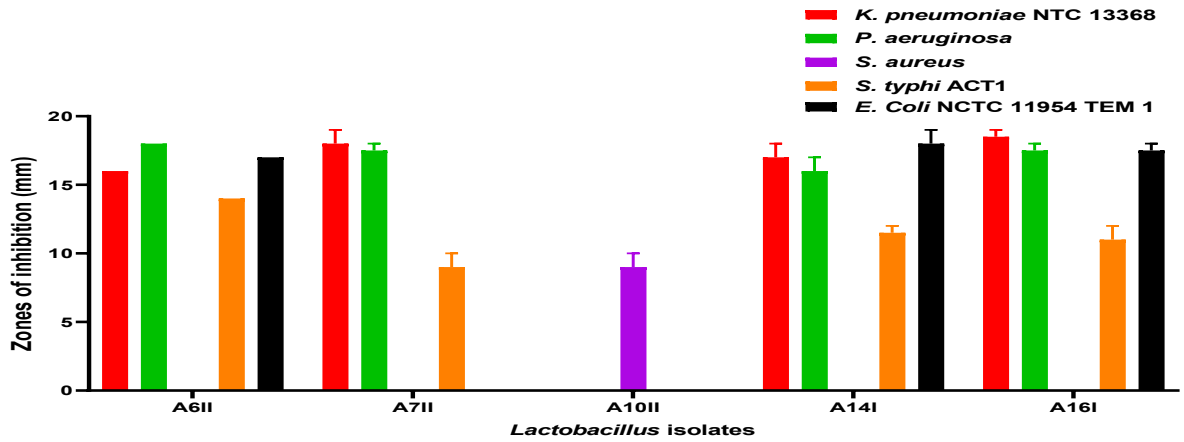


Figure 12.1b. Comparative antimicrobial activity of selected *Lactobacillus* isolates against five pathogenic strains.

This figure depicts the mean diameters of inhibition zones (in mm) exhibited by five *Lactobacillus* isolates (A6II, A7II, A10II, A14I, and A16I) against five clinically significant bacterial pathogens: *Klebsiella pneumoniae* NCTC 13368 (red), *Pseudomonas aeruginosa* (purple), *Staphylococcus aureus* (green), *Salmonella Typhi* ACT1 (orange), and *Escherichia coli* NCTC 11954 TEM-1 (black). Each bar represents the average result of duplicate measurements, with error bars indicating the corresponding standard deviation (SD), ensuring precision and reproducibility across assays. Statistical evaluation was conducted using two-way analysis of variance (ANOVA) to determine significant differences in antimicrobial efficacy among the *Lactobacillus* isolates across pathogen types. Post-hoc comparisons were performed using Tukey's test, with a significance threshold of $p < 0.05$. The analysis revealed isolate-specific antimicrobial activity, including differential potency against Gram-negative and Gram-positive organisms.

4.7.1 Antimicrobial activity of Lactic acid bacteria using cell free Supernatant

As shown in Figure 13.0, the cell-free supernatants (CFS) from *Lactobacillus* isolates exhibited varying degrees of antimicrobial activity against five pathogenic bacteria: *Klebsiella pneumoniae* NTC 13368, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi* ACT1, and *Escherichia coli* NCTC 11954 TEM 1. Isolates such as A15I and A16I demonstrated broad-spectrum inhibitory effects, with consistently large zones of inhibition across all tested pathogens, particularly against *S. aureus*, indicating strong anti-Gram-positive potential. In contrast, activity against *P. aeruginosa* was generally limited, reflecting its intrinsic resistance mechanisms. The inhibitory effects against *E. coli* and *K. pneumoniae* were variable, with some isolates like A4II and A15I showing moderate activity. Among all, A15I stood out for its consistent and potent antimicrobial performance, suggesting its promise as a probiotic candidate.

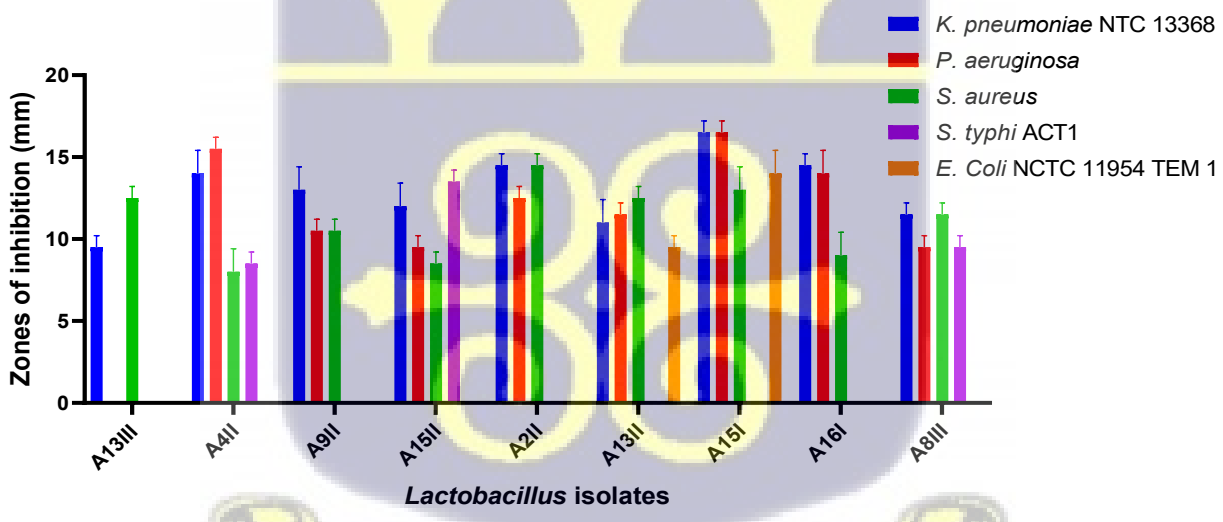


Figure 13.0: Antimicrobial activity of cell-free supernatants from *Lactobacillus* isolates against selected pathogenic bacteria.

Zones of inhibition (mm) were measured for nine LAB isolates (A13III, A4II, A9II, A15II, A21I, A13II, A15I, A16I, A8III) against five pathogens: *Klebsiella pneumoniae* NTC 13368 (blue),

Pseudomonas aeruginosa (red), *Staphylococcus aureus* (green), *Salmonella typhi* ACT1 (purple), and *Escherichia coli* NCTC 11954 TEM-1 (orange). Bars represent the mean ± SD from two independent biological replicates. Statistical analysis was performed using two-way ANOVA followed by Tukey's post-hoc test to compare inhibition zones across *Lactobacillus* isolates for each pathogen ($p < 0.05$ considered significant). The data reveal isolate-specific antimicrobial profiles, with certain *Lactobacillus* strains exhibiting broad-spectrum activity.

4.7.1 Growth Patterns of Lactic acid bacteria with cell free supernatant activity

Distinct growth kinetics among nine lactic acid bacteria (LAB) strains, based on OD_{600} measurements taken over a 24-hour period was observed in figure 14.0. Strain A15I recorded the shortest generation time (72 minutes), indicating the most rapid proliferation, closely followed by A4II (84 min), both of which suggest robust metabolic activity. Strains A15II (102 min), A2II (104 min), A13II (118 min), A9II (128 min), and A16I (132 min) exhibited moderate growth rates, reflecting balanced replication dynamics. In contrast, A8III and A13III showed the longest generation times (168 and 174 minutes, respectively), pointing to slower cell division and reduced growth efficiency.

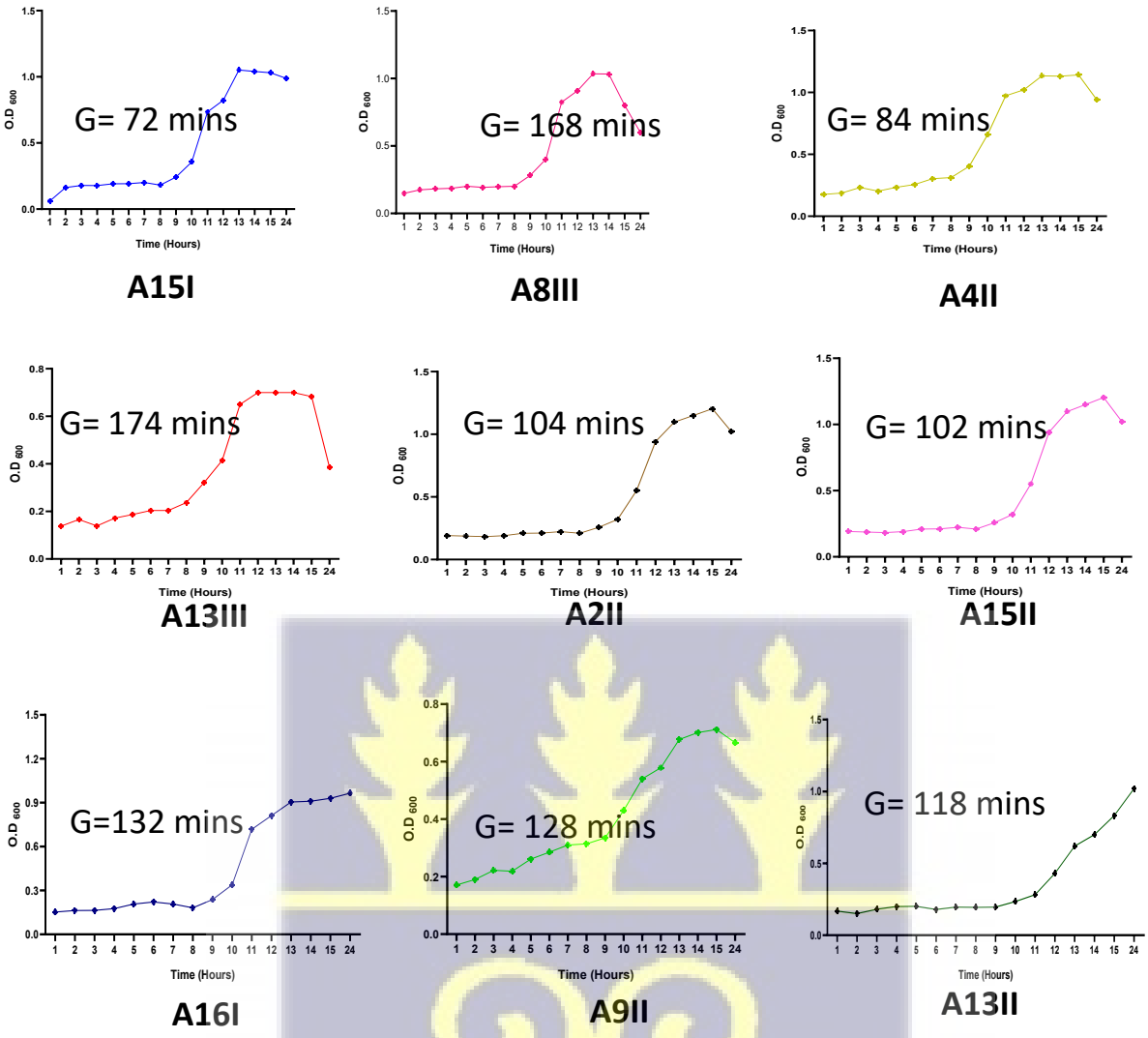


Figure 14.0. Growth kinetics of lactic acid bacteria (LAB) strains based on optical density measurements.

Growth curves of nine LAB isolates (A15I, A8III, A4II, A13III, A2II, A15II, A16I, A9II, A13II) were generated by monitoring optical density (OD_{600}) over a 24-hour period. Growth rates (G, in minutes) were calculated from the exponential phase using linear regression to estimate doubling time. Each data point represents the mean OD from two independent biological replicates, with error bars indicating standard deviation (±SD). Statistical comparison of growth rates across strains was performed using one-way ANOVA, followed by Tukey's multiple comparison test ($p < 0.05$

considered significant). Strain A15I exhibited the fastest growth (G = 72 mins), while A13III showed the slowest (G = 174 mins), highlighting inter-strain variability in metabolic activity and potential for biotechnological applications.

4.7.2 Molecular Identification of *Lactobacillus* strains

Clear, distinct bands corresponding to approximately 1500 bp were observed in all isolate lanes (01–10 and 11–20), confirming successful amplification of the target gene region as observed in figure 15.0. The presence of bands in the positive control (PC) lanes and their absence in the negative control (NC) lanes validate the specificity and reliability of the PCR conditions. Lanes labeled L and LL represent the molecular weight markers, which provided reference points for estimating fragment sizes.

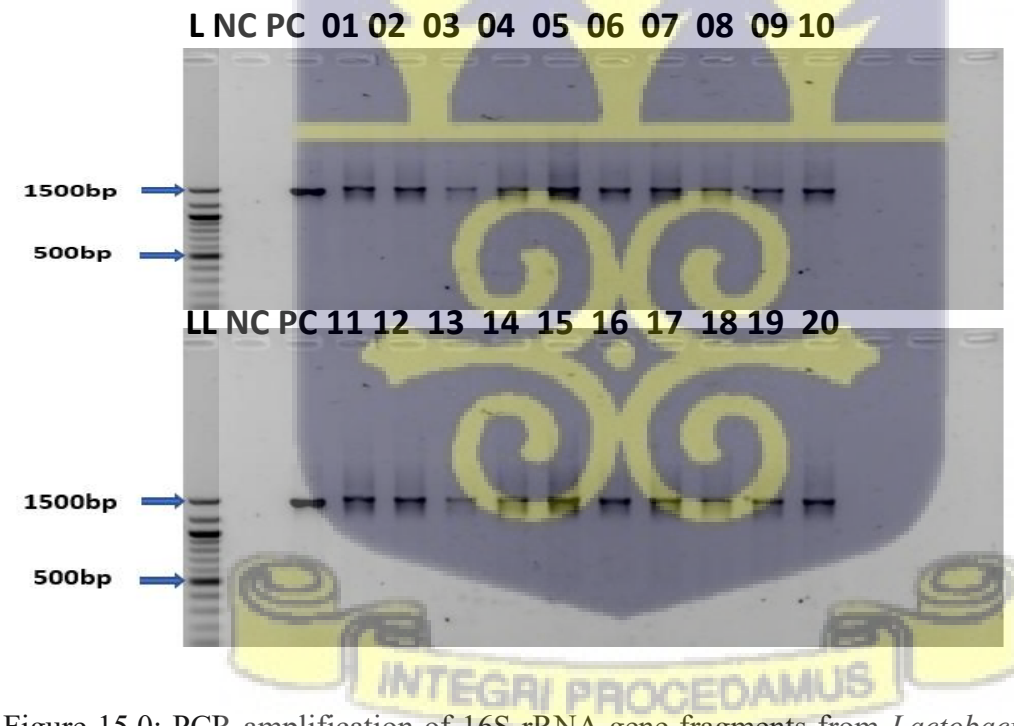


Figure 15.0: PCR amplification of 16S rRNA gene fragments from *Lactobacillus* isolates using universal primers 27F and 1492R.

Agarose gel electrophoresis showing PCR products from 20 *Lactobacillus* isolates (lanes 01–20), alongside a DNA ladder (L), negative control (NC), and positive control (PC). Bands at ~1500 bp confirm successful amplification of the 16S rRNA gene. Eleven isolates (lanes 01–11) yielded high-quality DNA suitable for Sanger sequencing. PCR reactions were performed in duplicate to ensure reproducibility. Band intensity and size were consistent across replicates, and no amplification was observed in the negative control. This confirms primer specificity and the integrity of the amplification protocol.

4.8 Concentrations of DNA samples

Quantification of PCR-amplified DNA samples showed concentrations ranging from 376.4 to 531.0 ng/µL, indicating sufficient yield for downstream applications such as sequencing. The A260/A280 purity ratios (1.52–1.94) suggested low levels of protein or phenol contamination, supporting the suitability of the samples for reliable molecular analysis.

Samples	ng/µl	A260/A280
1	485.6	1.67
2	401.4	1.88
3	531.0	1.56
4	437.5	1.94
5	495.5	1.81
6	440.7	1.52
7	501.1	1.71
8	488.3	1.84
9	463.3	1.79
10	414.9	1.91
11	502.0	1.81
12	472.2	1.87
13	399.8	1.77
14	453.2	1.86
15	376.4	1.82
P	485.1	1.73

4.8.1 Molecular determination of *Lactobacillus* species diversity and relative abundance

As observed from figure 16.0, prominent amplification of the 16S rRNA gene was observed across all twenty Lactobacillus isolates, with distinct bands aligning near the 1500 bp marker, confirming successful PCR using universal primers 27F and 1492R. The gel image reveals consistent band intensity and positioning in lanes 1-8 and 09-15, indicating uniform amplification efficiency among the isolates. Positive control lanes (P) showed expected amplification, while negative controls (N) lacked visible bands, validating the specificity of the reaction conditions.

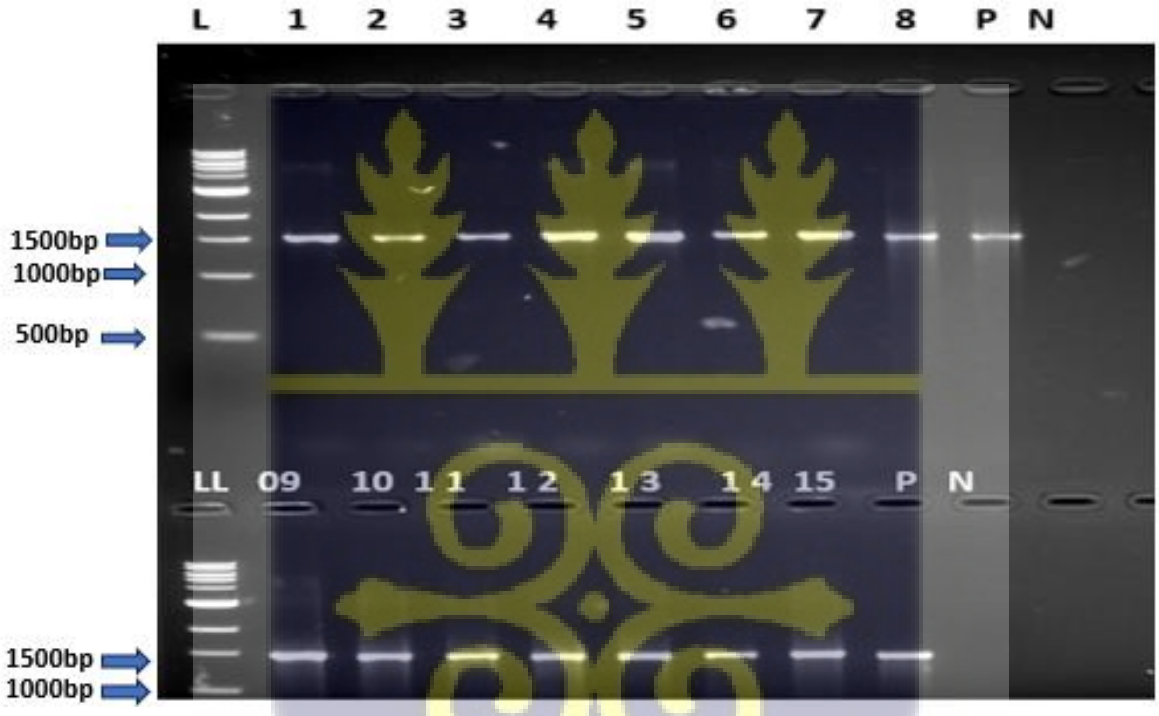


Figure 16.0: Agarose gel electrophoresis of PCR-amplified 16S rRNA gene from 20 *Lactobacillus* isolates using universal bacterial primers 27F and 1492R.

Distinct bands at ~1500 bp indicate successful amplification across several samples. L: DNA ladder; PC: positive control; NC: negative control. Eleven samples demonstrated adequate DNA yield and purity for downstream Sanger sequencing.

4.8.2 Molecular Identification

The eleven (11) bacterial isolates that produced reliable Sanger sequencing results were mainly characterized by their white or cream-colored pigmentation, irregular or circular shapes, and either flat or raised surfaces. Additionally, all of these isolates showed a positive result in Gram staining tests (Table 8)

Table 8: Characteristics of the colonial morphology of the isolated LAB.

Isolates	Species (16S rRNA gene analysis	Pigmentation	Shape	Elevation	Size	Gram Stain
A2 II	*Enterobacter hormaechei*	White	Irregular	Flat	Medium	+
A3 II	*Neobacillus fumarioli*	White	Circular	Convex	Medium	+
A5 I	Unidentified	White	Circular	Convex	Medium	+
A6 II	*Alishewanella agri*	White	Circular	Flat	Medium	+
A7 II	Unidentified	White	Circular	Flat	Small	+
A8 II	*Limosilactobacillus fermentum*	Creamy	Circular	Flat	Small	+
A8 III	*Limosilactobacillus fermentum*	White	Circular	Pulvinate	Large	+
A12 III	*Bacillus safensis*	Creamy	Irregular	Flat	Large	+
A13 III	*Faecalibaculum rodentia*	White	Circular	Flat	Large	+
A14II, A16I	*Limosilactobacillus fermentum*	White	Circular	Flat	Flat	+

4.8.3 Phylogenetic neighbors of the *Lactobacillus* strains

All the *Lactobacillus* strains had an E-value of 0.0 and a percentage identity greater or equal to 90%. The E-value indicated more significant alignments, suggesting a higher probability that the sequences share a common evolutionary origin. However, DNA sequencing revealed that not all the isolates belong to the *Lactobacillus* genus. Specifically, isolates A2II, A3II, A6II, A12III, and A13III were identified as non-Lactobacillus species.

Table 9: Phylogenetic neighbors of bacteria on the basis of similarity to the partial 16S rRNA

Sequence ID	E-value	Identity	Species (16S rRNA gene)	Accession
A2II	0.0	92.59%	*Enterobacter hormaechei*	ON989873.1
A3II	0.0	90%	*Neobacillus fumarioli* LMG 17489	NR_025370.1
A6II	0.0	98.81%	*Alishewanella agri* BL06	NR_116499
A8II	0.0	99.86%	*Limosilactobacillus fermentum* strain CIP 102980	NR_104927
A8III	0.0	99%	*Limosilactobacillus fermentum* strain CIP 102980	NR_104927
A12III	0.0	98.79%	*Bacillus safensis* DOK8	MK184539.1
A13III	0.0	100%	*Faecalibaculum rodentia* strain ALO17	NR_146011.1
A14II	0.0	99.85%	*Limosilactobacillus fermentum* strain NBRC 15885	NR_113335.1

A16I	0.0	99.85%	*Limosilactobacillus fermentum* strain NBRC 15885	NR_113335.1

4.8.4 Phylogenetic analysis

The phylogenetic relationships among the *Lactobacillus* isolates and reference bacterial species, as depicted in Figure 17.0, reveal distinct clustering patterns based on 16S rRNA gene sequences analyzed using PhyML. Isolates A6-I, A12-III, A2-II, A6-II, and A7-II clustered closely with reference strains of *Limosilactobacillus fermentum, L. reuteri*, and *L. gastricus*, supported by high branch confidence values, indicating strong phylogenetic affiliation. Isolates A5-I, A3-I, A4-II, and A8-II formed a separate clade that diverged from the main *Limosilactobacillus* group, suggesting potential taxonomic variation or unique lineage characteristics. Reference strains such as *Escherichia coli, Alishewanella aestuarii, Alishewanella fetalis*, and *Arrenibacterium perofaciens* served as outgroups, effectively rooting the tree and delineating the LAB clade. These trends show the genetic diversity among the *Brukina*-derived isolates and support their classification within the *Limosilactobacillus* genus, with implications for strain selection and functional characterization.

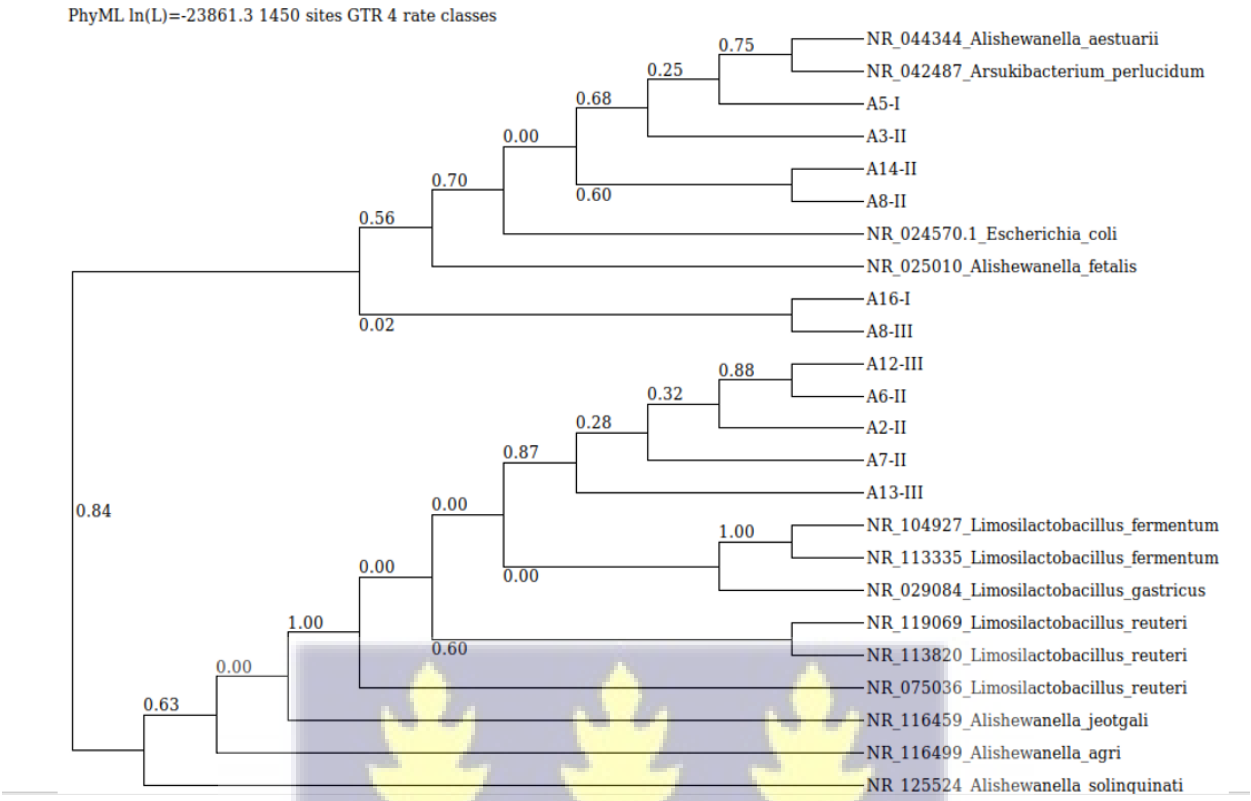
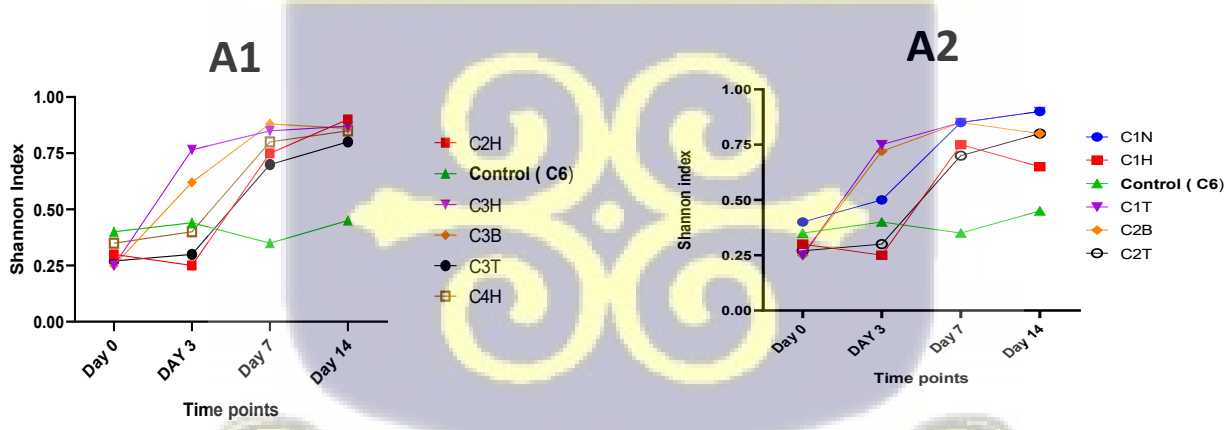


Figure 17.0: Phylogenetic tree of nucleotide sequence of 11 bacteria isolates from *Brukina*

Figure 17.0 shows the genetic relatedness of the eleven bacterial strains, namely, A2II, A3II, A5I, A6II, A7II, A8II, A8III, A12III, A13III, A14II, A16I. A total of thirteen reference strains used from GenBank were NR_125524, NR_024570.1, NR_025010, NR_029084, NR_042487, NR_044344, NR_075036, NR_104927, NR_113335, NR_113820, NR_116459, NR_116499 and NR_119069.

4.8.5 Percentage abundance and diversity of bacterial species in animal fecal samples

As shown in Figure 18.0, the Shannon Index trends across fecal samples from different groups reveal time-dependent increases in microbial diversity, with notable distinctions from the control group (C6). In Graph A1, groups C2H, C3H, and C4H exhibited progressive increases in diversity from Day 0 to Day 14, suggesting a positive impact of treatment, while C3B and C3T showed moderate gains. Graph A2 highlights marked diversity increases in C1H, C1T, and C2T, particularly by Day 14, whereas C1N and C2B remained relatively unchanged. In Graph A3, C5H and C5T demonstrated the most pronounced increases in Shannon Index, indicating strong microbial enrichment, while C4T, C4B, and C5B showed moderate shifts, and C5N remained variable. Across all graphs, the control group (C6) maintained a stable Shannon Index, reinforcing its role as a baseline and illustrating the influence of experimental treatments on gut microbiota diversity.



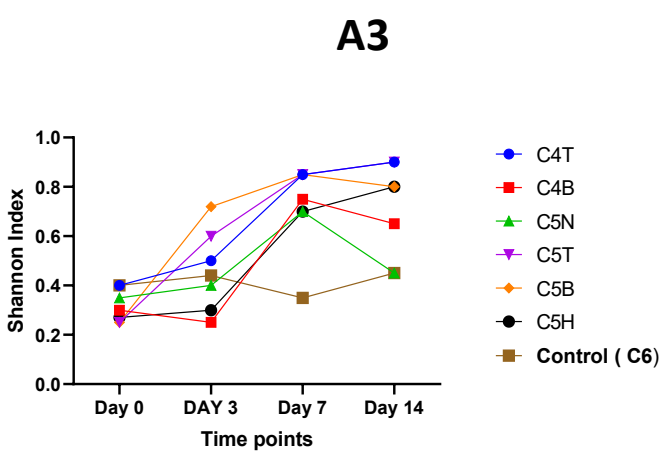


Figure 18.0: Percentage Abundance and Diversity of bacterial species in animal fecal samples.

The figures 18.0 (A1, A2, and A3) depict the variations in probiotic presence and diversity in human fecal samples taken at different intervals after consuming fermented millet-based milk beverage (*Brukina*) for 14 days. In most cases, the microbial abundance and diversity rose significantly as the duration of consumption increased. However, the control sample (C6) showed consistent levels of abundance and diversity throughout a 14-day period. Samples C1H and C2B experienced an initial increase in microbial prevalence and diversity from day 1 to day 7, followed by a decrease until day 14. Samples taken on day 0 displayed the lowest microbial abundance and diversity, as per the Shannon and Simpsons indices analysis.

4.8.6 Relative abundance of bacterial species in human fecal samples

Figure 19.0 illustrates the temporal dynamics of bacterial diversity in human fecal samples from two participant groups over a 14-day period, as measured by the Shannon Index. In graph X1, the fecal samples from participants P8 through P12 showed gradual increases in microbial diversity from Day 0 to Day 14, with P10 and P12 exhibiting the most pronounced upward trends, suggesting a strong response to intervention. P9 and P11 displayed moderate increases, while P8 maintained relatively stable diversity throughout the study period. In graph X2, participants samples P1 through P7 demonstrated more variable patterns; P2, P4, and P6 showed consistent increases in Shannon Index over time, indicating enhanced microbial richness, whereas P1 and P3 exhibited minimal changes. Participants samples P5 and P7 showed slight fluctuations but no sustained increase. Overall, the trends in Figure 19.0 suggest that bacterial diversity improved over time in several individuals, with inter-individual variability in response, highlighting the personalized nature of gut microbiota modulation.

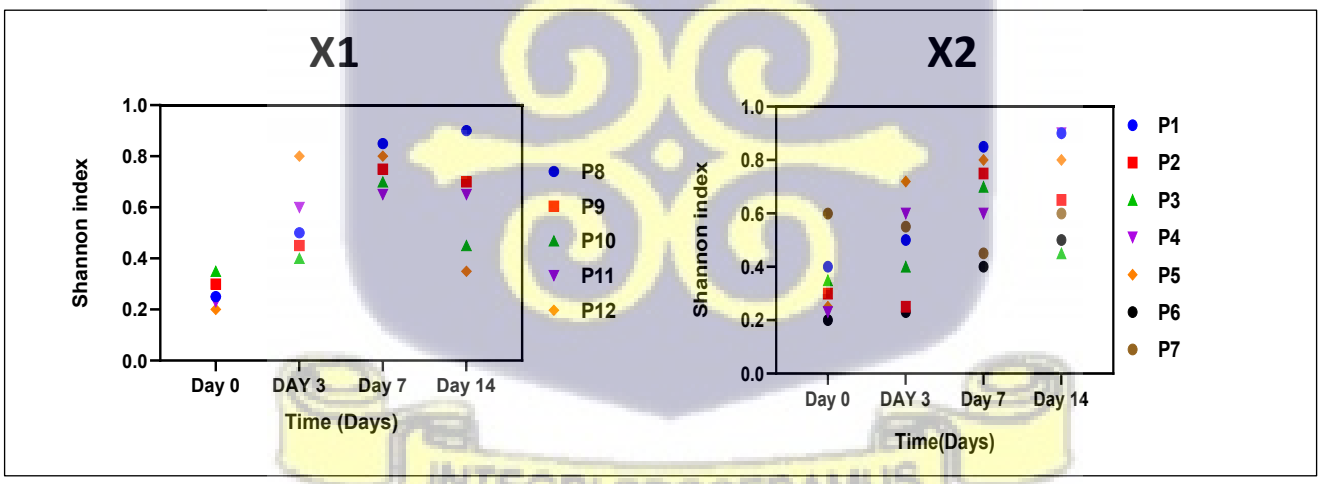


Figure 19.0: Temporal dynamics of bacterial diversity in human fecal samples from two participant groups (X1 and X2) over 14 days.

Shannon index values are plotted against sampling days (0, 3, 7, 14), with each individual represented by a distinct color and marker. Group X1 includes participants P8–P12; Group X2 includes participants P1–P7. Data reveal inter-individual variability and shift in microbial diversity across time, reflecting the response to experimental conditions and potential environmental or host factors.

4.8.7 Percentage abundance of bacterial species in *Brukina* samples

The microbial landscape of *Brukina* is dominated by *Lactobacillus delbrueckii,* which accounts for 50.6% of the total bacterial population, indicating its central role in the fermentation process and potential probiotic relevance as seen in figure 20.0. *Lactobacillus fermentum* and *Lactobacillus johnsonii* follow with 16% and 15.4% respectively, reinforcing the predominance of lactic acid bacteria (LAB) in the product.

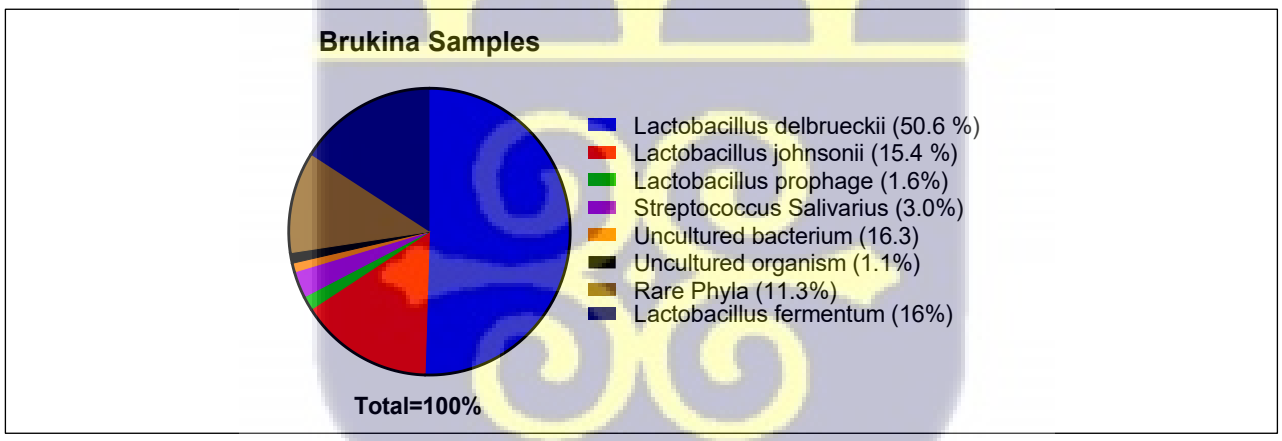


Figure 20.0: Percentage abundance of specific microbes in the *Brukina* samples

The sample analysis revealed significant presence of four key Lactobacillus species: *Lactobacillus delbrueckii, Lactobacillus johnsonii, Lactobacillus salivarius,* and *Lactobacillus fermentum*. Among these, *Lactobacillus delbrueckii* was the most abundant in all the fermented millet-based milk beverage (*Brukina*) samples studied, while uncultured organisms had the lowest abundance.

Additionally, a distinctive strain, *Streptococcus salivarius*, was found with a 3% abundance in the samples.

4.8.8 Percentage abundance of bacterial species in animal fecal samples

A distinct shift in microbial composition between animal fecal samples from the test and control groups, with the test group showing a pronounced dominance of lactic acid bacteria (LAB) was observed in figure 21.0. *Lactobacillus delbrueckii* (54%) and *Lactobacillus fermentum* (25%) were the most abundant species in the test group, collectively accounting for nearly 80% of the microbial population, alongside smaller contributions from *Lactobacillus taiwanensis* (8%), *Lactobacillus johnsonii* (5%), and *Lactobacillus prophage* (2%). This LAB-centric profile suggests a strong probiotic influence, likely due to dietary or experimental intervention. In contrast, the control group exhibited a more diverse microbial landscape, with reduced levels of *L. delbrueckii* (32.8%) and *L. fermentum* (17.3%), and increased representation of uncultured bacterium (23.6%), rare phyla (13.9%), and uncultured organism (6.1%). Additionally, *Proteus mirabilis* (1.1%) and *Streptococcus salivarius* (5.2%) were detected exclusively in the control group, indicating the presence of potentially opportunistic or commensal species in the absence of probiotic modulation.

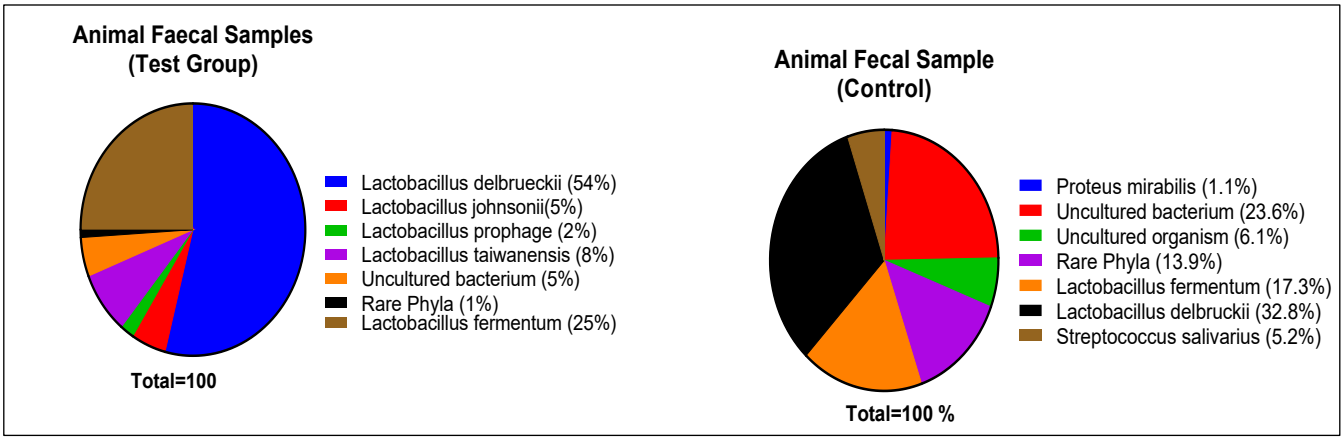


Figure 21.0: Percentage abundance of microbes in the animal fecal samples

The animal fecal sample contained five Lactobacillus species, with *Lactobacillus delbrueckii* being the most abundant and *Lactobacillus prophage* the least abundant. Notably, three Lactobacillus species (*Lactobacillus johnsonii, Lactobacillus prophage,* and *Lactobacillus taiwanensis*) were exclusive to the test group and absent in the control group. Conversely, the control group had other bacillus species, which were not found in the test group. Furthermore, the percentages of *Lactobacillus delbrueckii* and *Lactobacillus fermentum* in the test group were significantly higher compared to the control group.

4.8.9 Percentage abundance of bacterial species in human fecal samples

A comparative analysis of bacterial species abundance in animal fecal samples from a test group and a control group, revealing distinct shifts in microbial community structure is presented in figure 21.0.

In the test group, the microbial profile is heavily dominated by *Lactobacillus delbrueckii* (54%) and *Lactobacillus fermentum* (25%), together comprising nearly 80% of the total bacterial population. This pronounced enrichment of lactic acid bacteria (LAB) suggests a strong probiotic influence, likely driven by dietary or experimental intervention. Additional LAB species such as *Lactobacillus taiwanensis* (8%), *Lactobacillus johnsonii* (5%), and *Lactobacillus prophage* (2%) further reinforce the dominance of beneficial fermentative microbes. The presence of uncultured bacterium (5%) and rare phyla (1%) indicates minor contributions from less characterized taxa.

In contrast, the control group exhibits a more heterogeneous microbial composition. While *Lactobacillus delbrueckii* (32.8%) and *Lactobacillus fermentum* (17.3%) remain prominent, their relative abundance is markedly reduced compared to the test group. The control samples also show elevated levels of uncultured bacterium (23.6%) and rare phyla (13.9%), suggesting a more diverse and less LAB-centric microbiota. Notably, *Proteus mirabilis* (1.1%) and *Streptococcus salivarius* (5.2%) are present exclusively in the control group, potentially reflecting opportunistic or commensal species in the absence of probiotic modulation.

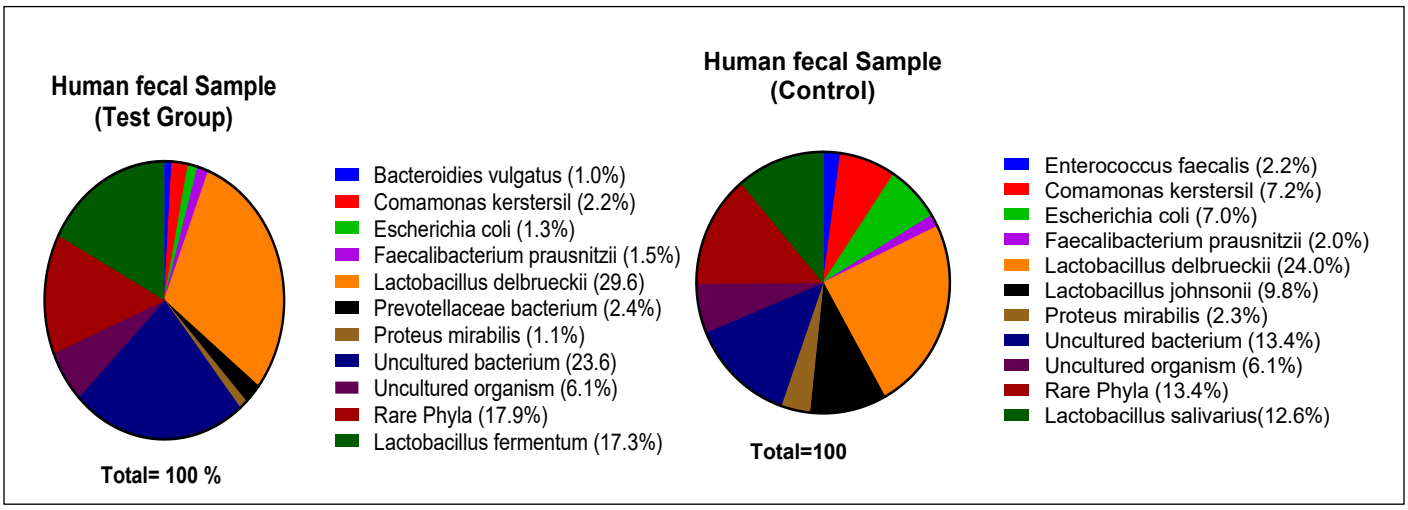


Figure 22.0: Comparative abundance of bacterial taxa in human fecal samples from test and control groups.

Pie charts illustrate relative percentages of identified species, with test samples dominated by *Lactobacillus delbrueckii* (29.6%) and uncultured bacterium (23.6%), while control samples showed higher levels of *Lactobacillus delbrueckii* (24.0%), *Lactobacillus johnsonii* (9.8%), and uncultured bacterium (13.4%). Minor taxa and rare phyla are also represented, highlighting differences in gut microbiota composition across groups.

CHAPTER FIVE

5.0 Discussion

From fifteen (15) FDA approved *Brukina* samples collected and processed, a total of 39 bacterial isolates were recovered and purified. Purified isolates that were found to be Gram positive, coccus, coccobacillus and rod shaped under microscope were taken through various tests to characterize them as potential probiotics. These tests included antimicrobial activity, host bio barrier tolerance (acid and bile tolerance), adhesion ability and antibiotic susceptibility investigation.

Acid tolerance test is one major criteria for probiotics characterization. Hydrochloric acid (HCl) found in the human stomach's gastric juice not only breaks down food but also eliminates bacteria (Smith, 2003). From this study, it is evident that, Lactic acid bacteria metabolism thrives at higher pH levels, leading to increased growth and viability (Batish et al., 1997). Other studies corroborated this, showing that exposure to gastric acid with a pH $\leq$ 2 for 3 hours significantly reduced the viability count of bacteria (Conway et al., 1987).

The findings align with the fact that, pH = 3 is the standard for probiotic culture acid tolerance (Sahadeva et al., 2011). Figures 5.1 to 6.1 demonstrated only a slight inhibition in viable bacteria numbers at pH = 2 and 3. Some of the Lactobacillus isolates exhibited decreased growth with longer incubation at pH = 2, and a similar pattern was observed at pH = 3 (Chou & Weimer, 1999). Most of the strains exhibited a significant increase in absorbance (optical density) over time at both pH 2 and 3 (Figures 5.1a, 5.1b, 6.1a & 6.1b). This suggests that Lactobacilli have an adaptive ability to acid conditions when present in an acidic environment (Siragusa et al., 2014). At pH 7, except for a few strains, there was a consistent trend of increasing Lactobacillus viability, indicating their preference for a neutral pH during the initial stages of survival, followed by acclimatization to lower pH environments as conditions become more acidic (Cotter & Hill, 2003).

The exposure of the Lactobacillus isolates to bile salts leads to disruptions in cellular balance, causing lipid bilayers and integral proteins in the cell membranes of few strains to dissociate resulting in bacterial cell content leakage (Wulandari et al., 2020). The leakage of bacterial content eventually results in death of few cells (Miao et al., 2016). However, most strains that are tolerant to varying pH levels (2.0, 3.0, and 7.0) are able to withstand exposure to 0.3% bile salt concentration (Anandharaj et al., 2015). This is probably due to the intricate ability of the Lactobacillus strains to counteract the negative effects of bile at the level of cell disorganization, oxidative stress, DNA damage and bacterial protein denaturation through overexpression of a battery of proteases and chaperones (Corcoran et al., 2008). A few strains (A7II, A14II, A6I) could not acclimatize, survive and thrive within the bile salt exposure despite their pH tolerance (Balasubramanian et al., 2021). This might be due to the imposition of oxidative stress on the bacteria as a result of production of reactive oxygen or nitrogen species (Zhang et al., 2021). Similarly, bile salt deconjugation might have significantly contributed to the release of protons thus, causing an intracellular acidification resulting in bacterial cell death (Ruiz et al., 2013). Additionally, mutations might have also been responsible for the downregulation of stress tolerance genes in the small number of these seemingly bile salt intolerant strains (Bove et al., 2012). Lactobacillus isolates A1I and A2II demonstrated that bile salt tolerance is largely predicated on the specific strain and duration of exposure. This was further authenticated by the increase in strain viability over time, although it began to decrease after the third hour (Caggia et al., 2015).

Isolates A8II and A14 do not cluster with the Lactobacillus genus based on phylogenetic analysis. As such, only isolates A8III and A13II were confirmed as Lactobacilli.

The Lactobacilli strains (A8III and A13II) recognized for their probiotic potential and their inhibitory action are associated with the production of lactic acid, bacteriocins, hydrogen peroxide, and deacetyl compounds (Wambugu, 2015). Other probable strains of Lactobacillus that were later identified as bacillus strains after sequencing also demonstrated antimicrobial activity against pathogenic microbes and this confirms the potential of microbial competition for survival in both food matrix and the GIT (Arqués et al., 2015). The antibacterial compounds secreted by the isolates such as organic acids, bacteriocins, or hydrogen peroxide may degrade over time, become unstable under storage or experimental conditions, or require specific pH or co-factors for optimal activity, which may not be preserved in the cell-free supernatant (Reis et al., 2012). Additionally, cell-to-cell contact or synergistic interactions between secreted compounds and surface-associated factors could also contribute to the observed activity in the whole-cell assays but be absent in the supernatant alone (Dsouza et al., 2024). This suggests the need for further investigation into the nature, stability, and mode of action of the antimicrobial substances produced. However, it is important to note that the results obtained in this *in vitro* study may not fully represent their performance in real-life situations, as various physiological conditions can affect strain survival (Marteau et al., 1997). Additionally, statistical analysis did not establish significant differences in the survival of Lactobacillus strains. Factors such as temperature during fermentation, inoculation, and transportation, as well as the protective effect of the food matrix might have had an effect on the microbiological load and inhibitory profile of these strains (Gálvez et al., 2007). Almost all the strains that were screened for antibiotics resistance property demonstrated resistance to one or more orally administered antibiotic drug. This was observed because, lactic acid bacteria (LAB) found in fermented products might have been carrying antimicrobial-resistance genes that can potentially be transferred to pathogens and commensals, either within the food chain or, more

importantly, in the digestive systems of humans and animals (Verraes et al., 2013) . However, there is disagreement regarding the susceptibility breakpoints for most antimicrobials in LAB, as indicated by various studies (Toomey et al., 2010). Differentiating between intrinsic, nonspecific, and acquired resistance is challenging, as it necessitates analyzing antimicrobial-resistance patterns across numerous LAB species (Moračanin et al., 2017).

Adhesion to the intestinal mucosa is considered a crucial step for colonization and plays a vital role in the interaction between probiotic strains and the host (Candela et al., 2008). This interaction serves to regulate the immune system and counteract potential harmful microorganisms (Candela et al., 2008). The results revealed that all tested samples exhibited varying levels of adhesion to Caco-2 cells, with different strains displaying different adhesion capacities, resulting in varying bacterial numbers per cell. This underscores the strain-specific nature of adhesion, consistent with findings from previous research (Ferreira et al., 2011). Despite the potential relevance of human intestinal epithelial cell lines to the *in vivo* scenario, Caco-2 cells were used as an *in vitro* model for assessing adhesion properties (Bermudez-Brito et al., 2013). This choice was motivated by the fact that Caco-2 cells, when cultured, form a uniform and cell monolayer, making them suitable for studying cellular interactions (Lozano-Ojalvo et al., 2019). The Caco-2 cell lines represent highly distinctive intestinal systems extensively used as models for investigating permeability, physiological functions, and the underlying mechanisms of diseases (Delie & Rubas, 1997). These findings align with previous reports highlighting the strong adhesion capacity of *Lactobacillus* to intestinal epithelial cells, which in turn aids in the competitive elimination of harmful pathogens (Rawal & Ali, 2023).

The identities of nine (9) bacterial isolates were correspondingly ascertained as members of the genera *Limosilactobacillus, Enterobacter, Neobacillus, Alishewanella* and *Faecalibaculum* as presented in Table 9. These unique genera contained 9 different species altogether, namely, *Limosilactobacillus fermentum* (four isolates, A8II, A8III, A14II & A6I), *Enterobacter hormaechei* (one isolate, A2II), *Neobacillus fumarioli* (one isolate, A3II), *Alishewanella agri* (one isolate, A6II), *Bacillus safensis* (one isolates, A12III), and *Faecalibaculum rodentia* (one isolate, A13III). The existence of enormously distinct microbes in the sample is indicative of the fact that, the microbial flora of fermented millet-based milk beverage (*Brukina)* was undoubtedly complicated and shown to include *Enterobacteriaceae*, aerobic mesophilic bacteria, lactic acid bacteria and *Bacillus* spp (Mehari & Ashenafi, 1995). DNA sequencing results revealed that not all the isolates in this study belong to the *Lactobacillus* genus, contrary to initial expectations based on their phenotypic characteristics (Herbel et al., 2013). Specifically, isolates A2II, A3II, A6II, A12III, and A13III were identified as non-*Lactobacillus* species. This finding underscores the limitations of relying solely on morphological and biochemical tests for bacterial identification, as such methods may lead to misclassification, particularly among closely related lactic acid bacteria (Sharma et al., 2020). This also has implications for interpreting the functional properties of the isolates, as different genera may vary significantly in their probiotic potential and safety profiles (Liu et al., 2023). Studies indicated that *Lactobacillus fermentum* is commonly found in the human intestinal tract which plays a key role in food fermentation by converting carbohydrates into acid, enhancing flavor, extending shelf life, and boosting nutritional value. *Limosilactobacillus fermentum* is also a very unique starter culture in the traditional fermentation of fruits and vegetables. Moreover, the US Food and Drug Administration recognized it as a safe organism in 2013 (Zhao et al., 2022). The phylogenetic grouping of the nine bacterial pure cultures sequenced

indicated that, strains having similar sequences were clustered in the same group and presumably were considered as close relatives as shown in Figure 17 (Siezen et al., 2010).

Probiotics should be viable, be able to traverse and persist in the GIT, adhere to, and colonize the intestinal epithelial cells in adequate amounts in order to confer their unique beneficial effects. These advantages are largely profound in the presence of abundant and diversified probiotic strains. Shannon index analysis demonstrated through this research that, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* were the predominant microbes in *Brukina*, animal and human fecal samples and are largely used as starter cultures for the manufacturing of dairy products, particularly yoghurts as illustrated in Figure 17. It was also clearly confirmed that, food substances significantly impact the gut microbiome in a matter of three days of consistent consumption (Kaczmarek et al., 2017). Some of the strains isolated demonstrated prolonged viability, persistence and colonization of the gut epithelial cells which is validated by the corresponding increase in the percentage abundance and diversity with respect to time as well as their short generational periods (Vaughan et al., 2005). The test group of human participants and animal models experienced a noteworthy reduction in *Comamonas kerstersil, Escherichia coli, Faecalibacterium prausnitzii, and Proteus mirabilis,* while lactic acid bacteria, such as *Lactobacillus fermentum* and *Lactobacillus delbrueckii*, demonstrated their ability to competitively eliminate these microorganisms. This may suggest some competitive dynamics situation not only between different species of enteric pathogens (like *Proteus mirabilis, Escherichia coli, Comamonas kerstersil*) but also with probiotics (such as *Faecalibacterium prausnitzii*) in the gut microbiome (Nair et al., 2017). *Faecalibacterium prausnitzii*, a gut probiotic, plays a crucial role in enhancing the body's self-defense against inflammatory responses (Leylabadlo et al., 2020). This defensive mechanism likely involves pro-inflammatory cytokines

and is instrumental in promoting the secretion of anti-inflammatory cytokines through active molecules (Shamoon et al., 2019). *Limosilactobacillus fermentum* was found in most of the *Brukina* sample because of its unique ability to catalyze specifically spontaneous fermentation reactions (Adesulu-Dahunsi et al., 2022). This is one unique strain that plays an essential role in both heterofermentation and homofermentation processes. Other microbes such as *Alishiwanella agri, Neobacillus fumarioli, Faecalibaculum rodentia* and *Bacillus safensis* had very low or zero relative abundance and this is possibly due to their elimination by probiotic strains in the gut.

CHAPTER SIX

6.1 Conclusion

This study showed that *Brukina*, a traditional millet-based fermented milk beverage, contains probiotic strains particularly *Limosilactobacillus fermentum* that exhibit strong acid and bile tolerance, antimicrobial activity, and the ability to adhere to intestinal cells. DNA sequencing revealed microbial diversity beyond *Lactobacilli*, with some non-LAB strains also showing antimicrobial potential.

Regular consumption of *Brukina* significantly modulated the gut microbiome, increasing beneficial LAB populations while reducing harmful pathogens like *E. coli* and *Proteus mirabilis*. These findings support *Brukina*'s potential as a functional food that can promote gut health and serve as a low-cost dietary intervention to improve microbiome balance and combat enteric infections in West African communities.

6.2 Recommendations

1. It would be necessary to isolate the active compounds produced by Lactic acid bacteria and investigate their impact on intestinal pathogens.
2. Conduct animal or human studies to confirm the survival, colonization, and health benefits of promising probiotic strains under real gastrointestinal conditions.
3. Perform genomic analysis to check for transferable antibiotic resistance genes and ensure the safety of probiotic strains for human consumption.

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Appendix

QUESTIONNAIRE ON THE CONSUMPTION PATTERNS OF *BRUKINA* IN GHANA

Dear respondent, this survey is a part of research being conducted at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana. The research principally seeks to investigate the effects of probiotics present in *Brukina* on the gut microbiome. However, this questionnaire will be limited to gathering information on your consumption pattern, knowledge about probiotics and the *Brukina* products you consume.

All responses provided here are treated with the highest confidentiality. You are free to ask for any clarifications regarding the project and questions asked in this questionnaire. In case of further inquiry, suggestions or comments, kindly contact the Principal Investigator on the addresses below.

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DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA

A. **PERSONAL INFORMATION**

PLEASE TICK OR FILL IN AS APPROPRIATE

Age: …….. Gender: Male ◯ Female ◯

Occupation: ……..

Tel/Mob: ..... email address: .....

Education:

Tertiary ◯ SHS ◯ JHS ◯ None ◯ Other ◯

B. BRUKINA CONSUMPTION PATTERN

Do you know Brukina?.....

Do you consume Brukina?.....

Name of Brukina products you consume often (Name in order of preference if more than one)

1. .....

2. .....

Product packaging: Bottle ◯ Sachet ◯ Other ◯

Please specify (if other): .....

Do you take milk?.....

If yes, how often do you take it?.....

Do you take millet?.....

If yes, how often do you take it?.....

C. KNOWLEDGE OF HEALTH BENEFITS OF *BRUKINA*

Do you know that brukina contains healthy microorganisms? .....

If yes, please name the microorganisms you know are in the brukina products.

.....

What practical health benefits do you get from your brukina?

.....

.....

Do you know any detrimental impact of Brukina?.....

How do you take brukina?

As a snack ◯

As a whole meal ◯

As a health food supplement ◯

How often do you drink brukina?

Daily ◯

Weekly ◯

Fortnight ◯

Monthly ◯

Rarely ◯

How do you keep your brukina?

.....

D. KNOWLEDGE OF PROBIOTICS

Do you have any knowledge about probiotics? Yes ◯ No ◯

If yes, how much knowledge do you have about probiotics?

Excellent ◯ Very good ◯ Fair ◯ Little ◯

Please state what you know .....

.....

.....

Do you know probiotics are used in manufacturing yoghurts?.....

Do you know probiotics can be killed by heat?.....

Do you know any health condition managed with probiotics?.....

E.

Are you taking any antibiotic currently? Yes/No

If yes, state the specific antibiotic……..

Do you take other probiotic products? Yes/No

If yes state the specific product……

Do you have any chronic debilitating health condition like HIV/AIDS, Diabetes etc. Yes/No

If yes state the condition……..

Are you willing to give us your stool sample intermittently? Yes/No

If you have given your consent to this project, sign here ……

Thank you for your responses. We will contact you again when the need arises.

Bless Hodasi

MPhil.

Adhesion Activity of Isolated Lactobacillus strains

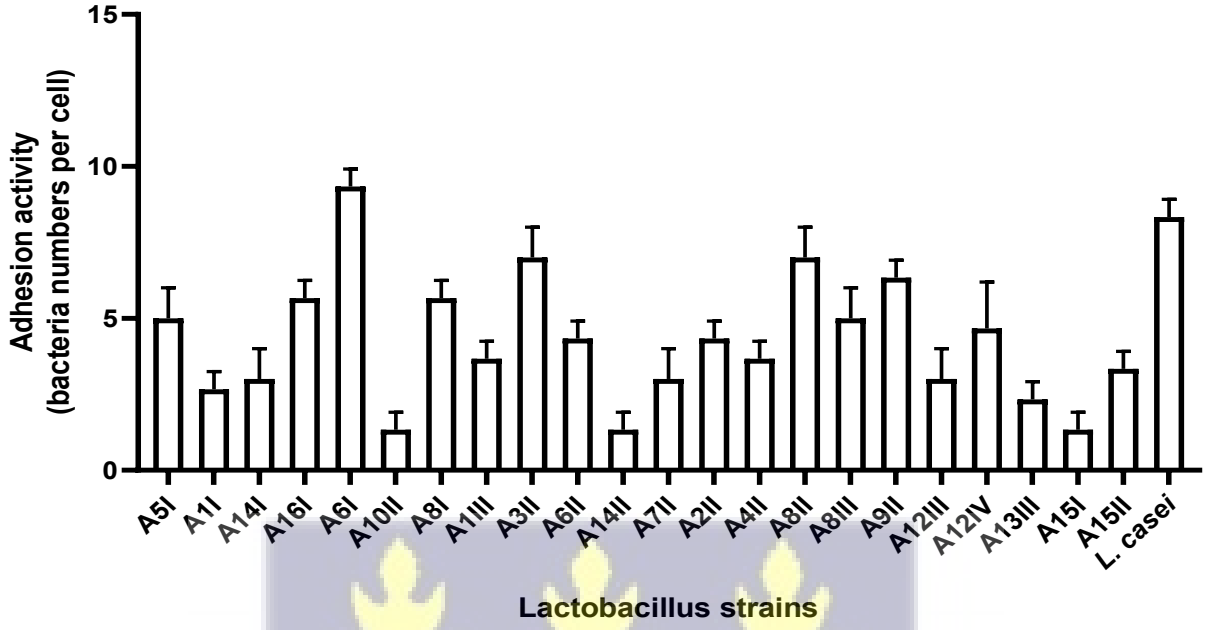


Fig 11.0 Adhesion activity of isolated lactic acid bacteria strains

The results from three independent experimental replicates were expressed as the mean ± standard deviation (SD). Every one of the 22 *Lactobacillus* strains displayed noticeable adherence to the Caco-2 cells (Fig 11.0). Among them, strain A6I exhibited the most substantial adhesion capacity, suggesting its effectiveness in competitively binding to intestinal epithelial cells thus eliminating enteric pathogens. Following closely in terms of adhesion were strains A3II, A8II, A9II, and A8I. In contrast, strains A10II, A14II, and A15I showed the lowest adhesion capacity to Caco-2 cells.

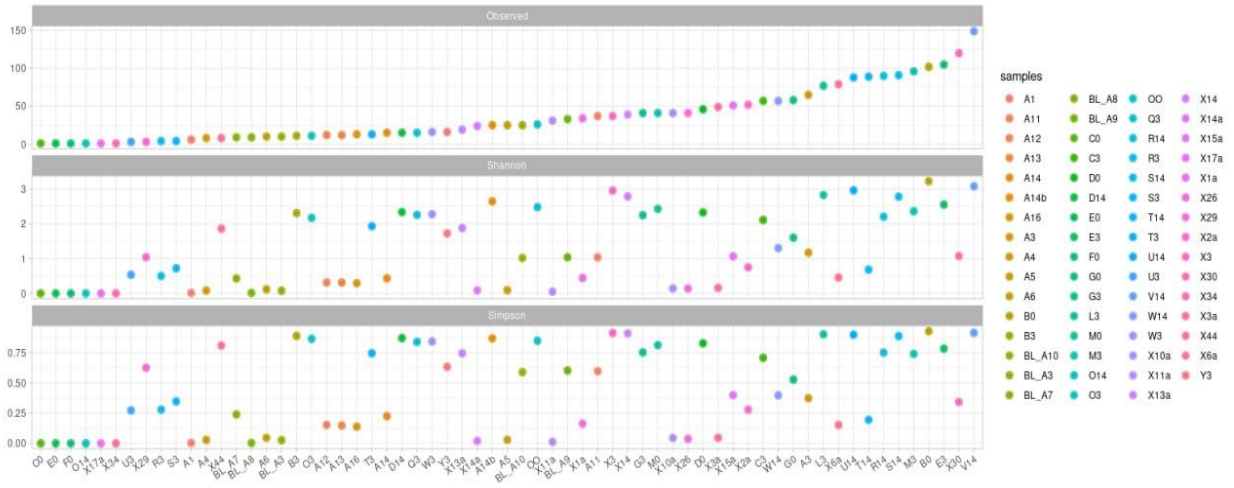


Fig 23.0: Microbial Diversity Measure of *Brukina*, animal and human fecal samples

Microbial diversity metrics across *Brukina*, animal, and human fecal samples assessed by 16S rRNA gene sequencing. Plots show species richness (Observed), Shannon diversity index (abundance and evenness), and Simpson diversity index (dominance) for each sample, with individuals color-coded as indicated in the legend. Samples exhibit substantial variation in alpha diversity, reflecting differences in community complexity and ecological structure among host groups.

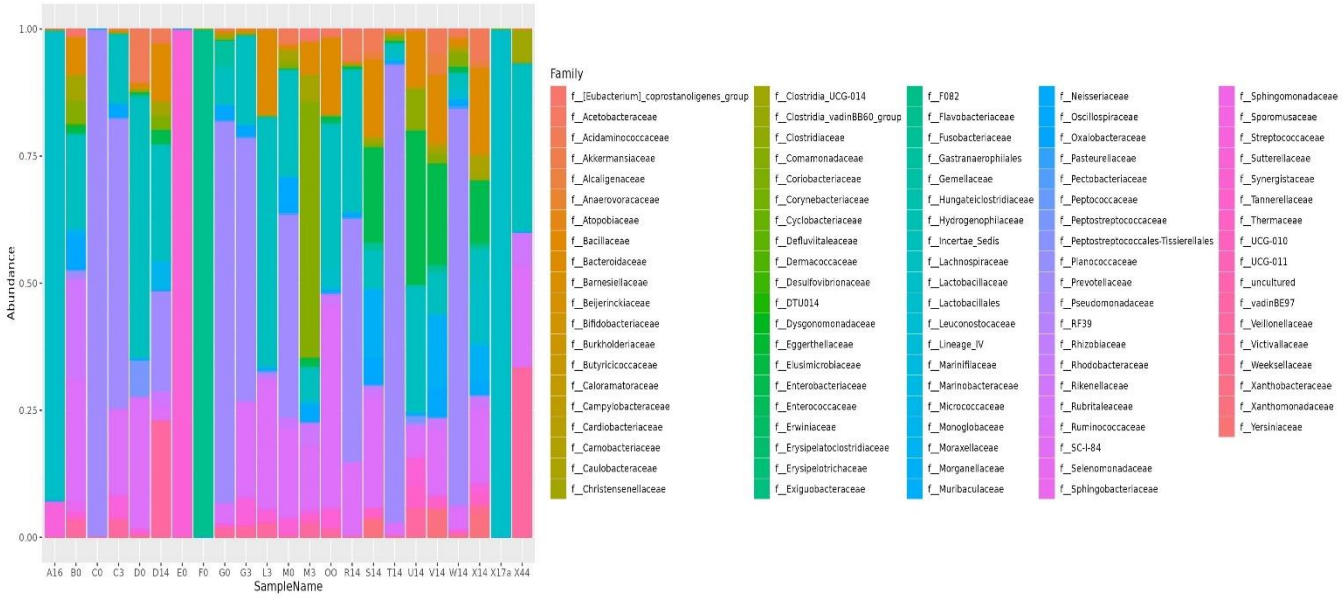


Fig 24.0: Family-level taxonomic distribution of gut microbiota in animal fecal samples based on 16S rRNA gene sequencing.

Stacked bar plots represent individual samples, with each colored segment indicating relative abundance of a specific bacterial family. Dominant families include *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Bacteroidaceae*, while numerous low-abundance taxa (e.g., *Erysipelotrichaceae*, *Christensenellaceae*, *Peptostreptococcaceae*) contribute to microbial diversity. The figure emphasizes inter-sample variability in community structure and highlights the prevalence of *Firmicutes*-associated families across samples.

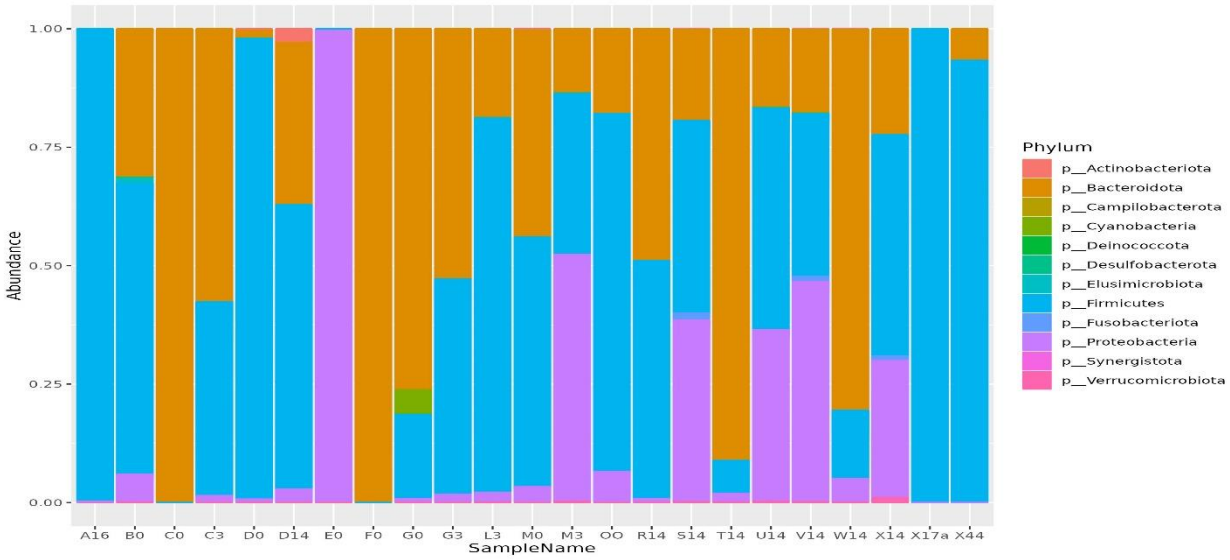


Fig 25.0: Relative abundance of bacterial phyla in human fecal samples as determined by 16S rRNA gene sequencing.

Stacked bar plots represent individual samples, each segmented by phylum-level taxonomy: *Actinobacteriota* (green), *Bacteroidota* (orange), *Campylobacterota* (yellow), *Cyanobacteria* (dark green), *Deinococcota* (dark blue), *Desulfobacterota* (light blue), *Elusimicrobiota* (purple), *Firmicutes* (brown), *Fusobacteriota* (dark purple), *Proteobacteria* (light purple), *Synergistota* (dark pink), and *Verrucomicrobiota* (pink). The distribution highlights inter-individual variability in gut microbiota composition, with *Firmicutes*, *Bacteroidota*, and *Proteobacteria* appearing as predominant phyla across samples.

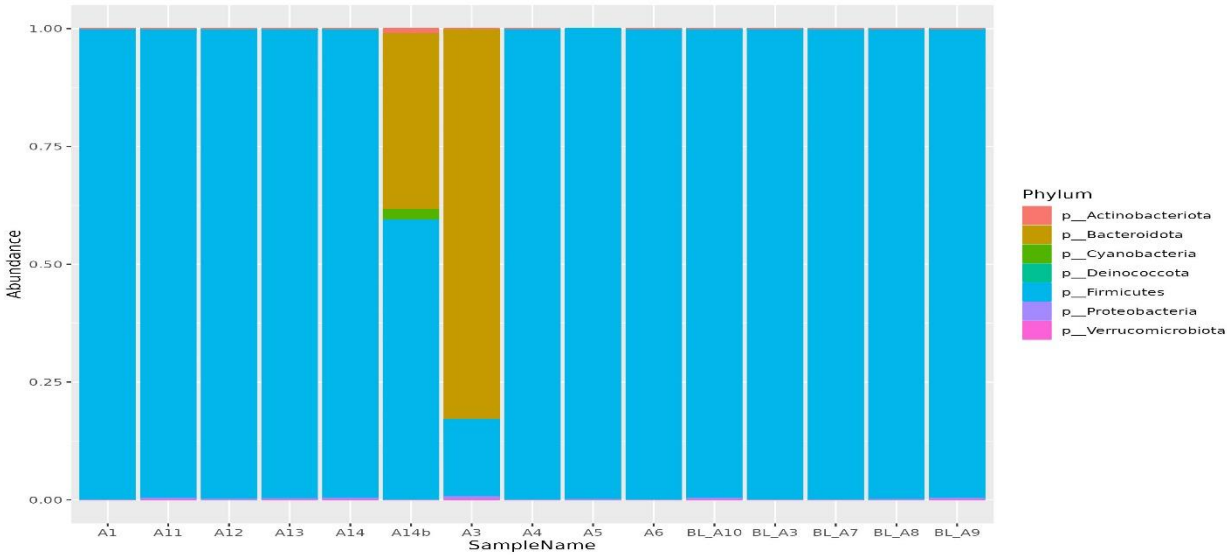


Fig 26.0: Relative abundance of microbial phyla in animal fecal samples based on 16S rRNA gene sequencing.

Bar plots represent taxonomic composition across individual samples (A1–BL9), with each color denoting a distinct phylum: *Actinobacteriota* (green), *Bacteroidota* (yellow), *Cyanobacteria* (blue), *Echinococcota* (red), Firmicutes (brown), *Proteobacteria* (purple), and *Verrucomicrobiota* (pink). Data illustrate inter-sample variability in gut microbiota structure, highlighting dominant phyla and shifts in community profiles across animals.

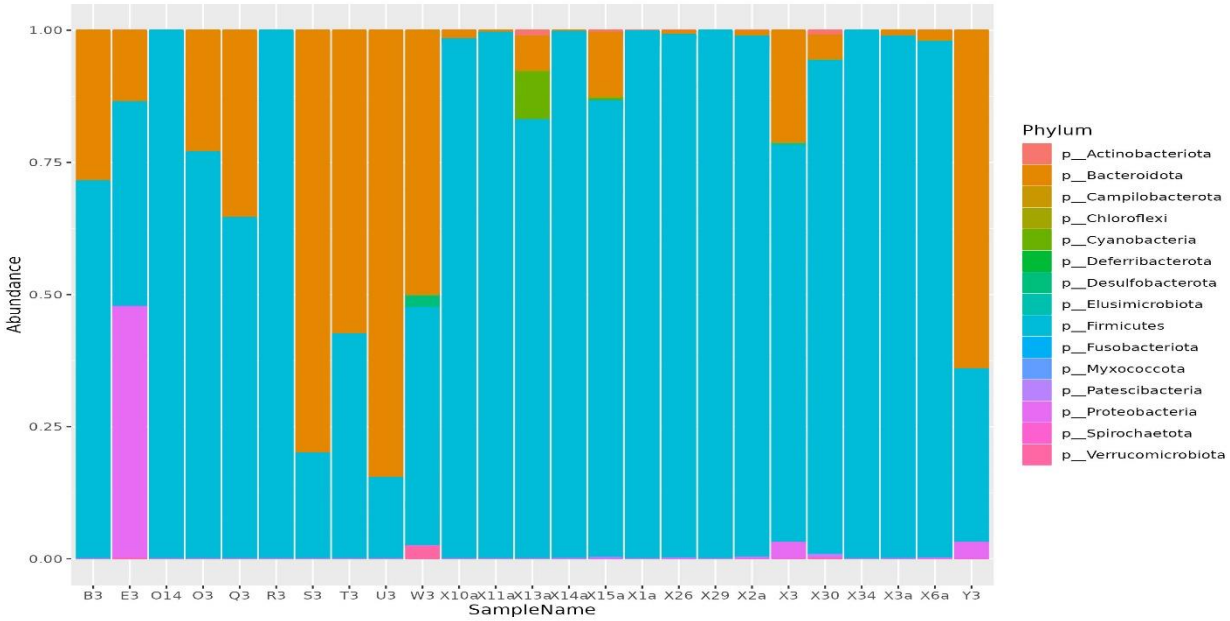
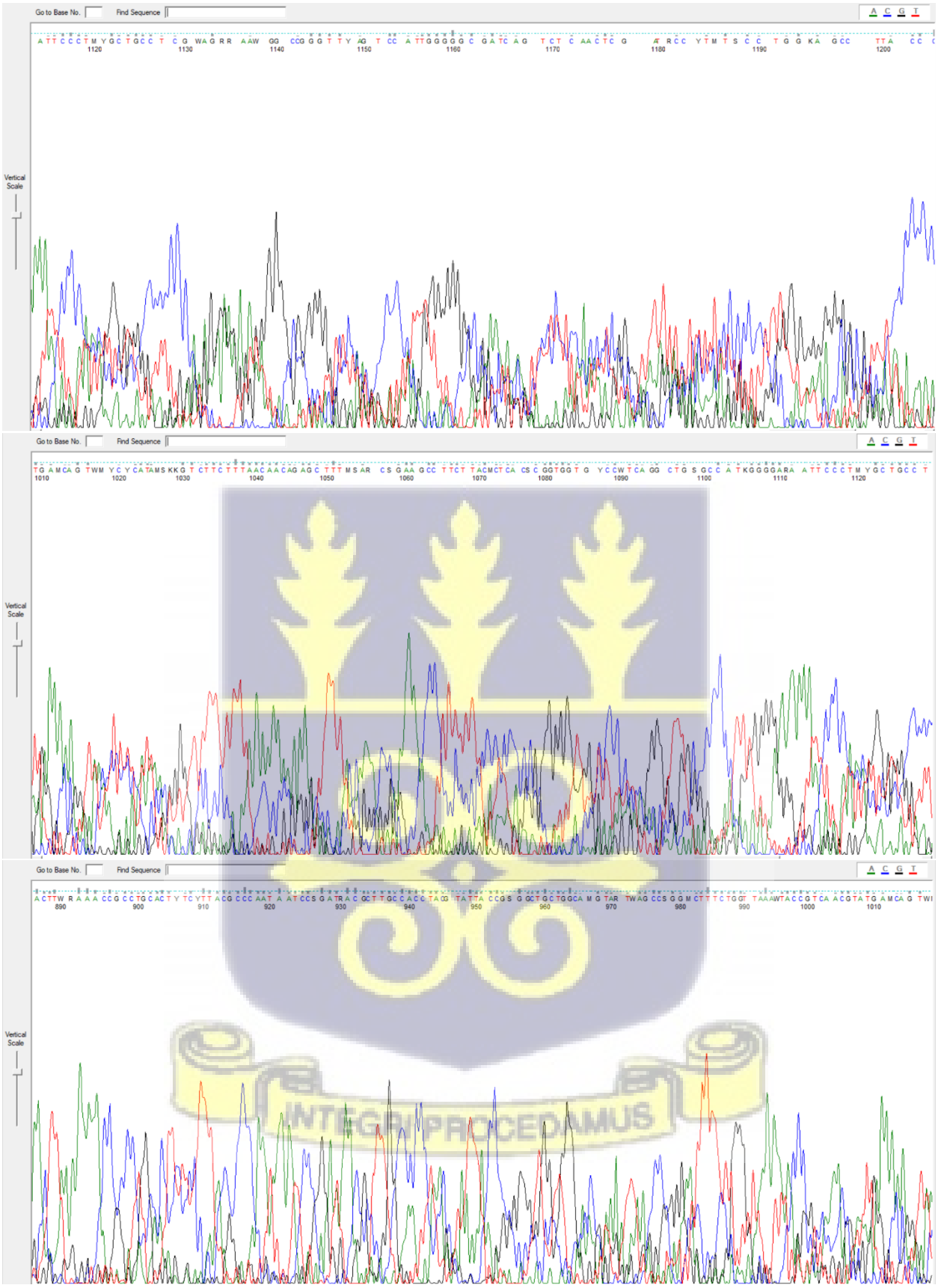
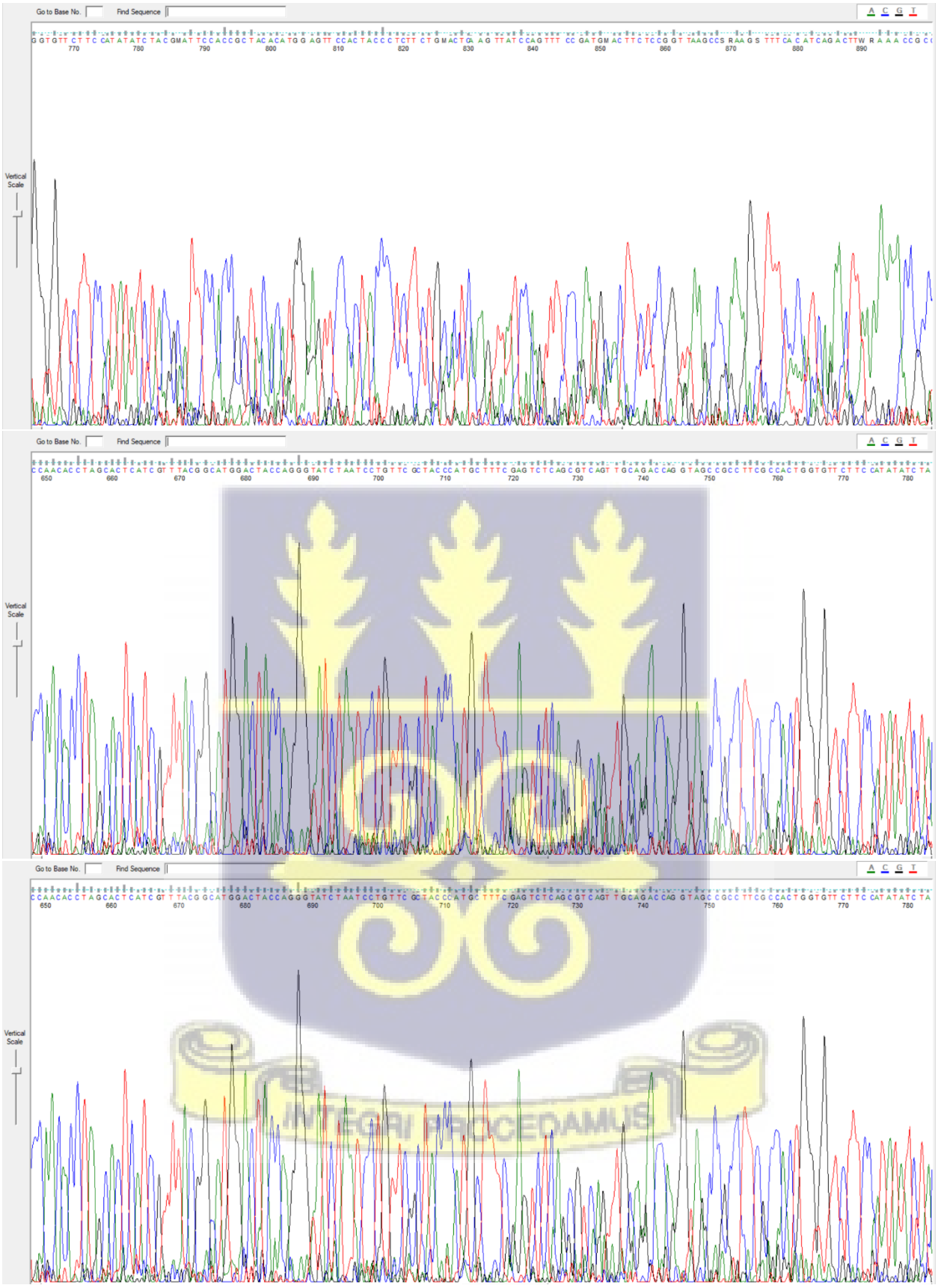
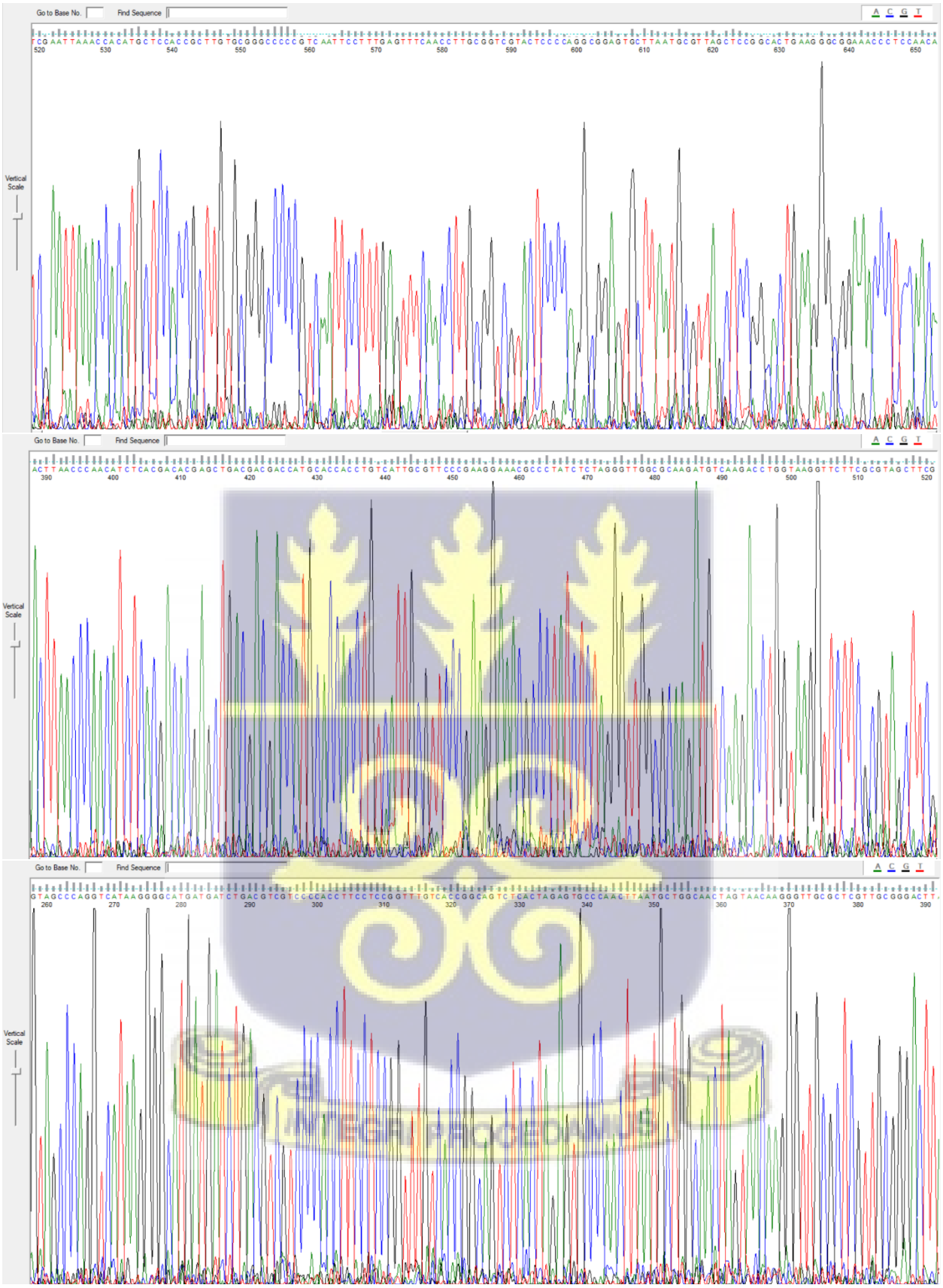


Fig 27.0: Relative abundance of bacterial phyla in *Brukina*

Stacked bar charts display phylum-level composition across individual samples (e.g., e3, e5, o1a, o3, r3–y3), with each color corresponding to a distinct phylum: *Actinobacteria* (pink), *Bacteroidota* (orange), *Campilobacterota* (green), *Chloroflexi* (brown), *Cyanobacteria* (blue), *Deferribacterota* (yellow), *Desulfobacterota* (dark green), *Elusimicrobiota* (light green), *Firmicutes* (purple), *Fusobacteriota* (dark purple), *Myxococcota* (light purple), *Patescibacteria* (light pink), *Proteobacteria* (red), *Spirochaetota* (dark red), and *Verrucomicrobiota* (magenta). The data illustrate substantial variation in microbial community structure among samples, with recurrent dominance of *Firmicutes*, *Bacteroidota*, and *Proteobacteria*, alongside low-abundance taxa contributing to overall diversity.






Go to Base No.
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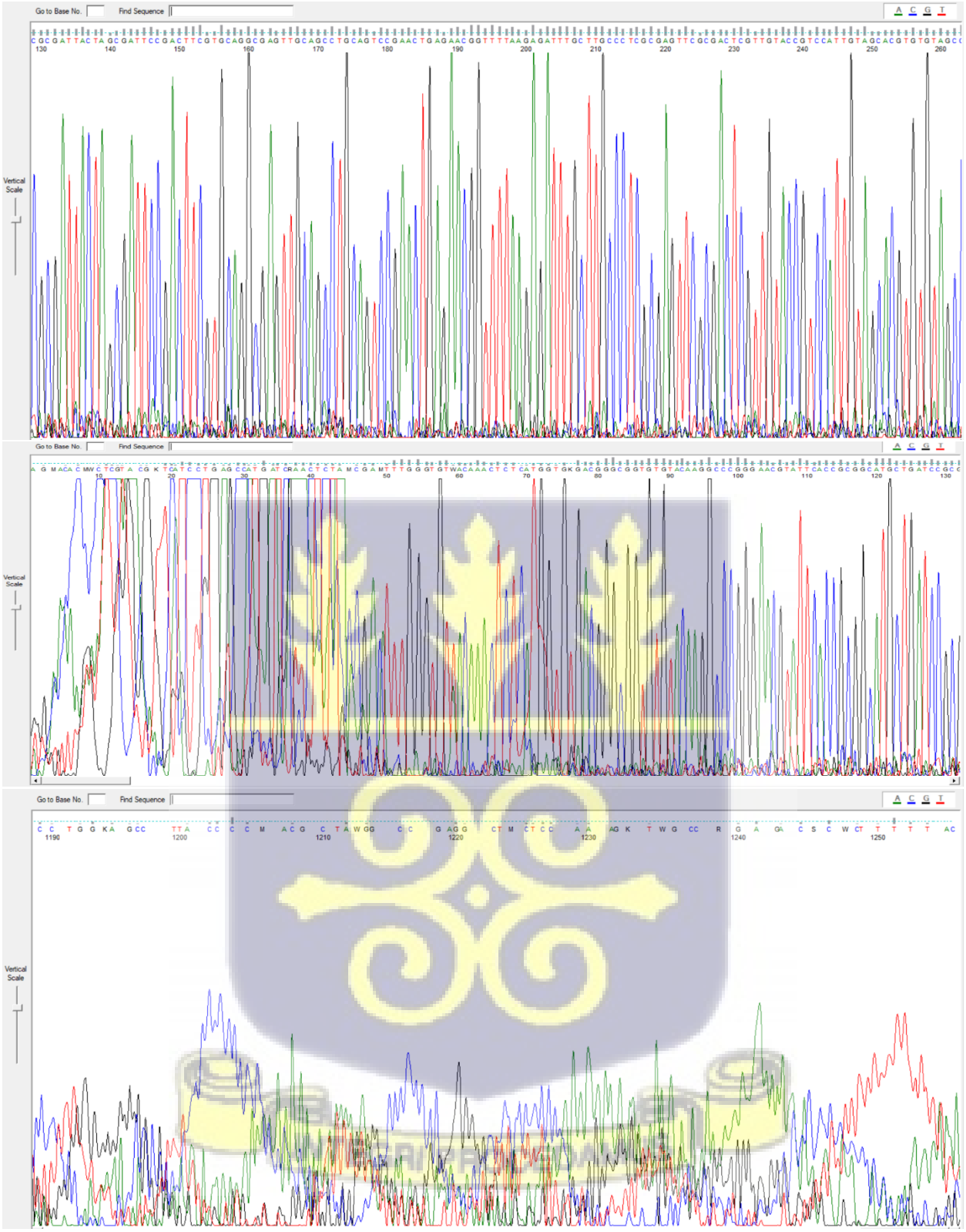

Fig 28.0: Representative chromatograms showing base-calling accuracy from Sanger sequencing of PCR-amplified 16S rRNA gene.

Clear, non-overlapping peaks correspond to high-confidence nucleotide assignments: adenine (green), thymine (red), cytosine (blue), and guanine (black). The sequences depicted illustrate high-quality read regions suitable for downstream analysis, with minimal background noise and sharp peak resolution across both panels.