Potential Probiotic Properties of Lactic Acid Bacteria Isolated from Gastrointestinal Tracts of Broiler Chickens

Submitted in fulfilment of the academic requirement for the degree of Master of Applied Sciences in Biotechnology at the Department of Biotechnology and Food Science

Faculty of Applied Sciences

Durban University of Technology, South Africa

By

Nwabisa Happiness Kokwe

Supervisor: Prof. Feroz Mahomed Swalaha

Co-supervisor: Dr Freedom Tshabuse

REFERENCE OF DECLARATION

We, Nwabisa Happiness Kokwe – Student number 22175189, Prof. Feroz Mahomed Swalaha and Dr Freedom Tshabuse hereby declare that in respect of the following dissertation:

Title: **Potential Probiotic Properties of Lactic Acid Bacteria Isolated from the Gastrointestinal Tract of Broiler Chickens**

1. To the best of our knowledge no other dissertation of a similar nature exists.

2. All references mentioned in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

31/10/2024

gnature of st Date

31-10-2024

Signature of supervisor Date

31/10/2024

Signature of co-supervisor Date

AUTHOR'S DECLARATION

I, Nwabisa Happiness Kokwe, hereby assert that this dissertation is an original piece of work by me. The content of this dissertation solely reflects my own research and findings, unless explicitly stated otherwise. Furthermore, I affirm that all references cited in this dissertation are accurately recorded to the best of my knowledge. This work has not been previously submitted to any other academic institution.

31/10/2024

Date

ABSTRACT

In poultry farming, antibiotics have been widely utilized as growth enhancers to promote intestinal health and reduce mortality in livestock from pathogenic microorganisms. Many countries have implemented bans on the excessive use of antibiotics because of increasing concerns regarding the resistance of bacteria to antibiotics and the presence of antibiotic residues in poultry products. To counteract this problem, probiotics could be used as adjuncts or as substitutes for preserving a diverse and balanced microflora to prevent the colonization and multiplication of pathogenic bacteria in the gastrointestinal (GI) tract, while also improving poultry performance. The functional properties of lactic acid bacteria (LAB) and their potential as poultry probiotics have been extensively recorded. Hence, this study aimed to assess the potential probiotic properties of LAB for the development of poultry probiotics by isolating lactic acid bacteria from the digestive tracts of broiler chickens.

To achieve this aim, a total of 66 LAB isolates were isolated from the crops and small intestines of broiler chickens, which were screened and evaluated for their probiotic properties, among which 11 strains exhibited excellent probiotic traits and were identified by 16S rDNA sequencing as *Enterococcus faecalis* strain ATCC 19433 (isolates C4 and C5), *Pediococcus pentosaceus* strain DSM 20336 (isolates C7, C13 C24, SI23 and SI38), *Streptococcus salivarius* strain ATCC 7073 (isolate SI4) and *Levilactobacillus brevis* ATCC 14869 (isolates SI6, SI8 and SI9). The selected strains inhibited tested pathogenic bacterial growth *Listeria monocytogenes* (ATCC 7644), *Salmonella enterica* (ATCC 13314), *Pseudomonas aeruginosa (*ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) with zones of inhibition ranging from 9.00 ±5.66 to 30.00 ±0 mm and survived in simulated gastric juice with a cell viability count greater than 7.0 CFU/ml. Furthermore, the isolates demonstrated remarkable auto-aggregation and coaggregation capabilities, along with α-glucosidase inhibitory activity ranging from 25.84 ± 3.08% to 61.77 ± 6.16%. Principal component analysis results indicated that *L. brevis* NKFS9, *P. pentosaceus* NKFS3, *P. pentosaceus* NKFS11 and *E. faecalis* NKFS1 are the most promising LAB candidates that can be utilized for the development of a multi-probiotic strain for broiler chickens. In conclusion, the lactic acid bacteria (LAB) strains isolated from the crops and small intestines of broiler chickens present a valuable prospect for the development of effective probiotics. These probiotics can be utilized as a supplementary

inclusion in poultry feed, reducing or obviating the need for antibiotics as growth promoters. Nevertheless, additional *in vivo* studies are essential to closely monitor and assess the beneficial effects of probiotics on the GI tract of chickens.

DEDICATION

With deep love and gratitude, I dedicate this work to my late mother, Bajabulile Elizabeth Kokwe, whose unwavering support, kindness, and sacrifices shaped every step of my journey. She was my guiding light, my greatest cheerleader, and the heart of my every success.

Through every challenge, she stood beside me, offering encouragement when I doubted myself and strength when I felt weak. She celebrated my victories, both big and small, and believed in me even when I struggled to believe in myself. Her love was boundless, her wisdom endless, and her presence irreplaceable. Though she is no longer here to see this moment, I carry her lessons, love, and warmth in my heart every single day. This achievement is as much hers as it is mine, and I will forever strive to honour her memory in all that I do.

Mom, this is for you. I love you always.

This work is also dedicated to my sister, Nomfuneko Priscilla Kokwe, who has been my pillar of strength through it all. In my darkest moments, she reminded me of my own resilience, lifting me up when I felt like falling. Her unwavering belief in me, her endless support, and her quiet strength gave me the courage to keep going. She has been my source of comfort, my rock, and my inspiration. I am endlessly grateful for her love, patience, and encouragement, and I could not have done this without her.

ACKNOWLEDGEMENTS

I would like to convey my profound gratitude to the following people for their contributions to the accomplishment of this effort.

- To begin with, I would like to express my heartfelt and profound gratitude to Prof. Feroz Mahomed Swalaha, my supervisor and mentor. His guidance, patience, motivation, support, and expertise have been invaluable throughout the entire process of completing this thesis. It has been an honour and privilege to have him as my supervisor. I am grateful for his constant encouragement and patience from the very beginning of this project. I also want to express my gratitude for educating me the significance of planning and for going beyond to ensure that I had the necessary resources to conduct my research. His constructive criticism has been instrumental in my growth as a researcher.

- I would like to express my gratefulness to my co-supervisor, Dr. Freedom Tshabuse, for his invaluable contributions to the writing of this thesis. Without his critiques, valuable suggestions, and thorough editing, completing this thesis would have been a challenging task. I am sincerely grateful for his enthusiasm, dedication, and extensive knowledge of the subject matter.

- • I also want to express my gratitude to my amazing sister and mother for their continuous support and affection. They have been a continual source of encouragement and inspiration.

- A special mention goes to Mr. Adedayo Lanrewaju for his continuous support and encouragement throughout this journey. I am truly grateful for his assistance, which has made this endeavor much smoother. We have been each other's pillars of support, and I deeply appreciate his presence.

- I extend my heartfelt appreciation to Mrs. Betty Ajibade, who has provided me with the assistance, encouragement and a nurturing company that I needed to succeed in achieving my Masters. Your contributions have been greatly appreciated.

- I express my sincerest gratitude to my lab technicians, Mr. Melvin Makolomakwa and Dr Adarsh Puri, who were consistently friendly and readily available for any assistance.

- I am sincerely grateful to my colleagues and friends whom I have had the privilege of meeting, for their consistent moral support, the intellectually enriching discussions we have had, and the delightful moments we have shared throughout our academic journey.

- Lastly, I would like to extend my thanks to the National Research Foundation for generously funding my master's program.

RESEARCH OUTPUTS

Conference Presentation

Kokwe, N.H., Tshabuse, F., and Swalaha, F.M. *In vitro* characterization and safety assessment of lactic acid bacteria isolated from the crops and small intestine of broiler chickens. 22nd Biennial Congress of the South African Society of Microbiology hosted by Stellenbosch University at the Protea Hotel, Techno Park, Stellenbosch, South Africa, 17 – 20 September 2023.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

U/ML: Units per millilitre

AGP: Animal Growth Promoters

ATCC: American Type Culture Collection

ANOVA: Analysis of Variance

BATH: Bacterial adherence to hydrocarbons

BHI: Brain Heart Infusion Broth

BLASTN: Basic Local Alignment Search Tool

bp: Base pair

CFS: Cell-Free Supernatant

D-value: Decimal Reduction Time

FCR: Feed Conversion Ratio

FDA: Food and Drug Administration

GDP: Gross Domestic Product

GIT: Gastrointestinal tract

GRAS: Generally Recognized as Safe

LAB: Lactic acid bacteria

Log CFU/mL: Log colony forming unit per millitre

MEGA: Molecular Evolutionary Genetic Analysis

MRS: De Man, Rogosa and Sharpe

NCBI: National Centre for Biotechnology Information

OD: Optical density

PBS: Phosphate buffered saline

PCA: Principal Component Analyses

PCR: Polymerase Chain Reaction

pNPG: *p*-nitrophenol-αD-glucopyranoside

SAPA: South African Poultry Association

W/V: Weight per volume

WHO: World Health Organization

1 CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION

The gastrointestinal tract (GIT) of poultry comprises of the oesophagus, gizzard, crop intestines (duodenum, jejunum, ileum), cecum, colon, and cloaca. It is populated with a variety microorganisms of different microbial communities (bacteria, fungi, protozoa, and viruses) that interact with the host and ingested feed (Pan and Yu 2014; Shang *et al.,* 2018). Intense interaction occurs between the host and gut microbiomes, such as pathogen exclusion, nutrients utilization and host immune system modulation (Shang *et al.,* 2018). Due to the variation of the microbiota in young chicks, adult chickens that are at least 20 weeks old are used for the definition of the core chicken microbiota (Rychlik, 2020). In the GI of chickens*, Lactobacillus* is the dominant microorganism found in the crop and gizzard (Gong *et al.,* 2007). A high microbial population is often observed in the ileum, the terminal segment of the small intestine, where *Lactobacillus, Clostridium, Enterococci,* and *Turicibacter* are found in high numbers compared to other compartments of the digestive tract (Han *et al.,* 2016; Seifert and Camarinha-Silva 2018; Torok *et al.,* 2008).

The GI microflora is essential for maintaining the health of the host by modulating several physiological functions including nutrition, metabolism and immunity (Diaz *et al.,* 2019). The gut microbiota protects the host by adhering to the mucus and epithelial walls, preventing the attachment of pathogenic bacteria to the intestinal cells. The microbiota accomplishes this by producing vitamins (vitamin K and vitamin B groups), short-chain fatty acids (SCFAs) such as acetic acid, butyric acid and propionic acid, organic acids (lactic acid) and antimicrobial compounds (i.e., bacteriocins), lower triglyceride, and inducing a non-pathogenic immune response, hence protecting both nutrition and the animal (Shang *et al.,* 2018). Characteristics such as age, diet and location of the host to determine the diversity of the microbiota, and each microorganism performs independent functions in each of the organs of the GI tract (Clavijo and Flórez, 2018). Most of the bacteria found in the GI of broiler chickens are not pathogenic organisms. However, specific microorganisms within that population may behave as opportunistic pathogens under certain conditions (Jeni *et al.,* 2021). The GI tract of broilers

serves as one of the protective barriers against pathogenic microorganisms that could affect the digestion and absorption of nutrients (Taheri *et al.,* 2009).

In the broiler industry, the management of intestinal health and function is crucial, as it influences the overall health and the use of nutrients by the bird (Eugenio and Santos 2021). Bacteria colonize the GI tract of young chicks during hatching and within the first hours of life. However, intensive poultry production prevents newly hatched chicks from maintaining contact with their mothers, resulting in slow colonization of microbial flora in their GI tracts. As a result, these chickens are susceptible to pathogens such as *Salmonella typhimurium (S. typhimurium), Escherichia coli O157:H7 (E. coli)*, and *Listeria monocytogenes* (*L. monocytogenes*), *Campylobacter jejuni* (*C. jejuni*) and *Salmonella enteritidis* (*S. enteritidis*) (Salehizadeh *et al.,* 2020).

The primary method for managing foodborne enteric infections has been the use of antibiotics, and some farmers, particularly those in high volume poultry production, use sub-therapeutic levels of antibiotics (such as bacitracin, virginiamycin and avoparcin) in animal feed as growth promoters (AGPs) (Tsega 2020). Unfortunately, increased antibiotic use has led to the widespread of antibiotic resistance among bacteria owing to the accumulation of antibiotic residues in animal carcasses and the transmission of these to consumers. Furthermore, antibiotics have been shown to alter the microflora including the beneficial LAB (Krysiak *et al.,* 2021). Interestingly, probiotics as alternatives to antibiotics have been defined as one of the potential strategies for preventing pathogen colonization and improving poultry production (Oyewole *et al.,* 2018).

Probiotics are defined as live microorganisms that are beneficial to the host when administered in sufficient amounts (Abd El-Hack *et al.,* 2020). They can regulate intestinal permeability, normalize the microflora, improve the gut microbiota barrier function, and enhance the balance between pro-inflammatory and anti-inflammatory cytokines. This is achieved through the synthesis of antimicrobial metabolites, including bacteriocins, organic acids, hydrogen peroxide, and diacetyl (Musikasang *et al.,* 2009; Oyewole *et al.,* 2018). Lactic acid bacteria (LAB) are commonly used microorganisms as probiotics, as they are considered generally recognized as safe (GRAS) (Hoque *et al.,* 2010). Recently, the role of these bacteria in the health and functionality of animal intestines has been emphasized due to their ability to increase

immunomodulation and prevent the adhesion of pathogenic microorganisms (Karimi Torshizi *et al.,* 2008, Shamsudin *et al.,* 2019).

Lactic acid bacteria, including species of *Lactobacillus*, *Enterococcus*, *Streptococcus, Lactococcus*, and *Pediococcus* are commonly used as probiotics as they are indigenous to the gut of animals and humans (Reuben *et al.,* 2019). Studies have demonstrated the ability of LAB-based probiotics to inhibit common poultry pathogenic microorganisms, such as *S. typhimurium*, *E. coli*, *L. monocytogenes*, *C. jejuni*, and *S. enteritidis* through competitive exclusion, enhancement of immunomodulation as a result of adhesion of the strain to the intestinal epithelium (Chen *et al.,* 2012, Gómez *et al.,* 2016, Makavchik *et al.,* 2019, Salehizadeh *et al.,* 2020). For strains to be potential probiotic candidates, they must withstand high levels of bile salt concentrations, survive in an acidic environment, produce antimicrobial compounds such as lactic acid, and successfully replicate in the GI tract of the host (Denkova *et al.,* 2017). In addition, they must be resistant to gastric fluids and adhere to the intestinal mucosa to increase their persistence and prevent the adhesion of pathogenic microorganisms via the formation of a protective barrier (Darmastuti *et al.,* 2021), which assists in maintaining the balance of the gut microflora. Furthermore, a cluster of bacteria can provide a stronger barrier against pathogenic microorganisms; hence, the ability of LAB to aggregate enhances their adhesion abilities (Darmastuti *et al.,* 2021).

The adhesion process is initially based on non-specific physical interactions between the bacterial cell and the surface to which it seeks to attach to (Reuben *et al.,* 2019). Specific interactions between adhesive components on the bacterial cell and complementary receptors on the surface take place after the initial physical interaction. As a result of specific interactions, a stronger and more stable attachment between the bacteria and the surface occurs as they are more targeted (Guan *et al.,* 2020). The cell surface of *Lactobacillaceae* bacteria comprises various components such as capsular polysaccharides, teichoic and lipoteichoic acids, surface proteins and lipoproteins. These components enable the ability of the bacteria to adhere to surfaces and form biofilms, which are crucial for their survival and colonization (Zawistowska-Rojek *et al.,* 2022).

According to Shokryazdan *et al.,* (2014), a strain intended to be used as a probiotic for chickens should be isolated from the indigenous microflora of the GI tract of chickens, as they can adapt to the gut environment. However, not all LAB can be potentially used as probiotics, hence, their properties and safety must be evaluated (Sirisopapong, 2023). Therefore, in this study, LAB were isolated from the crop and small intestine of chickens for the selection of the most suitable LAB strains with potential inhibitory effects against poultry pathogens to maintain the natural microflora of birds. In light of the aforementioned, this study aimed to evaluate the safety of LAB as a potential probiotic to reduce the dependence on antibiotics as prophylactics in poultry production.

1.2 RESEARCH PROBLEM

In South Africa, the poultry industry is essential in enhancing the country's economy, both in commercial concerns and in the supply of quality protein for subsistence farmers and rural communities (Kriel, 2022). According to the South African Poultry Association (SAPA) (2019), the poultry industry in SA is the largest agricultural sector, contributing more than 20% of its share of Gross Domestic Product (GDP) and 43% of animal product. However, the country does not produce enough poultry products to meet demand, and imports from other countries. Commercially reared chickens are usually exposed to numerous stressors, disrupting the gut microbiota, increasing disease susceptibility, and reducing production (Kimera *et al.,* 2020).

To counteract the losses due to diseases of poultry products, most farmers resort to the utilization of antibiotic therapy for the treatment and/or prevention of infections and to promote growth. Unfortunately, the overuse of antibiotics in animal husbandry has led to the emergence of antibiotic-resistant bacteria and the occurrence of residual antibiotics in food products (Reuben *et al.,* 2019) and has been further exacerbated by the slow introduction of novel antibiotics to the market. Furthermore, it was highlighted by Wu-wu *et al.,* (2023) that food safety and quality assurance cannot be guaranteed as these antibiotics can be obtained in the market without prescriptions and supervision of administration to chickens reared for food, leading to indiscriminate antibiotic use, not adhering to a proper withdrawal period of antibiotics, and incorrect dosage of antibiotics to promote chicken health by producers. Therefore, there is a need to find supporting therapies, such as probiotics, that may reduce dependence on antibiotics (Ardal *et al.,* 2020). Probiotics are beneficial microorganism

(FAO/WHO 2002) and are widely known to prevent diseases and improve host immunity. Since the gut microbiota is unique to each individual, this study will explore the probiotic microbial properties in South African chickens to exploit them to improve poultry health and yield.

1.3 AIM

To evaluate the probiotic potential of lactic acid bacteria isolated from the crop and small intestines of broiler chickens.

1.4 OBJECTIVES

- To isolate and characterize lactic acid bacteria from crops and small intestines of broilers using traditional microbiological methods.
- To preliminarily screen and evaluate the safety of lactic acid bacteria and their *in vitro* competitive activities against enteric poultry pathogens.
- To identify the selected LAB strains to species level using DNA sequencing.
- To assess the survival properties of the LAB strains in a simulated gastrointestinal tract.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 ANATOMY OF BIRD GASTROINTESTINAL TRACTS

Chickens comprise a simple, short, and extremely efficient digestive system that plays a significant role in converting feed into nutrients required for growth, maintenance, and reproduction (meat and eggs) (Salem *et al.,* 2023). The GI tract (Figure. 2.1) of birds is comprised of the beak, proventriculus, gizzard, duodenum, jejunum, ileum, two blind ceca, rectum, cloaca, liver and pancreas (Salem *et al.,* 2023). In the upper gastrointestinal tract, the beak accumulates the food, ingested saliva. After the feed is continuously not available, it moves through the oesophagus to the crop, where it is moistened; it further passes through the proventriculus, where it undergoes chemical digestion, much like in mammals' stomachs. It is then transferred into the gizzard, where a rhythmic contraction reduces the coarseness of its contents, preparing it for digestion and assimilation by the distal portion of the GI tract (Rodrigues and Choct 2018).

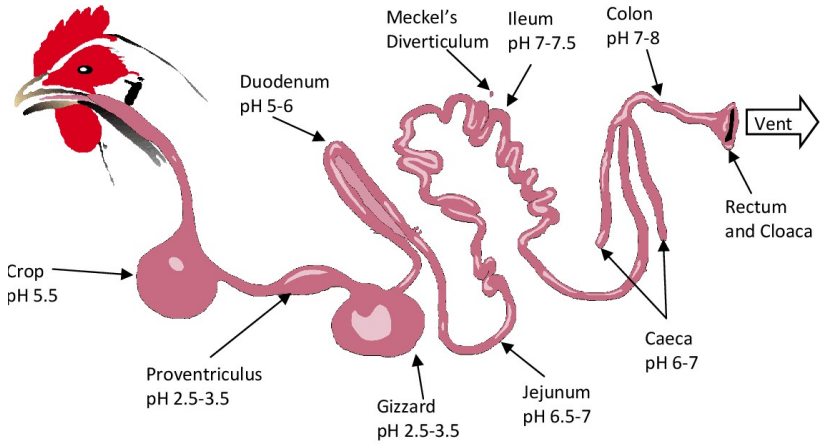


Figure 2.1: The gastrointestinal tract of birds (Bailey, 2010).

Structurally, the crop is a ventral diverticulum (sac) with the ability to disperse and store food due to longitudinal folds on its inner surface and it is not actively involved in the chemical digestion process because of the lack of enzyme secretion and absorption. However, it is essential for the activation of exogenous enzymes by moistening the feed and it is the seat of fermentation by lactic acid bacteria (Rodrigues and Choct 2018). Several studies have reported that the environment of the crop is highly variable depending on several factors, which include:

- pH, buffer capacity, and fibre content particle size of the feed;
- pH of the drinking water.
- feed retention and fermentation time, which depends on feed presentation, nature of the different substrates in the feed (Classen *et al.,* 2016; Cutler *et al.,* 2005; Fonseca *et al.,* 2010; Józefiak *et al.,* 2006;).

Beyond the crop, the thoracic oesophagus connects the gizzard and the proventriculus, also referred to as the ventriculus. The gizzard is composed of very dense muscular walls due to its mechanical function to break down and grind food (Salem *et al.,* 2023; Rodrigues and Choct 2018). The gizzard/proventriculus is also responsible for promoting feed regulation which is achieved through induction of satiety by vagal signals induced by stretch and muscular activity, humoral signals, such as ghrelin, gastrin, and cholecystokinin and limiting feed passage from the gizzard through the duodenum by a sieve-like structure (Rodrigues and Choct 2018).

According to Salem *et al.,* (2023), the low pH of the gizzard was found to make it a natural microbial barrier. The pH value is dependent on several elements, including physical and chemical characteristics of the feed (pH of the feed), the amount of feed in the organ (the feed intake), retention time and the methods used to measure the pH (Rodrigues and Choct 2018). A study by Lee *et al.,* (2017), reported the different pH values of the gizzards ranging from 1.2 to 2.1 captured by using real-time pH readings through the capsule retained from the gizzard in response to different dietary treatments.

Chicken small intestines are composed of a single layer of epithelium, which includes absorptive, goblet, and entero-endocrine cells. These cells line the villi and crypts, and goblet cells produce mucus through the secretion of mucins (Casteleyn *et al.,* 2010). The villi in the small intestine act as functional units and are extensions of the lamina propria into the lumen of the gut. The villi increase the surface area and vary in size and length from the proximal to the distal end (Rubin and Shaker, 2015). The small intestine facilitates primary digestion and absorption. It has a short retention time in the duodenum, which leads to the formation of a loop around the pancreas where digestive enzymes are received (Salem *et al.,* 2023). The duodenum is the primary location for minimal intestinal absorption, while the ileum is responsible for the absorption of nutrients, water, and minerals (Smith, 2022).

The avian large intestine is comprised of two blind caeca situated at the junction of the ileum and colon. These caeca are enveloped by an intricate network of elongated interdigitating villi, which serve as selective filters for sieving, fluid separation, and the passage of small particles from the ileum into the colon (Salem *et al.,* 2023). The caeca is responsible for the microbial carbohydrate degradation and fermentation, as well as the production of microbial vitamins and amino acids. Moreover, they contribute to compound synthesis, the conversion of urea into amino acids, and ensure water absorption and balance (Salem *et al.,* 2023).

2.2 THE GUT MICROBIOTA OF BROILER CHICKENS

Microorganisms adhere to the gut mucosal epithelium, gut lumen and caeca of chickens (Donaldson *et al.,* 2017). Characteristics such as age, diet and location of the host have been shown to contribute to the diversity of the microbiota, and each microorganism performs independent functions in each of the organs of the GI tract (Clavijo and Flórez 2018). Research on the identification of the chicken gut microbiota was initially limited by conventional microbiological methods. However, the emergence of 16S rRNA-based next-generation sequencing techniques has enabled the characterization of the chicken gut microbiota (Ngunjiri *et al.,* 2019; Shang *et al.,* 2018). Even though these techniques has been widely used in various scientific fields for several years, the initial study characterizing the gut microbiota of chickens was published in 2011 (Danzeisen *et al.,* 2011). The different microbes present in the GI tract of broiler chickens and most of the bacteria found in the GI tract of broilers are not pathogenic

organisms (Figure 2.2), but specific microorganisms within that population can behave as opportunistic pathogens in the presence of certain conditions (Jeni *et al.,* 2021).

According to Fathima *et al.,* (2022), *Streptococcus* and coliforms are the first species to colonize the GI tract of chickens by 3 days after hatching. By day 40, the microbiota of the small intestine is dominated by *Lactobacillus* species. The caecal microbiota is established between 6 and 7 weeks and is primarily composed of facultative and obligate anaerobic microbes including *Enterobacteria*, *Clostridia*, faecal *Streptococcus*, *Pediococcus*, and *Pseudomonas aeruginosa* (Donaldson *et al.,* 2017; Shang *et al.,* 2018).

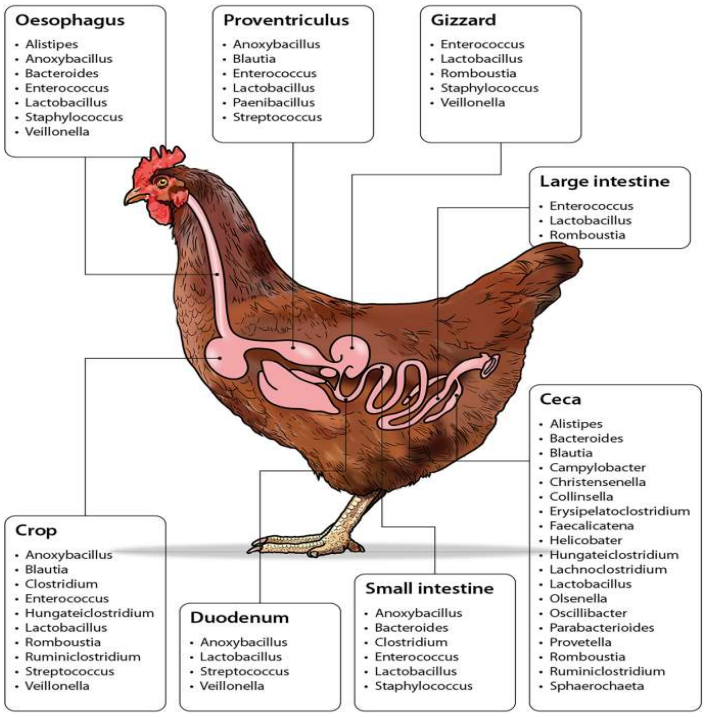


Figure 2.2: Diversity of the gut microbiota in broiler chickens (Ndotono *et al.,* 2022).

The gut microbiota of adult chickens that are at least 20 weeks (about four and a half months) old has been used for the definition of the gut microbiota, as the microbiota of young chicks is highly variable (Rychlik, 2020). The GI tract sections are essential for different metabolic functions that shape the microbial community (Shang *et al.,* 2018), it is therefore, crucial to study the chicken microbiota interactions, paying special attention to the sample location and study design (Rychlik, 2020). The crop microbiota consists of microbial communities of

bacterial cells ranging from 10^8 to 10^9 CFU/g, dominated by *Lactobacillus* spp (Ricke and Rothrock, 2020). Other bacterial phyla sequenced from the crops of chickens include *Enterococcus* (Choi *et al.,* 2014), *Bifidobacterium*, *Escherichia coli*, *Pseudomonas aeruginosa*, etc (Fathima *et al.,* 2022). In the intestinal section, in the duodenum, the density of bacteria is the lowest. This is due to the short passage time and bile dilutions, with clostridia, streptococci, enterobacteria, and lactobacilli present. The ileum is, however, dominated by *Lactobacillus*, *Clostridiaceae*, *Streptococcus*, and *Enterococcus* (Waite and Taylor, 2015). The microbiota of the ileum has been studied the most among the intestinal segments. Xiao *et al.,* (2021) assessed the microbial diversity and community variation in the intestine of chickens by existing 16S rRNA sequencing and found *Lactobacillus* as the major group (75.5%), followed by *E. coli* (60,5%), *Clostridium* (56,4%) and *Lachnospiraceae* (20.1%). According to Zhu *et al.,* (2019), environmental factors such as heat stress can significantly disrupt the composition of the intestinal microbiota during intensive poultry farming, therefore, the regulation of the intestinal microbiota is necessary.

2.3 ANTIBIOTICS USE IN POULTRY AS GROWTH PROMOTERS

Antibiotics are compounds (semi-synthetic or synthetic) that possess antimicrobial activity and have been actively used in the poultry industry for the treatment and prevention of diseases, growth promotion of livestock increased egg and meat production (Adams *et al.,* 2023; Mund *et al.,* 2017). The discovery of penicillin, streptomycin and chlortetracycline started the usage of antibiotics in chickens as these drugs were used to control and prevent disease outbreaks. Furthermore, these drugs were described to be beneficial to the host, including enhancing the microbiota of the GI tract of chickens and improving the overall health of the bird (John *et al.,* 2024). However, the increased utilization of antibiotics has led to several consequences, ranging from the presence of antibiotic residual contents in meant and eggs to the resistance of bacteria to antibiotics in both humans and animals (Thames and Sukumaran, 2020).

As a result, in 2006, the European Union banned the usage of antibiotics as growth promoters and global initiatives like the Global Action Plan on Antimicrobial Resistance were established to address the concerns about antibiotic resistance (African CDC, 2023). The framework highlighted the significance of addressing antibiotic resistance in poultry production to ensure

sustainable practices, animal welfare and food safety, aligning with global efforts to combat antimicrobial resistance and promote new alternatives in agriculture.

Antibiotics have been documented as being administered in poultry in three different ways, including parenterally, or intravenously and orally (Mund *et al.,* 2017). The mechanisms of action of antibiotics upon various target microorganisms involve the inhibition of different processes in microorganisms responsible for infections, including DNA replication, cell division, differentiation and development, folic metabolism, and cell membrane and cell wall synthesis (Diarra and Malouin, 2014).

In developing countries, commonly used antibiotics for treating respiratory diseases and necrotic enteritis include tetracycline, gentamicin, neomycin, tylosine, erythromycin, virginiamycin, ceftiofur and bacitracin (Filazi *et al.,* 2013; Fuoco, 2012). Fluoroquinolones and quinolone compounds have been used for the treatment of gastroenteritis and skin infections, while sulphonamide compounds have been used for managing coccidiosis together with amoxicillin, amprolium, ciprofloxacin and sulpha drugs (Fuoco, 2012). In certain countries such as China, the UK, Brazil and Europe, some classes of antibiotics have been approved for prophylactic use. These include tetracyclines, aminoglycosides and fluoroquinolones, while others such as glycolipids, macrolides and glycopeptides have been prohibited in the EU (Mund *et al.,* 2017).

The utilization of antibiotics in animals reared for food is greater than in humans to treat diseases (WHO, 2017), which poses a serious public threat because of the emergence of bacterial resistance to antibiotics that are transmitted between humans and animals via foodborne or indirect contamination through the environment (Ferdous *et al.,* 2019). Gram-negative foodborne pathogens such as *Acinetobacter* spp., *E. coli, Klebsiella* spp., and *Salmonella* spp. are some of the main bacteria responsible for poultry diseases (Carlet *et al.,* 2012). Several studies have reported a high level of antibiotic resistance bacteria in meat samples collected from birds during the slaughtering and processing of carcasses (Awad *et al.,* 2020; Elkenany *et al.,* 2019; Kanaan, 2023). Yang *et al.,* (2020) investigated the prevalence and levels of *Salmonella* contamination as well as the antibiotic profiles of raw meat samples in various regions of China. In this study, a total of 66 poultry meat samples from 39 cities were analyzed, and 249 samples were contaminated with *Salmonella*, indicating a rate of

37.5%, thereby highlighting the significant presence of *Salmonella* in poultry products available in retail markets.

Notably, concerning results of antibiotic profiles were reported, whereby out of the tested 318 non-duplicate *Salmonella* isolates, only a small portion (1.6%) showed susceptibility to the tested 22 antibiotics. A significant majority (60.1%) demonstrated resistance to at least three classes of antibiotics tested. The highest level of resistance was observed against nalidixic acid (72.3%), ampicillin (55,3%) and streptomycin (48.7%), thus indicating the presence of multi-drug resistant Salmonella strains in the poultry meat samples. These findings highlight the potential health risk associated with consuming poultry products contaminated with such multi-drug resistant *Salmonella* isolates, emphasizing the importance of controlling the utilisation of antibiotics in poultry farming (Yang *et al.,* 2020).

The prolonged use of antibiotics has also resulted in the presence of antibiotic residues in poultry products, which poses a significant health concern. A study by Sani *et al.,* (2023) aimed to determine the presence of antibiotic residues in poultry products using thin-layer chromatography for qualitative analysis and high-performance liquid chromatography (HPLC) for quantitative analysis. A total of 230 samples were tested, including 40 meat, 30 eggs, 140 feed and 40 faeces samples. It was found that 23.5% of the tested samples contained residues from six different classes of antibiotics, including tetracyclines, quinolones, beta-lactams, sulphonamides, aminoglycosides and macrolides. The highest antibiotic residues were detected in egg samples (50%) and meat samples (35%). It should be noted that these samples were also found to have exceeded the maximum residue limit (MRL) with a small percentage of 2.5% (meat) and 3.5% (eggs) for antibiotics. Oxytetracycline, followed by doxycycline and ciprofloxacin were the most commonly detected antibiotics in both the meat and the egg samples. While antibiotics below the maximum residue limits are considered safer for human consumption, their presence can lead to the development of antimicrobial resistance in pathogens, posing a potential health risk.

Jammoul and El Darra (2019) also analysed the presence of antibiotic residues in 80 chicken samples to assess the extent of antibiotic contamination in chicken meat consumed by the public. The quantification of antibiotic residues was done by liquid chromatography-mass

spectrometry, where 30 different antibiotics from distinct chemical classes; namely sulphonamides, tetracyclines, quinolones and beta-lactams, were detected. It was found that 77.5% of the samples were contaminated with antibiotic residues and within this subset, 53.7% of the samples demonstrated the presence of multidrug residues indicating a concerning level of co-occurrence. Ciprofloxacin (32.5%), amoxicillin (22.5%) and tetracyclines (17.5%) were found to have the highest occurrence, respectively. These findings confirm the presence of antibiotic residues in chicken samples, which further necessitates the importance of finding feasible products as substitutes to antibiotics.

2.4 HISTORY AND DEFINITION OF PROBIOTICS

The term probiotic was derived from the Greek word “Pro bios”, meaning “for life”, and it was first introduced by Lilly and Stillwell, (1965) to define substances secreted by microorganisms to promote growth. The term was later improved by Parker, (1974), who defined probiotics as microorganisms/substances that contribute to intestinal microbial balance. However, Fuller (1989) improved Parker’s definition due to the term substances which includes antibiotics, and later defined probiotics as “live microbial feed supplements which beneficially affect the host animal (referring to animals and humans) by improving the balance of the gut microbiota”. Havenaar *et al.,*(1992) broadened Fuller’s definition as a mono or mixed culture of live microorganisms that beneficially affect the host by enhancing the properties of indigenous microflora. In 1989, the USA Food and Drug Administration (FDA) suggested that the term direct-fed microbial (DFM) be used instead of probiotics and defined DFM as live microorganisms that are naturally occurring, including bacteria, fungi, and yeast (Jin *et al.,*. 1997).

In 2002, the Food and Agriculture Organization and World Health Organization defined the term "probiotics". According to their definition, probiotics are live microorganisms that can offer health advantages when administered in sufficient quantities. These microorganisms may not be permanent residents of the gastrointestinal tracts, but they should have a positive impact on the overall health of both humans and animals. In 2013, the definition was updated by the International Scientific Association as live microbes of strictly selected microorganisms that are advantageous to the host when administered in adequate amounts (Lambo *et al*., 2021). According to these definitions, numerous microbial species and genera are considered

probiotics. These include *Lactobacillus, Lactococcus, Bifidobacteria, Streptococcus, Pediococcus, Bacillus,* and *Enterococcus*. Among them, the most common representative species are *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus rhamnosus, Lactobacillus gasseri, Bifidobacterium bifidum, Bifidobacterium infantis, Enterococcus faecalis*, and *Enterococcus faecium* (Makete, *et al.,* 2017).

2.5 CLASSES OF MICROORGANISMS USED AS PROBIOTICS

2.5.1 Lactic Acid Bacteria

Probiotics are made from microorganisms belonging to various genera (Table 2.1). The heterogeneous group of lactic acid bacteria are commonly used as probiotics (Shokryazdan *et al.,* 2014). Lactic acid bacteria are Gram-positive, catalase-negative, non-spore-forming, and obligate anaerobes (non-respiring) but aerotolerant bacteria that produce lactic acid as a by-product and end-product after carbohydrate fermentation. In addition, the organisms' capacity to produce organic substances that enhance flavour, texture and aroma, giving them the distinct organoleptic characteristics (Ayivi *et al.,* 2020). Zheng (2024) reported that a monograph for the classification of lactic acid bacteria was published by Orla-Jensen in 1919 based on the following characteristics: carbohydrate fermentation characteristics, cell morphology, ability to use sugars and optimum growth temperature range. As a result, only four lactic acid bacteria from the genera of *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Leuconostoc* were recognized.

Lactic acid bacteria have been classified as homofermentative or heterofermentative, according to their capacity to ferment carbohydrates (Ayivi *et al.,* 2020). Homofermentative (ferment hexose) converts one glucose molecule into two lactate molecules. Due to their ability to produce high concentrations of lactic acid in fermentation, homofermentative LAB such as *Pediococcus*, *Streptococcus*, *Enterococcus* and *Lactobacillus* are widely used as starter cultures for dairy, meat and vegetables (de Souza *et al.,* 2023). Heterofermentative LAB (ferment pentoses) have been found to produce lactate, ethanol and carbon dioxide from one glucose molecule (Ayivi *et al.,* 2020). Lactic acid bacteria selected as probiotics must be generally recognized as safe, non-pathogenic, and non-toxic, and every strain must consist of special properties to be considered as a potential probiotic (Lambo *et al.,* 2021; Shokryazdan *et al.,* 2014).

Table 2.1: Common microorganisms used as probiotics.

***Lactobacillus* spp**	***Bifidobacterium* spp**	**Others**
L. acidophilus	*B. adolascentis*	*Enterococcus faecalis*
L. brevis	*B. animalis*	*Enterococcus faecium*
L. casei	*B. bifidum*	*Lactococcus subp. cremoris*
L. fermentum	*B. breve*	*Pediococcus acidilactici*
L. gasseri	*B. lactis*	*Streptococcus salivarius subsp. thermophilus*
L. johnsonni	*B. longum*	
L. reuteri		
L. rhamnosus		
L. salivarius		

Adapted from Mathew (2019).

2.5.1.1 *Lactobacillus*

Lactobacillus species are facultatively anaerobic, lactic acid-producing Gram-positive bacteria that are non-sporulating and commonly found in the human and animal gastrointestinal tracts and genitourinary tracts (Li *et al.,* 2020); they can also be isolated from dairy products, pickles, and fermented foods (Feng *et al.,* 2018). According to Bratcher (2018), the *Lactobacillus* species are considered to be both catalase-negative, can ferment carbohydrates and hydrolyze esculin. These microorganisms are ingested to recolonize the normal flora of the body and assist in improving growth factors and increasing the bioavailability of minerals (Aryana and Olson 2017). Lactobacilli have also been shown to play a role in stabilizing the mucus barrier and in reducing intestinal permeability (Bottari *et al.,* 2017). Lactobacilli are composed of 253 species, which vary depending upon the type of animal from which they are isolated and by the food they eat, such as herbivores, carnivores, and omnivores (Endo *et al.,* 2010). For example, in carnivores, the most dominant *Lactobacillus* spp are *L. johnsonii, L. reuteri, L. salivarius, L. vaginalis* and *L. ingluviei,* which are not commonly found in both omnivores and herbivores.

Several species from the *Lactobacillus* genus are usually administered as probiotics in both dairy and non-dairy food products produced for human consumption (Hammes *et al.,* 1999; Mbarga *et al.,* 2021). Furthermore, studies have reported the beneficial effect of the

Lactobacillus genus in the reduction of fish mortality (Newaj-Fyzul *et al.,* 2014; Ohashi and Ushida, 2009), improvement of growth performance in piglets (Huang *et al.,* 2004), and the improved production and quality of eggs in chickens (Bajagai *et al.,* 2016). Kim *et al.,* (2018) reported that *Lactobacillus* strains producing active dietary enzymes, including protease, amylase, lipase, and phytase, are essential potential probiotic strains, as these enzymes are involved in the digestion and absorption of nutrients.

In poultry, the administration *of Lactobacillus* strains as probiotics enhances both the digestion feed and absorption of nutrients (Sirisopapong *et al.,* 2023). A study by Betancur-Hurtado *et al.,* (2022) assessed the beneficial effect *of Lactobacillus salivarius* LP-40 on the health and performance of broiler chickens. This was achieved by administering *L. salivarius* LP-40 strain to broiler chickens through their drinking water and the effect of this supplementation on various performance parameters of the chickens for 42 days. It was found that the administration of this strain led to a significant ($p<0.05$) enhancement in body weight, and feed conversion efficiency of the broiler chickens, suggesting that *L. salivarius* LP-40 strain can potentially be a viable alternative to antibiotic treatment in poultry farming. The results obtained in this study were further compared to a control group that did not receive any treatment and another group that was treated with the antibiotic enrofloxacin.

Sureshkumar *et al.,* (2020) investigated the beneficial effect of orally administered *Lactobacillus salivarius* on various aspect on the health and performance of chickens. In this study, 10^9 CFU/ml of wild-type *L. salivarius* or a control substance, phosphate-buffered saline (PBS) were given to chickens over 5 weeks to observe any differences in outcomes. The microbial composition in the small intestine and caecum of the chickens was analyzed and a total of 286,331 bacterial species were identified across all experimental groups. The groups that received oral treatment of *L. salivarius* had 145, 012 bacterial species while the control group had 141, 319 species, suggesting the potential influence of the administered *L. salivarius* on the gut microbiota of chickens. Furthermore, the potential modulation of the immune system was also observed as the levels of certain cytokines (immune system signalling molecules), such as IL-8, TNF-α, IFN- γ and IL-4, were reduced in the *L. salivarius* group.

2.5.1.2 *Bifidobacterium*

Bifidobacteria are Gram-positive, catalase-positive anaerobic organisms that produce lactic and acetic acid from carbohydrates without the production of CO_2. Tissier first isolated Bifidobacteria in 1899-1990 and characterized them as rod-shaped, non-gas-producing anaerobic microorganisms. They are typically found in the natural flora of breastfed infants. A total of 30 species have been reported in the genus *Bifidobacterium*, 10 of which are were isolated from human sources such as faeces, vagina, and dental caries, 17 from the animal digestive tract or rumen, two from wastewater, and one from fermented milk (Makete *et al.,* 2017). They have been shown be responsible in the de-conjugation of bile acid, catabolism of dietary carbohydrates, and vitamin production (Jose, 2015). A large number of *Bifidobacteria* may prevent gut colonization by pathogenic bacteria and improve intestinal peristalsis, cancer prevention, cholesterol metabolism, and carbohydrate metabolism. This has led to the recognition of bifidobacteria to be used as probiotics (Hadebe, 2016).

In poultry, the species *Bifidobacterium animalis*, *Bifidobacterium thermophilum* and *Bifidobacterium longum* have been commonly used as feed additives (Arsène *et al.,* 2021). They have, respectively, previously demonstrated to reduce coccidiosis in broiler chickens infected with *Eimeria tenella (*Jungersen *et al.,* 2014), have antagonistic effect against *Salmonella* and *Listeria* species *in vitro* (Santini *et al.,* 2010) and against *E. coli* in chickens (Tanner *et al.,* 2016). Mortada *et al.,* (2020) investigated the effectiveness of *B. animalis* in reducing *Campylobacter coli* and the probiotic reduced the *in vitro* proliferation of *Campylobacter*. The administration of *Bifidobacterium* as probiotics in poultry feed has been shown to exert a positive effect and has been extensively tested for its application as a potential probiotic and as a supplement to antibiotics (Arsène *et al.,* 2021).

2.5.1.3 *Enterococcus*

Enterococci are Gram-positive, catalase-negative organisms that convert glucose to lactic acid as the main product of primary metabolism. They are non-spore-forming, oxidase-negative, and facultative anaerobes (Ramos *et al.,* 2020). The genus Enterococcus is composed of different species, but only two are often associated with probiotics and these are *E. faecalis* and *E. faecium* (Wang *et al.,* 2020). *Enterococcus faecium* is primarily used as an animal probiotic as it can produce lactic acid, survives in highly acidic conditions, is resistant to antibiotics and

inhibits the growth of pathogenic bacteria (Jose, 2015). E*nterococcus* spp has been used in poultry (Hanchi *et al.,* 2018) and pig nutrition (Pereira *et al.,* 2022). *E. faecium* has demonstrated the ability to stimulate other lactic acid bacteria and improve the weight gain and food conversion rate (FCR) in chickens (Arsène *et al.,* 2021). Moreover, a study by Cao *et al.,* (2013) reported improved growth performance and intestinal morphology in broiler chickens after supplementing the feed with *E. faecium.* However, this genus is associated with the pathogenesis of the urogenital tract and chicken endocarditis. In addition, these bacteria may transmit resistance to antibiotics; hence, this genus must be evaluated for safety before being used as an alternative probiotic (Arsène *et al.,* 2021).

2.6 CRITERIA FOR THE SELECTION OF PROBIOTICS

Microorganisms selected as potential probiotic candidates are expected to have desirable properties that are assessed during the development of new probiotics (Table 2.2). For the evaluation and assessment of potential probiotic strains, comprehensive approaches are required due to the probiotic range of the target functions and technological applications (de Melo Pereira *et al.,* 2018). For the selection of probiotic candidates, studies must adopt the selection criteria validated by WHO/FAO, including resistance to acid conditions, bile salts, epithelium adhesion ability and antagonistic activity (de Melo Pereira *et al.,* 2018; Kaur *et al.,* 2021). Figure 2.3 demonstrates preliminary ways to select probiotics as feed additives in the poultry industry. Different i*n vitro* experiments have been optimized for the initial screening of probiotic isolates, and the competitiveness of the strains selected *in vitro* must be further validated *in vivo* to monitor their persistence in chickens.

Table 2.2: Summary of desirable characteristics of isolated probiotic strains

Properties of probiotic strains	Functional properties
Adherence to mucus/ or epithelial cells and cell lines	Probiotic strains colonize the host's gut by adhering to intestinal cells. Adhesion can occur through either non-specific means, which are influenced by physiochemical factors, or through specific means that rely on adherent molecules on the surface of the bacteria and receptor molecules present in the epithelial cells.
Resistance to gastric acids, pancreas enzymes and bile	Probiotic strains must be able to resist low pH, different concentrations of bile and pancreatic enzymes to thrive and survive in the GI tract of the host.
Non-pathogenic	Probiotics should possess the status GRAS, as they are mostly marketed as foodstuffs or drugs.
Natural inhabitant of the targeted species	For probiotic strains to be more beneficial to a certain host, they must be isolated from that species.
Exert beneficial effects on host animal e.g. improved growth performance and enhanced immune system	The intake of probiotics results in several health benefits such as increased modulation and increased resistance to infections. Hence, the probiotic strains need to beneficial to the host.
Prolonged viability under storage and field conditions	During product manufacture and storage, the selected probiotic strains need to have characteristics such as strain viability.

Adopted from Makete *et al.,* (2017)

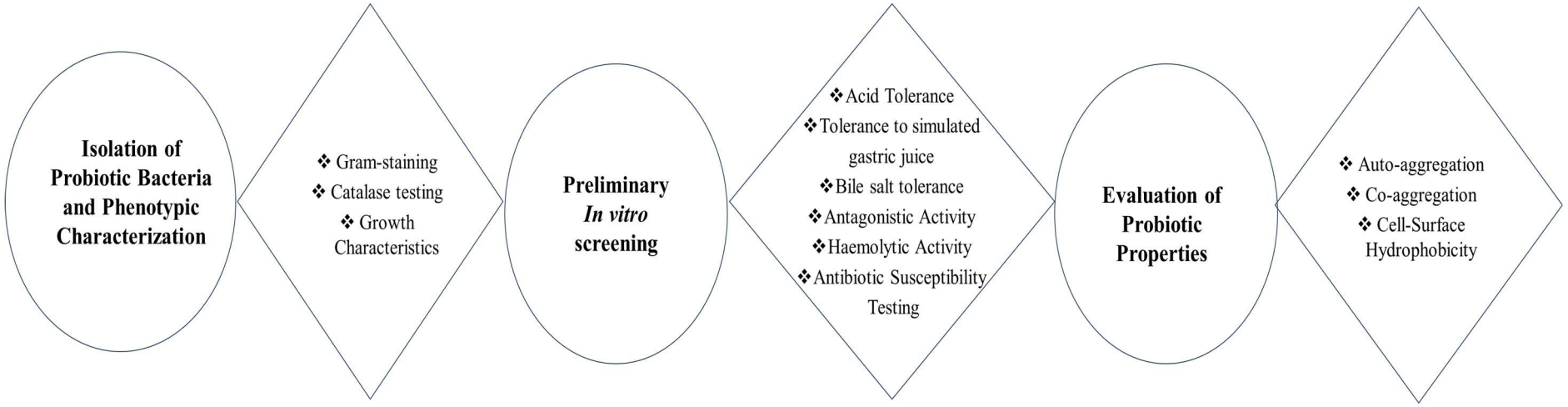


Figure 2.3: Criteria for the screening of probiotic strains in poultry (Adapted from Lutful, 2009)

2.6.1 Sources of Isolation

Probiotics can be obtained from several sources, including the digestive tracts of animals and humans, dairy and dairy-related products and fermented foods (Shokryazdan *et al*., 2017). To maximize the effectiveness of LAB as probiotics, it is encouraged to isolate these bacteria from the specific host species they will be used for. This can ensure that the strains selected are well-suited for the intended purpose and environmental use for the homologous host (Huang *et al.,* 2020). Probiotics targeted to human use, the GI tract and breast milk are identified as reliable sources of isolation (Ryan *et al.,* 2008). The GI tract of humans (the small intestine and the stomach) consists of diverse microbial communities that participate in a crucial role in enhancing human health (Nueno-Palop and Narbad, 2011). Similarly, breast milk, which is known for its nutritionally beneficial effects, also harbours bacteria that contribute to the colonization of the newborn's aseptic intestine with beneficial microflora (Damaceno *et al.,* 2023). This colonization is essential for the enhancement and development of the infant's immune system and overall health (Lyons *et al.,* 2020).

Apart from the GI tract and breast milk, several studies have isolated probiotic strains from faecal samples of individuals of different age groups including adults (Bazireh *et al.,* 2020), and children (Jomehzadeh *et al.,* 2020; Kook *et al.,* 2019). These probiotics have been reported to enhance and maintain gut health and support various physiological functions. Furthermore, the abundance of probiotic bacteria in faecal matter highlights the importance of exploring diverse sources for isolating potential probiotic strains for human benefits and consumption (Tulumoglu *et al.,* 2013).

Probiotic candidates have also been isolated from different animal species like wild boar (Li *et al.,* 2020a), poultry (Wang *et al.,* 2023) and pigs (Kaewchomphunuch *et al.,* 2022). For instance, (Shahbaz *et al.,* 2024) isolated four Lactobacillus strains, namely; *Lactobacillus delbrueckii*, *Lacticaseibacillus casei*, *Ligilactobacillus salivarius* and *Limosilactobacillus antri* strains from the gut of 10 broiler chickens that might be recommended as supplements for broiler diets with positive impact in broiler production. Another study by Kim *et al.,* (2007) isolated *Lactobacillus* amylovorus, *Lactobacillus salivarius* and *Bifidobacterium thermacidophilum* from the gut of healthy pigs after slaughter. The gut of animals, including

those mentioned above, can harbor beneficial bacteria that qualify as probiotics. This diversity in sources provides researchers with a broad spectrum to explore and potentially discover novel probiotics.

The isolation of probiotic strains is not only limited to the animal gut but also animal faeces as they have been reported to be contaminated with a high number of probiotic bacteria. Adetoye *et al.,* (2018) isolated a total of 88 LAB belonging to 15 different species from cattle faeces, where the most common species were *Streptococcus infantarius* (26), *Enterococcus hirae* (12), *Lactobacillus amylovorus* (10), *Lactobacillus mucosae* (10) and *Lactobacillus ingluviei* (9). It was further found that the isolated LAB strains demonstrated promising probiotic *In vitro* properties. Another study reported that the bacterial count of LAB obtained from the faeces of commercial pigs, native pigs and wild boar was found to be ranging from 4.64 to 7.39 $\log_{10}$ CFU/g, highlighting the prevalence of these bacteria in the faecal samples. Moreover, these LAB strains belonged to thirteen different species across *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Weissella* genera, indicating the variation of LAB found in the gut of microbiota of pigs (Huang *et al.,* 2020). However, more research must be done to provide valuable insight into practical applications of LAB strains isolated for probiotics used in livestock production and animal health.

Dairy products have been also used as a source of probiotic isolation (Fontana *et al.,* 2013). *Lactobacillus* strains and other lactic acid bacteria strains derived from fermented dairy products have been used have a long history of use and have been isolated in various regions like Iran (Vasiee *et al.,* 2022), Ethiopia (Taye *et al.,* 2021), and China (Liang *et al.,* 2021). In a study by Obioha *et al.,* (2021), LAB including *L. fermentum*, *S. thermophilus* and *E. thailandicus* isolated from dairy traditional fermented products for their potential use as starter cultures for controlled fermentation, were found to have the potential to stimulate the fermentation of dairy traditional fermented products. Further research, however, needs to be done to determine the appropriate conditions for upgrading these traditionally fermented products.

2.6.2 Isolation and Identification of Probiotic Strains

The isolation and identification of specific probiotic strains are essential to assess their potential beneficial effects on humans and animals (Fontana *et al.,* 2013). The conventional screening method for probiotic strains uses different types of selective media selected based on the specific purpose of probiotics (Qi *et al.,* 2021). For example, the isolation of potential probiotic strains from non-intestinal sources involves cultivating them on a high nutrient-rich medium that is modified from the conventional de Man, Rogosa and Sharpe (MRS) agar (Pramanik *et al.,* 2023). For instance, a medium containing mainly of glucose yeast extract and peptone (GYP) was utilized in the study by Kerry *et al.,* (2018) for the isolation of LAB from paddy rice silage, crop and silage fermentation. For the screening of LAB from different parts of animals, a modified medium is also utilized, these include modification of the pH conditions, nutrients and substrates (Yadav and Shukla, 2017). Jannah *et al.,* (2014) isolated LAB from chicken lumen contents (crop, ventriculus, ileum and cecum) from three 9-12 months old Cemani chickens. In this study, MRS agar glucose yeast extract peptone (GYP) agar supplemented with 0.5% (w/v) $CaCO_3$ was used to differentiate between acid-producing LAB. LAB isolated from fermented foods such as pickles were also isolated in acidic conditions by making use of a medium supplemented with glucose, yeast extract, peptone and 0.5% (w/v) $CaCO_3$ (Yu *et al.,* 2023).

The identification of isolated probiotic strains is crucial in probiotic research as the characteristics of probiotics are mostly strain-specific (Shokryazdan *et al.,* 2017). Phenotypic and genotypic techniques should be used in the identification of the isolated potential probiotic strains (FAO, 2017). Phenotypic methods, including Gram-staining, colony and cell morphologies growth characteristics, enzymatic production and metabolic activities have been widely used in several studies as the initial screening methods (García-Hernández *et al.,* 2016; Lim *et al.,* 2019; Reuben *et al.,* 2019; Vantsawa *et al.,* 2017). For the phenotypic identification of LAB isolated from different biotopes including sourdough and fermented vegetables (green olives, pepper, lemons and quinces), Atfaoui *et al.*, (2022) assessed the physiological characteristics such as tolerance to different growth temperatures of 10, 25, 37 and 45°C as well as salt concentrations of 2, 4, 6.5 and 10% NaCl. Furthermore, biogeochemical was also done to assess the fermentation types of the isolated LAB and their ability to ferment sugars. It was found that the isolated LAB were homo-fermentative, and fermented sugars such as glucose, galactose, fructose, xylose and maltose. It was further found that the isolates can

growth well at different temperatures as well as different NaCl concentrations. However, it was also found that there was no fermentation of sugars like sucrose, lactose, sorbisol and manitol. Based on the biochemical using an API CHL system results, the researchers were able to conclude that the isolated LAB belonged to the genus *Lactobacillus* and *Leuconostoc lactis*.

According to Shokryazdan *et al.,* (2017), an API system (BioMerieux, Marcy l'Etoile, France) identifies microorganisms based on their patterns of carbohydrate fermentation. However, this system is not always accurate in identifying the isolated LAB. This can be seen in the study by Reuben *et al.,* (2019), whereby results from both the API system and 16S rRNA sequences showed inconsistencies, and it was concluded that the API system can lead to misinterpretation and misidentification of the isolated LAB. Moreover, factors like the diverse nature and similarities of phenotypic characteristics can make the identification difficult as they are often based on observable traits such as shape, size and growth patterns, which can be influenced by environmental factors. As a result of these challenges, molecular techniques have gained popularity as they provide a more comprehensive understanding of microbial diversity from different sources, that can be used as potential probiotics.

Various contemporary techniques have been utilized to identify and differentiate the isolated microbial strains, including protein profiling, ribotyping, pulse field gel electrophoresis, conventional polymerase chain reaction (PCR), random amplification of polymorphic DNA, and repetitive element palindromic PCR (Shokryazdan *et al.,* 2017). However, due to the time-consuming nature and the need for a substantial reference strain collection, alternative methods are often considered less favorable. Consequently, 16S ribosomal rDNA/rRNA sequencing has emerged as a widely adopted technique for the accurate identification of isolated LAB strains. The identification is achieved through the analysis of the genetic material of bacteria to determine the specific makeup and evolutionary relationships (Raman *et al.,* 2022). The 16S DNA fragments used for phylogenetic classification are small, typically around 1500 base pairs (bp). In contrast, the whole bacterial genome is much larger, ranging from 30,000 to 40,000 bp, indicating the significant difference between the two (Fontana *et al.,* 2013). Several studies have identified different LAB strains from different sources through 16S rRNA sequencing, including *Lactobacillus* spp (*L. plantarum*, *L. rhamnosus* and *L. fermentum*) from the faeces of infants (Jomehzadeh *et al.,* 2020), *Enterococcus* spp (*E. faecium* and *E. hire*) from human

salivary and faecal sources (Bazireh *et al.,* 2020) and *Pediococcus* spp (*P. pentosaceus*) (Hamed, 2021). Although these methods are highly recommended by most researchers, they may not always provide sufficient information to differentiate between strains of the same bacterial species due to the limited base sequence diversity within the 16S DNA fragment. Therefore, this lack of diversity can make it challenging to distinguish between closely related organisms solely based on the 16S sequence (Fontana *et al.,* 2013).

2.6.3 Acid and Gastric Enzymes Tolerances

Acid tolerance is a fundamental criteria for the selection of probiotic strains to ensure their effective functionality and survival in acidic environments, such as the stomach which has a low pH due to the presence of gastric acid (Fontana *et al.,* 2013). *In vitro,* simulated gastrointestinal survival methods are commonly used for assessing tolerance to acid in the first steps of the selection of new probiotic strains. These methods include the inoculation of the prepared overnight culture onto an acidified medium (MRS broth/ buffer/peptone water; pH 2.0-3.0 for 1-4 hours) and the preparation of simulated gastric juices with pepsin with pH adjusted to 1.5-3.0 (Ayyash *et al.,* 2021).

Several studies have demonstrated the ability of probiotic strains to survive in acid conditions. Li *et al.,* (2020) evaluated acid tolerance of the isolated LAB by inoculating 100 µl of the overnight culture into MRS broth with pH adjusted to 3.0 and 6.5 using 1 M HCl and incubated at 37°C for 4 hours, where only five isolates showed tolerance to low acidic conditions. Among the selected, *Enterococcus durans* showed the highest tolerance, with a survivability percentage of 80,01%, followed by *Lactobacillus salivarius* with a survivability percentage of 70,67%. Another study by Vasiee *et al.,* (2022) evaluated acid resistance in acidic buffered solutions and after 3 hours of incubation, *Lactiplantibacillus plantarum* TW57-4 had a higher acid survivability as compared to *Lactobacillus brevis NS01*. However, the results also indicated that under pH 3, the observed reduction was only significant ($p<0.05$) for *L. plantarum TW57-4* and *L. brevis NS01* strains. Furthermore, in this study, pH 3.0 was considered a critical pH to ensure the viability of the selected probiotic strains. These studies highlight the criteria used to assess the viability and functionality of probiotic strains under acidic conditions.

The beneficial effect of probiotics depends on their resistance to challenging environments of the upper GI tract, which includes exposure to gastric acids, and proteolytic enzymes in the duodenum. The probiotics must reach the intestine in adequate quantities and maintain their activity for them to effectively provide their health benefit (Moussavi *et al.,* 2023). Reuben *et al.,* (2019) assessed the survivability of probiotic strains in simulated gastric juices formulated using glucose, NaCl, KH_2PO_4, $CaCl_2$ and KCl while the pH was adjusted to 2.0 using 1 M HCl. Additionally, porcine bile, lysozyme and pepsin were also added to mimic the entire gastric transit, simulating the entire journey of probiotic strains from the stomach to the intestine. The results of this study indicated that time also affected the survival of the tested strains. After 90 minutes of exposure to the simulated gastric juice, only six strains were able to survive with a viable count >2.0 CFU/ml. The tolerance to gastric acid by LAB from cereal-based Nigerian fermented products was assessed in a study by (Adesulu-Dahunsi *et al.,* 2018). In this study, the simulated gastric juice was prepared by making use of pepsin and 0.5% NaCl and the pH was adjusted to 2.0 using HCL. It was found that after exposure to gastric acid for 90-180 min, *Lactobacillus plantarum* and *Pediococcus pentosaceus* survived, except for *Weissella confusa* which lost its viability. These results indicated that the ability or probiotics to tolerate gastric juices is strain-dependent.

2.6.3.1 Mechanisms of Microbial Acid Tolerance

According to Ayyash *et al.,* (2021), when microbial cells are exposed to severe acidic conditions, such as pH 2 or 3, the proton leakage increases, reducing the ability of the cells to keep their normal homeostasis. As shown in Figure 4, this results in proton influx at low pH, disturbing the housekeeping pH homeostasis mechanisms and reducing the intracellular pH, and affecting physiological processes. Bacteria are more sensitive to changes in intracellular pH than extracellular ones. Despite the differences by which both organic and inorganic acids affect the microbial cells, both still lead to intracellular acidification and damage to biochemical processes (Chung *et al.,* 2006). According to Bearson *et al.,* (1997), when organic acids enter the cell membrane, they dissociate within the cell, releasing a proton and resulting in acidification of the cytoplasm. In response to the decrease in cytoplasmic pH, bacteria activate mechanisms responsible for protecting essential biological molecules such as DNA and proteins. These mechanisms include the upregulations of genes involved in repairing damaged DNA or stabilizing proteins under acidic conditions (Wang *et al.,* 2018).

Exposure to acidic environments, such as pH 2.0, the significant amount of undissociated HCl in the cell membrane environment, which is not charged and can easily move into the cytoplasm. Furthermore, the amount of undissociated HCl represents 5 to 10% of the dissociated acid form at pH 2.0 and due to high intracellular pH, the undissociated HCl can move into and dissociate in the cytoplasm, leading to the acidification of the cytoplasm (Ayyash *et al.,* 2021). Moreover, it is still not understood how protons can enter the bacterial cell as the membranes are not permeable to charged particles. It suggested protons may enter the cells via the use of membrane protein channels or damage to the lipid bilayer caused by high proton concentration (Foster, 2004). Bacterial mechanisms for tolerating acidic conditions have evolved (see Figure 2.4). These mechanisms include the activation of H+-ATPases, remodeling of the cell envelope, as well as intracellular proton consumption through glutamate decarboxylation, or alkali production through urease or arginine deiminase activities (Wang *et al.,* 2018).

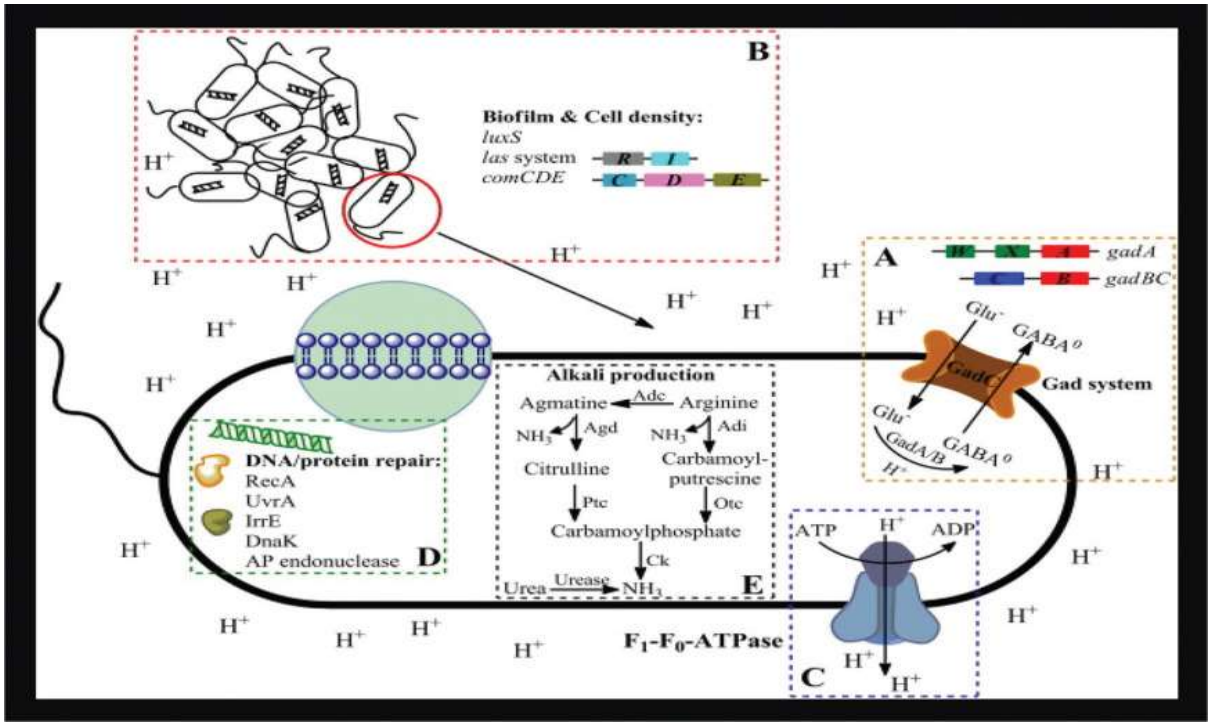


Figure 2.4: Demonstration of the mechanism of acid tolerance. A: The Gad system consists of gadA/B, which encodes glutamate decarboxylase (GadA/B), responsible for the conversion of glutamate (Glu) to gamma-aminobutyric acid (GABA), and GadC, encoded by gadC, which functions as the glutamate/GABA antiporter. B: Biofilm formation and cell density are regulated by the comCDE and luxS components, which are

integral to a quorum sensing system essential for biofilm development, while the las system is required for the activation of the exogenous Pseudomonas quinolone signaling molecule that facilitates biofilm maturation. C: The F1–F0–ATPase complex operates as a proton pump by expelling H+ ions, thereby increasing intracellular pH. D: The protection of macromolecules is mediated by proteins such as RecA, UvrA, and AP endonuclease, which are involved in DNA repair processes, while DnaK functions as a protein repair chaperone. Additionally, IrrE acts as a global regulatory protein that can enhance the transcription of recA. E: Alkali production occurs when urea is converted into ammonia (NH3) by urease, which functions to neutralize protons. The Adi and Agd systems are also capable of generating NH3 through various biochemical reactions. Abbreviations: Glu: glutamate; Adc: arginine decarboxylase; Agd: agmatine deiminase; Ptc: putative putrescine transcarbamylase; Adi: arginine deiminase; Otc: ornithine transcarbamylase; Ck: carbamate kinase *(Ayyash et al., 2021)*

2.6.4 Bile Salt Tolerance

Bile salt tolerance is one of the most important criteria for probiotic strain selection as it determines their ability to perform their probiotic function and remain viable in the small instetine. The presence of bile acids and pancreatic enzymes such as amylase, protease and lipase can result in significant killing of probiotics passing through the small intestine (Abuhelwa *et al.,* 2017; Brodkorb *et al.,* 2019). Bile salts are released to the small intestine and inhibit the growth of bacteria by destroying the cell membrane; however, most lactic acid bacteria used as probiotics consist of bile salt hydrolase (BSH), which hydrolyses and reduces their solubility; hence, the selected strains must be resistant to bile salts (Oyewole *et al.,* 2018; Tsega *et al.,* 2019).

2.6.4.1 Mechanisms of Tolerance to Bile Salts

The exposure of bacterial cells to bile acids leads to the disruption of the cell membrane, denaturing of cellular proteins, chelation of iron and calcium and triggering of oxidative DNA damage (Ayyash *et al.,* 2021). Bile salt hydrolysis results in the presence of unconjugated bile acids that have high inhibitory effects on bacterial cells compared with conjugated bile acids. Therefore, the selected probiotic strains need to resist bile and bile acids for their survival in the gastrointestinal tract (Prete *et al.,* 2020). Probiotic bacteria can also sense and develop

mechanisms to resist bile salt. For instance, Pfeiler *et al.,* (2007), reported that an eight-gene-operon encoding a TCS, a transporter, an oxidoreductase and four hypothetical proteins have been involved in sensing bile salts in *Lactobacillus acidophilus*. It was further highlighted that during exposure to high bile salt concentration, genes encoding multidrug transporters influencing the excretion of bile salts are unregulated in *Lactobacillus casei* and *Lactobacillus acidophilus* (Alcantara and Zuniga, 2012). In a study by Whitehead *et al.,* (2008), it was found that exposure of *Lactobacillus delbrueckii* to 0,1% bile salts concentrations resulted in an increased expression of clpP and clpE, which are involved in repairing and degrading damaged proteins.

Some probiotic bacteria can effectively express bile salt hydrolyzes (BSH), an enzyme responsible for conferring protection against bile salt deconjugation (Bustos *et al.,* 2018). It has been found that some other probiotic bacteria, like *Lactobacillus rhamnosus* GG, respond to bile salt exposure by changing the functions related to the cell envelope, such as disrupting pathways affecting the fatty acid composition cell surface charge, and thickness of the EPS layer (Whitehead *et al.,* 2008). In addition, the capacity of bacteria to metabolize carbohydrates and fatty acids using the expression of proteins, biosynthesize nitrogenous bases and amino acids and produce stress response proteins is essential for the mechanism of resistance to bile salts (Ayyash *et al.,* 2021). Although resistance to bile salts may vary depending on the type of strain, potential probiotic strains can be gradually adapted to the presence of bile salts through subculturing in a medium supplemented with increasing concentrations of bile salts.

2.6.5 Antimicrobial Activity Against Pathogens

Antimicrobial activity is one of the criteria to consider when selecting potential probiotic strains as it serves as one of the probiotic functions in promoting microbial balance in the gut and prevents the colonization of pathogenic bacteria (Shokryazdan *et al.,* 2017). Probiotic strains must be able to inhibit the growth of pathogenic bacteria and opportunist pathogens such as *Escherichia coli*, *Salmonella enterica*, *Salmonella typhimurium*, and *Campylobacter jejuni* as they are the most commonly encountered microorganisms in poultry farming (Vieco-Saiz *etal.,* 2019). This is achieved by antimicrobial activity that involves the production of microbial substances that may be considered to be antagonistic to other organisms (Table 2.3). The mechanism of these metabolites involves decreasing pH by producing consumption of available nutrients, decreasing the redox potential of pathogens, and producing antimicrobial compounds such as bacteriocins, thus helping probiotics to be effective (Kosin *et al.,* 2006).

The antimicrobial substances can be divided into three primary groups: (1) peptidic or proteinaceous bacteriocins; (2) organic acids (lactic acid, butyric, acetic acid); (3) other small molecules such as hydrogen peroxide, acetone, reutericyclin, diacetyl and acetaldehyde (Table 2.3) (Ibrahim *et al.,* 2021).

Table 2.3: Mechanism of antimicrobial substances in inhibiting pathogenic microorganisms.

Antimicrobial Substance	Mechanisms of Antagonistic activity
Hydrogen peroxide	Alters the redox potential of bacterial cell walls and disrupts the molecular structures of cell proteins.
Organic acids (lactic and acetic acid)	Reduces intracellular pH and dissipates the membrane potential. Furthermore, organic acids disrupt essential metabolic functions due to the undissociated form of the molecules, which diffuse across the cell membrane towards a more alkaline cytosol.
Bacteriocins	Inhibit the outgrowth and formation of spores in the cell membrane. The production of bacteriocins also inhibits the modulation of enzyme activity.

There are different methods used to determine a direct antagonistic effect between the isolated probiotic culture and that of a pathogenic strain (Silva *et al.,* 2020). The determination of microbial antagonism on a solid media is most appropriate when the study aims to detect the antagonism of one microorganism concerning another (Macaluso *et al.,* 2016). These methods involve the detection of inhibition of the growth of the tested pathogenic strain caused by the test culture. Several studies have demonstrated the antagonistic effect of Gram-positive probiotic bacteria against Gram-negative and Gram-positive pathogenic bacteria using different microbiological assays e.g. disc diffusion, agar spot test and agar well diffusion assays (Table 2.3) (Govindaraj *et al.,* 2021; Hesari *et al.,* 2017; Jafari-Nasab *et al.,* 2021; Kwun *et al.,* 2020).

The most common method is agar well diffusion using the cell-free supernatant (CFS). This method is preferred because the CFS contains metabolites, such as organic acids, which are known to possess antimicrobial properties (Fijan, 2023). In a study by Sirisopapong *et al.,* (2023), the potential of the cell-free supernatant (CFS) of *L. acidophilus*, *L. ingluviei, L. reuteri*, *L. salivarius* and *L. saerimneri* in inhibiting *E. coli* (ATCC 43888), *S. aureus* (ATCC 29213*), C. jejuni* (ATCC 33291) and *C. perfingens* was assessed and all isolates exhibited strong antibacterial activity against the tested pathogenic bacteria. Another study by Musikasang *et al.,* (2009) also assessed the antibacterial activity of LAB (*E. faecalis*, *E. durans*, *P. pentosaceus* and *E. faecium*) isolated from the digestive tract of chickens against *E. coli*, *S. aureus* and *Salmonella* spp and the isolates exhibited antibacterial activity with zones of inhibition ranging from 8-25 mm (*E. coli*), 13-40 mm (*Salmonella* spp) and 6- 24 mm (*S. aureus*). It was found that the isolated LAB confer inhibitory effects against Gram-positive and Gram-negative bacteria, however, more research needs to be conducted to explore and understand the specific mechanisms by which these isolates interact with and prevent the growth of pathogenic bacteria. This will assist in elucidating the specific pathways or mechanisms involved in the interactions.

Other methods, including agar spot tests, have also been investigated for assessing antimicrobial activity. In the agar spot test, the antagonistic effects of LAB against pathogens are measured by observing the area surrounding volumes of the spotted probiotics in liquid form onto lawned plates of target bacterial pathogens (Shokryazdan *et al.,* 2017). According to Silva *et al.,* (2020), there are two variations of this method, namely; simultaneous (or direct antagonism), in which the antagonistic effect is demonstrated by the release of substances that inhibit the growth of indicator culture as both the test and indicator culture grow together. Deferred antagonism is another variation of the agar spot test method whereby the growth of the probiotic bacteria is observed in the agar first, and then gets inactivated. The indicator strain is then placed on the agar, different incubation times are required, and the antagonist activity is determined by measuring either the size of inhibition or in arbitrary units.

Poimenidou *et al.,* 2023 evaluated the antagonistic activity of 45 *Lactobacillus* spp against *Listeria monocytogenes* using an agar spot test, where 3 μL of the active MRS culture of each *Lactobacillus* strain was spotted on a 45 ml MRS agar surface and incubated for 37°C for 24

hours. After the incubation, each strain was overlaid with 20 ml Brain Heart infusion (BHI) broth supplemented with 0,7% agar that was pre-warmed at 45°C and inoculated with 0,3 ml of an overnight culture of the indicator strain, *L. monocytogenes* and incubated for 24 hours. It was found that 21 Lactobacillus strains were able to inhibit the growth of *L. monocytogenes*, with *L. rhamnosus*, *L. paragasseri* and *L. crispatus* strain displaying the most pronounced antagonistic activity. It was concluded that each Lactobacillus strains used in this study had unique properties and the inhibitory effect was strain and treatment-dependent with competition resulting in greater inhibition.

Table 2.4: Antibacterial activity of LAB from different sources

LAB isolate	Source of Isolation	Cell-type	Method	Pathogen	Zones of inhibitions	Incubation time	Reference
Lactococcus lactis, Enterococcus faecalis, Weissella cibaria, Limolactobacillus fermentum	GI tract of freshwater fish species	Neutralized cell-free supernatant (pH 7)	Well diffusion assay	*Aermonas hydrophila* ATCC 7966, *Bacillus cereus* MTCC 430, *Staphylococcus epidermis* MTCC 435, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* MTCC 2453, *Escherichia coli* MTCC 443	Antimicrobial activity of the isolated strains against the tested strains ranged from 6,33-20,33mm.	37°C for 24 hours	(Govindaraj *et al.,* 2021)
Lactobacillus sakei	Fermented foods	Bacterial cell	Disc diffusion assay	*Bacillus cereus* KCTC 3634	Antibacterial activity of the isolated strain resulted in 8-10 mm zones of inhibitions against the tested pathogen.	30°C for 24 hours	(Kwun *et al.,* 2020)
Lactobacillus Fermentum, Lactobacillus plantarum, Bifidobacterium bifidum	Yogurt and buttermilk	Cell-free supernatant	Agar well diffusion, disc diffusion and pour plate method	*Escherichia coli* O157:H7	The antibacterial activity of the isolated probiotic bacteria varied from 4-8 mm in both the agar well and disc diffusion methods. Higher inhibition of E. coli was observed in the pour plate method with the reduced count from 143 to 13 CFU/ml.	37°C for 24 hours	(Hesari *et al.,* 2017)

Lactobacillus spp	Dairy products	Cell-free supernatant	Agar spot test	*Staphylococcus aureus MTCC 96, Enterococcus faecalis MTCC 439, Klebsiella pneumonia MTCC 432, Pseudomonas aeruginosa MTCC 7925, Escherichia coli MTCC 443,*	*Lactobacillus* spp inhibited the growth of indicator organisms with zones of inhibition ranging from 15-30 mm.	18°C for 24 hours	(Jafari-Nasab *et al.,* 2021)

2.6.6 Antibiotic resistance in LAB

One of the criteria for the selection of probiotics is to determine the nature of any antibiotic-resistant profiles in candidate microorganisms as the GRAS (generally recognized as safe) status also includes antibiotic resistance (Gueimonde *et al.,* 2013). The presence of antibiotic resistance is correlated with the existence of resistant genes that enable the deactivation of the active antimicrobial molecule, as well as the loss of susceptibility to modifications made by the antimicrobial at the target site or a decrease in the uptake of the antimicrobial agent (Table 2.5) (Yenizey and Edith 2018). With regards to antibiotic resistance, some probiotic strains such as *Lactobacillus* spp, have intrinsic resistance against vancomycin. This is defined as the natural or innate ability of a bacterium to withstand the effects of antibiotics (Sirichoat, 2020). Distinct factors influence this phenomenon, such as the chromosomal mutations or when vancomycin encounters the peptidoglycan precursors on the cell wall side of the cytoplasmic membrane and attaches to the D-alanine/ D-alanine terminus of the pentapeptide, preventing polymerization of peptidoglycan precursors (Gueimonde *et al.,* 2013).

Table 2.5: Summary of the mechanism of action of some antibiotics

Site of Action	Mode of Action	Antibiotics Class
Cell wall	Blocks the polymerization and cross-linking processes of wall peptidoglycan at the level of binding proteins (PBS's)	β -lactams (penicillin, derivatives, cephalosporins, etc.), glycopeptides (vancomycin)
Ribosome (50S)	Hinders the initiation of protein synthesis	Aminoglycosides
	Blocks the tRNA amino acids complex to ribosomes	Tetracyclines
	Blocks peptidyl transferase on the 50S ribosome subunit, modifying the protein's final elongation stages	Amphenicols, Macrolides< ketolides

Adapted from (Gueimonde *et al.,* 2013)

Most LAB has been proven to be resistant to some antibiotics such as vancomycin, streptomycin and oxacillin (Reuben *et al.,* 2019). Phenotypic methods such as antibiotic susceptibility tests by disc diffusion and molecular methods including PCR (polymerase chain reaction), have been utilised for the assessment of antibiotic resistance profiles and to determine antibiotic resistance genes present in LAB (Veerapagu *et al.,* 2022).

A study by Uezen *et al.,* (2023) reported high phenotypic resistance of *Pediococcus pentosaceus*, *Limosilactobacillus reuteri*, *Lactobacillus amylovorus* and *Lactobacillus johnsonii* to ampicillin, gentamicin, kanamycin, streptomycin, and tetracycline. This study further reported that 15 resistant genes (*erm (B), strA, strB and aadE*) were present in Lactobacilli confirming their resistance to erythromycin and streptomycin. Additionally, the Tet(M) gene was present in *L. reuteri* and *L. amylovorus.* In a study by Amenu and Bacha (2023), *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *Lactococcus lactis* were reported to show resistance against kanamycin, and susceptibility to ampicillin, tetracycline, chloramphenicol, and erythromycin (Table 2.6). The occurrence of antibiotic resistance of *Lactobacillus* spp and *Pediococcus* spp to aminoglycosides and tetracycline was also reported by (Stefańska *et al.,* 2021). However, it should be noted that the detection of the resistant genes in most reported studies is based on PCR analysis, which may miss certain genetic determinants and variations, potentially underestimating the true prevalence of resistance mechanisms.

Table 2.6: Summary of studies that have indicated antibiotic resistance of LAB isolated from different sources

Lactic Acid Bacteria	Antibiotic Resistance	Source of Isolation	Reference
Leuconostoc mesenteroides *Lacticaseibacillus rhamnosus*	Ceftazidime Vancomycin	Human milk	(Damaceno *et al.,* 2023)
Lactobacillus pentosus *Lactobacillus crispatus* *Pediococcus pentoceus* *Enterococcus hirae*	Streptomycin, kanamycin, ampicillin, vancomycin, trimethoprim-sulfamethoxazole	Broiler chicken faeces	(García-Hernández *et al.,* 2016)
Lactobacillus spp *Streptococcus thermophilus*	Nalidic acid, kanamycin, vancomycin, penicillin, erythromycin, tetracycline, gentamicin, streptomycin and chloramphenicol	Fermented foods	(Nawaz *et al.,* 2011)
Lactobacillus bulgaricus *Streptococcus thermophilus*	Ampicillins, chloramphenicol, chlortetracycline, tetracyclines, lincomycin, streptomycin, neomycin and gentamycin	Chinese yoghurt	(Zhou *et al.,* 2012)
Lactobacillus fermentum	Ampicillin, ciprofloxacin, vancomycin	Traditional fermented milk	(Ojha *et al.,* 2022)
Enterococcus spp *Lactobacillus* spp *Pediococcus lolii* *Lactobacillus plantarum*	Gentamicin, streptomycin, neomycin, amikacin, spectinomycin and kanamycin	Farm animals and commercial meat products	(Jaimee and Halami, 2016)

2.6.7 Adhesion Properties of Bacteria

Bacteria aggregate to provide protection against environmental stresses or to provide advantages in colonising substrates. The mechanism of bacterial adhesion is a complex process that involves two types of interactions: the non-specific hydrophobic group interaction and the specific adhesion receptor interaction (Darmastuti *et al.,* 2021). Non-specific interactions are reversible and occur due to physicochemical factors such as the hydrophobicity of the LAB cells. Conversely, specific irreversible interaction occurs due to mediating adhesins on the LAB surface cells. The mediating adhesins are proteins responsible for the adhesion process of LAB to the bacterial cell surfaces through the interaction with complementary receptors on host cells. Properties such as autoaggregation, co-aggregation and cell-surface hydrophobicity are associated with bacterial adhesion abilities (Tuo *et al.,* 2013).

2.6.7.1 Auto-aggregation and Co-aggregation

The capacity of bacteria to aggregate via auto-aggregation and co-aggregation is another prerequisite for the adhesion of probiotics in the gut. Auto aggregation is the ability of microorganisms of the same species to form self-forming groups, which is usually associated with adhesion to the intestinal mucosa. Co-aggregation, conversely, is the intracellular adhesion between microorganisms from distinct species, which is also linked to the ability to interact with pathogens (Zawistowska-Rojek *et al.,* 2022). The process of aggregation is associated with the surface of the bacterial cells and secreted substances such as exopolysaccharides. These factors influence the strength and speed of the interaction between cells.

Auto-aggregation and co-aggregation properties are also influenced by the length of the bacterial cells (Zawistowska-Rojek *et al.,* 2022). Longer cells have a greater surface area and, therefore, greater aggregation abilities. Various LAB strains, including *Lactobacillus* species, have been characterized for their aggregation-promoting factor (Apf) and the genes encoding them (García-Cayuela *et al.,* 2014). One of the most desirable characteristics of potential probiotics is their ability to attach and colonize the intestinal wall, which are dependent on the surface properties of the selected strains (Sharm *et al.,* 2019). Auto-aggregation and co-aggregation are important mechanisms used by probiotics to prevent surface colonization of the gastrointestinal tract by pathogens (Li *et al.,* 2020).

2.6.7.2 Hydrophobicity to aid aggregation

A cell surface characteristic known as hydrophobicity characterises the cell's capacity to repel and adhere to nonpolar substances (Darmastuti *et al.,* 2021). One of the techniques used to determine the hydrophobicity of lactic acid bacterial surfaces is known as microbial adhesion to hydrocarbons (MATH). This is assessed by adding a hydrocarbon solvent, such as xylene, n-hexadecane, or toluene, to a bacterial suspension and evaluating microorganisms' affinity to a solvent (Darmastuti *et al,.* 2021). The MATH method does not directly measure the cell surface hydrophobicity but rather reflects the interplay of van der Waals and electrostatic forces in overall adhesion capacity. The MATH method is sensitive to several factors, including the cultivation time, media composition, presence of certain acids and the type of solvents used. For example, the hydrophobicity of lactobacilli can decrease and change their surface properties due to the presence of elaidic acid. In addition, the hydrophobicity is also affected by the presence and concentration of saccharides in the cultivation medium, with lactose increasing it and bile salts and hydrochloric acid reducing it (Darmastuti *et al.,* 2021; Krausova *et al.,* 2019).

A range of factors, such as temperature, culture medium composition, pH and temperature, also influence the correlation between adhesion and hydrophobicity. The determination of the cell surface hydrophobicity is a pre-test of adhesion capacity and is an important property for improving the first contact between bacteria and the cells (Krausova *et al.,* 2019). These properties provide insight into the potential effectiveness of probiotic strains. Several studies have reported LAB to have positive hydrophobicity, indicating that they have hydrophobicity properties that may facilitate their persistence in the GI tract of broiler chickens (Darmastuti *et al.,* 2021; Reuben *et al.,* 2020).

A study by Krausova *et al.,* (2019) evaluated the adhesion properties of *Lactobacillus* and *Bifidobacterium*. To determine the cell surface hydrophobicity, hexane was used as a solvent, which was added to the bacterial suspension and mixed. The bacterial affinity was measured after phase separation and stabilization. The results indicated that the *B. thermophilum* T11B strain depicts the highest hydrophobicity with a percentage of 87.4%. In addition, *L. reuteri* K18 and *B. thermophilum* S4B also demonstrated high hydrophobicity of 76% and 78,2%

respectively, demonstrating their ability to adhere to the hydrocarbons. Another study by Tuo *et al.,* (2013) elucidated the hydrophobicity abilities of 22 *Lactobacillus* strains using a method called xylene extraction. In this study, xylene was added to the bacterial suspension, and the mixture was incubated at room temperature for an hour. Following the incubation, the aqueous phase was removed, and the affinity of the bacterial strains was determined. The results from this study indicated a significant difference in hydrophobicity, ranging from 16.90 to 96.62% of the tested strains.

2.6.8 Extracellular Enzymes of Probiotic Bacteria

Enzymes present in the animal gut play a key role in digestion and are either secreted from the pancreas or secretory cells in the gut wall (Durai 2019). Additionally, microorganisms present in the gut all have enzymatic activity that influences the metabolic activity of the host animal. Different enzymes are produced in the gut of animals including amylase, lipase and protease which are responsible for breaking down a wide range of proteins, carbohydrates and lipids (Durai 2019). Studies have shown that lactic acid bacteria such as *Lactobacillus* spp have the potential to produce these enzymes (Champ *et al.,* 1983; Sanni *et al.,* 2002; Yi *et al.,* 2020).

2.6.8.1 Amylase

Amylases are digestive enzymes that are essential in the gastrointestinal tract of chickens and mammals like pigs, horses, rabbits and human beings including infants in breaking down starch by breaking the bonds between sugar moieties through hydrolytic reaction resulting in the production of dextrin, maltose and glucose (Durai 2019; Padmavathi *et al.,* 2018). A study by Madhav *et al.,* (2011) detected the presence of amylase in *Bacillus* spp concerning time, temperature and pH of the media in which the microorganisms were inoculated. The results showed the maximum amylase activity ranging from 21,79-34,20%. Furthermore, an increase in amylase activity was observed with increasing time, pH and temperatures of incubation. Another study by Padmavathi *et al.,* (2018) reported amylase production by *Lactobacillus* sp. G341T02.

2.6.8.2 Protease

Proteases are a family of enzymes present in sources like animals, plants and humans that are responsible for the hydrolysis of peptide bonds of protein molecules, yielding amino acids

(Durai, 2019). These enzymes can be classified as endopeptidases (also known as proteinases responsible for cleaving the internal portions of polypeptide chains) and exopeptidases (responsible for the removal of amino acids from the ends of the protein chain, either from the C-terminal or N-terminal). In addition, these enzymes can further be classified as acidic, neutral or alkaline proteases depending on their optimal pH (Maske *et al.,* 2021).

The proteolytic system of LAB is composed of several components, including a cell envelope-associated proteinase, a specific transport system for peptides and amino acids, and various cytoplasmic peptides (Maske *et al.,* 2021). According to Kieliszek *et al.,* (2021), LAB require certain amounts of energy sources for growth and development due to their rapid intracellular metabolism. A study by Donkor *et al.,* (2007) assessed the proteolytic activity of *Lactobacillus acidophilus*, *Bifidobacterium* spp, *Lactobacillus casei* and *Streptococcus thermophilus* and reported that all tested strains exhibited proteolytic activity with intra-and extra-cellular peptidases including X-prolyl-dipeptidyl aminopeptidase. Furthermore, it was observed that the protease activity of the tested probiotic strains was time-dependent. In the meat industry, proteases play a significant role in the hydrolysis of muscle proteins; as a result, the meat becomes tender and the degree of hydration of the meat increases. Additionally, the protein digestibility of the meat increases, enhancing the texture, taste and colour of the meat while also preventing spoilage and foodborne illnesses (Kieliszek *et al.,* 2021).

2.7 MECHANISMS OF ACTION OF PROBIOTIC FUNCTIONS

The impact of biotechnology in poultry nutrition is essential for enhancing the quality and efficiency of poultry feed by improving their nutrition for the growth, health, and productivity of poultry birds. In broiler nutrition, studies have shown that probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium,* and *Enterococcus* have beneficial properties on broiler performance (Tortuero, 1973), modulation of the gut microflora (Mousapoor *et al.,* 2023), inhibition of pathogenic microorganisms (Chapman *et al.,* 2012), intestinal histological changes (Awad *et al.,* 2009), enhancing sensory characteristics of dressed broiler meat (Lutful 2009), and promoting microbiological quality of broiler meat (Awad *et al.,* 2009). As demonstrated in Figure 2.6, the mode of probiotic action includes:

(i) Maintaining normal intestinal flora by competitive exclusion and antagonisms

(ii) Modifying metabolism by enhancing the activity of the digestive enzymes, leading to better utilization of nutrients, decreasing bacterial enzyme activity and production of ammonia;

(iii) Improving the feed intake and digestion; and

(iv) Stimulating the immune system by inducing the production of immune cells, enhancing their activity and regulating the production of certain immune molecules such as cytokines (Kabir, 2009).

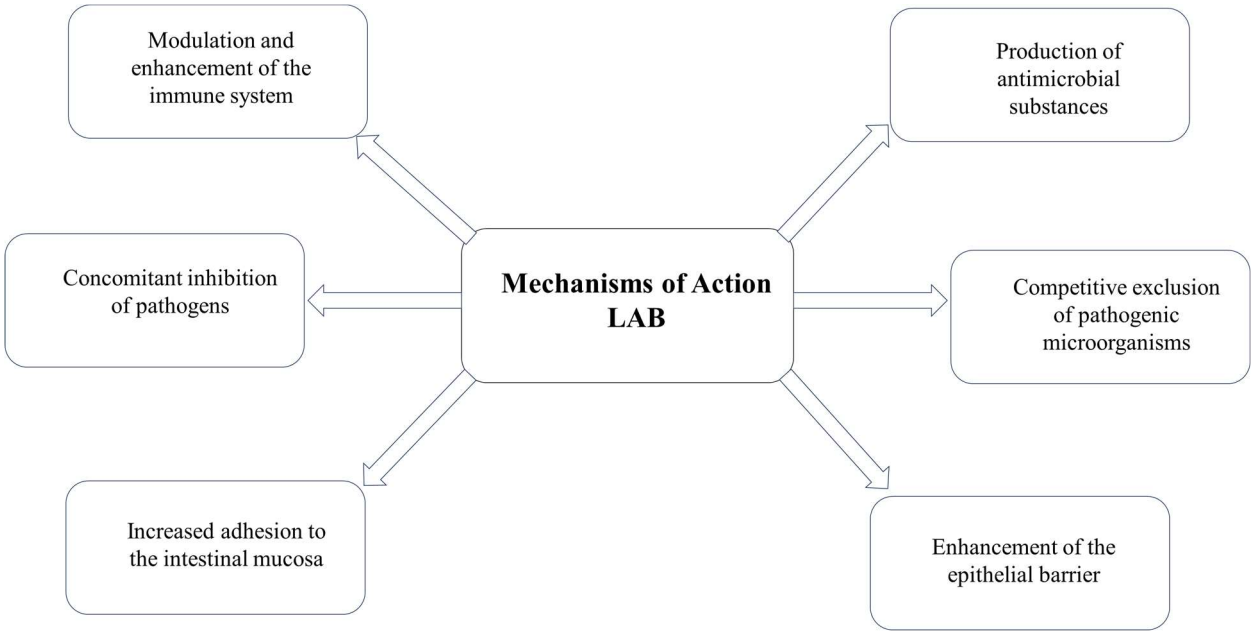


Figure 2.5: Mechanisms of action of probiotics *(Tiwari et al., 2012)*.

2.7.1 Enhancement of the Immune System

Patel *et al.,* (2015) highlighted that the action of probiotics in animal health is centred around their immunomodulatory mechanism, which works through the innate and adaptive immune systems. This mechanism is crucial for disease prevention and maintaining animal health. The gastrointestinal lumen contains various components, including essential nutrients, beneficial microorganisms, harmful microorganisms, toxins, and foreign antigens (Vancamelbeke. *et al.,* 2017). Probiotic bacteria can interact with different types of cells such as epithelial and dendritic cells, as well as monocytes/macrophages and lymphocytes (Mazziotta *et al.,* 2023**)**. The epithelial cells in the gastrointestinal mucosa form a selectively permeable barrier that

separates the lumen environment from the internal body tissues. This barrier plays a vital role in maintaining the overall balance and integrity of the digestive system.

The innate immunity of the animal gut functions as the primary defense mechanism against the infiltration of pathogenic microorganisms into the gastrointestinal tract (GIT) (Markowiak and Śliżewska, 2018).

Probiotics, such as *Lactobacillus plantarum*16 (Lac16) and *Paenibacillus polymyxa* 10 (BSC10), have been shown to improve the intestinal barrier. This is achieved by influencing the phosphorylation of cytoskeletal and tight junction proteins, which play a role in mucosal cell-cell interactions and cellular stability. In a study by Yanping *et al.,* (2019) on one-day-old broiler chickens, the researchers found that feeding the birds with Lac16 and BSC10 resulted in the maintenance of mucosal integrity and an increase in the number of goblet cells in the ileum. Goblet cells are responsible for producing and secreting mucins, which act as the body's first line of defense against pathogens. Additionally, BSC10 was found to regulate the expression of genes related to barrier function, such as claudin-1 (CLDN1), occludin (OCLN), and Zonula occludens-1 (ZO-1). This regulation enhanced the intestinal barrier function by influencing the structure of the intestines.

The adaptive immune system is facilitated by the B and T lymphocytes that induce antigen-specific responses and produce antibodies (Hernandez-Patlan *et al.,* 2020). In contrast, the innate immune system recognizes and responds to commonly found structures known as pathogen-associated molecular patterns (PAMPs). This response is initiated by pattern recognition receptors (PPRs) that bind to the PAMPs, triggering a complex stimulatory mechanism. Among the PPRs, toll-like receptors (TLRs) are the most extensively studied. Moreover, extracellular C-type lectin receptors (CLRs) and extracellular nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs) also transmit signals upon interactions with bacteria. (Bermudez-Brito *et al.,* 2012).

2.7.2 Production of Antimicrobial Substances

The health benefits of probiotics may be associated to the production of antimicrobial substances. These substances can be classified into two categories based on their molecular weight: high molecular weight (>1000 Da), such as bacteriocins, and low molecular weight (<1000 Da), such as organic acids. Bacteriocins are small antimicrobial peptides composed of amino acids synthesized by ribosomes of some LAB that confer antimicrobial activity against closely related Gram-positive bacteria (Sharma *et al.,* 2022; Šušković *et al.,* 2010). Bacteriocins are active against low G+C Gram-positive species such as *Listeria* spp, and *Staphylococcus* spp by inhibiting outgrowth spores and formation of pores in the cell membrane and interacting with anionic lipids found in the membrane, initiating the formation of pores in the membrane of susceptible cells.

The production of organic acids such as lactic and acetic acids inhibits the growth of enteric bacterial pathogens by decreasing the pH of the GI tract through the production and accumulation of acids released by antimicrobial compounds (Hernandez-Patlan *et al.,* 2020). The inhibitory effect of organic acids towards pathogens is caused by the diffusion of undissociated form of molecules into the cytoplasm, interfering with vital metabolite processes (Šušković *et al.,* 2010). In addition, acetic and lactic acids are lipophilic acids that can penetrate the cell membrane in undissociated form and can dissociate to produce hydrogen ions at intracellular pH, disrupting essential metabolic functions of pathogenic cells such as substrate translocation and oxidative phosphorylation (Denkova *et al.,* 2017).

2.7.3 Competitive Exclusion of Pathogens

Competitive exclusion of pathogens is another mechanism used by probiotics to exclude or inhibit the growth of pathogenic microorganisms (Yegani and Korver 2010). Greenberg (1969) first used the term “competitive exclusion” in which he described it as a scenario in which one species of bacteria actively competes with other species for receptor sites in the intestinal tract in his study assessing the complete exclusion of *Salmonella typhimurium* from maggots of blowflies. Probiotics use various mechanisms (Figure 2.7), including the development of antagonistic microecology, the elimination of available receptor sites for bacteria, production and secretion of antimicrobial substances and selective metabolites and the competitive depletion of essential nutrients to successfully exclude or inhibit the growth of other

microorganisms (Bermudez-Brito *et al.,* 2012). Probiotic cell surfaces consist of adherence factors, mostly proteins or polysaccharides that promote pathogen exclusion and *Lactobacillus* strains consist of mucus-binding proteins (Mub) that assist in intestinal adherence, as well as S-layer proteins that mediate the adhesion to different host surfaces to inhibit the growth of pathogens such as *Escherichia coli* by inducing MUC2 and MUC3 mucins (Denkova, 2017). These proteins may also facilitate the colonization of the gut through the degradation of the extracellular matrix of the cells by facilitating close contact with the epithelium.

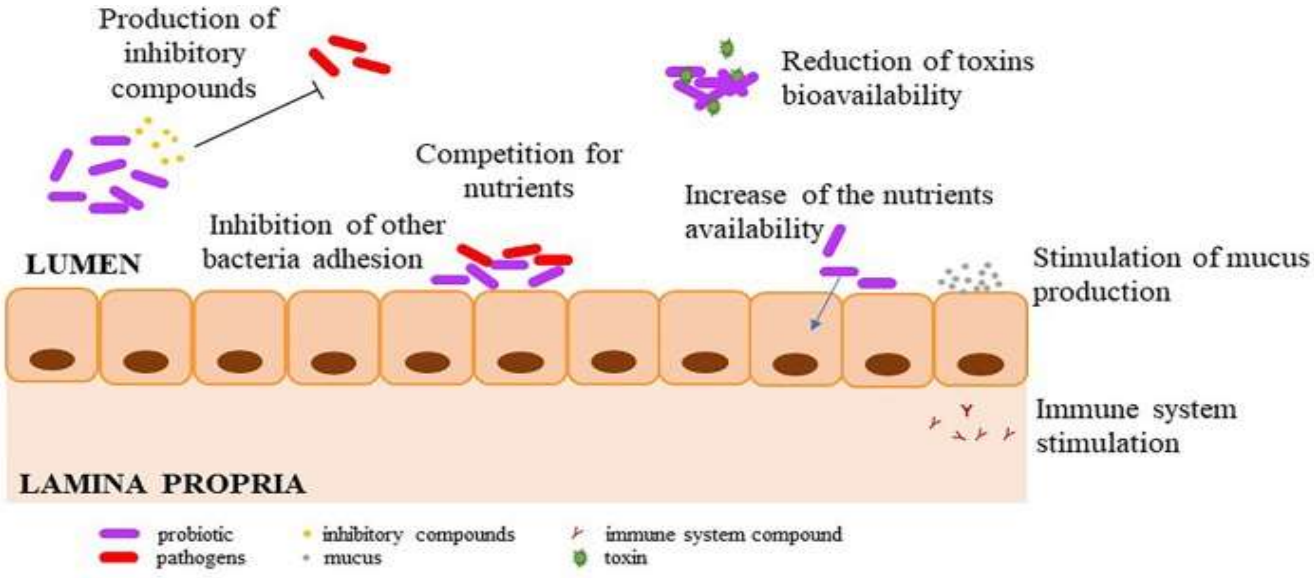


Figure 2.6: Mechanisms of action of pathogen inhibition by LAB through the production of antimicrobial compounds, competition for nutrients and stimulation of mucus production (Vieco-Saiz *et al.,* 2019).

2.8 EFFECT OF PROBIOTICS ON POULTRY MEAT QUALITY

Meat quality refers to the traits influencing consumer purchasing decisions and eating experiences, including the meat's colour, texture, and water-holding capacity (WHC). These traits are influenced throughout the stages of life of an animal and during the harvesting, fabrication, and preparation of the final meat product. In addition, the administration of probiotics for growth improvement and production capabilities has positively affected the meat quality of fresh and processed products (Al-Shawi *et al.,* 2020).

Atela *et al.,* (2019), investigated the effect of probiotic strains administered via drinking water on meat quality and growth performance. A total number of bacteria of 1.4×10^8 CFU/mL (*Bacillus safensis, Bacillus subtilis, Bacillus megaterium and Cupriavidus metallidurans*) were administered in five-week-old chickens for 12 weeks. The results indicated that probiotics improved the meat's pH, which is affected by factors such as stress, handling, and inclement temperatures. In another study by Wang *et al.,* (2017), a total of six strains, including *Pediococcus pentosaceus, Bacillus cereus var.albolactis, Bacillus macerans, Bacillus subtilis, Lactobacillus plantarum* and *Issatchenkia orientalis* were used as candidate probiotic strains, in which the results showed beneficial effects in body weight. Flavour compounds such as (E)-2-heptanol, (E, E)-2,4-nonradial, and certain C6-C9 unsaturated fatty acids were detected in high concentrations, resulting in a stronger chicken-fatty or fatty odour, which directly improved the flavour, indicating that the administration of probiotics could enhance chicken meat flavour and increase the diversity of the gut microbiota. In addition, the administration of *Bacillus subtilis* was shown to improve the quality of the meat, while *Bifidobacterium bifidu* and *Bacillus toyonensis* enhance the growth rate and meat quality in quails (Mohammed *et al.,* 2021). The level of high-density lipoprotein (HDL) was found to be increased whilst reducing the low-density level (LDL) through the administration of *Lactobacillus casei* in broiler chickens (Ma *et al.,* 2020).

3 CHAPTER THREE: METHODS

3.1 ETHICS AND APPROVAL

This study required no ethical approval as the collection of samples was from animals slaughtered for food purposes. All other experiments were conducted *in vitro*.

3.2 BACTERIAL STRAINS AND CULTURE CONDITIONS

The culture medium used in this study for the isolation of LAB was de Man, Rogosa and Sharpe (MRS) medium and all LAB isolates were incubated anaerobically at 37°C in anaerobic jars using AnaeroGen ™ 2.5 L sachet (Thermo Scientific) to generate an anaerobic environment. The pathogens, including *Listeria monocytogenes* (ATCC 7644), *Salmonella typhimurium*, *Salmonella enterica* (ATCC 13314), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213), were used for the evaluation of antagonistic activities, and coaggregation abilities of the isolates. These pathogenic strains were grown in Brain Heart Infusion (BHI) medium and incubated at 37°C. *Lactobacillus casei* (ATCC 393) was used as the reference strain in our study for comparison.

3.3 ISOLATION OF LACTIC ACID BACTERIA FROM GASTROINTESTINAL TRACTS OF CHICKEN

Gastrointestinal crops and small intestines of four healthy 23-week-old locally bred chickens were collected immediately after slaughter from a poultry farm in the KwaZulu-Natal province, South Africa. The collected samples were transported and delivered to the laboratory for microbial analysis at 4°C. The samples were washed with sterile phosphate-buffered saline (PBS) at pH 7.2 to remove intestinal contents and mucus, to allow only adhering bacteria to remain on the intestinal surfaces. Both crop and small intestine samples (10 g of each) were subjected to enrichment in 40 ml deMan, Rogosa Sharpe (MRS) broth (Merck) in sterile 100 ml flasks, followed by homogenization and incubation under anaerobic conditions in anaerobic jars using AnaeroGen ™ 2.5 L sachets (Thermo Scientific) to generate an anaerobic environment at 37°C for 24 hours with continuous shaking (at 180 rpm). Serial dilutions were carried out on tubes exhibiting turbidity by adding 1 ml of each sample to 9 ml of saline solution

(0.8% w/v). One loopful of the sixth dilution (10^{-6} CFU/ml) was subsequently streaked on an MRS agar (Merck) plate and incubated under anaerobic conditions at 37°C for 24-48 hours. Individual distinct white and creamy colonies from the cultures were purified by streaking through three MRS medium subcultures. After purification, the initial characterization of the presumptive isolates was done through Gram-straining and catalase testing (Reuben *et al.,* 2019).

3.4 PRELIMINARY SCREENING OF LACTIC ACID BACTERIA ISOLATES

3.4.1 Classification According to Gram Reaction

The initial morphological examination and tentative identification of the isolated LAB was done using Gram staining as described in Bergey's Manual of Systematic Bacteriology (deMan *et al.,* 1960). As LAB are Gram-positive, this step was to determine whether the MRS medium did indeed select for LAB.

3.4.2 Catalase Production

For the catalase test, a drop of 15% hydrogen peroxide was placed on a slide surface containing the smear of the bacterial colony. As LAB are catalase-negative, this step was used to determine whether the MRS medium was indeed selective for LAB. Catalase-negative isolates, were distinguished by the absence of bubble formation, and were selected for further screening. All Gram-positive, catalase-negative isolates were stored at -80°C in MRS broth supplemented with 28% v/v glycerol (Reuben *et al.,* 2019)

3.4.3 Screening for Acid Tolerance

Acid tolerance testing was done as described by Shamsudin *et al*., (2019) to assess the capacity of the isolated LAB to resist and persist in low pH conditions of 3 and 4. In summary, LAB isolates that were grown in MRS broth at 37°C for 24 hours were subjected to centrifugation at 10, 000 ×g for 5 minutes. The bacterial cells were then resuspended in 0,9% NaCl (w/v) and adjusted to an optical density (OD_{600}) of 0,2 OD units to achieve a concentration of approximately 1×10^8 CFU/mL. The diluted samples were then inoculated in MRS broth with pH levels of 3 and 4 adjusted using 1 M hydrochloric acid (HCl). The mixture was then incubated for 3 hours at 37°C in an anaerobic jar using AnaeroGen ™ 2.5 L sachet (Thermo Scientific). Following a 3-hour anaerobic incubation period, cell viability was evaluated by

plating onto MRS agar plates and incubating at 37°C for 48 hours, anaerobically. The survivability percentage was calculated using the following equation:

$$\text{pH survivability \%} = \frac{\text{Viable LAB count } \left(\frac{\text{CFU}}{\text{mL}}\right) \text{after acid exposure}}{\text{Initial viable LAB count } \left(\frac{\text{CFU}}{\text{mL}}\right)} \times 100 \ldots\ldots\ldots\ldots \text{(1)}$$

3.4.4 Screening for Bile Salt Tolerance

Resistance to bile salts was assessed to determine the isolates' capacity to withstand intestinal bile salts, following the method outlined by Reuben *et al*., (2019). In summary, the LAB isolates were grown in MRS broth at 37°C for 24 hours and centrifuged at 10, 000 x g for 5 minutes. The bacterial cells were then resuspended in 0,9% NaCl (w/v) and adjusted to an optical density (OD_{600}) of 0,200 OD units to achieve a concentration of 1×10^8 CFU/mL. The adjusted isolates were then inoculated into fresh MRS broth containing 0.3% and 0.6% bile salt (Merck) (w/v) and were left to incubate for 3 hours at 37°C, anaerobically. Cell viability was evaluated by plating the samples on MRS agar and subjecting them to anaerobic incubation at 37°C for 48 hours in an anaerobic jar using AnaeroGen ^{TM} 2.5 L sachet (Thermo Scientific). The equation below was used to determine the survivability percentage.

$$\text{Bile salt survivability \%} = \frac{\text{Viable LAB count } \left(\frac{\text{CFU}}{\text{mL}}\right) \text{after acid exposure}}{\text{Initial viable LAB count } \left(\frac{\text{CFU}}{\text{mL}}\right)} \times 100 \ldots\ldots\ldots \text{(2)}$$

3.5 SAFETY EVALUATION OF SELECTED LAB

3.5.1 Haemolytic Assay

The isolates were subjected to haemolytic activity testing to ascertain the production of haemolysin as previously described (Maragkoudakis *et al*., 2006). The LAB isolates cultivated in MRS broth were streaked onto the surfaces of Blood agar supplemented with 5% defibrinated (v/v) sheep blood (Merck) and incubated anaerobically at 37°C for 48 hours in an anaerobic jar using AnaeroGen ^{TM} 2.5 L sachet (Thermo Scientific). Following the incubation period, the plates were assessed for haemolytic activity by observing the zones of hydrolysis around the colonies, which were classified as either haemolytic, partially haemolytic, or non-haemolytic based on the presence of beta (β), alpha (α), and gamma (γ) haemolysis,

respectively. The presence of β-haemolysis is characterized by a clear, colourless/light yellow zone surrounding the colonies, indicating complete lysis of red blood cells. α-haemolysis is characterized by a limited greenish to brownish discolouration of the media surrounding the colonies, indicating the conversion of haemoglobin to methaemoglobin and subsequent diffusion into the medium. Finally, γ -haemolysis exhibits no observable changes in the media with no discernible clearing around colonies, indicating non-haemolysis. Further *in vitro* screening was conducted exclusively on non-haemolytic isolates.

3.5.2 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed by employing the agar disc diffusion method according to Reuben *et al.,* (2019). In summary, the LAB isolates were cultured in MRS broth and left to incubate at 37°C overnight. The bacterial suspension was adjusted using McFarland's standard of 0.5 (10^8 CFU/mL) as a reference. Using a sterile cotton swab, the LAB suspension (100 µL) was spread evenly onto the surface of the MRS agar plate. Different antibiotic discs, such as penicillin G (10 µg), ceftriaxone (30 µg), ampicillin (10 µg), vancomycin (30 µg), oxacillin (1 µg), streptomycin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), erythromycin (15 µg), tetracycline (20 µg), novobiocin (5 µg), and ciprofloxacin (5 µg), were placed on the agar plate surface. This is because different bacterial response to antibiotics are concentration-dependent, with higher concentration exhibiting stronger antimicrobial activity, while lower, subinhibitory concentration can trigger diverse biological responses and even promote resistanceThe plate was then incubated at 37°C for 24 hours. A digital caliper was used to measure the clear areas around each antibiotic disc. As per the CLSI Guidelines (Clinical & Laboratory Standards Institute) indicated in Appendix 8.1, the results were categorized as resistant, intermediate, or susceptible.

3.5.3 Extracellular Enzymatic Activity

To detect the production of amylase and protease enzymes by LAB, the isolates were subjected to enzymatic activity tests following the method outlined by Salehizadeh *et al.,* (2020). To assess amylase activity, the isolates were sub-cultured in MRS broth containing 0.25% starch and incubated under anaerobic conditions at 37°C for 24 hours. Following incubation, 30 µL of the overnight culture was transferred onto a paper disc and placed on a Nutrient agar plate. The plate was then incubated in an anaerobic environment at 37°C for 48 hours. After incubation, the halo zone surrounding each paper disc was measured using a calliper.

To measure protease activity, the isolates were sub-cultured in MRS broth and incubated under anaerobic conditions at 37°C for 24 hours. Following the incubation period, a volume of 30 µL of the overnight culture was applied to a paper disc, which was then positioned on Nutrient agar plates containing 1% skim milk. Subsequently, the plates were and incubated anaerobically at a temperature of 37°C for 48 hours. The halo zone surrounding each paper disc was measured using a digital calliper.

3.5.4 Antagonistic Activity Assays of LAB

3.5.4.1 Agar well diffusion assay

The inhibitory effect of the isolates was assessed using an agar-well diffusion assay to evaluate the potential of the LAB isolates to suppress prevalent poultry pathogens. The isolated LAB were cultured in MRS broth for 18-24 hours at 37°C, whilst the pathogens of interest (*Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium*, *Salmonella enterica* (ATCC 13314)*, Staphylococcus aureus* (ATCC 29213) and *Listeria* monocytogenes (ATCC 7644) were pre-cultured under the same conditions in Brain Heart Infusion (BHI) broth (Merck). The test pathogens were adjusted according to McFarland's standard 0,50 (10^8 CFU/mL) at an optical density of 600 OD units, and 200 µL were evenly spread onto the Mueller Hinton agar plates (Merck). The LAB isolates were centrifuged at 10,000 x g for 10 minutes, and about 100 µL of cell-free supernatants (CFS) was inoculated into a seven diameters wells punctured into the agar plates using sterile micropipette tips. The plates were

then incubated at 37°C for 24 hours and the zone of inhibition was measured using a digital calliper (Reuben *et al.,* 2019).

3.5.4.2 Agar spot test

Agar spot test was done to assess the antagonistic activity of the isolated LAB following the method by Armas *et al.,* (2017). Test pathogens grown in Brain Heart Infusion (BHI) broth for 24 hours at 37℃ were adjusted to McFarland's standard 0,50 (10^8 CFU/mL) using PBS (pH 7.2) after centrifugation at 10 000 x g for 5 minutes. One-hundred microlitres (100 µL) of test pathogen was then evenly spread onto the BHI agar plate and allowed to dry for 30 minutes. The MRS broth containing the isolated LAB isolates was centrifuged at 15,000 x g for 10 minutes, and 3 µL of the resulting cell-free supernatant (CFS) were spotted in duplicates on agar surfaces and incubated under aerobic conditions at 37℃ for 24 hours. The test was considered positive if the measured zones of inhibitions were > 1 mm.

3.6 ASSESSMENT OF GROWTH CHARACTERISTICS

3.6.1 Tolerance evaluations of temperature

Temperature tolerance of LAB isolates was assessed by introducing 1% v/v of LAB isolates cultivated at 37°C for 24 hours into MRS broth and subsequently incubating them at varying temperatures (25°C, 30°C, 40°C, 60°C, and 70°C) for 24 hours. The growth was assessed by quantifying turbidity in a 1 mL cuvette at a wavelength of 600 nm utilizing a spectrophotometer. The survivability % of the isolated LAB was determined as follows:

$$Survival\ \% = \left[1 - \left(\frac{OD_{24h}}{OD_i}\right)\right]\ x\ 100 \text{..... (3)}$$

3.6.2 Tolerance evaluation against sodium chloride, and phenol

Phenol and NaCl tolerance tests were conducted by diluting the overnight LAB cultures in maximum recovery diluent up to 10^{-8} CFU/mL. These diluted cultures were then inoculated into MRS broth containing various concentrations of NaCl (0.5, 2.0, 4.0, 6.5, and 10.0%) and phenol (0.2, 0.3, and 0.4%). The samples were incubated at 37°C for 24 hours. The growth was monitored by quantifying the turbidity 1 mL cuvette at a wavelength of 600 nm using a spectrophotometer (Reuben *et al.,* 2019).

3.7 MOLECULAR IDENTIFICATION OF LAB BY 16S rDNA SEQUENCING

The isolated LAB was further identified based on 16S rDNA gene sequencing using the universal primer. Briefly, total DNA was isolated using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005) according to the manufacturer's instructions. A PCR was performed with a reaction volume of 20 µL, which included 10 µL of GoTaq 2× Green Master Mix, 0.5 µL (10 µM) of both forward and reverse universal primers (Inqaba Biotech), 6 µL of nuclease-free water, and 2 µL of DNA template. The 16S target region was amplified, as shown in Table 3.1, and the amplification conditions were as follows; The initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and 68°C for 1 min, and a final extension at 68°C for 10 min.

The amplified DNA fragments were observed using agarose gel electrophoresis with a 1% (w/v) agarose gel, running at 80 V for 50 minutes. A 100 bp DNA ladder was used as a molecular weight marker. The amplicons were visualized by UV transillumination. The amplicon with the desired size was further purified and sequenced at Inqaba Biotech (Africa's Genomics Company). BLASTn analysis (with default parameters) was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the database matches the query sequence above a certain threshold (99% query coverage; 99% identity) (Accessed on 21 January 2024). The software package MEGA 11 was used to contrast the phylogenetic tree using the neighbour-joining method.

Table 3.1: 16S universal PCR primers used for the identification of LAB isolated from the crops and small intestines of broiler chickens

Name of Primer	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

3.8 ASSESSMENT OF PROBIOTIC CHARACTERISTICS

3.8.1 Simulated Gastric Juice Survivability Test

3.8.1.1 Preparation of simulated gastric juice

Simulated gastric acid (1 L) was prepared using glucose (3.5 g/L), NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), $CaCl_2$ (0.11 g/L), and KCl (0.37 g/L),1 M HCl to adjust the pH to 2.5 to mimic the low pH environment of the chicken stomach, and the mixture was autoclaved at 121℃ for 15 minutes (Beumer *et al.,* 1992; Corcoran *et al.,* 2005) . Before lysis, the prepared gastric juice was supplemented with filter sterile porcine bile (0.05 g/L), lysozyme (0.1 g/L), and pepsin (13.3 mg/L) that were prepared by diluting in sterile distilled water and filter sterilized using a 45-micron filter Millipore.

3.8.1.2 Survivability in Simulated gastric juice (with and without) lysozyme (pH 2.5)

Simulated gastric juice survivability testing was done by plate count method as previously described by Reuben (2019). Briefly, 1 ml of LAB isolates that were cultured in MRS broth at 37°C for 24 hours was suspended in an equal amount of PBS (pH 7.2) and then centrifuged at 3,500 x g for 25 minutes. The pelleted cells were mixed with approximately 5 mL of simulated gastric juice (with and without lysozyme) after centrifugation. The mixture was then incubated at 37˚C with continuous stirring for 90 minutes. The samples were serially diluted in maximum recovery diluent up to 10^{-8} CFU/mL, and incubated at different intervals of 0, 30, 60, and 90 minutes at 37°C. The isolates were further plated on MRS agar plates and incubated anaerobically at 37°C for 48 hours in an anaerobic jar using AnaeroGen ™ 2.5 L sachet (Thermo Scientific) (Reuben *et al.,* 2019). After incubation, the number of bacteria that survived was counted and reported as log CFU/mL. The D-values (decimal reduction time) which is the time required to achieve a log reduction after exposure to lysozyme, were estimated graphically from the slop of the regression line obtained by plotting the log of survival counts versus their corresponding time as explained by Majeed *et al.,* (2020).

3.8.2 Cell Surface Hydrophobicity

Cell surface hydrophobicity was done to assess the bacterial adhesion of LAB isolates to solvents (n-hexadexane) according to the method previously described by Abbasiliasi *et al.,* (2017). Briefly, LAB (10 ml) isolates were grown on MRS broth at 37°C for 24 hours, then 5 ml of the LAB isolates were centrifuged at 4,000 x g for 15 minutes and the pellet was washed twice and resuspended PBS (pH 7.2) and the OD_{600} was adjusted to 0.2 OD units. Three millilitres of the cell suspension were mixed with 1 mL of n-hexadecane, and vortexed for 1 minute. The mixture resulted in two-phase separation after standing for 15 minutes, and the absorbance of 1 ml of the aqueous phase was measured at 600 nm using a spectrophotometer. Bacterial affinity to the solvent (BATS) was determined using the following equation:

$$BATS\ \% = [1 - \left(\frac{A_{30min}}{A_{0min}}\right)\ x\ 100 \quad \ldots\ldots\ldots\ldots\ (4)$$

3.8.3 Auto-aggregation Assay

The isolates' capacity for auto-aggregation was evaluated following the protocol outlined by Li *et al.,* (2020). This was done to assess their cell-binding abilities which helps in the colonization of the host's epithelial cells and enhancement of the host's defence mechanisms against the gut pathogens. Briefly, 10 ml of the LAB isolates were cultured in MRS broth and incubated at 37°C for 18 hours. Centrifugation at 5,000 x g for 15 minutes was used to pellet the cultures, followed by three washes with PBS (pH 7.2). The cells were further resuspended in 5 mL PBS to adjust to an absorbance of 0.500 OD units. The cultures were then incubated for 4, 8, 16 and 24 hours at 37°C, and the absorbance of the supernatant was measured at 600 OD units. The auto-aggregation coefficient (AC) was determined according to the following formula:

$$AC(t)\ \% = \left[1 - \left(\frac{OD_{tf}}{OD_i}\right)\right]\ x\ 100 \ldots\ldots\ldots\ldots\ldots\ (5)$$

Where OD_i represents the initial optical density of the microbial suspension at 600 OD units, while OD_{tf} represents the optical density of the microbial suspension after incubation (4, 8, 16, and 24 hours).

3.8.4 Co-aggregation Assay

To evaluate the isolates' capacity to prevent pathogen colonization, a co-aggregation assay was performed, following the protocol described by Armas *et al.,* (2017). The LAB cultures (10 ml) were incubated in MRS broth at 37°C for 24 hours, then 4 ml was centrifuged at 6,000 x g for 15 minutes. After centrifugation, the bacterial cells were washed twice with sterile PBS (pH 7.2) and the optical density was (OD_{600}) adjusted to 0.5 using PBS. The mixture containing 2 mL of each LAB isolate and test pathogen culture (*Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium*, *Salmonella enterica* (ATCC 13314)*, Staphylococcus aureus* (ATCC 29213) and *Listeria monocytogenes* (ATCC 7644) were incubated for 4 hours at 37°C. Tubes comprising 4 mL of each sample, the tested pathogens, and LAB isolates were used as control. The absorbance of the mixture was measured at 600 OD units (OD_{mix}) by adding 1 ml of the mixture in cuvettes and compared to the absorbance of the control tube containing the probiotic strains (OD_{strain}) after the 2-hour incubation period. The equation below was used to calculate the co-aggregation percentage:

$$Co - aggregation\ \% = [1 - \ OD_{mix} / (OD_{strain\ +} OD_{pathogen})/2] \times 100 \ldots\ldots\ldots (6)$$

3.8.5 α-Glucosidase Inhibitory Activity Assay

The α-glucosidase inhibitory assay was performed to assess the ability of the LAB isolates to inhibit the carbohydrate hydrolysing enzyme; α-glucosidase, using the method described by Kim *et al.,* (2018). Briefly, 5 ml of the overnight cultures of LAB isolated growth on MRS broth were centrifuged at centrifuged at 4,000 x g for 15 minutes and resuspended in potassium phosphate buffer (pH 6.5) and the optical density (OD_{600}) of the bacterial cells was adjusted to 0.2 OD units. One hundred microlitres of the LAB isolate was then resuspended in 50 µl of 0.2 M potassium phosphate buffer (pH 6.5). Fifty-microlitre of α-glucosidase (0. 75 U/mL) was added to the suspension and pre-incubated for 15 min at 37°C. After pre-incubation, 3 mM *p*-nitrophenol-α-D-glucopyranoside (*p*NPG, 100 µL) was added and the enzymatic reaction was allowed to proceed for 15 minutes at 37°C. The reaction was then stopped by the addition of 50 µL of 0.1 M Na_2CO_3. The release of 4-p-n-Nitrophenol was measured at 405 OD units using a microplate reader. The α-glucosidase inhibitory activity was determined using the following formula:

$$\alpha - glusidase\ inhibitory\ activity = 1 - \frac{A}{B} \times 100 \ldots\ldots\ldots (7)$$

Where A was the absorbance of the reactants with the sample, and B was the absorbance of the reactants without the sample (negative control). Acarbose was used as the standard reference (positive control)

3.9 STATISTICAL ANALYSES

All experimental measurements were done in triplicates to validate the obtained results. The results were expressed as means ± standard errors of the mean (SEM) calculated in Microsoft Excel 365 for Windows, where possible to demonstrate the variance in the means. In addition, all percentage values presented in this study were also calculated in MS Excel. Statistical analysis of the acquired data was performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to compare the means of obtained data, with a significance level of $p < 0.05$ to differentiate among statistically significant means.

Additionally, the student's independent t-test was used in the simulated gastric juice survivability test of the isolated LAB to discriminate differences between the means of the control **(**simulated gastric juice without lysozyme) and LAB isolate. Differences were considered statistically significant at $p < 0.05$. The correlation between auto-aggregation and cell surface hydrophobicity percentages was determined using the Pearson correlation coefficient using GraphPad Prism Software.

Principal Component Analysis (PCA) was performed on XLSTATS (for Windows) to obtain the statistical differences among the isolates for probiotic properties (acid, bile and temperature tolerance, auto-aggregation, co-aggregation, cell-surface hydrophobicity and α-glucosidase inhibitory activity).

4 CHAPTER FOUR: RESULTS

4.1 ISOLATION AND PHENOTYPIC CHARACTERIZATION OF LACTIC ACID BACTERIA (LAB) ISOLATES FROM THE CROPS AND SMALL INTESTINES OF BROILER CHICKENS

In this study, 66 LAB strains were isolated from the GI tract (27 from the crop and 39 from the small intestine) of four healthy 23-week-old locally bred broiler chickens. Based on their morphological characteristics; creamy white colonies as well as the cocci and bacilli characteristics were observed. They showed strong growth in MRS media under anaerobic conditions at 37°C. Only 29 colonies (Tables 4.1 and 4.2) were found to be Gram-positive and catalase-negative.

Table 4.1: Phenotypic characterization of LAB isolates from the crops of broiler chickens. *Lactobacillus casei* (ATCC 393) was used as a positive control

	C4	**C5**	**C7**	**C10**	**C12**	**C13**	**C14**	**C20**	**C24**	**C27**	**C34**	**C38**	***L. casei* (ATCC 393)**
Morphology	Cocci	Cocci	Cocci	Bacilli	Bacilli	Cocci	Cocci	Cocci	Cocci	Bacilli	Bacilli	Cocci	Bacilli
Gram-stain	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2: Phenotypic characterization of LAB isolates from the small intestines of broiler chickens.

	SI2	**SI3**	**SI4**	**SI6**	**SI7**	**SI8**	**SI9**	**SI21**	**SI22**	**SI23**	**SI25**	**SI27**	**SI30**	**SI31**	**SI32**	**SI38**	**SI42**
Morphology	Cocci	Cocci	Cocci	Bacilli	Bacilli	Bacilli	Bacilli	Cocci	Cocci	Cocci	Bacilli	Cocci	Bacilli	Bacilli	Bacilli	Cocci	Cocci
Gram-stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.2 PRELIMINARY SCREENING OF LAB ISOLATES FROM THE CROPS AND SMALL INTESTINES OF BROILER CHICKENS

4.2.1 Acid Tolerance Assay

Figure 4.1 displays the results of the survivability percentage of lactic acid bacterial strains isolated from the crops and small intestines of broiler chickens after exposure to acidic MRS broth at pH 3 and 4. Among the 29 isolates that were tested for their ability to survive, only 19 isolates (7 from crops and 12 from small intestines) along with the reference strain, *L. casei* (ATCC 393), met the criteria for the acid tolerance test by demonstrating a survivability percentage equal to or greater than 50%. The survivability percentage of the isolates ranged from 22.12% to 94.06% and 22.37% to 95.01%, in both pH 3 and 4, respectively. Notably, a significant difference ($p < 0.0001$) was observed in the survivability percentage of n=10 isolates and that of the reference strain under acidic conditions of pH 3 and 4. This observation indicates that the isolates did not closely mimic the acid-tolerance capability of the reference strain, *L. casei* ATCC 393.

Importantly, the survivability percentage of Isolates SI3 (94,06 ± 0,66%), SI22 (83,12 ± 2,38%), and SI42 (82,84 ±6,41%) at pH 3 and SI27 (95,01 ±3,74%), C38 (89, 98 ± 6,19%), and SI32(88,95 ± 2,89%) at pH 4 showed no statistical difference to the survivability percentage of the reference strain, *L. casei* ATCC 393. This indicates that these isolates closely mimic the reference's acid-tolerance capabilities.

A higher survivability percentage was observed at pH 4, even the isolates that showed survivability less than 50% at pH 3 were able to tolerate pH 4. For instance, isolate C20 and C34 showed the survivability percentage of 22,39 ± 0,53% and 42,52 ± 4,4% at pH 3, and 72,91 ±1,96% and 86, 28 ± 5,10% at pH 4, respectively. However, in this study, only isolates that exhibited a survivability percentage of 50% or higher, particularly at pH 3 (C4, C5, C7, C13, C14, C24, C38, SI2, SI3, SI4, SI6, SI7, SI8, SI9, SI22, SI23, SI27, SI38 and SI42) were deemed to have survived and were chosen for additional *in vitro* testing, as pH 3 is considered as the standard pH for acid tolerance of potential probiotics. Based on the obtained results in this study, it can be concluded that the survival of probiotics in acidic pH depends on the type of strain being tested, as it was observed that all isolates behaved differently from each other.

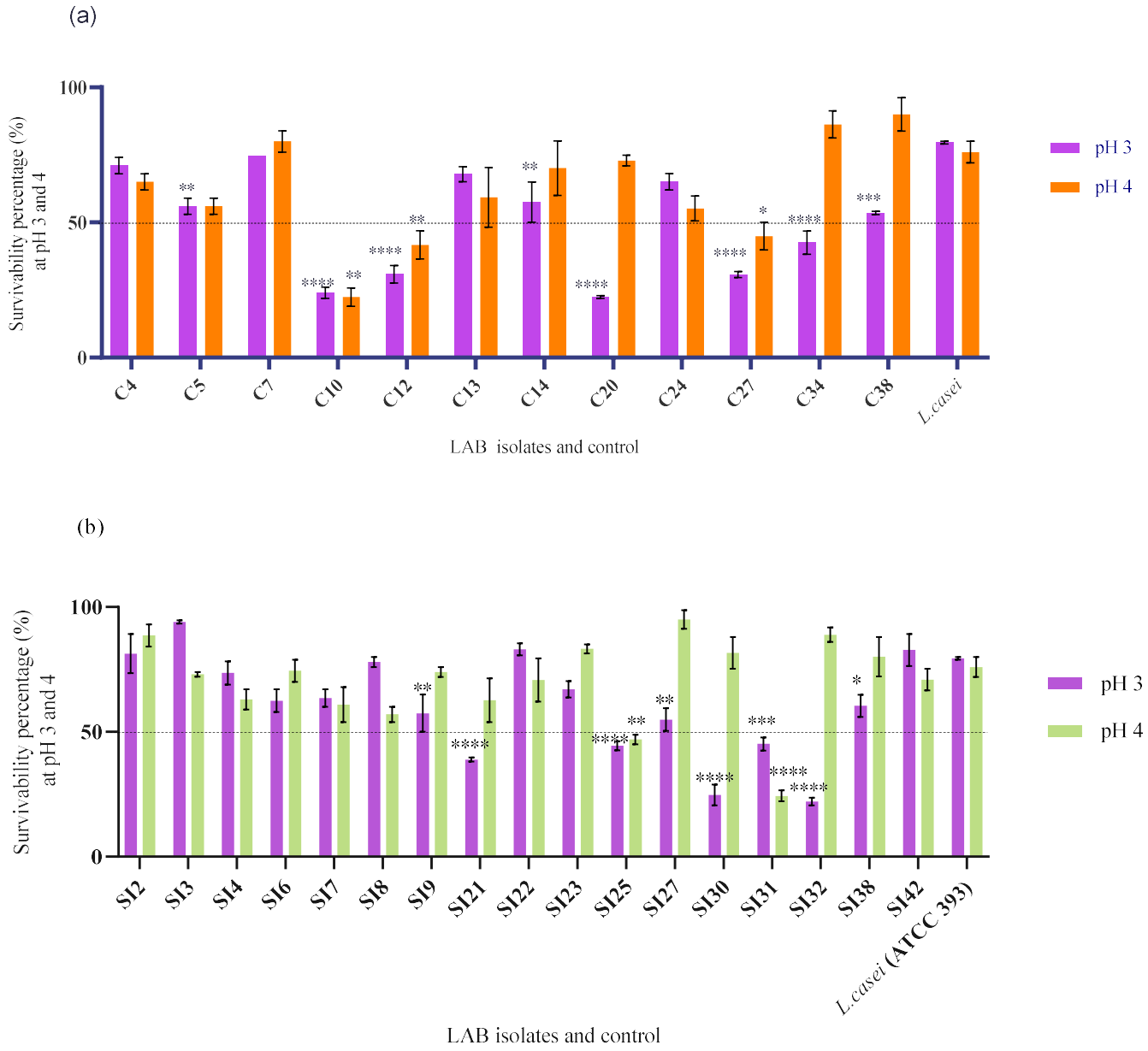
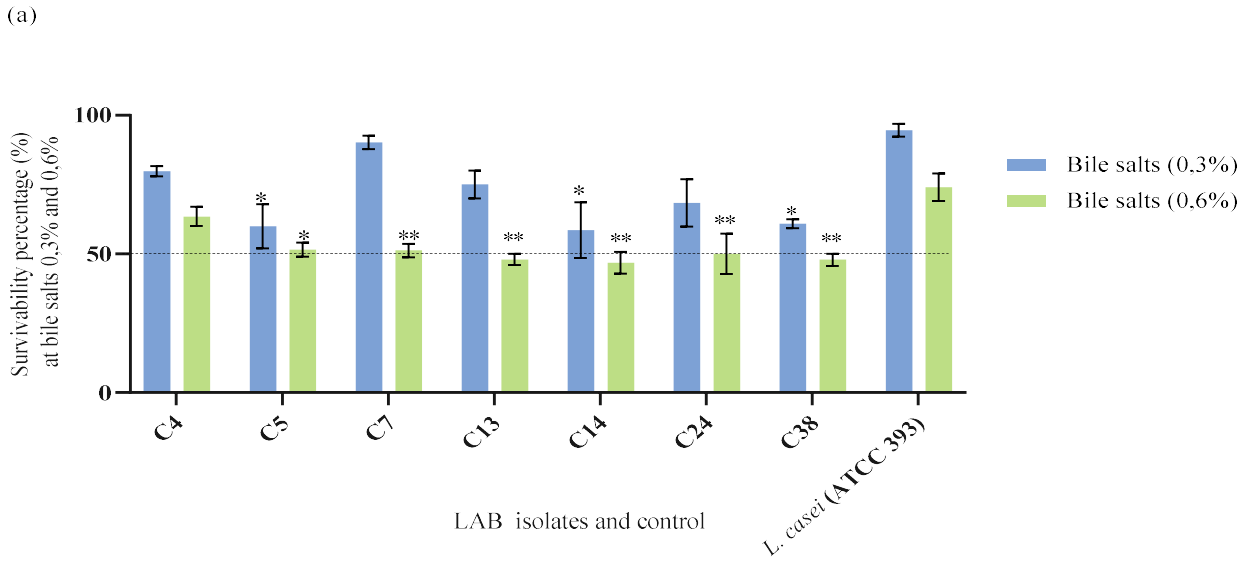


Figure 4.1: Survivability percentages of bacterial strains isolated from the (a) crops of broiler chickens and (b) small intestines of broiler chickens after 3-hour exposure to pH 3 and pH 4. Reference strain: *Lactobacillus casei* (ATCC 393). n = 3 replicates. Values are means ± S.E.M. Significant differences between the means of LAB isolates and the reference strain are displayed as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

4.2.2 Bile Salts Tolerance Assay

The survivability percentage of LAB, obtained from crops and small intestines of broiler chickens, is illustrated in Figure 4.2 after being exposed to MRS broth supplemented with 0.3% and 0.6% (w/v) bile salts for 3 hours. Out of the 19 isolates tested (7 from crops and 12 from small intestines), all exhibited the highest level of tolerance at 0.3% bile salts. The level of tolerance differed among the isolates ($p<0.05$), with C7, SI23, and SI38 exhibiting the greatest growth and compatibility, with survival rates of 90.23%, 81.95%, and 80.47%, respectively. With increasing bile salt concentrations, a significant decrease in the survivability of isolates was observed, with isolate SI3 showing the lowest significant survivability percentage of 27,20% ($p < 0.0001$). Isolates SI23, SI28 and SI42 demonstrated the highest survivability percentage of 64,69%, 68,61% and 68,18% respectively. Furthermore, no statistically significant difference was observed when compared to the survivability percentage of the of the reference strain *L. casei* ATCC 393, suggesting that these isolates closely mimic the bile salt-tolerance traits of the reference strain.

According to Oyewole *et al.,* (2018), probiotic bacteria must grow in bile salt concentrations ranging from 0, 15-0,30% to be efficient and effective in promoting gut health. Hence, in this study, isolates that showed a survivability percentage greater or equal to 50%, especially at 0,30% bile salt concentrations were selected for further *in vitro* screening.



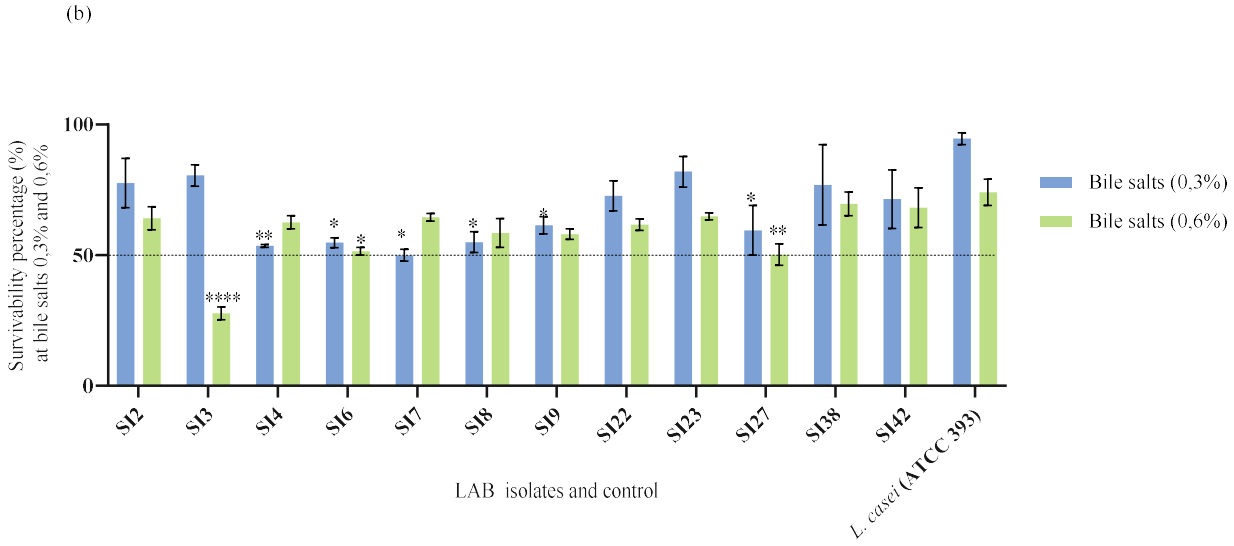


Figure **4.2:** Survivability of lactic acid bacterial strains isolated from the (a) crops and (b) small intestines of broiler chickens after 3-hour exposure to bile salts concentrations of 0,3% and 0,6%. Reference strain: *Lactobacillus casei* (ATCC 393). n = 3 replicates. Values are means ± SEM. Significant differences between the means of LAB isolates and the reference strain are displayed as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

4.3 SAFETY EVALUATION OF THE ISOLATES LAB ISOLATES

4.3.1 Haemolysis Activity

Nineteen (19) isolates (7 from the crops and 12 from the small intestines) were tested for haemolytic activity to assess their pathogenic nature, among which 14 isolates were characterized as non-haemolytic, while five (n=5) isolates showed *β*-haemolytic activity. *Lactobacillus casei* (ATCC 393) was used as the positive control for the selection of all non-haemolytic isolates, while *E. coli* and *S. aureus* were used as references for *α*-haemolytic and *β*-haemolytic activity, respectively (Figure 4.3). All isolates that demonstrated *β*-haemolytic activity (C14, C38, SI2, SI3 and SI27) were removed from the subsequent screening for probiotics.

A. B. C.

Figure 4.3: (a) (γ) haemolysis of LAB isolate C7 from the crop isolated from the broiler chicken indicating non-haemolysis, a typical example of all isolates examined (indicated by no zone surrounding the colony). (b) *α*-haemolysis of *E. coli* indicated by green colour change in the medium. C. *β*-haemolysis activity of *S. aureus* indicated by a yellow colour change in the medium.

4.3.2 Antibiotic Susceptibility Testing

Fourteen (14) isolates were examined for antibiotic activity against 12 commonly used antibiotics as shown in Table 4.3 by measuring the zones of inhibition as indicated in Figure 4.4. As a result, the LAB isolates showed varying responses to the antibiotics used (zones of inhibition shown in Appendix 8.2). All isolates demonstrated 100% resistance to vancomycin and ciprofloxacin. A total of thirteen (13) isolates showed resistance (86,67%) to novobiocin and oxacillin, while 11 LAB isolates showed 80% resistance to gentamicin. In addition, 73,30% resistance to streptomycin was observed in 10 isolates. Thirteen (13) isolates were sensitive (93,33%) to ampicillin and chloramphenicol, while nine LAB isolates were sensitive (86,67%) to ceftriaxone, 10 isolates were sensitive (80%) to erythromycin and 10 were intermediate to penicillin.

It was also observed in this study that isolate SI23 was resistant to all tested antibiotics, while C5 and *L. casei* (ATCC 393) exhibited resistance against eight out of the twelve antibiotics tested. Isolates C24 and SI22 were resistant to seven antibiotics, while isolates C4, C7, C13, SI6, and SI38 were resistant to six of the antibiotics tested in this study. Isolates SI7, SI9, SI42 exhibited resistance against five of the tested antibiotics. The combination of resistance, along with high susceptibility to other antibiotics that are used as therapeutic agents, indicates a favourable safety profile for these strains in terms of antibiotic resistance.

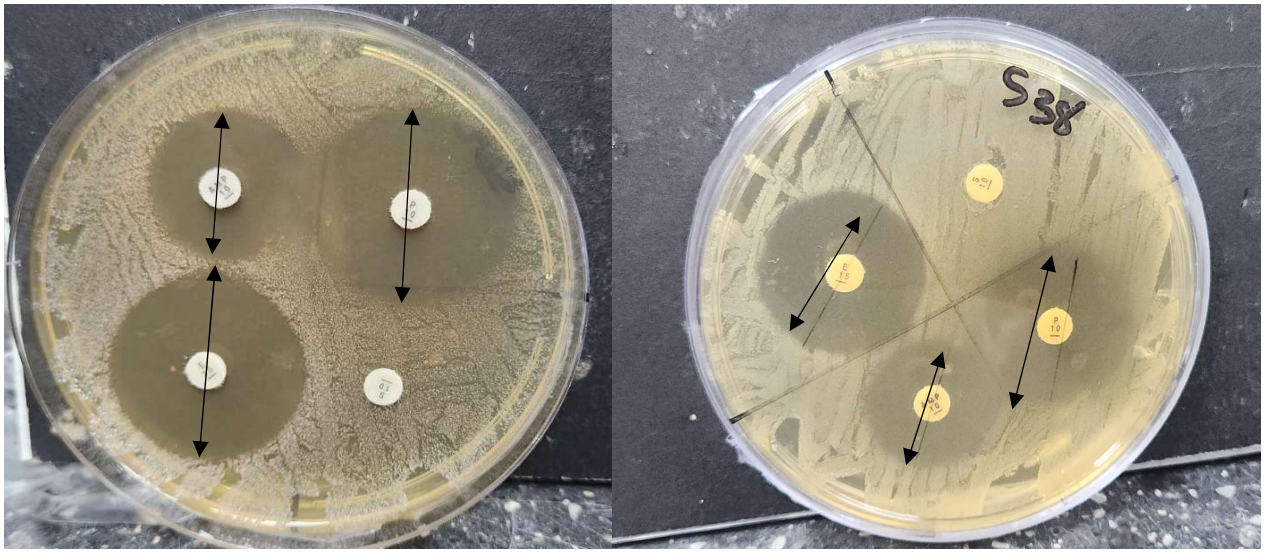


Figure 4.4: MRS agar plate showing zones of inhibition (measured in mm) of LAB isolate (A.) S42 and S38 (B) against erythromycin (15 µg), ampicillin (10 µg), penicillin and resistance against streptomycin (10 µg).

Table 4.3: Antibiotic resistance profiles of LAB isolates isolated from the crops and small intestines of broiler chickens

Antibiotic class	Antibiotics	C4	C5	C7	C13	C24	SI4	SI6	SI7	SI8	SI9	SI22	SI23	SI38	SI42	*L. casei (ATCC 393)*
Aminoglycosides	Gentamicin	R	R	R	I	R	I	R	R	S	R	R	R	R	R	R
	Streptomycin	R	R	R	R	R	I	R	S	S	I	R	R	R	R	R
Amphenicols	Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
Cephalosporins	Ceftriaxone	S	S	I	I	R	S	S	S	S	S	I	R	S	S	R
Fluoroquinolones	Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Glycopeptides	Vancomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Macrolides	Erythromycin	I	R	S	R	S	S	S	S	S	S	S	R	S	S	I
Penicillin	Ampicillin	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
	Penicillin	R	I	I	I	I	I	I	I	I	I	I	R	R	S	S
	Oxacillin	S	R	R	R	R	R	R	R	R	R	R	R	I	R	R
Tetracyclines	Tetracycline	S	R	S	S	S	S	S	S	S	S	R	R	S	S	R
Aminocoumarin	Novobiocin	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R

Resistant (R); Intermediate (I); Susceptible (S)

4.3.3 Extracellular Enzymatic Activity

Fourteen isolates were tested for their ability to produce extracellular enzymes, protease and amylase. Thirteen isolates were able to produce protease with zones of clearance ranging from 8.50 ± 2.12 to 12.50 ± 0.71 mm diameter except for isolate SI42. Among them, isolate C7 (12,5±0,71 mm) exhibited the highest production of the protease enzyme. For amylase production, only 12 isolates were able to produce amylase with zones of clearance ranging from 9,5±0,71 to 12,0±1,41 mm in diameter. The amylase enzyme was most abundantly produced by isolates C4 and SI6 with an average diameter of 11,0±1,41 mm. (Table 4.4). These dietary enzymes play a crucial role in the hydrolysis of starch and proteins into short oligosaccharides and smaller peptides or amino acids. Therefore, potential probiotic strains must be able to produce them. The zones of clearance of SI22 and SI42 showed a significant difference of $p < 0,0001$ when compared with that of the reference strain, *L. casei* (ATCC 393), indicating that these isolates did not mimic the reference strain's ability to produce extracellular enzymes. Therefore, these isolates were excluded from further *in vitro* screening.

Table 4.4: Amylase and protease activity of LAB isolates from the crops and small intestine of broiler chickens. Zones of clearance are shown as are mean ± SEM. n = 3 replicates with * indicating P< 0.05 and ***** p < 0,0001

	C4	C5	C7	C13	C24	SI4	SI6	SI7	SI8	SI9	SI22	SI23	SI38	SI42	*L. casei* (ATCC 393)
Amylase activity	11,0±1,41	9,50±2,12*	10,5±0,71	9,5±0,71*	10,0±2,83	10,0±2,83	11,0±1,41	10,5±2,12	11,5±2,12	12,0±1,41	0,0±0,00****	10,5±0,71*	9,5±0,71*	0±0,00****	15,0±1,41
Protease activity	10,5±0,71	10,0±1,41	12,5±0,71	10,5±2,12	11,5±0,71	9,5±0,71	10,5±0,71	8,5±2,12	8,5±4,95	10,0±1,41	9,5±2,12	9,5±2,12	9,5±2,12	0±0,00****	12,5±0,71

4.3.4 Antagonistic Activity Assay of Cell-Free Supernatant LAB

4.3.4.1 Agar well diffusion assay

Twelve LAB isolates were tested for their capacity to inhibit the growth of common poultry pathogens, which comprised *Listeria monocytogenes* (ATCC 7644), *Salmonella typhimurium, Salmonella enterica* (ATCC 13314), *Pseudomonas aeruginosa (*ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Only 11 of these isolates exhibited the ability to inhibit pathogen growth. As shown in Table 4.5, antimicrobial activity of the isolates indicated by the zones of inhibition varied among the isolates, ranging from 9,00 ±5,66 to 30,00 ±0 mm. Isolate SI9 showed the highest inhibition activity against *L. monocytogenes* (25,00 ±7,07 mm), *S. enterica* (30,00 ±0 mm), and *P. aeruginosa* (24,50 ±0,71mm). Isolate C4 (23,50 ±3,54 mm) and SI23 (23,00 ±7,07 mm) showed the highest inhibition activity against *S. typhimurium*, while SI6 (23,00 ±4,24 mm) showed the highest inhibitory effect against *S. aureus*. When compared to the reference strains, *L. casie* ATCC 393, it can be concluded that a significant decrease ($p < 0.0001$) in antimicrobial activity was observed against *P. aeruginosa,* with isolates C24, C7 and C13 demonstrating the least inhibition activity with the zones of inhibition (mm) of 9,00 ±5,66, 11,00 ±1,41 and 11,00 ±1,41, respectively.

Table 4.5: Antimicrobial activity of lab isolates from the crops and small intestines of broiler chickens against poultry pathogens (zones in mm) by agar well diffusion assay**.** Zones of inhibition are shown as mean ± SEM. n = 3 replicates with * indicating statistically significant differences ($p < 0.05$)

	C4	**C5**	**C7**	**C13**	**C24**	**SI4**	**SI6**	**SI8**	**SI9**	**SI23**	**SI38**	***L. casei (ATCC 393)***
L. monocytogenes (ATCC 7644)	13,5±4,95	13,00 ±0	16,5±2,12	15,00 ±0	20,5±0,71	14,5±0,71	20,5±0,71	19,0 ±2,83	25,0±7,07	23,0±4,24	19,00 ±0	21,0±1,41
S. typhimurium	20,5±0,71	22,5±6,36	15,5±0,71	20,5 ±3,54	23,5±3,54	18,0±4,24	17,5±0,71	18,5 ±2,12	21,0±1,41	23±7,07	15,5±3,54	25,5±2,12
S. enterica (ATCC 13314)	14,0 ±0*	13,5±0,71*	14,5±0,71	14,5 ±0,71	15,5±2,12	28,5±3,54	29,0±1,41	28,5±12,02	30,0 ±0	13,5±0,71*	13,0±1,41*	27,0±4,24
Ps. aeruginosa (ATCC 27853)	12,5±3,54	15,5±0,71	11,0±1,41	11,0 ±1,41	9,0 ±5,66*	23,5±2,12	21,5±0,71	21,0 ±0	24,5±0,71	13,5±0,71	14,0±1,41	17,5±3,54
S. aureus (ATCC 29213)	15,5±0,71*	16,0±5,66*	19,5±3,54	14,5±3,54*	18,5±0,71	18,5±2,12	23,0±4,24	20,0 ±4,24	19,5±4,95	14,5±0,71*	20,5±0,71	27,0±2,82[a]

4.3.4.2 Agar spot test

Table 4.6 shows the inhibition of pathogens by the isolated LAB isolates from the crops and small intestines of broiler chickens, and as in agar well diffusion assay, out of the 12 isolates tested for antimicrobial activity, only 11 (5 from the crops and 6 from the small intestine) were able to inhibit the growth of the tested pathogens. The zones of inhibition observed in this study varied among the isolates, ranging from 1,00 ±0 to 5,50 ±0,71. Notably, the zones of inhibition of isolates SI23 (5,50 ±0,71 mm) and SI4 (5,00 ±1,41 mm) against *S. aureus* **(**ATCC 29213**)** and *S. typhimurium* were significantly higher ($p < 0.05$) than that of the reference strains which exhibited inhibition of 2,0±1,4 and 3,0±1,41 mm against *S. aureus* **(**ATCC 29213**)** and *S. typhimurium,* respectively.

Table 4.6: Inhibitory effect of LAB isolates from the crops and small intestines of broiler chickens against pathogens (zones in mm) by agar spot test. Zones of inhibition are shown as mean ± SEM. n = 3 replicates with * indicating statistically significant differences ($p < 0.05$)

	C4	C5	C7	C13	C24	SI4	SI6	SI8	SI9	SI23	SI38	*L. casei* (ATCC 393)
S. typhimurium	4,5 ±0,71	3,5 ±0,71	1,5 ±0,71	4,0 ±0	4,0±1,41	5,0±1,41	4,5±0,71	3,0±1,41	2,5±0,71	3,0±1,41	4,5±0,71	3,0±1,41
L monocytogenes (ATCC 7644)	2,5 ±0,71	3,5 ± 0,71	2,5 ±0,71	1,5 ±0,71	3,0±1,41	3,0±1,41	4,0±1,41	3,0±1,41	2,5±2,12	1,5±0,71	3,0 ±0	3,5±0,71
S. enterica (ATCC 13314)	2,0 ±0	4,0 ±1,41	2,0 ±1,41	1,5±0,71	2,0±1,41	3,5±0,71	4,5±0,71	3,5±0,71	4,5±0,71	4,5±0,71	3,0±1,41	2,0±1,41
Ps. aeruginosa (ATCC 27853)	2,0 ± 1,41	3,5 ±0,71	5,0 ±1,41	3,5 ±0,71	4,0 ±0	2,5±0,71	3,5±0,71	4,0±1,41	2,0 ±0	2,5±0,71	2,5±0,71	3,5±0,71
S. aureus (ATCC 29213)	3,0 ± 1,41	3,0 ±0	1,5 ±0,71	1,0 ±0	2,5±0,71	3,0±1,41	3,5±0,71	3,5±0,71	4,5±0,71	5,5±0,71*	3,5±0,71	2,0±1,4

4.4 GROWTH CHARACTERISTICS

4.4.1 Phenol Tolerance Assay

The effect of phenol (0,2%, 0,3% and 0,4%) on LAB isolates after 24 hours of incubation at 37°C is shown in Figure 4.5. All isolates showed resist to phenol at 0,2%, 0,3% and 0,4% concentrations with OD values that are greater than the initial values ranging from the OD_{600} of 0,49 ± 0,13 to 1,89 ± 0,011 OD units. A significant increase ($p < 0.05$) in growth was observed in phenol concentrations of 0.2%, with isolate C5 demonstrating the highest phenol tolerance of 1.89 ± 0.011 OD units which was much higher than that of the reference strain, *L. casei* ATCC 393. This increase was also observed in phenol concentrations of 0.3%, with a slight decrease when compared to the growth exhibited in phenol concentrations of 0,2%. Interestingly, isolate C5 also demonstrated the highest resistance to 0.2% phenol concentration with the OD of 0,884 ±0,003 OD units.

Nevertheless, notable alterations were detected in MRS supplemented with 0.4% phenol, with C4 isolates exhibiting the lowest growth tolerance to phenol with a growth of 0.49 ± 0.13 OD units. It should be noted that the isolates showed no statistically significant difference ($p > 0.05$) at phenol concentrations of 0,4% when compared to the growth phenol tolerance of the reference strain, indicating that the isolates were able to mimic the phenol-tolerance abilities of the known probiotic strain. Furthermore, even though there was a decrease in growth when compared to the growth exhibited by isolates in phenol concentrations of 0,2 and 0,3%, the growth tolerance in phenol concentrations of 0,4% was still greater than the initial values. Therefore, all isolates were selected for further probiotic screening. Based on the data obtained in this experiment, it can be concluded that increasing phenol concentration, results in a decrease in the viability of LAB.

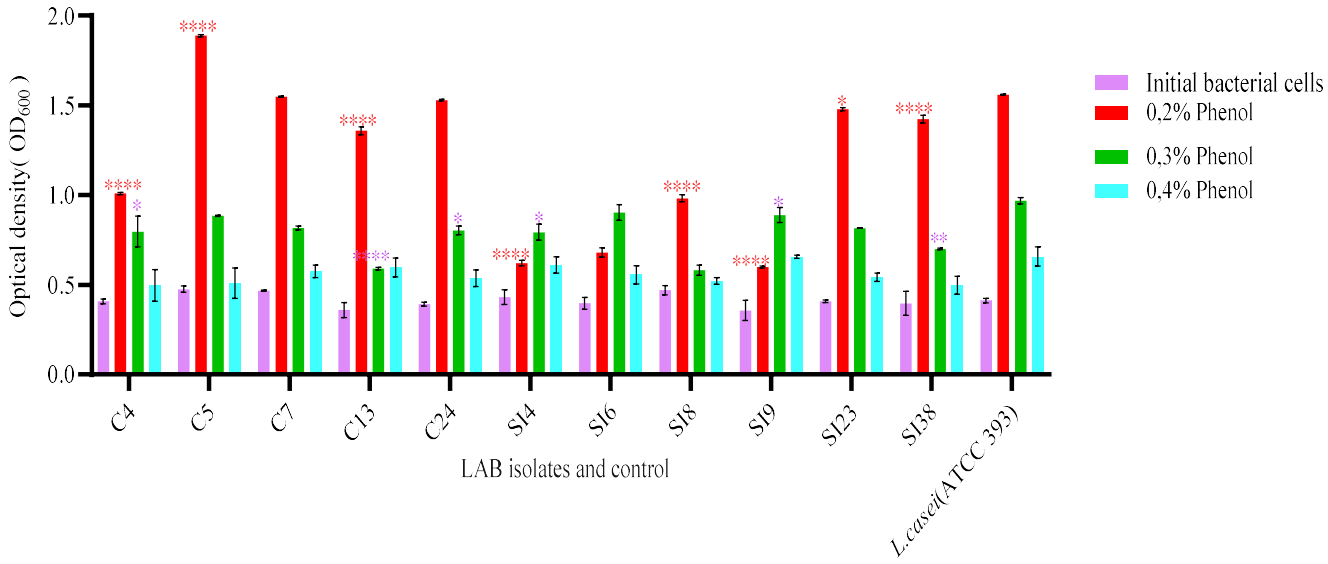


Figure 4.5: Growth tolerance to different concentrations of 0,2%, 0,3% and 0,4% phenol by LAB isolates from the crops and small intestines of broiler chickens after 24 hours of incubation. The values are expressed in means ± S.E.M of three replicates. Reference strain: *Lactobacillus casei* (ATCC 393). Significant differences between the means of LAB isolates and the reference strain are shown as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

4.4.2 NaCl Tolerance Assay

Figure 4.6 depicts the growth capability of 11 potential LAB isolates in an MRS medium with varying concentrations of NaCl (0,5%, 2%, 5%, and 10%) after being incubated at 37°C for 24 hours. In this study, the significant increase ($p < 0,0001$) in the growth tolerance of LAB exposed to MRS broth supplemented with NaCl concentrations of 0,5%, 2% and 5% was observed with optical density ranging from 0,35 ± 0,02 to 1,99 ± 0,04 OD units. Isolate C5 exhibited the highest growth tolerance in the three observed concentrations with the of 1,96 ± 0,026 OD units at 0,5% concentration, 1,84 ± 0,001 OD units at 2% concentration and 1,73 ± 0,019 OD units at 5% concentration. However, the growth was reduced at a NaCl concentration of 10%, with isolates C24 and SI38 exhibiting the lowest tolerance with an OD of 0,030 OD units. It was observed that the isolates grew best at NaCl concentrations below 5% as the increase in NaCl concentrations resulted in a significant decrease in LAB growth.

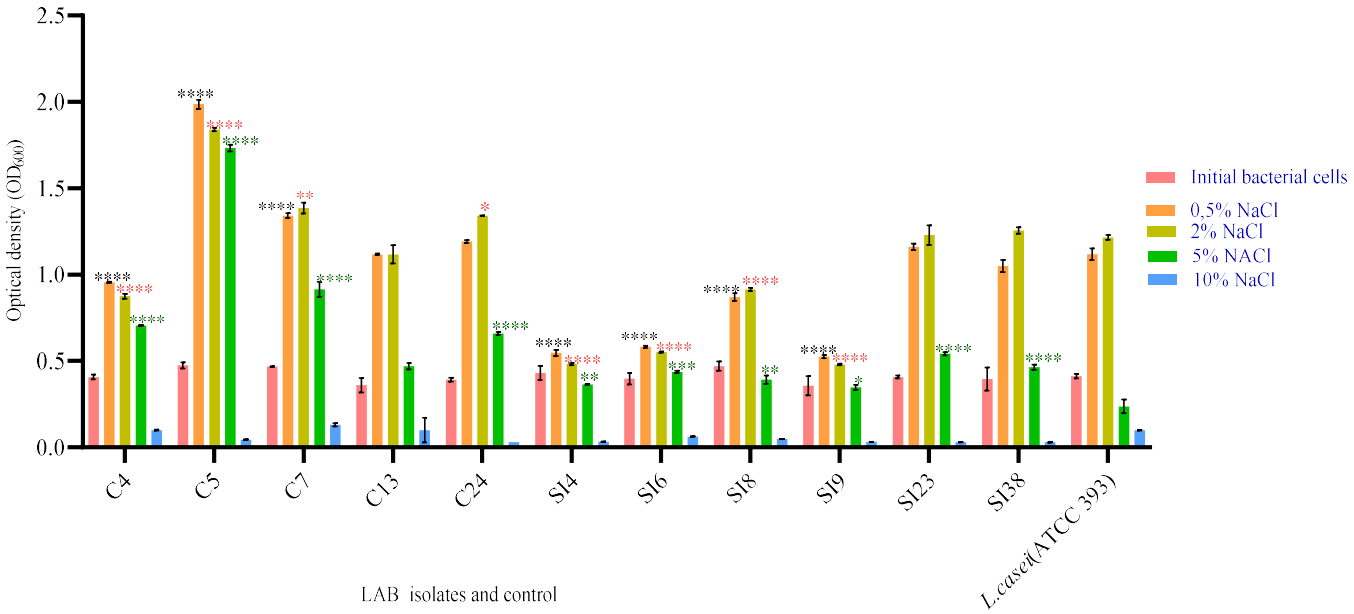


Figure 4.6: Growth tolerance of isolated LAB to different concentrations of sodium chloride. The values are expressed as means ± S.E.M. of three replicates. Reference strain: *Lactobacillus casie* (ATCC 393). Significant differences between the means of LAB isolates and the reference strain are displayed as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

4.4.3 Temperature Tolerance Assay

Figure 4.7 illustrates the survivability percentage for 11 LAB isolates in MRS broth incubated at different temperatures (25 °C, 30°C, 40°C, 60°C, and 70°C) for 24 hours. The survivability percentage of all 11 LAB isolates varied between 24.28% and 82.09% when exposed to temperatures of 25°C, 30°C, and 40°C. In this study, the assessment of growth tolerance to 40°C was very crucial as it is the normal body temperature of broiler chickens, and interestingly, the most significant increase occurred at temperatures of 30°C and 40°C, where isolates C13 and SI4 showed the highest survival rates of 82.09% ± 5.82% and 76.82% ± 1.47%, respectively. Additionally, the growth tolerance of these isolates further showed a statistically significant difference of $p < 0,001$ and $p < 0,05$ when compared to the growth tolerance of the reference strain, *L. casie* ATCC 393, as their temperature was even higher than that of the reference strain. Conversely, a reduced survivability percentage of the LAB isolates at 60°C and 70°C was recorded, with isolate SI4 exhibiting the lowest percentage of 7,10 ± 0,54%. Based on these observations, it was noted that with increasing temperatures, the growth tolerance decreases.

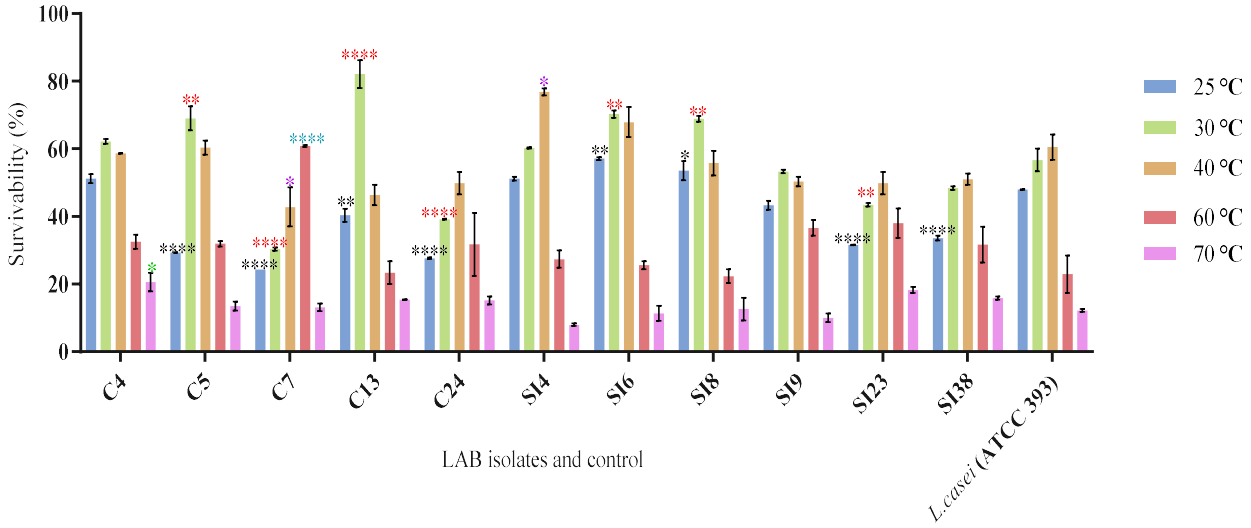


Figure 4.7: Growth tolerance of isolated LAB to different temperatures of 25°C, 30°C, 40°C, 60°C and 70°C. The values are expressed as means ± S.E.M of three replicates. Reference strain: *Lactobacillus casie* (ATCC 393). Significant differences between the means of LAB isolates and the reference strain are shown as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

4.5 GENOTYPIC IDENTIFICATION OF LAB

The identification of the isolated LAB was confirmed using the 16S LAB-specific primers, 27F and 1492R with species-level identification based on the 16S rDNA. Figure 4.8 shows the amplified PCR products of all LAB isolates that were positive with 1500 bp size ran on a 1% agarose gel. The amplicon sizes corresponded with the amplicon size of the reference strain *L. casei*. Table 4.7 shows the accession numbers of the 16S rDNA sequences of the isolated LAB that were blasted and deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank). The results showed that the isolated eleven LAB strains belonged to four species, *Pediococcos* spp., *Enterococcus* spp., *Lactobacillus* spp., and *Streptococcus* spp (Figure 4.8-4.12). The identified isolates were *Enterococcus faecalis* strain (isolates C4 and C5) with a 99,86% similarity, *Pediococcus pentosaceus* strain (isolates C7, C13 C24, SI23 and SI38) with 99,58% similarity, *Streptococcus salivarius* strain (isolate SI4) with 100% similarity and *Levilactobacillus brevis* (isolates SI6, SI8 and SI9) with 99,85% similarity.

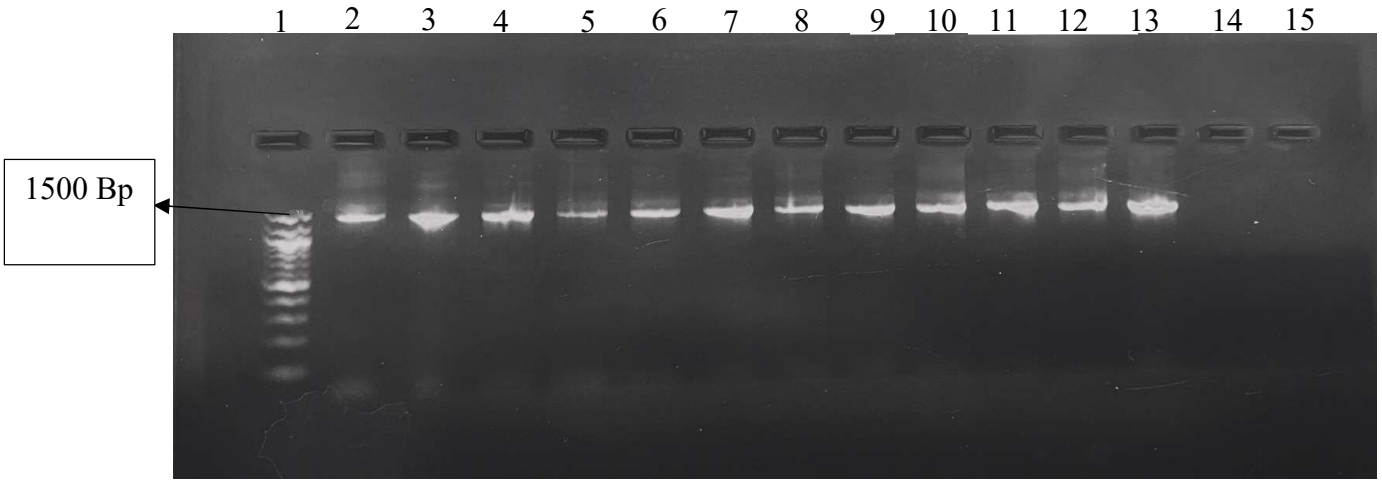


Figure 4.8: 1% agarose gel electrophoresis of PCR amplicons of the 16s rDNA genes of bacterial isolates. lane 1: 100 bp ladder, lane 2: *L. casei* (ATCC 393), lane 3-13: LAB isolates (C4, C5, C7, C13, C24, SI4, SI6, SI8, SI9, SI23, SI38) at 1500 bp, lane 14: negative control (PCR mixture without any isolate).

Table 4.7: Identification of LAB isolated from the crops and small intestines of broiler chickens by the 16S rDNA sequencing

Isolate code	**Phylogenetic affiliation**	**GenBank accession No.**	**Similarity**
C4	*Enterococcus faecalis* strain NKFS1	PP446703	99.86%
C5	*Enterococcus faecalis* strain NKFS2	PP446719	99.93%
C7	*Pediococcus pentosaceus* strain NKFS3	PP446720	99.58%
C13	*Pediococcus pentosaceus* strain NKFS4	PP446760	99.64%
C24	*Pediococcus pentosaceus* strain NKFS5	PP446764	99.44%
SI4	*Streptococcus salivarius* strain NKFS6	PP446765	100%
SI6	*Levilactobacillus brevis* NKFS7	PP446788	99.85%
SI8	*Levilactobacillus brevis* NKFS8	PP446791	99.85%
SI9	*Levilactobacillus brevis* NKFS9	PP446797	99.85%
SI23	*Pediococcus pentosaceus* strain NKFS10	PP446799	99.58%
SI38	*Pediococcus pentosaceus* strain NKFS11	PP446802	99.51%

(A)

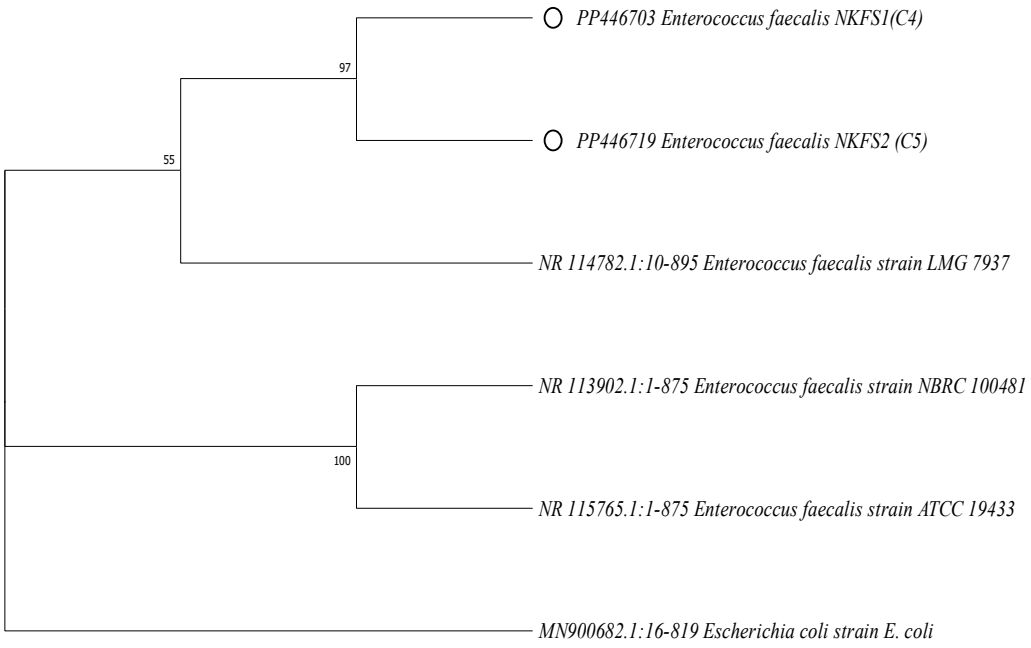


Figure 4.9: Evolutionary relationship of taxa of the isolated LAB from the crop's broiler chickens using the neighbour-joining method. The positions of C4 (PP446703) and C5 (PP446719) among selected *Enterococcus* spp are shown in the figure. *E. coli* was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes of the tree.

(B)

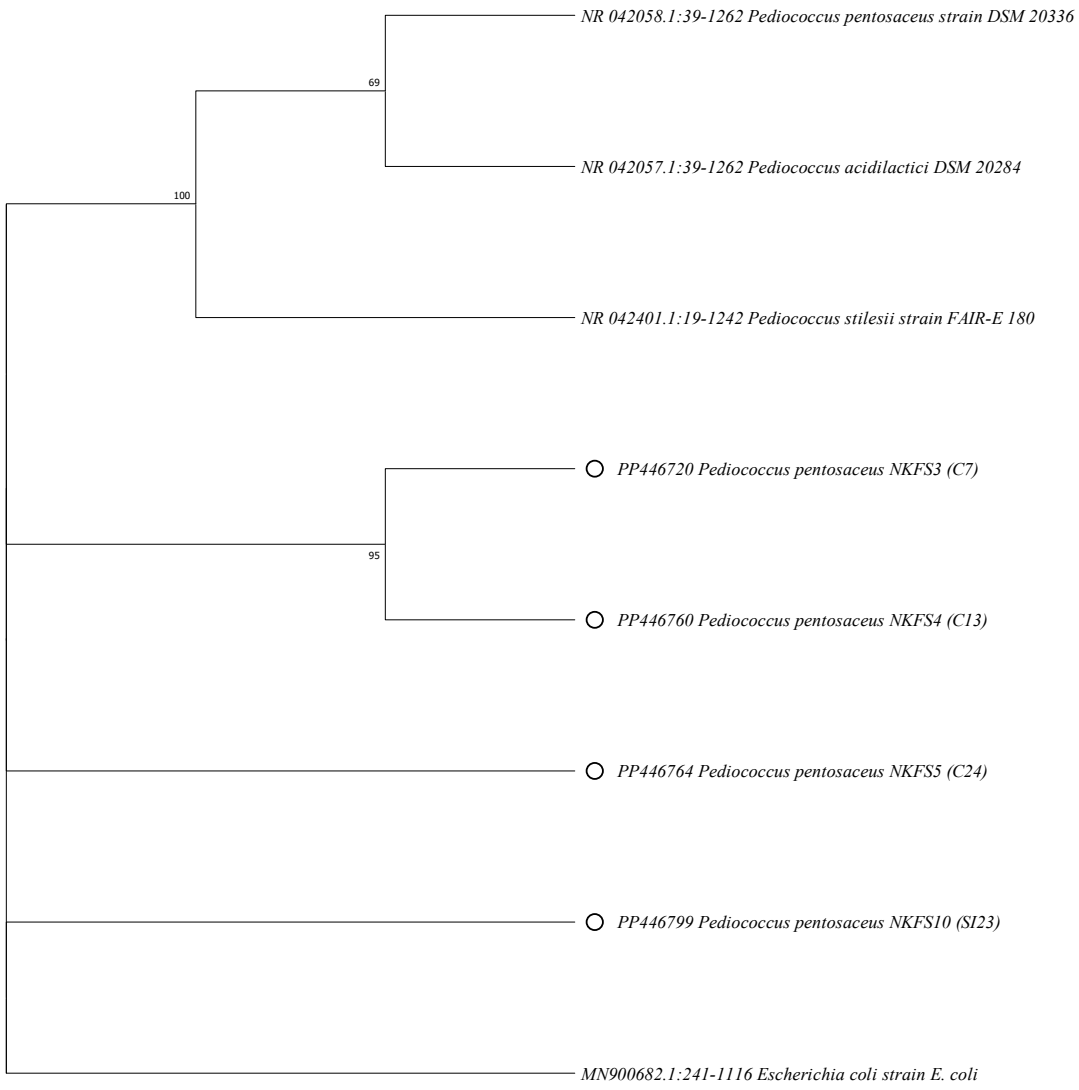


Figure 4.10: Evolutionary relationship of taxa of the isolated LAB from the crop and small intestines broiler chickens using the neighbour-joining method. The positions of C7 (PP446720), C13 (PP446760), C24 (PP446764) and SI23 (PP446719) among selected *Pediococcus* spp are shown in the figure. *E. coli* was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes of the tree.

(C)

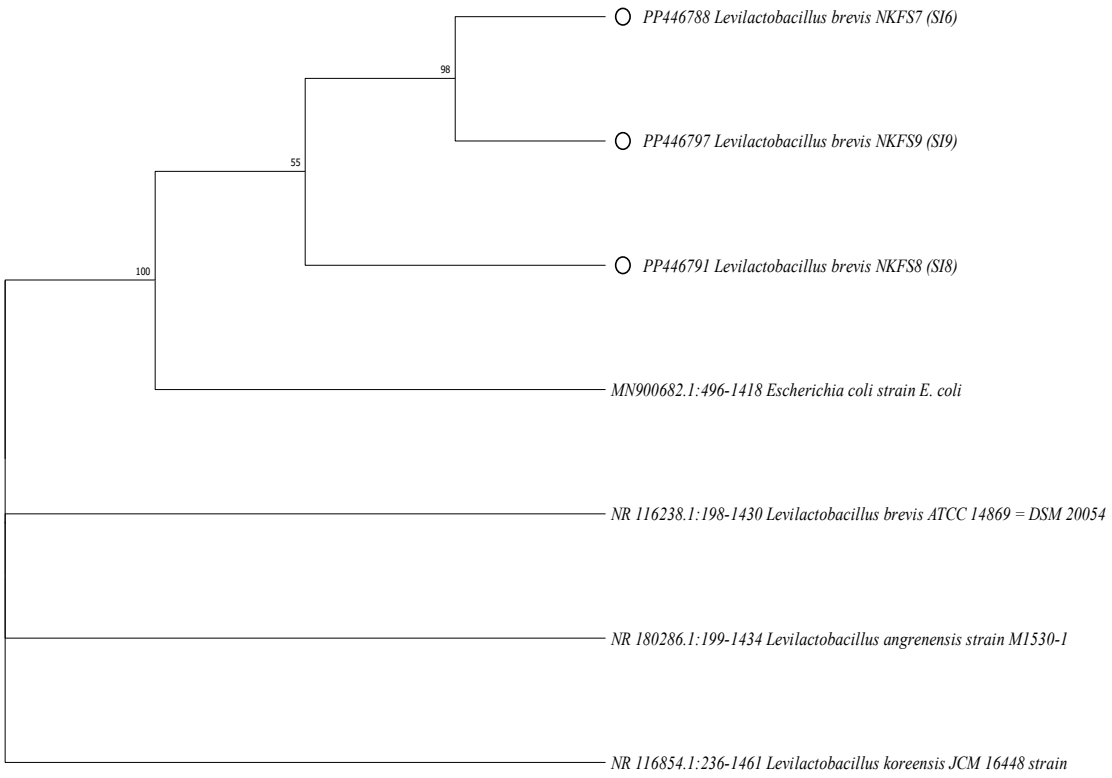


Figure 4.11: Evolutionary relationship of taxa of the isolated LAB from the small intestine broiler chickens using the neighbour-joining method. The positions of SI6 (PP446788), SI8 (PP446791), and SI9 (PP446797) among selected *Levilactobacillus* spp are shown in the figure. *E. coli* was used as an outgroup**.** Bootstrap values based on 1000 replications are indicated at the nodes of the tree.

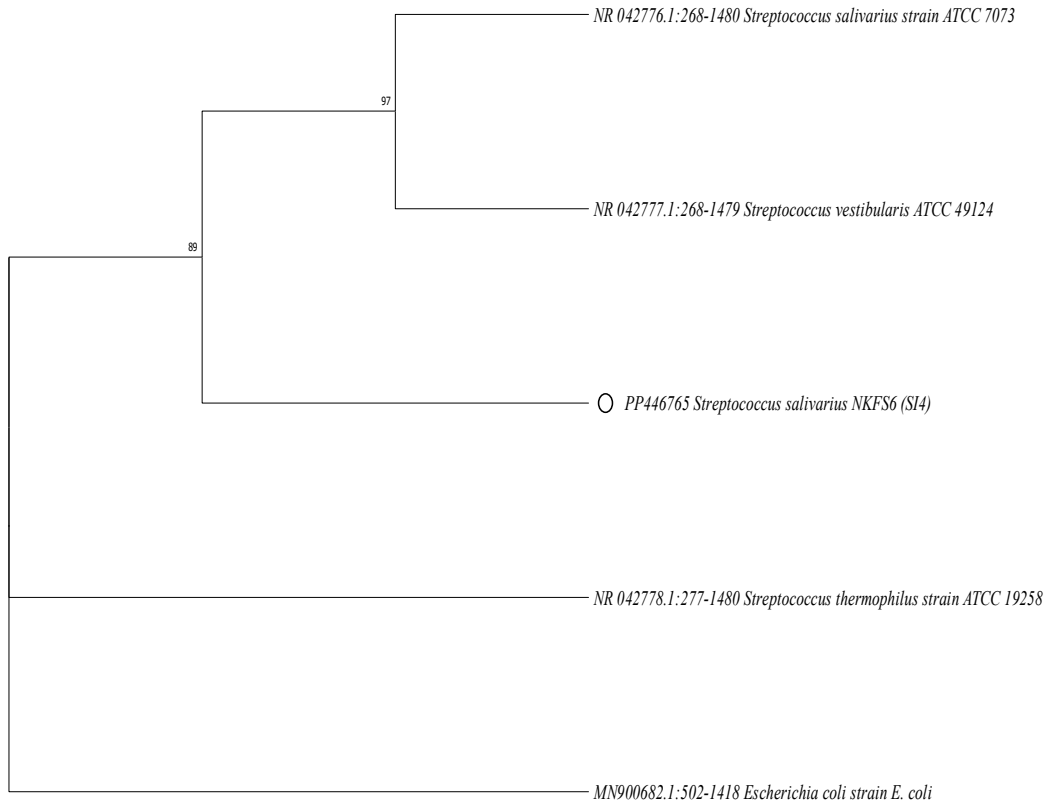


Figure 4.12: Evolutionary relationship of taxa of the isolated LAB from the small intestine broiler chickens using the neighbour-joining method. The position of SI4 (PP446765) among selected *Streptococcus* spp is shown in the figure. *E. coli* was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes of the tree.

4.6 ASSESSMENT OF THE PROBIOTIC PROPERTIES OF LAB STRAINS

4.6.1 Simulated Gastric Juice Survivability Testing

The survival of the isolated 11 LAB strains in gastric juice with lysozyme at pH 2.5 was examined to determine their ability to survive in simulated GI transits, and the cell viability was measured at different time points (0, 30, 60, and 90 minutes). The survival of LAB isolates in simulated gastric juice with and without lysozyme after 90 minutes of exposure is depicted in Figure 4.13, and it can be observed that there was a reduction ($p < 0,05$) in cell viability in the presence of lysozyme when compared to the control (without lysozyme) even though the tested LAB isolates exhibited a cell viability count that is greater than 7.0 CFU/ml. After 90 minutes of exposure, the highest cell viability was observed in *Pediococcus pentosaceus NKFS4* with $9,83 \pm 0,02$ $\log_{10}$ CFU/ml, while *Streptococcus salivarius* showed the lowest cell viability of $8,00 \pm 0,00$ $\log_{10}$ CFU/ml.

There were notable variations ($p < 0.05$) in isolates *Streptococcus salivarius* NKFS6, *Levilactobacillus brevis* NKFS7, and *Levilactobacillus brevis* NKFS8 compared to the control after 90 minutes of exposure. Importantly, there was no significant difference ($p > 0.05$) observed in the LAB isolates *Levilactobacillus brevis* NKFS9, *Pediococcus pentosaceus* NKFS10, *Enterococcus faecalis* NKFS1, *Enterococcus faecalis* NKFS2, *Pediococcus pentosaceus* NKFS3, *Pediococcus pentosaceus* NKFS4, and *Pediococcus pentosaceus* NKFS5 following a 90-minute exposure.

This study further determined the D-values (decimal reduction time) of each probiotic strain from the slope of the regression line (shown in Fig. 4.14) obtained by plotting the log of viable count after exposure to lysozyme against time (min). The D-values obtained ranged from 36,10-172,91 minutes as indicated in Table 4.8. *Pediococcus pentosaceus* NKFS4, *Pediococcus pentosaceus* NKFS3, *Enterococcus faecalis* NKFS2 and *Pediococcus pentosaceus* NKFS10 demonstrated the highest D-values of 172,91, 138,89, 103,09 and 126,58 minutes, while *Streptococcus salivarius* showed the lowest D-value of 36,10 minutes followed by *Levilactobacillus brevis* NKFS9 and *Levilactobacillus brevis* NKFS8 with D-values of 9,53 and 45,05 minutes respectively. Based on the data obtained in this study, it can be concluded that the resistance to lysozyme is strain-specific, and the longer the incubation time, the lower the cell viability.

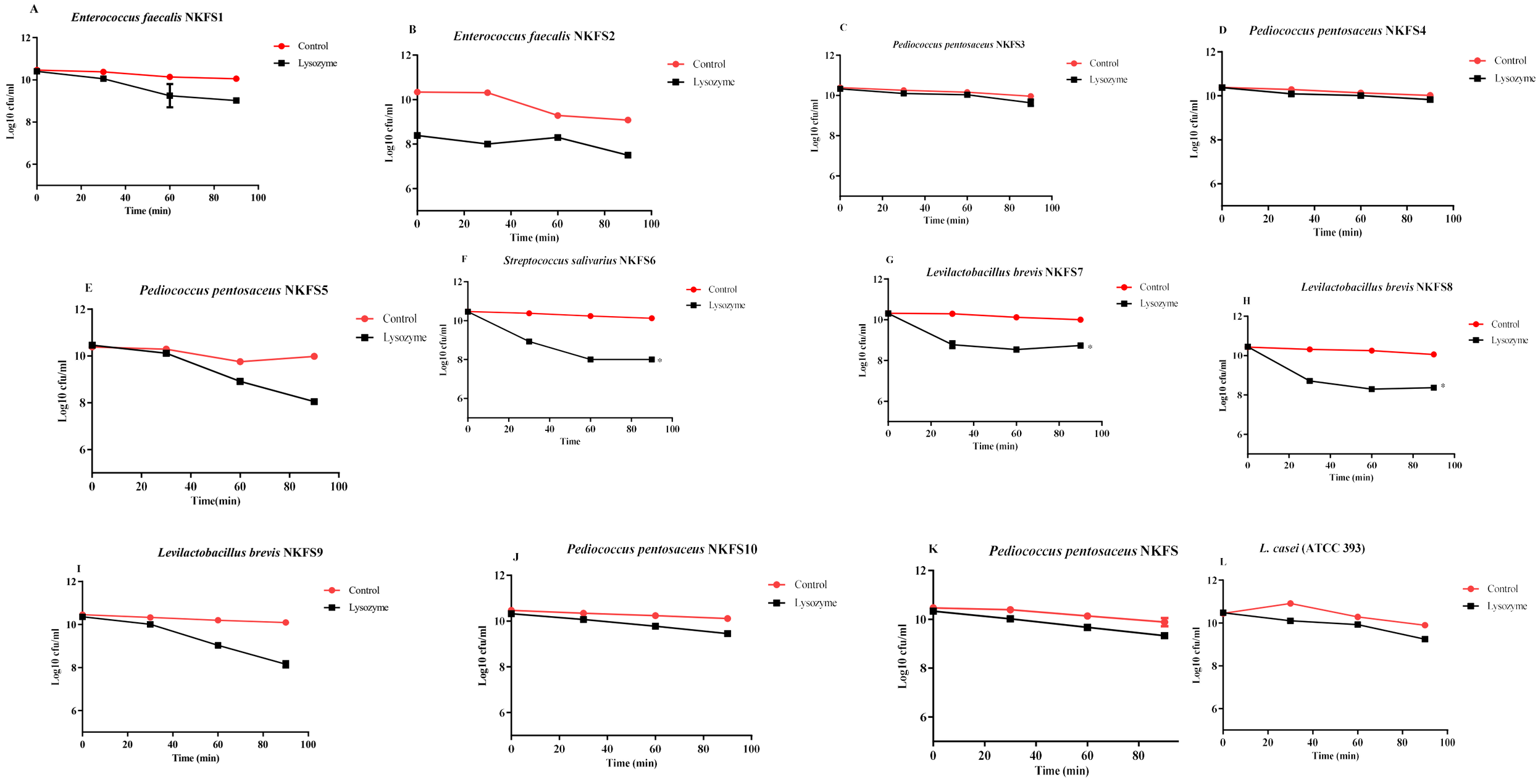
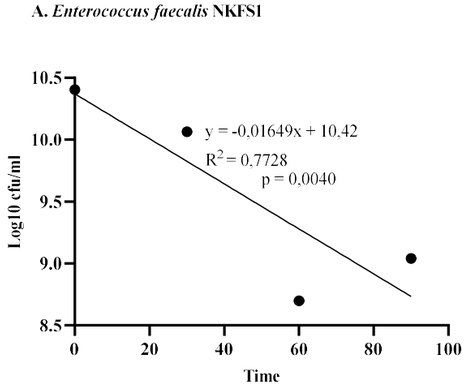
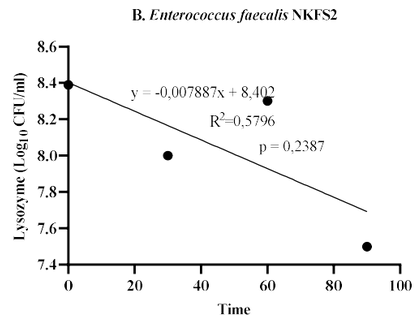
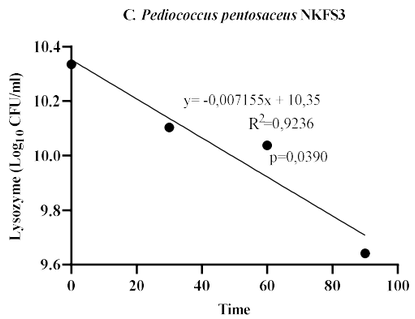
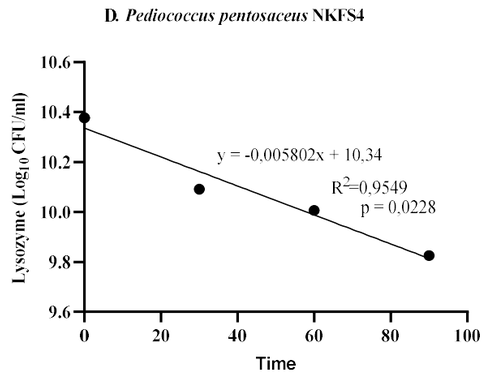


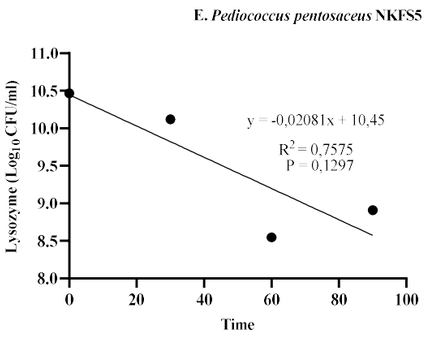
Figure 4.13: Lysozyme resistance of LAB isolates from the crops and small intestine of broiler chickens at pH 2.5 after 0, 30, 60 and 90 min of incubation. Reference strain: *Lactobacillus casei* (ATCC 393). n = 3 replicates. Values are means. Significant differences between the means of LAB isolates and the reference strain are presented as * $p < 0.05$. A-K are LAB isolates

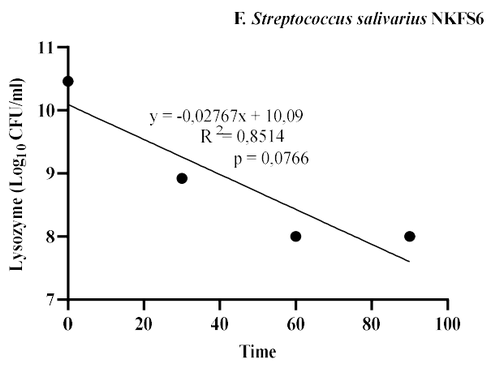


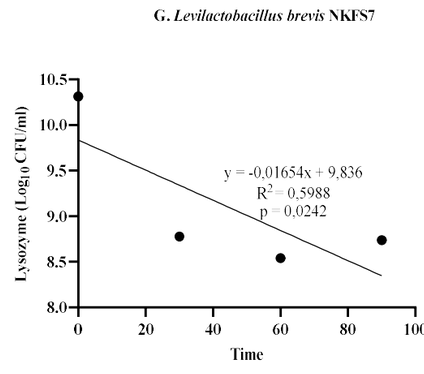


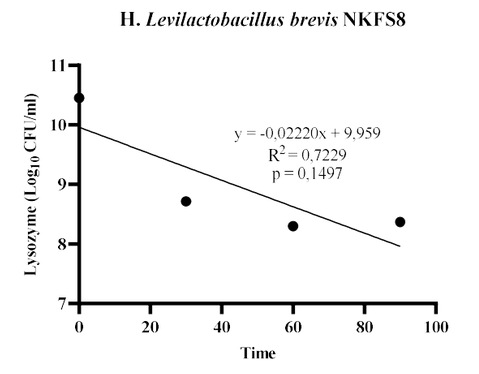


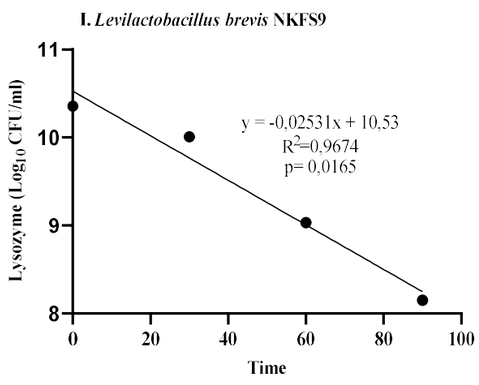


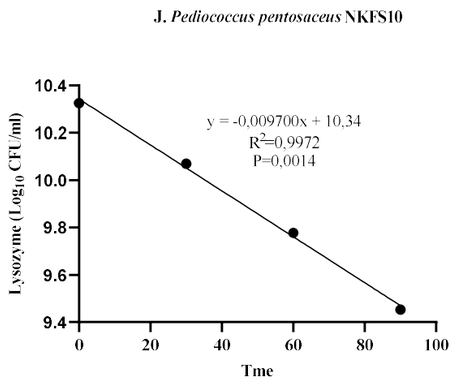


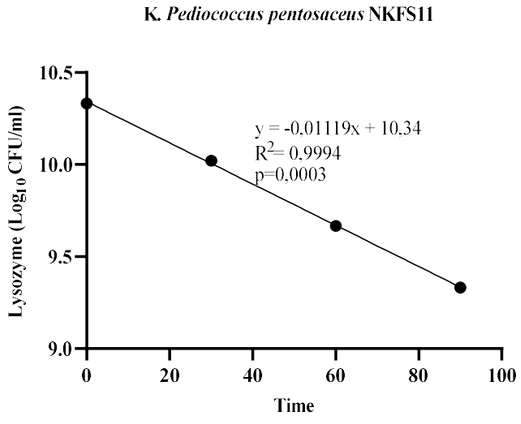












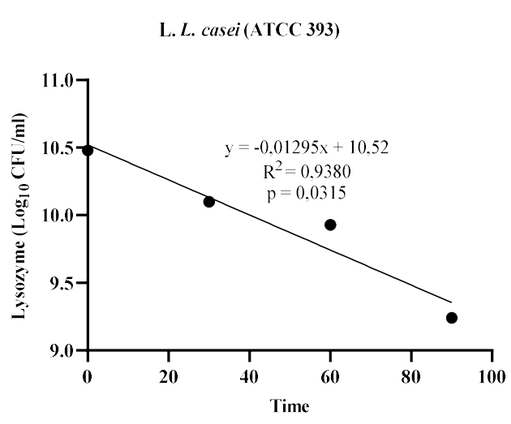


Figure 4.14: Regression lines and associated constants of twelve probiotic LAB strains obtained to demonstrate survivability in simulated gastric juice with lysozyme

Table 4.8: D-values of LAB isolates to measure their survivability after exposure to simulated gastric juice with lysozyme at different time intervals

LAB isolate	**Slope (m)**	**D-value (min)**
Enterococcus faecalis NKFS1	-0,0165	60,61
Enterococcus faecalis NKFS2	-0,0079	126,58
Pediococcus pentosaceus NKFS3	-0,0072	138,89
Pediococcus pentosaceus NKFS4	-0,0058	172,41
Pediococcus pentosaceus NKFS5	-0,0208	48,08
Streptococcus salivarius NKFS6	-0,0277	36,10
Levilactobacillus brevis NKFS7	-0,0165	60,61
Levilactobacillus brevis NKFS8	-0,0222	45,05
Levilactobacillus brevis NKFS9	-0,0253	39,53
Pediococcus pentosaceus NKFS10	-0,0097	103,09
Pediococcus pentosaceus NKFS11	-0,0112	89,29
L. casei (ATCC 393)	-0,0129	77,52

4.6.2 Cell Surface Hydrophobicity

Cell-surface hydrophobicity testing was done by assessing the capacity of the isolated LAB to attach to hydrocarbons. The bacterial adhesion of LAB to n-hexadecane was measured after 30 minutes of incubation at 37°C. All isolates adhered to the hydrocarbon tested, and the hydrocarbon % ranged from 27,64% to 73,15%. As illustrated in Figure 4.15, the hydrophobicity percentage varied among the isolates, with *L. brevis* isolates demonstrating the highest hydrophobicity percentage, with *L. brevis* NKFS7 showing the highest adhesion% of 73,15% followed by *L. brevis* NKFS8 with a percentage of 70,72%. Furthermore, a hydrophobicity percentage of 70, 86%, 63,78% and 64,50% were observed in *P. pentosaceus* NKFS3, *E. faecalis* NKFS1 and *L. brevis* NKFS9, respectively. A statistically significant difference of p= 0,0001 was observed in the hydrophobicity percentage of *E. faecalis* NKFS2, *P. pentosaceus* NKFS5*, P. pentosaceus* NKFS10 and *S. salivarius* NKFS6 as they obtained the lowest hydrophobicity percentages of 27,64%, 32,18%, 29, 70% and 38,49% respectively.

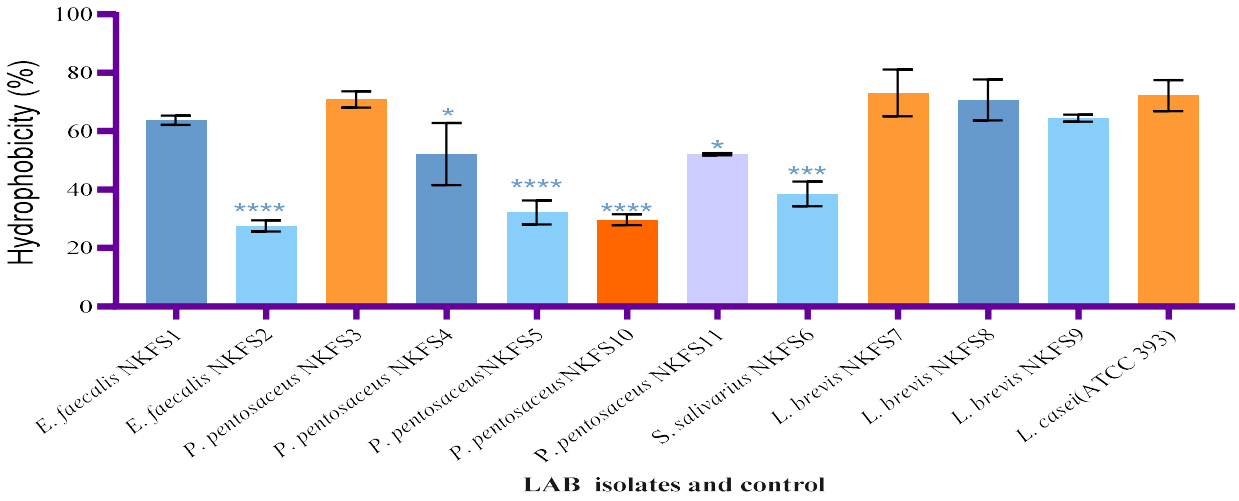


Figure 4.15: Adhesion of LAB isolates from the crops and small intestines to n-hexadecane. Reference strain: *Lactobacillus casei* (ATCC 393). n = 3 replicates. Values are means ± SEM. Significant differences between the means of LAB isolates and the reference strain are shown as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

4.6.3 Auto aggregation

The auto-aggregation properties of 11 LAB isolates, which were obtained from both crop and small intestine samples of broiler chickens, were assessed using a spectrophotometric assay to

evaluate the ability of the isolates to adhere to the same bacteria (Figure 4.16). The percentage of auto-aggregation was measured at 4, 16, and 24 hours of incubation, and the findings indicated that each isolate was able to auto-aggregate with a range of 33,12 ± 1,86% to 81.89 ± 11.93%.

After four hours of incubation, there was no significant difference between the auto-aggregation percentage of the reference strain, *L. casei* ATCC 393 and the tested LAB isolates. However, at 16 hours, an increase in the auto-aggregation percentage (p = 0,0001) was observed with *L. brevis* NKFS9 isolate demonstrating the highest auto-aggregation percentage amongst all isolates with a percentage of 66, 32 ± 8,16%. After 24 hours of incubation, the highest auto aggregation was displayed by isolates *L. brevis* NKFS8 (81,89 ± 11,93%), *L. brevis* NKFS9 (81, 89 ± 11,90%) and *L. brevis* NKFS7 (77, 25 ± 1,53%) and when compared to the auto-aggregation percentage of the reference strain, *L. case* ATCC 393 (77, 57 ± 2,45%), no statistically significant difference was observed, indicating that the isolates were able to closely mimic the auto-aggregation abilities of the known probiotic strain. The observed results demonstrate that the auto-aggregation increases with increasing incubation time.

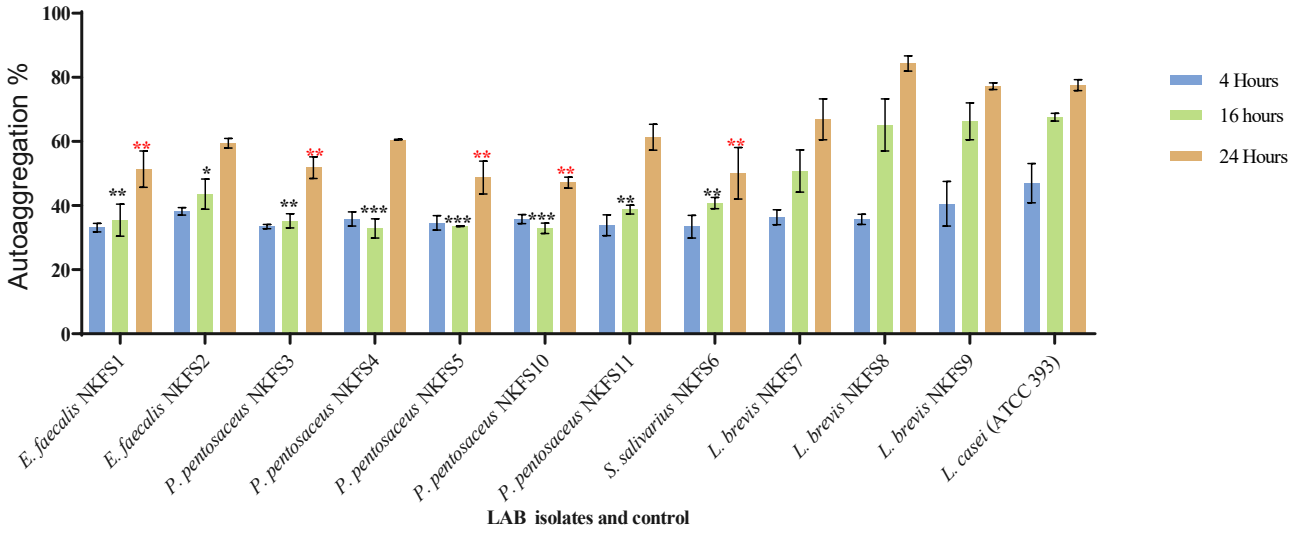
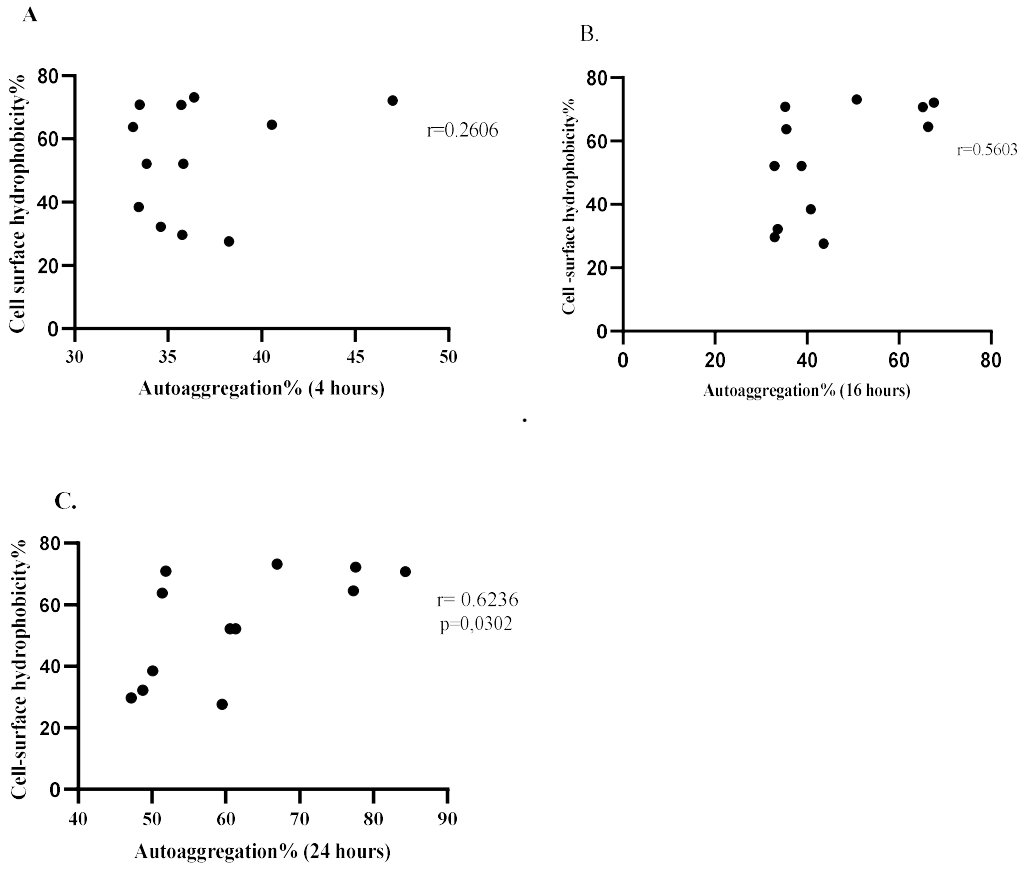


Figure 4.16: Auto aggregation abilities (%) of LAB isolates from the crops and small intestine of broiler chickens after 4, 16 and 24 hours of incubation at 37°C. Reference strain: *Lactobacillus casei* (ATCC 393). n =3 replicates. Values are means ± SEM. Significant differences between the means of LAB isolates and the reference strain are displayed as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

The measured auto-aggregation percentages were plotted against cell-surface hydrophobicity to determine the correlation between the two probiotic traits. Since the percentage of auto-aggregation was measured at 4, 16, and 24 hours, we determined the correlation between the cell surface hydrophobicity and auto-aggregation at different time intervals.

As indicated in Figure 4.17, the correlation (r) of 0,2606 and 0,5603 were observed at 4 and 16 hours of auto-aggregation against cell surface hydrophobicity, and no statistically significant correlation was observed. However, a fairly strong positive correlation was observed between cell surface hydrophobicity and auto-aggregation percentage after 24 hours of incubations with a statistically significant correlation of p= 0,0302 and correlation coefficient (r) of 0,6236. Figure 4.17 (D) demonstrates the fairly strong positive correlation between auto-aggregation and cell surface hydrophobicity. It can be concluded that there is a directly proportional relationship between the auto-aggregation percentage at different time intervals and cell-surface hydrophobicity, meaning that the higher the auto-aggregation percentage of the isolates, the stronger the adhesion of the isolates to the hydrocarbons.



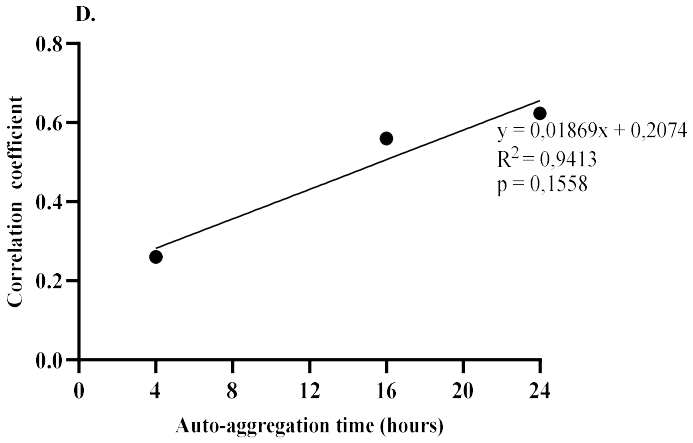


Figure 4.17: Correlation of auto-aggregation at (A)4, (B)16, and (C)24 hours against cell surface hydrophobicity. The summary, (D) demonstrates the direct positive relationship between auto-aggregation and cell-surface hydrophobicity indicated by the correlation coefficient observed after 4, 16 and hours of incubation.

4.6.4 Co-aggregation

The co-aggregation abilities of the isolated LAB were done to evaluate the ability of the isolates to adhere to pathogens as it is essential for the inhibition of pathogens from adhering to the GI tract of the host. The co-aggregation percentage (%) of 11 LAB isolates from the crops and small intestines of broiler chickens with common poultry pathogens (*L. monocytogenes*, *S. typhimurium*, *P. aeruginosa*, *S. aureus* and *S. enterica*) is shown Figure 4.18. As shown in the results, the co-aggregation% varied among the isolates, with isolate *E. faecalis* NKFS1 exhibiting the lowest coaggregation percentage ($p < 0,0001$) of 31,52 ± 2,69 against *L. monocytogenes*, and *P. pentosaceus* NKF3 with coaggregation% of 41,79 ± 9,99% against *S. typhimurium*. However, *E. faecalis* NKFS1 demonstrated the highest co-aggregation abilities with a coaggregation% of 88,32 ± 2,0% and 82,05 ± 04 against *S. aureus* and *Ps. aeruginosa*, and *P. pentosaceus* NKF3 with a coaggregation% of 83,52 ± 3,86% against *S. aureus*. It should be noted that no statistically significant difference was observed in the coaggregation percentages of the isolates against *S. typhimurium*, *P. aeruginosa*, *S. aureus* and *S. enterica* when compared to the coaggregation percentage of the reference strain, L. *casei* ATCC393, indicating that the isolates were able to closely mimic the coaggregation abilities of the known probiotic strain.

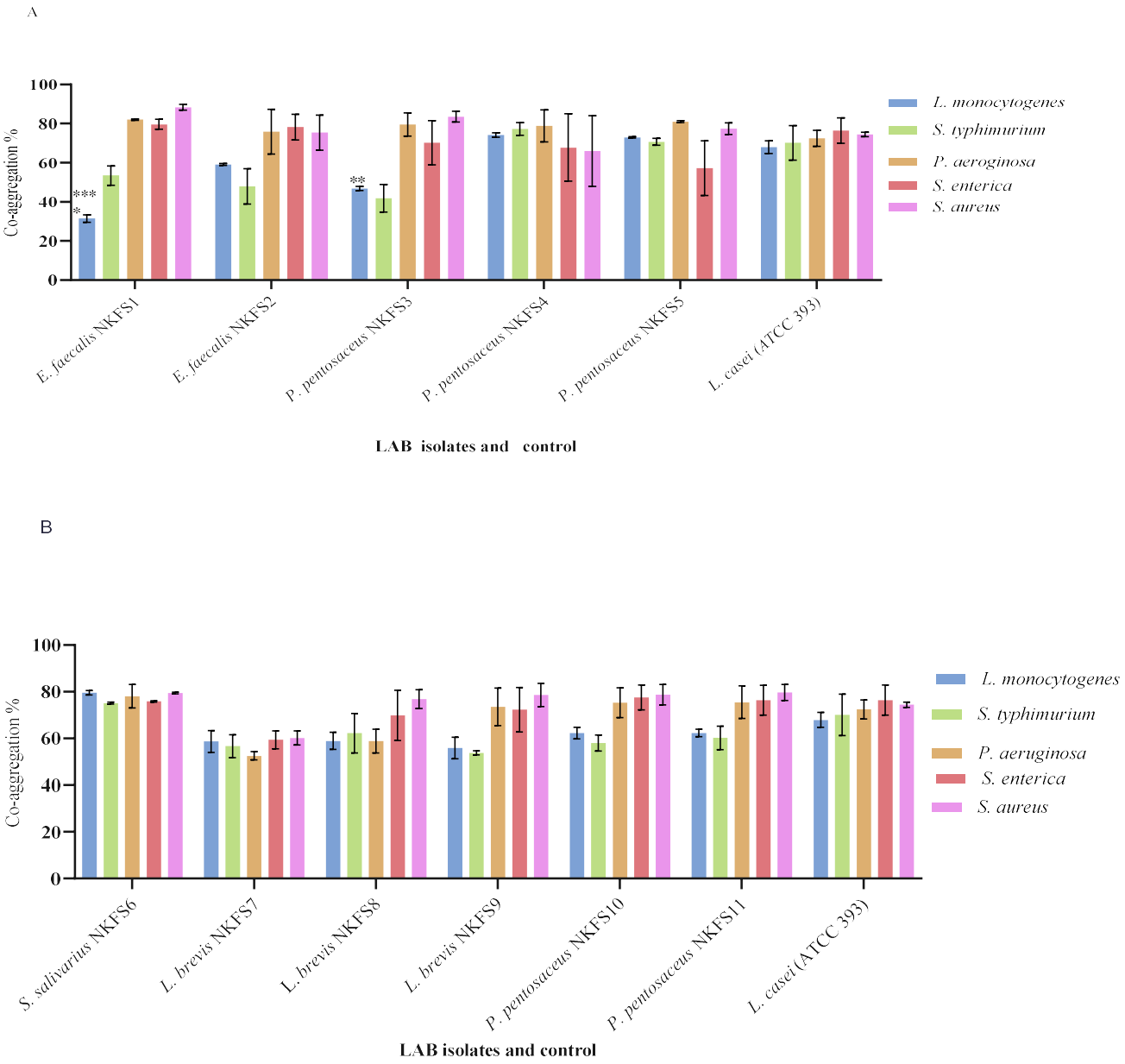


Figure 4.18: Co-aggregation percentage (%) of LAB isolates from the (A) crops and (B) small intestines of broiler chickens with five pathogens (*L. monocytogenes*, *S. typhimurium, P. aeruginosa, S. aureus* and *S enterica*) after four hours of incubations. Reference strain: *Lactobacillus casei* (ATCC 393). n = 3 replicates. Values are means ± SEM. Significant differences between the means of LAB isolates and the reference strain are shown as ** p <0.01 and **** p < 0.0001.

4.6.5 α-Glucosidase Activity

The *α*-glucosidase inhibition activity of cell-free extract from 11 LAB isolates is presented in Figure 4.19 as it was performed to assess the capacity of isolates to inhibit α-glucosidase, an enzyme involved in the metabolism of carbohydrates. All isolates tested showed different

patterns of inhibition of the α-glucosidase with the inhibition percentage ranging from 25,84 ± 3,08% to 61,77 ± 6,16%, with *P. pentosaceus* NKFS3 showing the highest inhibitory activity of 61,77 ± 6,16% and *S. salivarius* showing the lowest inhibitory activity of 25,84 ± 3,08%.

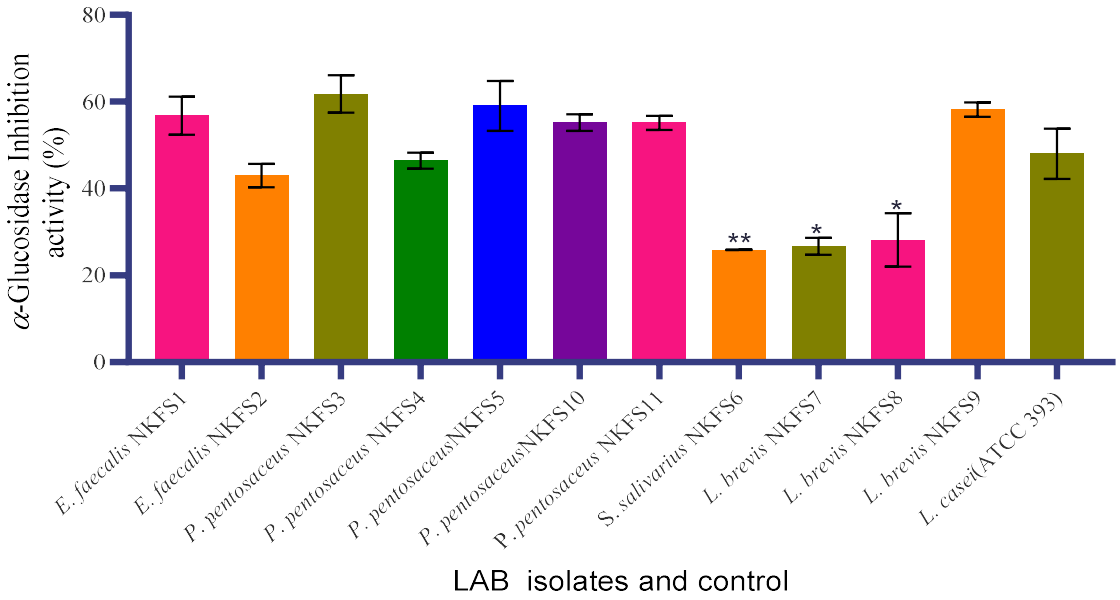


Figure 4.19: α-glucosidase inhibition activity (%) of LAB isolates from the crops and small intestines of broiler chickens. Reference strain: *Lactobacillus casei* (ATCC 393). N = 2 replicates. Values are means ± sem. Significant differences between the means of lab isolates and the reference strain are shown as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

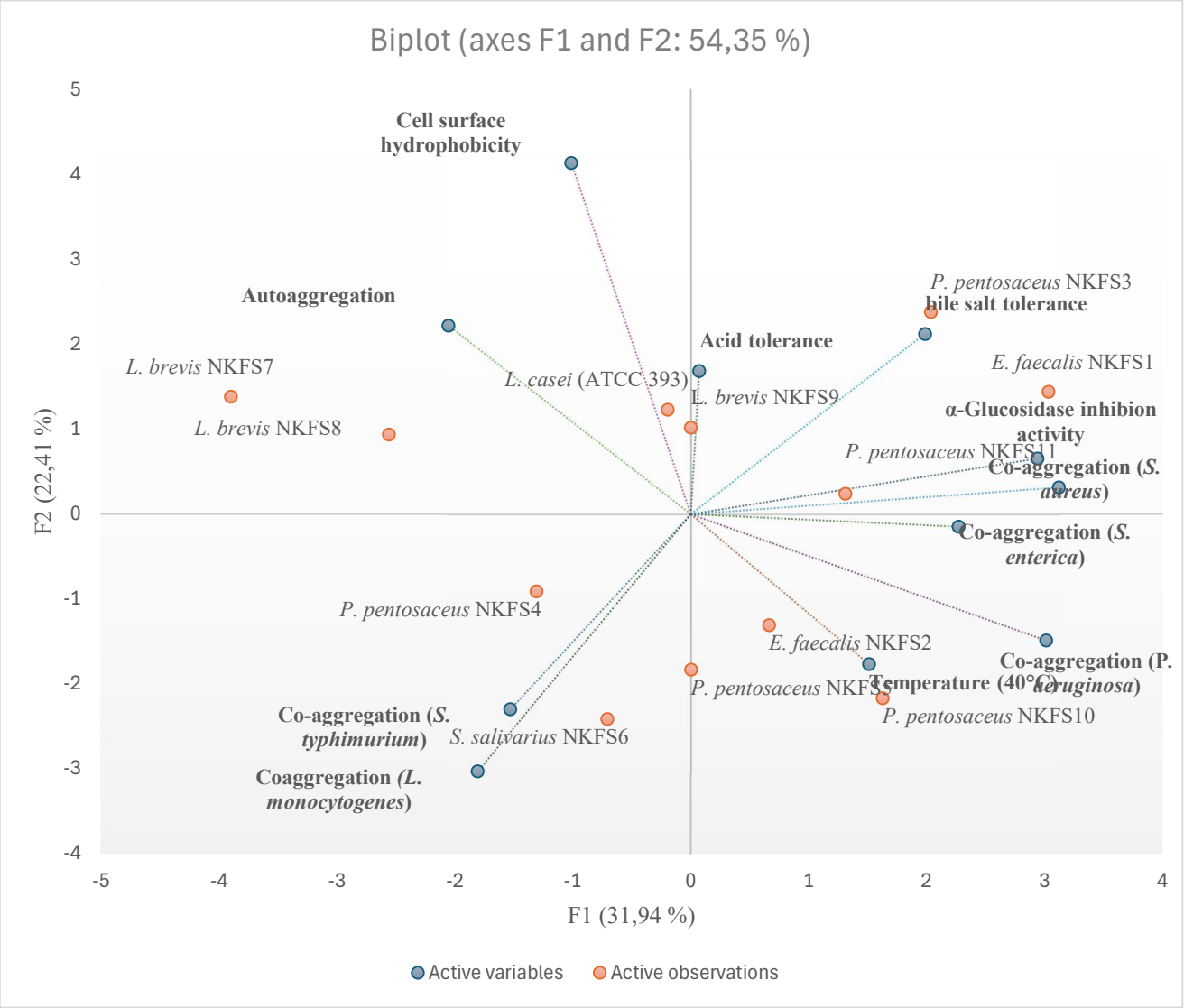
4.6.6 Principal Component Analysis

Principal Component Analysis (PCA) was conducted in our study to provide a conclusive assessment of the selection of promising probiotic strains. The probiotic attributes were analyzed by assessing the correlation between the tested probiotic properties, which were acid, bile and temperature (40°C) tolerance, cell-surface hydrophobicity, auto-aggregation and co-aggregation. The PCA demonstrated a total variation of 54,35% across principal components, and the variable homogenous distribution on the principal plane indicated that F1 and F2 accounted for 31,94% and 22.41% of the variation (Figure 4.20) respectively.

The analysis demonstrated that the factorial space can be classified into four main groups. The first group which is located in quadrant one (positive side of both F1 and F2) consists of *L. brevis* NKFS9, P. *pentosaceus* NKFS3, *P. pentosaceus* NKFS11 and *E. faecalis* NKFS1, which exhibited the highest values for acid and bile salt tolerance, α-glucosidase inhibitory activity and co-aggregation against *S. aureus*. The second group in quadrant two (positive for F2 and negative for F1) consists of *L. brevis* NKFS7, *L. brevis* NKFS8 and *L. casei* ATCC 393) which expressed high values for auto-aggregation and cell-surface hydrophobicity attributes. The third group located in quadrant three (negative for both F1 and F2) consists of *S. salivarius* NKFS5 and *P. pentosaceus* NKFS4 which exhibited the highest co-aggregation values against *S. typhimurium* and *L. monocytogenes*. The fourth group located in quadrant four (positive for F1 and negative for F2) consists of *P. pentosaceus* NKFS10 and *P. pentosaceus* NKFS5 which exhibited the highest values in temperature tolerance and coaggregation against *P. aeruginosa.*

Based on the observed findings, it can be concluded that *L. brevis* NKFS9, *P. pentosaceus* NKFS3, *P. pentosaceus* NKFS11 and *E. faecalis* NKFS1 present in quadrant one are the most promising probiotic candidates as they exhibited the highest correlation to variables with maximum factor scores indicated by different colours as shown in Table 4.9.

A.


Biplot (axes F1 and F2: 54,35 %)
Cell surface hydrophobicity
Autoaggregation
Acid tolerance
L. brevis NKFS7
L. casei (ATCC 393)
L. brevis NKFS9
L. brevis NKFS8
P. pentosaceus NKFS3
bile salt tolerance
E. faecalis NKFS1
α-Glucosidase inhibion activity
P. pentosaceus NKFS11
Co-aggregation (S. aureus)
Co-aggregation (S. enterica)
P. pentosaceus NKFS4
E. faecalis NKFS2
Co-aggregation (P. aeruginosa)
P. pentosaceus NKFS5
Temperature (40°C)
P. pentosaceus NKFS10
Co-aggregation (S. typhimurium)
S. salivarius NKFS6
Coaggregation (L. monocytogenes)
F2 (22,41 %)
F1 (31,94 %)
Active variables
Active observations

B.

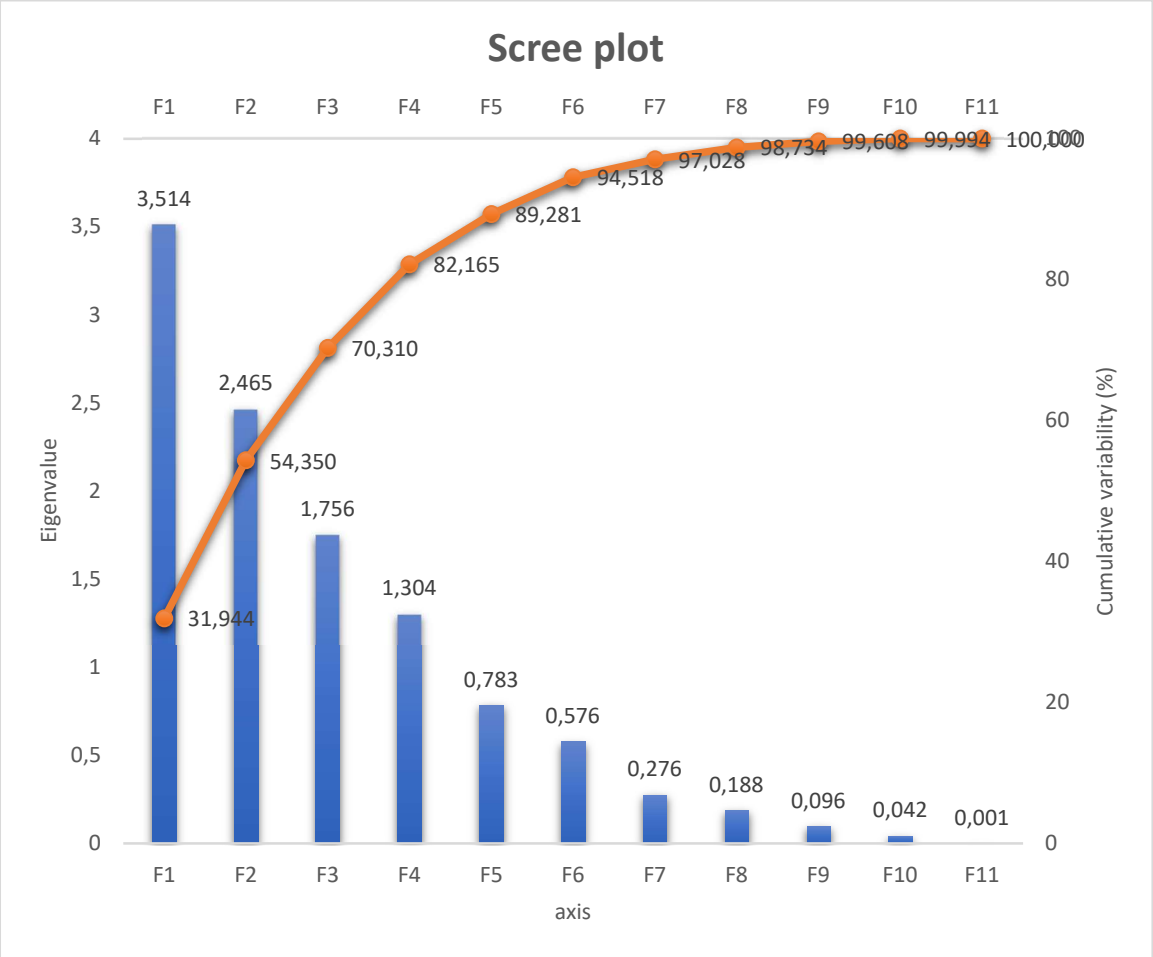


Figure 4.20: Principal Component Analysis (PCA) of the probiotic attributes (acid, bile, cell surface hydrophobicity, α-glucosidase inhibitory activity, temperature (40°C) tolerance, co-aggregation and auto-aggregation) of 11 LAB isolates from the crops and small intestine of broiler chickens and the control (*L. casei* ATCC 393) for the selection of the most promising probiotic strains. (A)The PCA biplot projection on probiotic attributes the percentage of variance is explained by F1 and F2, reported after each exists. (B) Scree plot of eigenvalues of principal components (F1-F11) for potential probiotic properties of LAB from the crops and small intestine of broiler chickens.

Table 4.9: Factor scores of principal components (F1 and F2) of potential probiotic strains.

	Factor scores:	
Isolates	F1	F2
E. faecalis NKFS1	3,034	1,441
E. faecalis NKFS2	0,664	-1,309
P. pentosaceus NKFS3	2,035	2,383
P. pentosaceus NKFS4	-1,308	-0,911
P. pentosaceus NKFS5	0,002	-1,833
P. pentosaceus NKFS10	1,627	-2,168
P. pentosaceus NKFS11	1,311	0,240
S. salivarius NKFS6	-0,708	-2,412
L. brevis NKFS7	-3,901	1,383
L. brevis NKFS8	-2,559	0,938
L. brevis NKFS9	0,001	1,019
L. casei (ATCC 393)	-0,196	1,230

Green: Positive, Red: Negative

Table 4.10: Summary of the probiotic properties of the selected LAB isolates from the crops and small intestine of broiler chickens

SELECTED LAB ISOLATES	PROBIOTIC PROPERTIES								
	Acid Tolerance (pH 3 and 4)	**Bile salt Tolerance (0,3% and 0,6%)**	**Phenol Tolerance (0,4%)**	**Temperature Tolerance (40°C)**	**Antibiotic Susceptibility Testing (critical antibiotics)**	**Simulated Gastric Juice Survivability Testing (D-values)**	**Cell Surface Hydrophobicity**	**Auto-aggregation (24 Hours)**	**Co-aggregation**
***E. faecalis* NKFS1**	71.65% (pH 3) and 65% (pH 4)	79.78% (0,3%) and 63.5% (0,6%)	0.496 OD units	58.66%	resistance to ceftriaxone, vancomycin, and gentamycin, but sensitive to tetracycline and erythromycin	60.61 minutes	63.77%	51.37%,	*S. aureus (*88.32%), *Ps. aeruginosa* (82.05%) and *S. enterica* (79.62%)
***P. pentosaceus* NKFS3**	74.64% (pH 3) and 80.00% (pH 4)	90.23% (0,3%) and 51.22% (0,6%)	0.575 OD units	42.83%	intermediate to ceftriaxone, resistant to vancomycin and gentamycin, and sensitive to tetracycline and erythromycin, which are	70.86 minutes	79.86%,	51.87%	*S. aureus* (83.52%), *Ps. aeruginosa* (79.52%), and *S. enterica* (70.21%).
***P. pentosaceus* NKFS11**	60.44% (pH 3) 80.00% (pH 4)	76.92% (0,3%), and 69.61% (0,6%)	0.498 OD units	51.05%.	resistance to vancomycin and gentamycin, while showing sensitivity to tetracycline, ceftriaxone, and erythromycin.	89.29 minutes	52.10%.	61.33%,	*S. aureus* (83.19%), *Ps. aeruginosa* (82.53%), and *S. enterica* (82.82%).
***L. brevis* NKFS9**	57.50% (pH 3) 74% (pH 4)	61.39% (0,3%) 58.00% (0,6%)	0.657 OD units	56.31%.	resistant to vancomycin and gentamycin, but sensitive to tetracycline, ceftriaxone, and erythromycin	39.53 minutes	64.50%.	77.25%	*S. aureus*, (83.60%). against *Ps. aeruginosa* (81.63%) and *S. enterica (*81.87%)

5 CHAPTER FIVE: DISCUSSION

Probiotics have gained considerable attention in the poultry industry as a viable substitute to antibiotics or as an addition to enhance yield of chicken products (Reuben *et al.,* 2019). Currently, lactic acid bacteria (LAB) have been used as probiotics to prevent intestinal infections, as they promote host intestinal health by developing a balance of the intestinal microbiota (Tsega *et al.,* 2019). Therefore, the isolation of indigenous probiotic strains from the GI is preferred, as they are more accustomed to the environment of the GI tract of the respective animals. In addition, the strains can easily proliferate in the GI tract, leading to better colonization and expression of the desired beneficial effect (Reuben *et al.,* 2019). Hence, in the present study, 66 isolates were obtained using MRS (de Man, Rogosa and Sharpe) medium from the crops (27 colonies) and small intestines (39 colonies) of broiler chickens. However, as shown in Table 4.1, only 29 (12 from the crops and 17 from the small intestines) colonies displayed the typical characteristics of LAB, Gram-positive cocci or bacilli-shaped. Our study also found that the isolated LAB were catalase-negative, the negative catalase results obtained in the present study are a crucial characteristic of the potentially isolated probiotic bacteria indicating their inability to cannot break down hydrogen peroxide into water and oxygen as this was demonstrated by the absence of bubbles during the test. Furthermore, it was affirmed in a study conducted by Bazireh, (2020) that this is a widely recognized trait in *Lactobacillus* spp.

5.1 ACID TOLERANCE

The potential probiotic strains must possess the ability to withstand acidic pHs, which is a fundamental criterion for their selection. This characteristic is necessary for them to successfully navigate the stomach and intestine, ultimately colonizing the host's gastrointestinal tract (Gupta *et al.,* 2023). As indicated in Figure 4.1, Nineteen (7 from the crop and 12 from the small intestines) LAB isolates could survive in MRS broth of pH 3 and 4 after 3 hours of exposure, with the survivability of the isolates varied from 22,12%- 94,06% and 22,37-95,01% respectively. This survivability is attributed to the fact that Gram-positive bacteria possess multiple acid resistance systems that aid in their ability to survive in acidic

conditions (Sirisopapong *et al.,* 2023). The most common mechanisms involve proton pumps, which assist in moving protons out of the cell, thereby reducing intracellular acidity. Additionally, the bacteria employ methods such as protecting macromolecules to help shield important molecules from damage, altering the cell membrane to adapt to acidity; producing alkali substances that neutralize acids; regulating pathways that activate specific processes to combat acidity and adjusting metabolism to optimize energy utilization in acid environments (Shamsudin, 2019). These mechanisms play an essential role in the prevention of harm caused by low pH, like enzyme inactivation and damage to DNA and protein. Papadimitriou *et al.,* (2016) further highlighted that the survivability of probiotic bacteria in an acidic environment can be enhanced in the presence of metabolized sugars such as glucose. This is because glucose is believed to facilitate proton exclusion by providing ATP to F-ATPase.

The findings (Fig. 4.1) observed in this study are in agreement with the findings reported in a study by Tian *et al.,* (2024), which reported a survivability percentage of 86.19% in *Lactobacillus pentosus* R26 isolated from 72-day-old yellow feathered broiler chickens. The observations also aligns with the results of a previous study (Wu *et al.,* 2021), which also indicated that the LAB isolates could survive acidic conditions at pH 3 and 4, however, they exhibited a greater survivability percentage at pH 4. This observation was also shown in our study, as the highest survivability percentage was observed at pH 4 by isolates SI27 (95,01 ±3,74%), C38 (89, 98 ± 6,19%), and SI32(88,95 ± 2,89%). A study has, however, reported probiotic strains that were sensitive to low pHs of 3 and 4, including *Lactobacillus rhamnosus* (Karu and Sumeri, 2016), and *Bifidobacterium animalis* (Saarela *et al.,* 2011). It must be noted that the ability of potential probiotic strains to survive at pH 3 is considered a more stringent test for their acid tolerance than surviving at pH 4 as it indicates that they can adapt and colonize the digestive tract (Gauvry *et al.,* 2021). Hence, in our study LAB isolates that displayed a survival percentage greater or equal to 50% at pH 3 were selected for further *in vitro* screening of probiotic properties as they exhibited traits to survive the unfavourable conditions of the digestive tract and reach lower parts of the chicken intestines.

5.2 BILE SALT TOLERANCE

The persistence of potential probiotic strains inside the gastrointestinal tract of the host is determined by their ability to withstand bile salts released into the small intestine (Vasiee *et al.,* 2022). Bile salts play a pivotal role in specific and non-specific mechanisms of the gut, such as the absorption of dietary fats and digestion. Bile salts inhibit the growth of pathogenic microorganisms and toxins that are indigested through water and food. In the gastrointestinal tract of chickens, the fluid in the duodenum, cecum, and jejunum comprises of 0,008%, 0,175% and 0,7% of bile salts respectively. Hence, LAB need to tolerate different concentrations of bile salts as concentrations vary in the different areas of the digestive system (Padmavathi *et al.,* 2018). In this study, the presumptive LAB isolates were tested for their capacity to grow in bile salt concentrations of 0.3% and 0.6% after 3 hours of exposure. These concentrations were selected as the concentration of 0.3% bile salts has been used as a standard concentration to determine the tolerance of LAB to bile salts as potential probiotics (Reuben *et al.,* 2019), and 0.6% was selected to aid in the differentiation of the isolated LAB strains with varying levels of bile salt tolerances. This improves the selection of strains, as higher bile salt concentrations reveal their stress responses and adaptability. According to Liu *et al.,* (2013), potential probiotic isolates can be categorized as being tolerant to bile salts of different concentrations if they have a survivability percentage greater or equal to 50%, hence in this study all LAB isolates demonstrating a survivability percentage higher than or equal to 50% were said to exhibit bile salt-resistant at bile salt concentrations of 0,3% and 0,6%.

As indicated in Figure 4.2, it was observed that all 19 (7 from the crops and 12 from the small intestines) LAB isolates could tolerate a bile salt concentration of 0.3%, with isolate C7 exhibiting the highest survival percentage of 90.23%. It was reported that this tolerance to bile salts may due to LAB producing bile salt hydrolase, an enzyme which deconjugates bile acids (Nallala *et al.,* 2017). Furthermore, the resistance of LAB to bile salt concentrations is a function of the expression of bile salts-resistance-related proteins such as Hsp 1, Hsp 3 and ClpP in the bacterial cell (Cele *et al.,* 2022). It was also observed that there was a significant decrease ($p < 0.05$ or 0.01 or 0.0001) of the survival percentage at a bile salt concentration of 0,6% after 3 hours of exposure. Doubling the concentration of bile salts resulted in the inhibition of all bacteria, except for the most resistant strains. A significantly lower number of bacteria were able to survive at a concentration of 50% in 0.6%, and all bacteria exhibited a decrease in their ability to survive at the higher concentration. This decrease in increasing bile

salt concentrations is largely because bile salts prevent the growth of bacteria, especially Gram-positive as a result of probiotic organisms binding with bile salts (Oyewole *et al.,* 2018).

5.3 HAEMOLYTIC ACTIVITY

According to the WHO/FAO (2002), the safety of potential probiotic candidates must be evaluated by haemolysis testing as a requirement for the selection of probiotics. Haemolysis is a known virulence aspect among pathogenic microorganisms; hence, the potential probiotic strains must be non-haemolytic (Kumar *et al.,* 2023). The study revealed that 15 isolates did not haemolyse blood suggesting that they are non-pathogenic due to their inability to produce haemolysin. This aligns with the study by Reuben *et al.,* (2019) that reported similar findings of LAB isolates with no haemolytic activity. The presence of haemolysin serves as an indicator of pathogenicity, as it breaks down the defence of the epithelial layer, disrupting its normal function and subsequently initiates pathways for infections (Oyewole *et al.,* 2018).

5.4 ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotic resistance allows LAB to survive in the intestine even in their presence. Resistance to antibiotics is a desirable factor among LAB as it allows for LAB to function in synergy with antibiotics. Antibiotic susceptibility evaluation showed a high variation amongst the LAB isolates (Table 4.3). In this study, the resistance of the LAB isolates against vancomycin (100%), streptomycin (73,30%), ciprofloxacin (100%), gentamicin (80%), novobiocin (86,67%) and oxacillin (86,67%) was observed. It was also observed that isolate SI23 exhibited resistance to all tested antibiotics. This resistance might be due to the ability of the LAB to modify antibiotic enzymatic complexes that prevent the antibiotic-target interactions, enzymatic degradations of intracellular or extracellular antibiotics, and reduction in intracellular antibiotic concentration through the activation of flow pumps or due to the change in cell wall permeability (Yenizey *et al.,* 2018).

According to Jose *et al.,* (2015), some LAB, especially the *Lactobacillus* species such as *L. casei, L. plantarum, L. brevis, L. fermentum* and *L. curvatus* have intrinsic resistance to some antibiotics that are not transferrable to other microorganisms. Due to the potential exposure of probiotic strains to animals treated with antibiotics for growth promotion purposes, the inherent resistance of the probiotic is considered to be advantageous (Tsega *et al.,* 2019). Most LAB are

resistant to aminoglycosides such as gentamicin and streptomycin (Li *et al.,* 2020). Specifically, *Lactobacillus* and *Pediococcus* species, have been observed to be intrinsically resistant to glycopeptides, such as vancomycin (commonly used to treat infections caused by Gram-positive bacteria) (Stefańska *et al.,* 2021). This resistance is partly due to the presence of specific genes in the chromosome of LAB strains, which are responsible for encoding proteins that prevent the mechanism of action of glycopeptides (Wang *et al.,* 2021). The resistance of LAB to different antibiotics differs among species, suggesting that specific gene factors determine the susceptibility or resistance of LAB strains to antibiotics. Furthermore, the resistance of LAB to antibiotics such as ciprofloxacin and gentamicin has been reported previously (Li *et al.,* 2020). Hameed and Sattar Salman (2023) further reported that factors such as bacterial cellular wall constructions, permeability and influx mechanism are responsible for resistance against quinolones.

There was also moderate and complete sensitivity of LAB to some of the tested antibiotics, including ampicillin (93,33%) and chloramphenicol, ceftriaxone (86,67%) and erythromycin (80%). It has been reported that some LAB strains are frequently susceptible to antibiotics such as erythromycin, tetracycline, and chloramphenicol (Wang *et al.,* 2021). Our results are in agreement with the results reported in the study by Paul *et al.,* (2024), which reported complete sensitivity of *P. pentosaceus* and *S. thermophilis* to ampicillin, chloramphenicol and tetracycline. While intrinsic resistance to antibiotics is generally seen as a benefit for bacteria during antibiotic therapy, it is worth noting that the simultaneous use of antibiotics and probiotics can also offer therapeutic and preventive benefits (Zommiti *et al.,* 2017), it is essential to identify and characterize probiotic strains that are both safe and effective for animal use.

5.5 EXTRACELLULAR ENZYMATIC ACTIVITY

Lactic acid bacteria produce enzymes such as proteases, amylases, polysaccharides degrading enzymes, ureases, lipases, phenol oxidases and esterases (Padmavathi *et al.*,2018), which play an essential role in the digestion and absorption of nutrients in the GI tract (Noohi *et al.* 2021; Kumar *et al.,* 2023). Potential probiotics producing these enzymes can also reduce antinutritional compounds in poultry feed, thereby enhancing body weight gain and feed conversion efficiency (Nallala *et al.,* 2017). In this study, out of the 14 tested isolates for the

production of protease and amylase, only 12 isolates were able to produce both enzymes (Table 4.4), with zones of clearance ranging from 8.50 ± 2.12 to 12.50 ± 0.71 for protease activity and 9,5±0,71 to 12,0±1,41 for amylase activity.

LAB producing enzymes are beneficial to the GI tract of the host, as these enzymes are helpful in the digestion of carbohydrates and proteins (Nandi *et al.,* 2017). Our findings are similar to Noohi *et al.,* (2021), where all isolated lactobacilli produced protease enzymes, however, the isolated lactobacilli lacked extracellular amylase activity, which contrasts with our study, where only isolate SI42 and S22 did not show amylase activity. LAB-produced enzymes are gaining significant attention due to their potential ability to promote nutritional benefit in poultry, hence, only twelve LAB isolates were selected for further probiotic screening as they were strong producers of protease and amylase.

5.6 ANTAGONISTIC ACTIVITY OF PROBIOTICS

Potential probiotic strains must have the ability to prevent or inhibit the growth of pathogenic bacteria to maintain a healthy balance inside the GI tract of the host, promoting overall health (Cele *et al.,* 2022). Moreover, the antagonistic effect of probiotic bacteria has been identified as the primary influential factor in preventing the colonization of heterochthonous bacteria in the GI tract. Therefore, the utilisation of indigenous bacteria as potential probiotics is expected to play a crucial contribution to the control of invading pathogens (Li *et al.,* 2024). Jose *et al.*, (2015), reported that probiotic strains from animal rumen yield a better antagonistic effect against pathogens than those derived from dairy products. Hence, it is essential to develop probiotics that are specific to the host to enhance animal health and optimal performance (Dowarah *et al.,* 2018).

Table 4.5 and Table 4.6 show the zones of inhibition against common poultry pathogens by LAB isolated from the crops and small intestines of broiler chickens. In this study, 11 out of 12 tested LAB isolates demonstrated a broad spectrum against five tested pathogens. Comparable antagonistic effects against Gram-positive (*L. monocytogenes and S. aureus*) and Gram-negative bacteria (*Ps. aeruginosa*, *S. typhimurium* and *S. enterica*) were observed amongst the isolates with the zones of inhibition ranging from 9,00 ±5,66 to 30,00 ±0 mm (agar well diffusion assay) and 1,00 ±0 to 5,50 ±0,71 (agar spot test) but the least antagonistic activity

was observed against *Ps. aeruginosa*. Some studies have reported that LAB from the GI tract have a high antagonistic effect against Gram-positive pathogens, but a low antagonistic effect against negative pathogens (García-Hernández *et al.,* 2016; Melara *et al.,* 2023; Nallala *et al.,* 2017).

The antagonistic activity of LAB is mainly because of the production of metabolites such as organic acids (lactic acid and acetic acid), hydrogen peroxide, diacetyl and bacteriocins (Rastogi *et.al.,* 2021), and de Almeida Júnior *et al.,* (2015) reported that antagonism was independent of the Gram-type of pathogen test. This can be observed in our study as well as some of the LAB isolates were able to exhibit a highly antagonistic effect against *S. enterica,* and *S. typhimurium, L. monocytogenes* and *S. aureus.* The results obtained in our study are comparable to a previous study that demonstrated a stronger inhibition against *S. aureus*, *L. monocytogenes,* and *S. typhimurium* (Mulaw *et al.,* 2019).

This study also found that LAB isolates obtained from the small intestines demonstrated significantly greater inhibitory activity compared to the isolates obtained from the crops of broiler chickens. These observations are consistent with those from the study by Reuben *et al.,* (2019), which also reported that LAB isolates from the small intestines displayed wider zones of inhibition against indicator organisms tested. Admittedly, the ability of probiotics to inhibit a wide range of pathogenic bacteria is a noteworthy characteristic, as it holds a promising prospect for using them as feed additives or in veterinary medicine (Li *et al.,* 2024). Consequently, the eleven LAB isolates were selected for further assessment due to their favourable antagonistic activity against tested pathogens.

5.7 ASSESSMENT OF GROWTH CHARACTERISTICS OF LAB

Phenol is a toxic metabolite secreted by microbes in the GI tract because of the deamination of aromatic amino acids derived from dietary and indigenous proteins (Reuben *et al.,* 2019). Probiotics with resistance to phenol have a significant advantage as they can withstand the inhibitory effect of phenol, potentially improving their survival and effectiveness in the gut of the host (Divya *et al.,* 2012). In this study, phenol resistance of the isolated LAB was assessed by exposing the LAB isolates to an MRS medium supplemented with phenol of different concentrations (0,2%, 0,3% and 0,4%).

As shown in Figure 4.5, the isolated LAB were able to withstand the different concentrations with OD values ranging from 0,49 ± 0,13 to 1,89 ± 0,011 OD units. This is indicative that the bacteria were able to tolerate phenol concentrations, when compared to the initial inoculum. Strain C5, showed particularly strong growth, outperforming the reference strain as well, at lower concentrations. Several factors such as the type of strain, as some bacteria are more tolerant than others, the type of medium used as well as the growth conditions (incubation period) may have influenced the variation observed in this study (Yang *et al.,* 2024). However, we observed that with increasing phenol concentrations, the cell viability decreased.

Several factors can contribute to this decrease in cell viability during exposure to high phenol concentrations. These include the disturbance of the bacterial cell membrane caused through phenol dissolving in the lipid bilayer of the cell membrane, leading to structural changes, increased permeability and leakage of cellular contents. Additionally, high phenol concentrations can result to cell lysis, which subsequently results in cell death (Lobiuc *et al.,* 2023). Despite this, growth was observed in LAB growth in MRS supplemented with 0,4% of phenol, demonstrating their ability to persist in the GI tract. Phenol and other harmful metabolites are produced during specific digestive processes, hence, LAB that can persist in such conditions can therefore be considered as potential probiotic (Huligere *et al.,* 2023). Also, El-Sayed *et al.,* (2022), reported good tolerance of tested strains (*Lactobacillus acidophilus* SAM1 and *Lactiplantibacillus plantarum* SAM2) towards phenol. However, the viability rate of the tested LAB isolates significantly decreased gradually by increasing phenol concentration.

The GI tract of broiler chickens has an osmolarity equivalent to 0.3 mol/L hence, the potential probiotic LAB must tolerate high salt concentrations to maintain its osmotic balance to survive and proliferate inside the GI tract (Tsega *et al.,* 2019). Moreover, high salt concentrations enable bacteria to initiate metabolism, thus producing acid responsible for the inhibition of the growth of undesirable organisms (Sathyabama *et al.,* 2012). Hence, in this study, we evaluated the ability of potential probiotic strains to survive in different NaCl concentrations of 0,5%, 2%, 5% and 10%. The results shown in Figure 4.6 indicate that the highest growth tolerance of LAB to NaCl was observed at 0,5% and 2% concentration, with isolate C5 displaying the highest growth at 1,99 ± 0,04 OD unit. This can be a result of the adaptive strategies that are

employed by LAB when exposed to high concentrations of NaCl that involve the accumulation of Osmo-protectants, particularly betaine, choline, and carnitine which enhances the cells' tolerance threshold (Braschi *et al.,* 2021). Furthermore, osmotic stress triggers the production of chaperons like DnaK, GroEL, and GroES, which are stress proteins that are specially synthesized in response to osmotic stress. These stress proteins play a crucial role in protecting cellular proteins and structures during exposure to high salt concentrations.

Conversely, weak growth was observed in 10% NaCl concentrations, with isolates C24 and SI38 exhibiting the lowest tolerance with an OD of 0,030 OD units. This reduction in cell growth can be explained by the fact that in high salt concentrations, the change in the osmotic pressure causes water molecules to move out of the bacterial cell into the extracellular environment, reducing metabolic activity within the cell. Furthermore, exposure to hypertonic solutions leads to cell dehydration, reducing turgor pressure, subsequently altering cell volume and ion concentration as well as plasmolysis which is the separation of the protoplast from the bacterial cell wall (Derunets *et al.,* 2024). All these phenomena significantly reduce cell viability as it is widely believed that maintaining a consistent turgor is the key driving force behind cellular development and growth. Our results agree with the findings of Reuben *et al.,* (2019), where weak growth in 10% of NaCl concentrations was reported. Simultaneously, salt tolerance of the LAB is essential to industrial and food preservation applications (Tawab *et al.,* 2023).

The growth trends of LAB isolates incubated at various temperatures are shown in Figure 4.7. In this study, it was observed the LAB isolates were able to withstand all temperatures (25°C, 30°C, 40°C, 60°C, and 70°C), with maximum growth observed at temperatures of 25°C, 30°C, and 40°C. This means that the isolated LAB isolates could survive as bacteria are exposed to a series of environmental factors, temperature changes being one of the variable factors that allow them to live and withstand temperature ranges. Furthermore, the resistance of LAB isolates to 40°C was considered a good trait in this study, as it signifies that the isolated LAB will be able to survive and proliferate in the GI tract of chickens as this is the optimum body temperature and digestive tract of chickens. These results are in collaboration with the findings of Srifani *et al.,* (2024), who also reported good temperature tolerance of four LAB isolates from soymilk waste for potential use in poultry at 40°C. We also observed that with increasing

temperatures, the growth survivability percentage of LAB significantly reduced at 60°C and 70 °C. The reduction in growth survivability percentage can be because exposure to high temperatures disrupts the balance within the bacterial cell, resulting in various changes within the bacterial cell (Derunets *et al.,* 2024).

Exposure to high temperatures triggers the production of heat stress proteins (HSPs), including chaperones, chaperonins and proteases that are responsible for the correct folding of the newly produced polypeptide chains and the reorganization of damaged proteins. Furthermore, these proteins promote the assembly of protein complexes, degradation and translocation of proteins (Tawab *et al.,* 2023). Our results are similar to those of Reuben *et al.,* (2019) who reported reduced growth of the LAB isolates at increasing temperatures. LAB with high tolerance to different temperatures could translate to increased growth rate and lactic acid production, whilst reducing contaminations by other microorganisms (Ibourahema *et al.,* 2008). Microbes can be classified into psychrotrops (2°C-19°C), mesophiles (20°C-40°C), and thermophiles (> 45°C) (Pellissery *et al.,* 2020). Based on the obtained results, it can be concluded that the isolated LAB can be categorized as mesophile bacteria as they demonstrated high growth survivability percentage at temperatures 25°C, 30°C and 40°C. Thus, all the LAB isolates that were able to tolerate different growth temperatures can live and survive in the digestive temperatures in chickens and can be tested further for probiotic screening.

5.8 GENOTYPIC IDENTIFICATION OF LAB

In this study, 16S rDNA sequencing was conducted to identify the species of the isolates. Sequences that showed ≥ 98% homology with previously published sequences from NCBI were selected for species identification. The 11 isolates were identified as *Enterococcus faecalis* (C4 and C5), *Pediococcus pentosaceus* (C7, C13, C24, SI23, and SI38), *Streptococcus salivarius* (SI4), and *Levilactibacillus brevis* (SI6, SI8, SI9). Similar studies have identified similar organisms from the gut of chickens (Jannah *et al.,* 2014; Oyewole *et al.,* 2018; Reuben *et al.,* 2019). Furthermore, these sequences were observed to have a molecular weight ranging from 1000 to 1500 bp (Figure 4.8). Phylogenetic trees were constructed for each identified organism using 16S rDNA gene sequence analysis. The results demonstrated that the isolated LAB shared homology with nucleotide sequences of another probiotic LAB from the NCBI database (Figure 4.9-4.12).

5.9 SIMULATED GASTRIC JUICE SURVIVABILITY TESTING

Simulated gastric juice survivability testing of LAB was performed to detect the ability of the isolates to thrive in gastrointestinal environments such as low pH, bile acids and enzyme digestion. It has been reported by Xu *et al.,* (2023) that the pH of avian gastric juice normally ranges from 2.00 to 2.60. Hence, in this study, we evaluated the ability of LAB isolates to survive in simulated gastric juice at pH 2.5.

As shown in Figure 4.13, it was observed that the tolerance of LAB to simulated gastric juice at low pH, bile salt conditions and in the presence of lysozyme was nearly a two-log reduction in CFU count from the initial count of 10 $\log_{10}$ CFU/ml after 90 minutes of incubation. Interestingly, all isolated LAB were able to survive in simulated gastric juice of pH 2.5, with cell viability that is greater than 7.0 CFU/ml and a significant reduction ($p < 0.05$) observed when compared to the initial bacterial count. This reduction might be due to the fact in Gram-positive bacteria, lysozyme breaks down the peptidoglycan cell wall by cleaving the β (1-4) bond of N-acetylmuramic acid and N-acetyl glucosamine, subsequently leading to cell death (Dias *et al.,* 2015). It was also noted that each strain behaved differently when exposed to lysozyme. For instance, *Pediococcus pentosaceus* NKFS4 demonstrated the highest tolerance while *Streptococcus salivarius* NKFS6 demonstrated the lowest tolerance.

The D-value, which is the time required at a given condition to achieve a one-log reduction of relevant microorganisms was also determined in this study (Majeed *et al.,* 2020). This was done to determine the amount of time required for lysozyme to 90% of LAB strains. As indicated in Table 4.8, the D-values of the isolated probiotic strains ranged from 36.10-172.41 minutes, with *Pediococcus pentosaceus* NKFS4 demonstrating the highest D-value of 172.41 and *Streptococcus salivarius* demonstrating the lowest D-value of 36,10 minutes. The recorded data shows that the crop has a pH of 4.8 and a retention time of 30 minutes, the small intestine has a pH of 6.2 and a retention time of 90 minutes, the large intestine has a pH of 6.3 and a retention time of 15 minutes, the proventriculus has a pH of 4.4 and a retention time of 15 minutes, and the gizzard has a pH of 2.6 and a retention time of 90 minutes (Sirisopapong *et al.,* 2023). This means that the total recorded time through the intestinal tract is 225 min. The highest surviving organism *Pediococcus pentosaceus* NKFS4 will thus only reduce from 10

log to 7.8 log, being able to survive for 172.91 minutes. The lowest surviving organism, *Streptococcus salivarius,* will be reduced from 10 log to 1,6 log at the end of the intestine.

Coulon *et al.,* (2012) reported that the resistance of *Pediococcus* spp in lysozyme may be due to the presence of *β*-glucan that develops around the cell, serving as a protective layer against anti-bacterial agents. It was further reported that the presence of lysozyme in the growth medium reduced the efficacy of cell lysis of *Streptococcus* and *Lactobacillus* spp. It was also observed that even in the absence of lysozyme there was still a reduction in cell growth. This might be due to the effect of low pH, porcine bile and pepsin that were also used in the solution of the simulated gastric juice. A similar trend was reported in a study by Reuben *et al.,* (2019). The resistance of probiotic bacteria to low pH is mainly due to the reduction of F_1F_0-ATPase proton pump activity, which is affected by the decrease of glycolytic enzymes caused by the influx of hydrogen (H^+) (Yao *et al.,* 2020). The results observed in this study indicate that the resistances of probiotic isolates to simulated gastric juice varies amongst different probiotic strains

5.10 CELL SURFACE HYDROPHOBICITY

The capacity of probiotic strains to adhere to the epithelial cells of the host is directly influenced by cell surface characteristics, including cell-surface hydrophobicity and aggregation properties (co-aggregation and auto aggregation) (Wang *et al.,* 2021). Cell adhesion is one of the important properties of probiotic strains as it aids the colonization of the GI tract by the probiotic strains and prevents the colonization of the GI tract by pathogens via competitive exclusion (Collado *et al.,* 2008; Reuben *et al.,* 2019). Cell surface hydrophobicity is commonly measured using the MATH method, which evaluates the hydrophobicity as the affinity of microorganisms to a solvent (e.g., chloroform, xylene and hexadecane) (Krausova *et al.,* 2019). In this study, n-hexadecane was used for the evaluation of cell surface hydrophobicity of the isolated LAB strains. As illustrated in Figure 4.15, the highest hydrophobicity percentage was observed in *L. brevis* isolates (*L. brevis* NKFS7, *L. brevis* NKFS8 and *L. brevis* NKFS9), *P. pentosaceus* NKFS3 and *E. faecalis* NKFS1 indicating the greater ability of these strains to adhere to the epithelial cells that is attributed to the electron donor property of the bacterial surface, which is characterized by the carboxylic groups and the Lewis-acid-base interactions (Escobar-Sánchez *et al.,* 2023). In a study by Kariyawasam *et al.,* (2020), the cell surface hydrophobicity of *L. brevis* KU15006 has been reported to be 48,07%, which is lower when compared to the hydrophobicity percentage obtained in this study.

According to de Paula *et al.,* (2015), several factors influence cell surface hydrophobicity, such as the type of probiotic strains and different nutrients or carrier food matrices that may influence the expression of adhesion genes in the microorganisms. This can be also seen in our study, as *E. faecalis* NKFS2*, P. pentosaceus* NKFS5*, P. pentosaceus* NKFS10 and *S. salivarius* NKFS6 demonstrated the lowest hydrophobicity, indicating that cell surface hydrophobicity is indeed strain-dependant. A study by Wang *et al.,* (2022) also reported weak hydrophobicity of 23,22% and 21,18% in *P. pentosaceus*. However, *S. salivarius* was reported to exhibit the highest hydrophobicity percentage of 95,27%. These differences in findings can be due to the type of compound (n-hexadecane, xylene and toluene) used and the specificity of the strains, resulting in different responses between the isolated strains (Rocha-Ramírez *et al.,* 2021). Furthermore, factors like the cultivation medium used, the duration of cultivation time, and the presence of acids can result in different hydrophobicity based on the MATH method. We also observed that LAB isolated from the small intestine showed good hydrophobicity, as compared with those from the crops. These results, however, are not in agreement with those from the study by Reuben *et al.,* (2019), which observed good hydrophobicity from isolates from the crops. According to Shi *et al.,* (2020), bacteria exhibiting strong hydrophobicity move toward the intestinal wall to avoid hydrophilic electrolytes in the intestinal environment.

5.11 AUTO-AGGREGATION

Auto-aggregation is an essential attribute that facilitates the adherence and colonization of probiotic strains to intestinal cells (Li *et al.,* 2024b). Nallala *et al.,* (2017) reported that potential probiotic strains exhibiting higher auto-aggregation and hydrophobicity will have a higher chance of adhering to the intestinal epithelial cell. As shown in Figure 4.16, all tested strains demonstrated some degree of auto-aggregation abilities from the 4-hour incubation period, which continued to increase over time. The highest auto-aggregation percentage was observed after 24 hours, with *Lactobacillus* spp (*L. brevis* NKFS 8, *L. brevis* NKFS 9 and *L. brevis* NKFS 7) exhibited a high auto-aggregation percentage 81,89% and 77, 25%while *E. faecalis, P. Pentosaceus* and *S. salivarius* showed moderate aggregation abilities. Nishiyama *et al.,* (2015) reported that self-aggregation occurs as a result of aggregation-promoting factors (APF), which are proteins secreted by aggregating bacteria and are essential for facilitating the maintenance of cell shape.

These findings indicate that these different strains consist of different levels of auto-aggregation characteristics due to the complex interaction among bacteria surface molecules, such as proteins and secreted factors, etc. Similar finding were indicated in a study by Alizadeh *Behbahani et al.,* (2024), where the *L. brevis* HL6 showed the highest auto-aggregation percentage 35,20%. In addition, it has been reported that obligatory homofermentative lactobacilli exhibit higher auto-aggregation values (Krausova *et al.,* 2019). Furthermore, our finding indicates that the auto-aggregation percentage increased with increasing incubation time. This observation was also found in the study by Rastogi *et al.,* (2021), which reported auto-aggregation percentages ranging between 7.59 and 75.45% and observed the values increased with increasing periods of incubation.

Studies have found a correlation between cell surface hydrophobicity and auto-aggregation and reported that some bacterial species with a high cell surface hydrophobicity would normally show high auto-aggregation abilities (Pinto *et al.,* 2007; Todorov *et al.,* 2011; Wang *et al.,* 2021). By analyzing the relationship between the auto-aggregation percentage and hydrophobicity percentage, it has been possible to verify the significant diversity in the cells' surface properties among probiotic strains (Collado *et al.,* 2008). The findings observed in this study also support the hypothesis that strains demonstrating higher auto-aggregation abilities, also show high cell surface hydrophobicity. Interestingly, *L. brevis* isolates also demonstrated the highest hydrophobicity percentage and the relation between the two probiotic properties was also investigated in our study. The correlation coefficients between auto-aggregation abilities and cell surface hydrophobicity are shown in Figure 4.17. No statistically significant correlation was observed between auto-aggregation after 4 and 16 hours of incubation and hydrophobicity of the tested LAB strains. However, the value (r= 0,62) increased when strains showing higher aggregation percentages that were recorded after 24 hours of incubation were consistent. These results show a good correlation between auto-aggregation ability and cell surface hydrophobicity.

5.12 CO-AGGREGATION

The coaggregation properties of LAB prevent adherence of pathogenic bacteria to the intestinal cell wall barrier (Vasiee *et al.,* 2022). Inconsistent opinions exist in the literature regarding the importance of co-aggregation. A study has indicated that probiotics must have a lower coaggregation with pathogens to avert the formation of biofilms, subsequently reducing the adhesion of pathogens to the GI tract (Mohanty *et al.,* 2019). Conversely, de Souza *et al.,* (2019) reported that increased coaggregation between probiotic strains and test pathogens provides potential means to exclude pathogens from the GI tract. Therefore, in this study, co-aggregation of the 11 isolated LAB strains were evaluated against Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative bacteria (*Ps. aeruginosa*, *S. typhimurium* and *S. enterica*) with co-aggregation percentages ranging from 31,52 ± 2,69 to 88,32 ± 2,0%. As shown in Figure 4.18, the highest co-aggregation percentage was observed against *S. aureus* and *Ps. aeruginosa* by *E. faecalis* NKFS1 and *P. pentosaceus* NKF3. These results are in agreement with the results by Li *et al.,* (2020) that reported 80% coaggregation percentage of *Enterococcus durans* M2-3 isolated from wild boar with *S. aureus* after 4 hours of incubation. Alp *et al.,* (2020) explained that the co-aggregation abilities of probiotic bacteria are attributed to an S-layer protein. This protein interacts with a particular cellular position on the surface of the pathogen, resulting in modifications that cause pathogenic bacterial cells to cluster together with the probiotic bacteria. This process effectively prevents the manifestation of infections.

It was also observed in this study that when compared to the reference strain *L. casei* (ATCC 393), the coaggregation of LAB with Gram-positive *S. aureus* was stronger when compared to other isolates. This is influenced by the exposed surface molecules and the cell wall structure facilitating the direct interactions (Fonseca *et al.,* 2021). However, some LAB isolates were able to show high coaggregation abilities against Gram-negative bacteria (*P. aeruginosa*, *S. enterica* and *S. typhimurium*), while exhibiting lower coaggregation abilities towards *L.* monocytogenes. These findings were also observed by Nami *et al.,* (2019), who reported a lower coaggregation of LAB isolates towards Gram-positive pathogens than Gram-negative ones. This might be facilitated by the differences observed in the structure of their cell wall and surface properties (Malfa *et al.,* 2023). The overall effectiveness of coaggregation with Gram-negative and Gram-positive can vary, with Gram-positive exhibiting stronger interactions with probiotics, while Gram-negative require more specialized or selective mechanisms for co-aggregation

Other factors contributing to the co-aggregation of probiotic bacteria in addition to the S-layer protein include lectin-like proteins present on the surface of numerous bacterial cells that influence cellular interactions. Moreover, the secretion of a 32 kDa protein by probiotic bacteria, along with the involvement of specialized peptides plays a crucial role in co-aggregation (Rashad *et al*., 2023). Li *et al.,* (2020) also reported a higher co-aggregation percentage of *Enterococcus* spp against *S. aureus*. According to Sophatha *et al.,* (2020), strains with high coaggregation abilities with pathogenic microorganisms may have great advantages, as they cannot be easily removed through the GI tract of the host, unlike those with non-coaggregation abilities. LAB strains have a major influence on the GI tract micro-environment and around the pathogen's process of coaggregation, increasing the metabolites secreted by LAB, such as bacteriocins and organic acids that inhibit the proliferation of pathogens (Abbasiliasi *et al.,* 2017).

5.13 α-GLUCOSIDASE INHIBITORY ACTIVITY

α-Glucosidase is an enzyme involved in breaking down complex carbohydrates in the intestinal brush border into their constituents (Huligere *et al.,* 2023). Inhibiting these enzymes delays the absorption of carbohydrates and the rise in glucose levels after eating. Consequently, this increase in blood sugar leads to a decrease in insulin secretion after a meal (Fonseca and John-Kalarickal 2010). Therefore, the evaluation of the specific probiotic isolates to inhibit the *α*-glucosidase enzyme involved in carbohydrate metabolism is crucial.

Figure 4.19 demonstrated the *α*-glucosidase inhibition activity of 11 LAB strains from the crops and small intestines of broiler chickens with the inhibition percentage ranging from 25,84 ± 3,08% to 61,77 ± 6,16%. A higher inhibition percentage observed in *P. pentosaceus* NKFS3 relative to the reference strain is similar to the report of (Kim *et al.,* 2021) which reported the *α*-glucosidase of *P. pentosaceus* KI62 of 94 ±3,30%. The inhibition of *α*-glucosidase is achieved by differing the release and absorption of glucose, thereby preventing postprandial hyperglycaemia, hindering carbohydrate metabolism, and inhibiting extreme glucose absorption. Consequently, inhibiting carbohydrate hydrolases, including *α*-glucosidase, is considered an effective approach for managing postprandial hyperglycaemia in individuals with type 2 diabetes mellitus (T2DM) (Won *et al.,* 2021). We also observed that *S. salivarius*

and *L. brevis* isolates exhibited the lowest inhibitory activity. Simultaneously, another study by Son *et al.,* (2017) reported a low inhibition of *α*-glucosidase of the CFS of *L. brevis* to be 24,11%. In our study, the highest inhibition activity was observed from the crop isolates (*E. faecalis* NKFS1*, E. faecalis* NKFS2, *P pentosaceus* NKFS3, *P. pentosaceus* NKKFS4, *P. pentosaceus* NKFS5, P. *pentosaceus* NKFS10). This may be due to the adaptations of chickens to plant-based diets and higher production of bioactive compounds such as short-chain-fatty acids (Ben-Miled *et al.,* 2023). In contrary, Reuben *et al.,* (2019) reported the highest inhibitory activity of 92% LAB isolates from the intestine. From our results, it is clear that the isolated LAB can reduce the absorption of carbohydrates in the intestines of the host.

According to Ben-Miled *et al.,* (2023), optimal percentage of the inhibition of *α*-glucosidase vary depending on the type of probiotic strain being tested. However, in general, an inhibition of 50% or higher is deemed as significant in studies evaluating antidiabetic properties of probiotic strains. Based on our observation, *P. pentocaseus* NKFS3 (61,77%), *P. pentosaceus* NKFS5 (59,05%), *L. brevis* NKFS9 (58,16%), *E. faecalis* NKFS2 (56,77%), *P. pentosaceus* NKFS10 (55,19%) and *P. pentosaceus* NKFS11 (55,11%) are LAB isolates with strong potential for managing blood glucose as they performed better when compared to the reference strain *L. casei* (ATCC 393) with the inhibition percentage of 48%.

5.14 PRINCIPAL COMPONENT ANALYSIS (PCA)

Principal component analysis (PCA) is a commonly used method to identify the most effective probiotic strains for reducing the number of dependent variables. This selection is done by the observing the correlation values between the variables (Kumar *et al.,* 2023). PCA is characterized by two principal components, the first principal component (F1) signifies the highest variation of the data, while the second principal component (F2) encompasses the remaining variation concerning the first component (Baliyan *et al.,* 2021).

As demonstrated in Figure 4.20, the total variation of both F1 (34,94%) and F2 (22.41%) resulted in 54,35% where F1 was primarily influenced by GI tolerance, enzyme inhibition, as well co-aggregation, and F2 was influenced by functional properties such as temperature tolerance, adhesion characteristics (auto-and co-aggregation and hydrophobicity). In this study, *E. faecalis* NKFS1, *P. pentosaceus* NKFS3, *P. pentosaceus* NKFS11 and *L. brevis* NKFS9,

located on the positive sides of both F1 and F2 were selected as the most promising probiotics, with probiotics characteristics including resistance to both acid and bile salts, α-glucosidase inhibition activity and co-aggregation abilities. Jung *et al.,* (2019) also applied PCA to select the most promising probiotic strains to be used as a starter culture for fermented foods and reported a total variation of 81,26% with the first factor accounting for 51,35% and the second factor accounting for 29,91%. *L. plantarum* was selected as the most suitable probiotic strain to be used with functional properties such as enzyme activity, acid and bile resistance, simulated gastric juice resistance as well as adhesion abilities.

Table 4.10 presents a summary of the probiotic characteristics of the selected strains from PCA. Interestingly, all of these strains exhibited the ability to co-aggregate with pathogens, particularly *S. aureus*, *P. aeruginosa,* and *S. enterica*, with co-aggregation percentages equal to or greater than 70%. Moreover, these isolates demonstrated the capability to inhibit clinically used antibiotics such as erythromycin and tetracycline, while exhibiting resistance to gentamicin, vancomycin, and ceftriaxone. These characteristics could be advantageous in probiotic-antibiotic therapy. The survivability of these isolates during GI transit was also observed, suggesting their ability to endure longer periods in the GI tract while providing beneficial effects. From the performed experiments, it can be concluded that PCA is an essential tool for analysing potential probiotic strains, and it can be concluded that acid, bile and co-aggregation are some of the essential attributes for the selection of probiotic strains.

6 CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

- The study focused on the isolation of potential probiotic LAB from the crops and small intestines of broiler chickens, intending to identify probiotic candidates. Eleven LAB met the criteria for consideration as probiotic strains and were identified as *Enterococcus faecalis* (C4 and C5*)*, *Pediococcus pentosaceus* (C7. C13. C24. SI23 and SI38*)*, *Streptococcus salivarius* (SI4) and *Levilactibacillus brevis* (SI6. SI7 AND SI9).

- The LAB strains exhibited good tolerance to GI transits conditions, including growth at pH values of 3 and 4, tolerance of bile salt concentrations of 0,3% and 0,6% and simulated gastric juice acid (containing lysozyme, pepsin and porcine bile). This resilience allows them not only to survive but also thrive in the challenging gut conditions of broiler chickens, thereby offering their beneficial effects as probiotic strains.

- The results obtained from these experiments demonstrated that tolerance of probiotic strains into stressful conditions is rather strain-specific, as there was a variation in cell viability observed in each strain. Additionally, prolonged exposure to these conditions was found to rapidly reduce cell viability.

- The LAB isolates were non-haemolytic, indicating their non-pathogenic nature.

- *L. brevis*, *E. faecalis* and *P. pentosaceus* displayed the highest antagonism against the tested pathogens (*S. enterica*, *S. typhimurium, P. aerogenosa* and *L. monocytogenes*). This antagonism was induced by the production of antimicrobial substances, subsequently enhancing the probiotic bacteria's capacity to inhibit the growth of pathogens.

- The investigation of antibiotic susceptibility profiles revealed diverse responses of LAB to the tested antibiotics. Resistance was observed against vancomycin, gentamicin, ciprofloxacin, and novobiocin. To our knowledge, this resistance might be

due to their intrinsic nature, as studies published indicated that some species of LAB have intrinsic resistance to the mentioned antibiotics. This intrinsic resistance may be advantageous if an antibiotic-probiotic combination therapy is desired.

- Among the isolates, *E. faecalis* NKFS2 showed the highest growth tolerance to both salt and phenol concentrations, while *S. salivarius* NKFS6 and *P. pentosaceus* NKFS4 exhibited good tolerance when exposed to different temperatures. These qualities are likely to contribute to the enhanced bacterial growth and the production of metabolites. Additionally, these attributes make isolates suitable for various industrial and technological applications such as preservatives.

- *E. faecalis* NKFS1 and *P. pentosaceus* NKF3 exhibit the highest co-aggregation against the tested pathogens. This suggests that these probiotic strains could be utilized as effective bio-control agents in the poultry industry, particularly for combating poultry infections.

- A fairly strong correlation between auto-aggregation and cell surface hydrophobicity in this study. This correlation implies that the isolated LAB have a higher likelihood of adhesion. Consequently, this promotes the successful colonization of these strains within the chicken's gut to adhere to the intestinal epithelial cells.

- It was discovered that *P. pentosaceus* NKFS3 exhibits potent α-glucosidase inhibitory activity. This allows the conclusion that it can interfere with or delay the absorption process of dietary carbohydrates leading to the suppression of increased levels of glucose.

- Based on the results observed in Principal component analysis, it can be concluded that *L. brevis* NKFS9, *P. pentosaceus* NKFS3, *P. pentosaceus* NKFS11 and *E. faecalis* NKFS1 have the potential to be used for the development of a multistrain probiotic. This probiotic can be utilized as a supplement for poultry feed, serving as a sustainable alternative to the use of antibiotics as growth promoters.

6.2 RECOMMENDATIONS

The study primarily focused on LAB isolates found in the crop and small intestine of broiler chickens, which may not fully represent the entire range of LAB diversity in other body sites or populations. Consequently, the generalizability of the findings is limited. Therefore, future research must conduct a pilot study that investigates the diversity of LAB present in various body sites of broiler chickens.

Our study solely focused on *in vitro* research. Therefore, future studies should give priority to conducting *in vivo* research to monitor and evaluate the effect of probiotics on the chicken gut. To produce the isolated LAB on a large scale, it is imperative to optimize cell growth and ensure that the probiotics used as feed additives have a stable shelf life.

The study did not investigate the resistant genes and specific mechanisms underlying the acquisition of resistance genes in LAB isolates. Future research can further investigate this aspect as understanding these mechanisms could yield valuable insights into the evolution and spread of antibiotic resistance in these bacteria.

7 REFERENCES

ABBASILIASI, S., TAN, J. S., BASHOKOUH, F., IBRAHIM, T. A. T., MUSTAFA, S., VAKHSHITEH, F., SIVASAMBOO, S. AND ARIFF, A. B. 2017. *In vitro* assessment of *Pediococcus acidilactici* Kp10 for its potential use in the food industry. *BioMed Central of Microbiology*, 17, 121.

ABUHELWA, A. Y., WILLIAMS, D. B., UPTON, R. N. AND FOSTER, D. J. 2017. Food, gastrointestinal pH, and models of oral drug absorption. *European Journal of Pharmaceutics and Biopharmaceutics*, 112, 234-248.

ADAMS, J. R. G., MEHAT, J., LA RAGIONE, R. AND BEHBOUDI, S. 2023. Preventing bacterial disease in poultry in the post-antibiotic era: a case for innate immunity modulation as an alternative to antibiotic use. *Frontiers in Immunology*, 14,1664-3224.

ADESULU-DAHUNSI, A. T., JEYARAM, K. & SANNI, A. I. 2018. Probiotic and technological properties of exopolysaccharide producing lactic acid bacteria isolated from cereal-based nigerian fermented food products. *Food Control,* 92, 225-231.

ADETOYE, A., PINLOCHE, E., ADENIYI, B. A. & AYENI, F. A. 2018. Characterization and anti-*salmonella* activities of lactic acid bacteria isolated from cattle faeces. *BioMed Central of Microbiology*, 18, 96.

ALCANTARA, C. & ZUNIGA, M. 2012. Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23. *Microbiology*, 158, 1206-1218.

ALIZADEH BEHBAHANI, B., JOOYANDEH, H., HOJJATI, M. & GHODSI SHEIKHJAN, M. 2024. Evaluation of probiotic, safety, and anti-pathogenic properties of *Levilactobacillus brevis* HL6, and its potential application as bio-preservatives in peach juice. *Food Science and Technology*, 191, 115601.

ALP, D., KULEAŞAN, H. & KORKUT ALTıNTAŞ, A. 2020. The importance of the S-layer on the adhesion and aggregation ability of Lactic acid bacteria. *Molecular Biology Reports*, 47, 3449-3457.

AMENU, D. AND BACHA, K. 2023. Probiotic potential and safety analysis of lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages. *Annals of Microbiology*, 73, 37.

ARSÈNE, M. M., DAVARES, A. K., ANDREEVNA, S. L., VLADIMIROVICH, E. A., CARIME, B. Z., MAROUF, R. AND KHELIFI, I. 2021. The use of probiotics in animal feeding for safe production and as potential alternatives to antibiotics. *Veterinary World*, 14, 319.

ARYANA, K. J. AND OLSON, D. W. 2017. A 100-Year Review: Yogurt and other cultured dairy products. *Journal of Dairy Science*, 100, 9987-10013.

ATFAOUI, K., OMAR, B., ABDESSAMAD, E., RACHID, I., IMANE, O., HICHAM, H. & MOHAMMED, O. 2022. Phenotypic and Genotypic Identification of the most acidifiers LAB strains isolated from fermented food. *Biology Bulletin*, 49, 260-270.

AWAD, W. A., GHAREEB, K., ABDEL-RAHEEM, S. AND BÖHM, J. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. *Poultry Science*, 88, 49-56.

AWAD, A., GWIDA, M., KHALIFA, E. & SADAT, A. 2020. Phenotypes, antibacterial-resistant profile, and virulence-associated genes of *Salmonella serovars* isolated from retail chicken meat in Egypt. *Vetenary World*, 13, 440-445.

AYIVI, R. D., GYAWALI, R., KRASTANOV, A., ALJALOUD, S. O., WORKU, M., TAHERGORABI, R., SILVA, R. C. D. AND IBRAHIM, S. A. 2020. Lactic acid bacteria: food safety and human health applications. *Dairy*, 1, 202-232.

AYYASH, M. M., ABDALLA, A. K., ALKALBANI, N. S., BAIG, M. A., TURNER, M. S., LIU, S.-Q. AND SHAH, N. P. 2021. Invited review: Characterization of new probiotics from dairy and nondairy products—Insights into acid tolerance, bile metabolism and tolerance, and adhesion capability. *Journal of Dairy Science*, 104, 8363-8379.

BAILEY, R., 2010. Intestinal microbiota and the pathogenesis of dysbacteriosis in broiler chickens (Doctoral dissertation, University of East Anglia). (Accessed 19 October 2024).

BAJAGAI, Y. S., KLIEVE, A. V., DART, P. J. AND BRYDEN, W. L. 2016. Probiotics in animal nutrition: production, impact and regulation. *Food and Agricultural Organization*. 179, 0254-6019.

BALIYAN, N., DINDHORIA, K., KUMAR, A., THAKUR, A. & KUMAR, R. 2021. Comprehensive substrate-based exploration of probiotics from undistilled traditional fermented alcoholic beverage 'lugri'. *Frontiers in Microbiology*, 12. 302-1664.

BAZIREH, H., SHARIATI, P., AZIMZADEH JAMALKANDI, S., AHMADI, A. & BOROUMAND, M. A. 2020. Isolation of novel probiotic *Lactobacillus* and *Enterococcus* strains from human salivary and fecal sources. *Frontiers in Microbiology*, 4. 11-59.

BEN-MILED, H., BENOIT-BIANCAMANO, MO., BEN-MAHREZ, K. 2023. Alpha-amylase and alpha glucosidase inhibitory properties, beta-galactosidase activity, and probiotic potential of lactic acid bacteria and *bifidobacteria* from *Apis mellifera* intermissa and its products. *World Journal of Microbiology and Biotechnology*. 39, 205.

BERMUDEZ-BRITO, M., PLAZA-DÍAZ, J., MUÑOZ-QUEZADA, S., GÓMEZ-LLORENTE, C. AND GIL, A. 2012. Probiotic mechanisms of action. *Annals of Nutrition and Metabolism*, 6, 160-174.

BOTTARI, B., FELIS, G. E., SALVETTI, E., CASTIONI, A., CAMPEDELLI, I., TORRIANI, S., BERNINI, V. AND GATTI, M. 2017. Effective identification of *Lactobacillus casei* group species: genome-based selection of the gene *mutL* as the target of a novel multiplex PCR assay. *Microbiology (Reading)*, 163, 950-960.

BRATCHER, D. F., LONG, S. S., PROBER, C. G. AND FISCHER, M. 2018. Other Gram-Positive Bacilli. principles and practice of pediatric infectious diseases (Fifth Edition). *Elsevier*, 784, 786-790.

BRODKORB, A., EGGER, L., ALMINGER, M., ALVITO, P., ASSUNÇÃO, R., BALLANCE, S., BOHN, T., BOURLIEU-LACANAL, C., BOUTROU, R. AND CARRIÈRE, F. 2019. Infogest static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols*, 14, 991-1014.

BUSTOS, A. Y., FONT DE VALDEZ, G., FADDA, S. AND TARANTO, M. P. 2018. New insights into bacterial bile resistance mechanisms: the role of bile salt hydrolase and its impact on human health. *Food Research International*, 112, 250-262.

CASTELEYN, C., DOOM, M., LAMBRECHTS, E., VAN DEN BROECK, W., SIMOENS, P. AND CORNILLIE, P. 2010. Locations of gut-associated lymphoid tissue in the 3-month-old chicken: a review. *Avian Pathology*, 39, 143-150.

CHOI, A.-R., PATRA, J. K., KIM, W. J. AND KANG, S.-S. 2018. Antagonistic activities and probiotic potential of lactic acid bacteria derived from a plant-based fermented food. *Frontiers in Microbiology*, 9.

CHUNG, H., BANG, W. AND DRAKE, M. 2006. Stress response of *Escherichia coli. Comprehensive Reviews in Food Science and Food Safety*, 5, 52-64.

CLASSEN, H., APAJALAHTI, J., SVIHUS, B. AND CHOCT, M. 2016. The role of the crop in poultry production. *World's Poultry Science Journal*, 72, 459-472.

CLAVIJO, V. AND FLÓREZ, M. J. V. 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. *Poultry Science*, 97, 1006-1021.

COLLADO, M. C., MERILUOTO, J. & SALMINEN, S. 2008. Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology*, 226, 1065-1073.

CORCORAN, B., STANTON, C., FITZGERALD, G. AND ROSS, R. 2005. Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology*, 71, 3060-3067.

COULON, J., HOULÈS, A., DIMOPOULOU, M., MAUPEU, J. & DOLS-LAFARGUE, M. 2012. Lysozyme resistance of the ropy strain *Pediococcus parvulus* IOEB 8801 is correlated with *β*-glucan accumulation around the cell. *International Journal of Food Microbiology*, 159, 25-29.

CUTLER, S., RASMUSSEN, M., HENSLEY, M., WILHELMS, K., GRIFFITH, R. AND SCANES, C. 2005. Effects of Lactobacilli and lactose on *Salmonella typhimurium* colonisation and microbial fermentation in the crop of the young turkey. *British Poultry Science*, 46, 708-716.

DAMACENO, Q. S., GALLOTTI, B., REIS, I. M. M., TOTTE, Y. C. P., ASSIS, G. B., FIGUEIREDO, H. C., SILVA, T. F., AZEVEDO, V., NICOLI, J. R. AND MARTINS, F. S. 2023. Isolation and identification of potential probiotic bacteria from human milk. *Probiotics and Antimicrobial Proteins*, 15, 491-501.

DARMASTUTI, A., HASAN, P. N., WIKANDARI, R., UTAMI, T., RAHAYU, E. S. AND SUROTO, D. A. 2021. Adhesion Properties of *Lactobacillus plantarum* Dad-13 and *Lactobacillus plantarum* Mut-7 on Sprague Dawley Rat Intestine. *Microorganisms*, 9, 2336.

DANZEISEN, J. L., KIM, H. B., ISAACSON, R. E., TU, Z. J. AND JOHNSON, T. J. 2011. Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PloS One*, 6, 27949.

DE MELO PEREIRA, G. V., DE OLIVEIRA COELHO, B., JÚNIOR, A. I. M., THOMAZ-SOCCOL, V. AND SOCCOL, C. R. 2018. How to select a probiotic? A review and update of methods and criteria. *Biotechnology Advances*, 36, 2060-2076.

DE PAULA, A. T., JERONYMO-CENEVIVA, A. B., SILVA, L. F., TODOROV, S. D., FRANCO, B. D. G. M. AND PENNA, A. L. B. 2015. *Leuconostoc mesenteroides* SJRP55: a potential probiotic strain isolated from Brazilian water buffalo mozzarella cheese. *Annals of Microbiology*, 65, 899-910.

DE SOUZA, E. L., DE OLIVEIRA, K. Á. AND DE OLIVEIRA, M. E. G. 2023. Influence of lactic acid bacteria metabolites on physical and chemical food properties. *Current Opinion in Food Science*, 49, 100981.

DENKOVA, R., GORANOV, B., TENEVA, D., DENKOVA, Z. AND KOSTOV, G. 2017. Antimicrobial activity of probiotic microorganisms: Mechanisms of interaction and methods of examination. *Antimicrobial Research: Novel Bioknowledge and Educational Programs*, 1, 201-212.

DE ALMEIDA JÚNIOR, W. L. G., DA SILVA FERRARI, Í., DE SOUZA, J. V., DA SILVA, C. D. A., DA COSTA, M. M. & DIAS, F. S. 2015. Characterization and evaluation of lactic acid bacteria isolated from goat milk. *Food control*, 53, 96-103.

DE SOUZA, B. M. S., BORGONOVI, T. F., CASAROTTI, S. N., TODOROV, S. D. & PENNA, A. L. B. 2019. *Lactobacillus casei* and *Lactobacillus fermentum* strains isolated from mozzarella cheese: probiotic potential, safety, acidifying kinetic parameters and viability under gastrointestinal tract conditions. *Probiotics and Antimicrobial Proteins*, 11, 382-396.

DERUNETS, A. S., SELIMZYANOVA, A. I., RYKOV, S. V., KUZNETSOV, A. E. & BEREZINA, O. V. 2024. Strategies to enhance stress tolerance in lactic acid bacteria across diverse stress conditions. *World Journal of Microbiology and Biotechnology*, 40, 126.

DIARRA, M. S. AND MALOUIN, F. 2014. Antibiotics in Canadian poultry productions and anticipated alternatives. *Frontiers in Microbiology*, 5, 282.

DIAS, R., VILAS-BOAS, E., CAMPOS, F. M., HOGG, T. AND COUTO, J. A. 2015. Activity of lysozyme on *Lactobacillus hilgardii* strains isolated from Port wine. *Food Microbiology*, 49, 6-11.

DONALDSON, E. E., STANLEY, D., HUGHES, R. J. AND MOORE, R. J. 2017. The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs. PeerJ, 5, 3587.

DIVYA, J., K K, V. & NAMPOOTHIRI, K. M. 2012. Newly isolated lactic acid bacteria with probiotic features for potential application in food industry. *Applied Biochemistry and Biotechnology*, 167, 1314-24.

DONKOR, O. N., HENRIKSSON, A., VASILJEVIC, T. AND SHAH, N. P. 2007. Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and *in vitro* angiotensin-converting enzyme inhibitory activity in fermented milk. *Le Lait*, 87, 21-38.

DOWARAH, R., VERMA, A. K., AGARWAL, N., SINGH, P. & SINGH, B. R. 2018. Selection and characterization of probiotic lactic acid bacteria and its impact on growth, nutrient digestibility, health and antioxidant status in weaned piglets. *PloS One*, 13, 0192978.

DURAI, N. S. P. 2019. Isolation and characterization of potential probiotic bacterium *Bacillus amyloqufaciens* cs4 from gut of fish Channa Striata Bloch 1793 and the biological evaluation of the bacterium produced metabolites and nanoparticles. Article IDPeriyar University, 83-108.

ELKENANY, R., ELSAYED, M. M., ZAKARIA, A. I., EL-SAYED, S. A. & RIZK, M. A. 2019. Antimicrobial resistance profiles and virulence genotyping of *Salmonella enterica* serovars recovered from broiler chickens and chicken carcasses in Egypt. *BioMed Central of Vetinary Research*, 15, 124.

EL-SAYED, A. I. M., EL-BORAI, A. M., AKL, S. H., EL-AASSAR, S. A. AND ABDEL-LATIF, M. S. 2022. Identification of *Lactobacillus* strains from human mother milk and cottage cheese revealed potential probiotic properties with enzymatic activity. *Scientific Reports*, 12, 22522.

ENDO, A., FUTAGAWA-ENDO, Y. AND DICKS, L. M. T. 2010. Diversity of *Lactobacillus* and *Bifidobacterium* in feces of herbivores, omnivores and carnivores. *Anaerobe*, 16, 590-596.

ESCOBAR-SÁNCHEZ, M.; CARRASCO-NAVARRO, U.; JUÁREZ-CASTELÁN, C.; LOZANO-AGUIRRE BELTRÁN, L.; PÉREZ-CHABELA, M.L.; PONCE-

ALQUICIRA, E. 2023. Probiotic Properties and Proteomic Analysis of *Pediococcus pentosaceus 1101*. *Foods*, 12, 46.

FOOD AND AGRICULTURAL ORGANIZATION 2017. FAOSTAT: Livestock primary. Food and Agriculture Organization Rome, Italy.

FATHIMA, S., SHANMUGASUNDARAM, R., ADAMS, D. & SELVARAJ, R. K. 2022. Gastrointestinal microbiota and their manipulation for improved growth and performance in chickens. *Foods*, 11, 1401.

FENG, J., JIANG, Y., LI, M., ZHAO, S., ZHANG, Y., LI, X., WANG, H., LIN, G., WANG, H., LI, T. AND MAN, C. 2018. Diversity and evolution of *Lactobacillus casei* group isolated from fermented dairy products in Tibet. *Archives of Microbiology*, 200,1111-1121.

FIJAN, S. 2023. Probiotics and Their Antimicrobial Effect. *Microorganisms*, 11, 528.

FIJAN, S., KOCBEK, P., STEYER, A., VODIČAR, P. M. AND STRAUSS, M. 2022. The antimicrobial effect of various single-strain and multi-strain probiotics, dietary supplements or other beneficial microbes against common clinical wound pathogens. *Microorganisms*, 10, 2518.

FILAZI, A., ŞIRELI, U. T., PEHLIVANLAR-ONEN, S., ÇADIRCI, Ö. AND AKSOY, A. 2013. Comparative pharmacokinetics of gentamicin in laying hens. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 19, 495-498.

FONSECA, B. B., BELETTI, M. E., SILVA, M. S. D., SILVA, P. L. D., DUARTE, I. N. AND ROSSI, D. A. 2010. Microbiota of the cecum, ileum morphometry, pH of the crop and performance of broiler chickens supplemented with probiotics. *Revista Brasileira De Zootecnia*, 39,1756-1760.

FONSECA, V. AND JOHN-KALARICKAL, J. 2010. Type 2 diabetes mellitus: epidemiology, genetics, pathogenesis, and clinical manifestations. *Principles of Diabetes Mellitus*, Article ID: 203-220.

FONSECA, H.C., DE SOUSA MELO, D., RAMOS, C.L. 2021. Probiotic Properties of Lactobacilli and Their Ability to Inhibit the Adhesion of Enteropathogenic Bacteria to Caco-2 and HT-29 Cells. *Probiotics & Antimicrobials*. 13, 102–112.

FONTANA, L., BERMUDEZ-BRITO, M., PLAZA-DIAZ, J., MUÑOZ-QUEZADA, S. & GIL, A. 2013. Sources, isolation, characterisation and evaluation of probiotics. *British Journal of Nutrition*, 109, S35-S50.

FOSTER, J. W. 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nature Reviews Microbiology*, 2, 898-907.

FULLER, R. 1989. Probiotics in man and animals. *The Journal of Applied Bacteriology*, 66, 365-378.

FUOCO, D. 2012. Classification framework and chemical biology of tetracycline-structure-based drugs. *Antibiotics (Basel)*, 1, 1-13.

GARCÍA-CAYUELA, T., KORANY, A. M., BUSTOS, I., DE CADIÑANOS, L. P. G., REQUENA, T., PELÁEZ, C. AND MARTÍNEZ-CUESTA, M. C. 2014. Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. *Food Research International*, 57, 44-50.

GARCÍA-HERNÁNDEZ, Y., PÉREZ-SÁNCHEZ, T., BOUCOURT, R., BALCÁZAR, J. L., NICOLI, J. R., MOREIRA-SILVA, J., RODRÍGUEZ, Z., FUERTES, H., NUÑEZ, O., ALBELO, N. AND HALAIHEL, N. 2016. Isolation, characterization and evaluation of probiotic lactic acid bacteria for potential use in animal production. *Research in Veterinary Science*, 108,125-132.

GAUVRY, E., MATHOT, A.-G., COUVERT, O., LEGUÉRINEL, I. & COROLLER, L. 2021. Effects of temperature, pH and water activity on the growth and the sporulation abilities of *Bacillus subtilis* BSB1. *International Journal of Food Microbiology*, 337, 108915.

GONG, J., SI, W., FORSTER, R. J., HUANG, R., YU, H., YIN, Y., YANG, C. AND HAN, Y. 2007. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiology Ecology*, 59,147-157.

GOVINDARAJ, K., SAMAYANPAULRAJ, V., NARAYANADOSS, V. & UTHANDAKALAIPANDIAN, R. 2021. Isolation of lactic acid bacteria from intestine of freshwater fishes and elucidation of probiotic potential for aquaculture application. *Probiotics and Antimicrobial Proteins*, 13, 1598-1610.

GUAN, C., CHEN, X., JIANG, X., ZHAO, R., YUAN, Y., CHEN, D., ZHANG, C., LU, M., LU, Z. AND GU, R. 2020. *In vitro* studies of adhesion properties of six lactic acid bacteria isolated from the longevous population of China. *Royal Science of Chemistry Advances*, 10, 24234-24240.

GUEIMONDE, M., SÁNCHEZ, B., DE LOS REYES-GAVILÁN, C. G. AND MARGOLLES, A. 2013. Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology*, 4, 202.

GUPTA, M., RAUT, R., MANANDHAR, S., CHAUDHARY, A., SHRESTHA, U., DANGOL, S., G, C. S., BUDHA, K. R., KARKI, G., DÍAZ-SÁNCHEZ, S., GORTAZAR, C., DE LA FUENTE, J., RAJBHANDARI, P., MANANDHAR, P., NAPIT, R. AND KARMACHARYA, D. 2023. Identification and characterization of probiotics isolated from indigenous chicken (Gallus domesticus) of Nepal. *PLoS One*, 18, 0280412.

HAMED, E. 2021. Isolation, characterization and identification of lactic acid bacteria as probiotic. *Annals of Agricultural Science, Moshtohor*, 59, 311-322.

HAN, G. G., KIM, E. B., LEE, J., LEE, J.-Y., JIN, G., PARK, J., HUH, C.-S., KWON, I.-K., KIL, D. Y. AND CHOI, Y.-J. 2016. Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. *Springerplus*, 5, 1-9.

HAVENAAR, R., BRINK, B., HUIS IN'T VELDT, J. AND FULLER, R. 1992. Probiotics: the scientific basis. *London: Chapmann and Hall*, Article ID: 209-224.

HANCHI, H., MOTTAWEA, W., SEBEI, K. & HAMMAMI, R. 2018. The Genus *Enterococcus*: between probiotic potential and safety concerns-an update. *Frontiers in Microbiology*, 9, 1791.

HERNANDEZ-PATLAN, D., SOLIS-CRUZ, B., HARGIS, B. M. AND TELLEZ, G. 2020. The use of probiotics in poultry production for the control of bacterial infections and aflatoxins. *Prebiotics and Probiotics-Potential Benefits in Nutrition and Health*, Article ID: 217-238.

HESARI, M. R., DARSANAKI, R. K. & SALEHZADEH, A. 2017. Antagonistic activity of probiotic bacteria isolated from traditional dairy products against E. coli O157: H7. *Journal of Medical Bacteriology*, 6, 23-30.

HUANG, C., QIAO, S., LI, D., PIAO, X. AND REN, J. 2004. Effects of Lactobacilli on the performance, diarrhea incidence, VFA concentration and gastrointestinal microbial flora of weaning pigs. *Asian-Australasian Journal of Animal Sciences*, 17, 401-409.

HUANG, J., ZHANG, W., HU, Z., LIU, Z., DU, T., DAI, Y. AND XIONG, T. 2020. Isolation, characterization and selection of potential probiotic lactic acid bacteria from feces of wild boar, native pig and commercial pig. *Livestock Science*, 237, 104036.

HULIGERE, S. S., CHANDANA KUMARI, V., ALQADI, T., KUMAR, S., CULL, C. A., AMACHAWADI, R. G. AND RAMU, R. 2023. Isolation and characterization of lactic acid bacteria with potential probiotic activity and further investigation of their activity by *α*-amylase and *α*-glucosidase inhibitions of fermented batters. *Frontiers in Microbiology*, 13, 1042263.

IBOURAHEMA, C., DAUPHIN, R. D., JACQUELINE, D. AND THONART, P. 2008. Characterization of lactic acid bacteria isolated from poultry farms in Senegal. *African Journal of Biotechnology*, 7, 2006-2012.

IBRAHIM, S. A., AYIVI, R. D., ZIMMERMAN, T., SIDDIQUI, S. A., ALTEMIMI, A. B., FIDAN, H., ESATBEYOGLU, T. AND BAKHSHAYESH, R. V. 2021. Lactic Acid Bacteria as Antimicrobial Agents: Food safety and microbial food spoilage prevention. *Foods*, 10, 3131.

JAFARI-NASAB, T., KHALEGHI, M., FARSINEJAD, A. & KHORRAMI, S. 2021. Probiotic potential and anticancer properties of *Pediococcus* sp. isolated from traditional dairy products. *Biotechnology Reports*, 29, e00593.

JAIMEE, G. AND HALAMI, P. M. 2016. High level aminoglycoside resistance in *Enterococcus*, *Pediococcus* and *Lactobacillus* species from farm animals and commercial meat products. *Annals of Microbiology*, 66, 101-110.

JAMMOUL, A. & EL DARRA, N. 2019. Evaluation of antibiotics residues in chicken meat samples in Lebanon. *Antibiotics* (*Basel*), 8, 69.

JANNAH, S. N., DINOTO, A., WIRYAWAN, K. G. & RUSMANA, I. 2014. Characteristics of lactic acid bacteria isolated from gastrointestinal tract of Cemani chicken and their potential use as probiotics. *Media Peternakan*, 37, 182-189.

JENI, R. E., DITTOE, D. K., OLSON, E. G., LOURENCO, J., SEIDEL, D. S., RICKE, S. C. AND CALLAWAY, T. R. 2021. An overview of health challenges in alternative poultry production systems. *Poultry Science*, 100, 101173.

JIN, L. Z., HO, Y. W., ABDULLAH, N. AND JALALUDIN, S. 1997. Probiotics in poultry: modes of action. *World's Poultry Science Journal*, 53, 351-368.

JOHN, A., MAKINDE, J., ACHENEJE, E., FELIX, M. & ADEWARA, O. 2024. Antibiotics in broilers chicken production: a review of impacts, challenges and potential alternatives. 559-578.

JOMEHZADEH, N., JAVAHERIZADEH, H., AMIN, M., SAKI, M., AL-OUQAILI, M. T., HAMIDI, H., SEYEDMAHMOUDI, M. & GORJIAN, Z. 2020. Isolation and identification of potential probiotic *Lactobacillus* species from feces of infants in southwest Iran. *International Journal of Infectious Diseases*, 96, 524-530.

JOSE, N. M., BUNT, C. R. & HUSSAIN, M. A. 2015. Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and animal rumen contents. *Microorganisms*, 3, 198-212.

JÓZEFIAK, D., RUTKOWSKI, A., JENSEN, B. AND ENGBERG, R. 2006. The effect of *α*-glucanase supplementation of barley-and oat-based diets on growth performance and fermentation in broiler chicken gastrointestinal tract. *British Poultry Science*, 47, 57-64.

JUNG, J. H., KIM, S. J., LEE, J., YOON, S. R., YOU, S.-Y. & KIM, S. 2019. Multifunctional properties of *Lactobacillus plantarum* strains WiKim83 and WiKim87 as a starter culture for fermented food. *Food Science & Nutrition*, 7, 2505-2516.

JUNGERSEN, M., WIND, A., JOHANSEN, E., CHRISTENSEN, J.E., STUER-LAURIDSEN, B. AND ESKESEN, D., 2014. The Science behind the Probiotic Strain *Bifidobacterium animalis subsp. lactis BB-12*. *Microorganisms*, *2*(2), pp.92-110.

KAEWCHOMPHUNUCH, T., CHAROENPICHITNUNT, T., THONGBAIYAI, V., NGAMWONGSATIT, N. & KAEOKET, K. 2022. Cell-free culture supernatants of *Lactobacillus* spp. and *Pediococcus* spp. inhibit growth of pathogenic *Escherichia coli* isolated from pigs in Thailand. *BioMed Central of Veterinary Research*, 18, 60.

KANAAN, M. H. G. 2023. Prevalence and antimicrobial resistance of *Salmonella enterica* serovars, *Salmonella enteritidis* and *Salmonella typhimurium* isolated from retail chicken meat in Wasit markets, Iraq. *Vetinary World*, 16, 455-463.

KAUR, S., KAUR, R., RANI, N., SHARMA, S. AND JOSHI, M. 2021. Sources and selection criteria of probiotics. *Advances in Probiotics for Sustainable Food and Medicine*, Article ID: 27-43.

KARIYAWASAM, K.M.G.M.M., YANG, S.J., LEE, N.K. AND PAIK, H.D., 2020. Probiotic properties of *Lactobacillus brevis* KU200019 and synergistic activity with

fructooligosaccharides in antagonistic activity against foodborne pathogens. *Food Science of Animal Resources*, 40, 297.

KARU, R. & SUMERI, I. 2016. Survival of *Lactobacillus rhamnosus* GG during simulated gastrointestinal conditions depending on food matrix. *Journal of Food Research*, 5, 56.

KERRY, R. G., PATRA, J. K., GOUDA, S., PARK, Y., SHIN, H.-S. & DAS, G. 2018. Benefaction of probiotics for human health: A review. *Journal of Food and Drug Analysis*, 26, 927-939.

KIELISZEK, M., POBIEGA, K., PIWOWAREK, K. AND KOT, A. M. 2021. Characteristics of the Proteolytic Enzymes Produced by Lactic Acid Bacteria. *Molecules*, 26, 1858.

KIM, S., HUANG, E., PARK, S., HOLZAPFEL, W. AND LIM, S. D. 2018. Physiological Characteristics and Anti-obesity Effect of *Lactobacillus plantarum* K10. *Korean Jornal of Food Science and Anim Resources*, 38, 554-569.

KIM, S., HONG, S. P. & LIM, S. D. 2021. Physiological Characteristics and Anti-Diabetic Effect of *Pediococcus pentosaceus* KI62. *Food Science and Animimal Resources*, 41, 274-287.

KIM, P. I., JUNG, M. Y., CHANG, Y.-H., KIM, S., KIM, S.-J. & PARK, Y.-H. 2007. Probiotic properties of *Lactobacillus* and *Bifidobacterium* strains isolated from porcine gastrointestinal tract. *Journal of Applied Microbiology and Biotechnology*, 74, 1103-1111.

KIMERA, Z.I., MSHANA, S.E., RWEYEMAMU, M.M. 2020. Antimicrobial use and resistance in food-producing animals and the environment: an African perspective. Antimicrobial Resistance and Infection Control 9, 37.

KOOK, S.-Y., CHUNG, E.-C., LEE, Y., LEE, D. W. & KIM, S. 2019. Isolation and characterization of five novel probiotic strains from Korean infant and children faeces. *PLoS One*, 14, 0223913.

KRAUSOVA, G., HYRSLOVA, I. & HYNSTOVA, I. 2019. *In Vitro* evaluation of adhesion capacity, hydrophobicity, and auto-Aggregation of newly isolated potential probiotic strains. *Fermentation*, 5, 100.

KRIEL, G. 2022. Biosecurity in Poultry Production Poultry Farming in South Africa. *South Africa Online* Available: https://southafrica.co.za/biosecurity-in-poultry-production.html (Accessed 04 February 2024).

KUMAR, S., VARADA, V. V., BANAKAR, P. S., TYAGI, N., CHOURADDI, R., HOGAREHALLI MALLAPA, R. & TYAGI, A. K. 2023. Screening and characterization of Sahiwal cattle calves-origin lactic acid bacteria based on desired probiotic attributes for potential application. *Animal Biotechnology*, 34, 1612-1625.

KWUN, S. Y., BAE, Y. W., YOON, J. A., PARK, E. H. & KIM, M. D. 2020. Isolation of acid tolerant lactic acid bacteria and evaluation of α-glucosidase inhibitory activity. *Food Science and Biotechnology,* 29, 1125-1130.

LAMBO, M. T., CHANG, X. AND LIU, D. 2021. The recent trend in the use of multistrain probiotics in livestock production: an overview. *Animals*, 11, 2805.

LIANG, T., XIE, X., ZHANG, J., DING, Y. & WU, Q. 2021. Bacterial community and composition of different traditional fermented dairy products in China, South Africa, and Sri Lanka by high-throughput sequencing of 16S rRNA genes. *Food and Technology*, 144, 111209.

LI, M., WANG, Y., CUI, H., LI, Y., SUN, Y. & QIU, H.-J. 2020. Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. Frontiers in veterinary science, 7, 49.

LI, X., LI, W., ZHAO, L., LI, Y., HE, W., DING, K. & CAO, P. 2024. Characterization and assessment of native lactic acid bacteria from broiler intestines for potential probiotic properties. *Microorganisms*, 12, 749.

LI, T., TENG, D., MAO, R., HAO, Y., WANG, X. AND WANG, J. 2020. A critical review of antibiotic resistance in probiotic bacteria. *Food Research International*, 136, 109571.

LIM, Y.-S., KIM, J. & KANG, H. 2019. Isolation and identification of lactic acid bacteria with probiotic activities from kimchi and their fermentation properties in milk. *Journal of Dairy Science and Biotechnology*, 37, 115-128.

LIU, W. J., CHEN, Y. F., KWOK, L. Y., LI, M. H., SUN, T., SUN, C. L., WANG, X. N., DAN, T., MENGHEBILIGE, ZHANG, H. P. & SUN, T. S. 2013. Preliminary selection for potential probiotic Bifidobacterium isolated from subjects of different Chinese ethnic groups and evaluation of their fermentation and storage characteristics in bovine milk. *Journal of Dairy Science*, 96, 6807-6817.

LOBIUC, A., PAVĂL, N. E., MANGALAGIU, II, GHEORGHIȚĂ, R., TELIBAN, G. C., AMĂRIUCĂI-MANTU, D. & STOLERU, V. 2023. Future Antimicrobials: Natural and functionalized phenolics. Molecules, 28, 1114.

LUTFUL KABIR, S. M. 2009. The role of probiotics in the poultry industry. *International Journal of Molecular Science*, 10, 3531-3546.

LYONS, K. E., RYAN, C. A., DEMPSEY, E. M., ROSS, R. P. & STANTON, C. 2020. Breast milk, a source of beneficial microbes and associated benefits for infant health. *Nutrients*, 12, 1039.

MOHANTY, D., PANDA, S., KUMAR, S. & RAY, P. 2019. *In vitro* evaluation of adherence and anti-infective property of probiotic *Lactobacillus plantarum* DM 69 against *Salmonella enterica. Microbial Pathogenesis*, 126, 212-217.

MACALUSO, G., FIORENZA, G., GAGLIO, R., MANCUSO, I. & SCATASSA, M. L. 2016. *In vitro* evaluation of bacteriocin-like inhibitory substances produced by lactic acid bacteria isolated during traditional Sicilian cheese making. *Italian Journal of Food Safety*, 5, 5503.

MAJEED, M., MAJEED, S., ARUMUGAM, S., ALI, F. & BEEDE, K. 2020. Comparative evaluation for thermostability and gastrointestinal survival of probiotic *Bacillus coagulans* MTCC 5856. *Bioscience, Biotechnology, and Biochemistry*, 85, 962-971.

MAKETE, G., AIYEGORO, O. A. AND THANTSHA, M. S. 2017. Isolation, identification and screening of potential probiotic bacteria in milk from south african saanen goats. *Probiotics and Antimicrobial Proteins*, 9, 246-254.

MALFA, P., BRAMBILLA, L., GIARDINA, S., MASCIARELLI, M., SQUARZANTI, D.F., CARLOMAGNO, F. AND MELONI, M., 2023. Evaluation of antimicrobial, antiadhesive and co-aggregation activity of a multi-strain probiotic composition against different urogenital pathogens. *International Journal of Molecular Sciences*, 2.1323.

MARKOWIAK, P. AND ŚLIŻEWSKA, K. 2018. The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathogens*, 10, 21.

MASKE, B. L., DE MELO PEREIRA, G. V., DA S. VALE, A., DE CARVALHO NETO, D. P., KARP, S. G., VIESSER, J. A., DE DEA LINDNER, J., PAGNONCELLI, M. G., SOCCOL, V. T. AND SOCCOL, C. R. 2021. A review on enzyme-producing lactobacilli associated with the human digestive process: From metabolism to application. *Enzyme and Microbial Technology*, 149, 109836.

MATHEW, S. 2019. Isolation and characterizatin of probiotic bacteria and develop probiotic enriched food. Article IDKannur University. Available: http://hdl.handle.net/10603/339395 . (Accessed 20 May 2024).

MAZZIOTTA, C., TOGNON, M., MARTINI, F., TORREGGIANI, E. & ROTONDO, J. C. 2023. Probiotics Mechanism of Action on Immune Cells and Beneficial Effects on Human Health. *Cells*, 12.

MOUSAPOOR, M., LOSACCO, C., SEIDAVI, A., NOSRATI, M., DE ARAÚJO, W. A. G., ALBINO, L. F. T., LAUDADIO, V., TUFARELLI, V. AND DE MARZO, D. 2023. Effect of a probiotic blend in broiler chicken diets and its effect on growth performance, carcass traits, and haematological profile. *South African Journal of Animal Science*, 53, 493-502.

MOUSSAVI, M., BAROUEI, J., EVANS, C., ADAMS, M. C. & BAINES, S. 2023. Viability and *in vitro* gastrointestinal transit tolerance of multispecies probiotic combinations incorporated into orange juice and drinking water. *Foods*, 12, 2249.

MULAW, G., SISAY TESSEMA, T., MULETA, D. AND TESFAYE, A. 2019. *In vitro* evaluation of probiotic properties of lactic acid bacteria isolated from some traditionally fermented ethiopian food products. *International Journal of Microbiology*, 2019, 7179514.

MUND, M. D., KHAN, U. H., TAHIR, U., MUSTAFA, B.-E. AND FAYYAZ, A. 2017. Antimicrobial drug residues in poultry products and implications on public health: A review. *International Journal of Food Properties*, 20, 1433-1446.

MUSIKASANG, H., TANI, A., H-KITTIKUN, A. AND MANEERAT, S. 2009. Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. *World Journal of Microbiology and Biotechnology*, 25, 1337-1345.

NAWAZ, M., WANG, J., ZHOU, A., MA, C., WU, X., MOORE, J. E., CHERIE MILLAR, B. AND XU, J. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Current Microbiology*, 62, 1081-1089.

NALLALA, V., SADISHKUMAR, V. & JEEVARATNAM, K. 2017. Molecular characterization of antimicrobial *Lactobacillus* isolates and evaluation of their probiotic characteristics *in vitro* for use in poultry. *Food Biotechnology*, 31, 20-41.

NAMI, Y., VASEGHI BAKHSHAYESH, R., MOHAMMADZADEH JALALY, H., LOTFI, H., ESLAMI, S. AND HEJAZI, M.A., 2019. Probiotic properties of *Enterococcus* isolated from artisanal dairy products. *Frontiers in Microbiology*, 10, 300.

NANDI, A., DAN, S. K., BANERJEE, G., GHOSH, P., GHOSH, K., RINGØ, E. & RAY, A. K. 2017. Probiotic potential of autochthonous bacteria isolated from the gastrointestinal tract of four freshwater teleosts. *Probiotics and Antimicrobial Proteins*, 9, 12-21.

NDOTONO, E.W., KHAMIS, F.M., BARGUL, J.L. AND TANGA, C.M., 2022. Gut microbiota shift in layer pullets fed on black soldier fly larvae-based feeds towards enhancing healthy gut microbial community. *Scientific Reports*, 12, 16714.

NGUNJIRI, J. M., TAYLOR, K. J., ABUNDO, M. C., JANG, H., ELAISH, M., KC, M., GHORBANI, A., WIJERATNE, S., WEBER, B. P. & JOHNSON, T. J. 2019. Farm stage, bird age, and body site dominantly affect the quantity, taxonomic composition, and dynamics of respiratory and gut microbiota of commercial layer chickens. *Applied and Environmental Microbiology*, 85, 03137-18.

NISHIYAMA, K., NAKAZATO, A., UENO, S., SETO, Y., KAKUDA, T., TAKAI, S., YAMAMOTO, Y. & MUKAI, T. 2015. Cell surface-associated aggregation-promoting factor from *Lactobacillus gasseri* SBT 2055 facilitates host colonization and competitive exclusion of *Campylobacter jejuni*. *Molecular Microbiology*, 98, 712-726.

NOOHI, N., PAPIZADEH, M., ROHANI, M., TALEBI, M. AND POURSHAFIE, M. R. 2021. Screening for probiotic characters in lactobacilli isolated from chickens revealed the intra-species diversity of *Lactobacillus brevis. Animal Nutrition*, 7, 119-126.

NUENO-PALOP, C. & NARBAD, A. 2011. Probiotic assessment of *Enterococcus faecalis* CP58 isolated from human gut. *International Journal of Food Microbiology*, 145, 390-394.

OBIOHA, P. I., OUOBA, L. I. I., ANYOGU, A., AWAMARIA, B., ATCHIA, S., OJIMELUKWE, P. C., SUTHERLAND, J. P. & GHODDUSI, H. B. 2021. Identification and characterisation of the lactic acid bacteria associated with the traditional fermentation of dairy fermented product. *Brazilian Journal of Microbiology*, 52, 869-881.

OJHA, A. K., SHAH, N. P. AND MISHRA, V. 2022. Characterization and transferability of *erm* and *tet* antibiotic resistance genes in *Lactobacillus* spp. isolated from traditional fermented milk. *Current Microbiology*, 79, 339.

OYEWOLE, O. A., MARIA, C., TOPE, P. AND FUNMI, O. 2018. *In vitro* study of potential probiotic lactic acid bacteria isolated from the gut of chickens in Abeokuta, Nigeria. *Alexandria Journal of Veterinary Sciences*, 58, 73-84.

PADMAVATHI, T., BHARGAVI, R., PRIYANKA, P. R., NIRANJAN, N. R. AND PAVITRA, P. V. 2018. Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification. *Journal of Genetic Engineering and Biotechnology*, 16, 357-362.

PAPADIMITRIOU, K., ALEGRÍA, Á., BRON, P. A., DE ANGELIS, M., GOBBETTI, M., KLEEREBEZEM, M., LEMOS, J. A., LINARES, D. M., ROSS, P., STANTON, C., TURRONI, F., VAN SINDEREN, D., VARMANEN, P., VENTURA, M., ZÚÑIGA, M., TSAKALIDOU, E. & KOK, J. 2016. Stress physiology of lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, 80, 837-90.

PAN, D. AND YU, Z. 2014. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, 5, 108-119.

PAUL, S., HOSSAIN, T. J., ALI, F., HOSSAIN, M. E., CHOWDHURY, T., FAISAL, I. K. & FERDOUSE, J. 2024. Assessment of the *in vitro* probiotic efficacy and safety of *Pediococcus pentosaceus* L1 and *Streptococcus thermophilus* L3 isolated from Laban, a popular fermented milk product. *Archives of Microbiology*, 206, 82.

PATEL, S., SHUKLA, R. & GOYAL, A. 2015. Probiotics in valorization of innate immunity across various animal models. *Journal of Functional Foods*, 14, 549-561.

PELLISSERY, A. J., VINAYAMOHAN, P. G., AMALARADJOU, M. A. & VENKITANARAYANAN, K. 2020. Spoilage bacteria and meat quality. Meat Quality Analysis.

PEREIRA, W. A., FRANCO, S. M., REIS, I. L., MENDONÇA, C. M. N., PIAZENTIN, A. C. M., AZEVEDO, P. O. S., TSE, M. L. P., DE MARTINIS, E. C. P., GIERUS, M. & OLIVEIRA, R. P. S. 2022. Beneficial effects of probiotics on the pig production cycle: An overview of clinical impacts and performance. *Veterinary Microbiology*, 269, 109431.

PFEILER, E. A., AZCARATE-PERIL, M. A. & KLAENHAMMER, T. R. 2007. Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. *Journal of Bacteriology*, 189, 4624-4634.

PINTO, M. G. V., SCHUSTER, T., BRIVIBA, K., WATZL, B., HOLZAPFEL, W. H. AND FRANZ, C. M. 2007. Adhesive and chemokine stimulatory properties of potentially probiotic *Lactobacillus* strains. *Journal of Food Protection*, 70, 125-134.

POIMENIDOU, S. V., SKARVELI, A., SAXAMI, G., MITSOU, E. K., KOTSOU, M. & KYRIACOU, A. 2023. Inhibition of *Listeria monocytogenes* growth, adherence and invasion

in Caco-2 cells by potential probiotic lactic acid bacteria isolated from fecal samples of healthy neonates. *Microorganisms*, 11, 363.

PRAMANIK, S., VENKATRAMAN, S., KARTHIK, P. & VAIDYANATHAN, V. K. 2023. A systematic review on selection characterization and implementation of probiotics in human health. *Food Science and Biotechnology*, 32, 423-440.

PRETE, R., LONG, S. L., GALLARDO, A. L., GAHAN, C. G., CORSETTI, A. AND JOYCE, S. A. 2020. Beneficial bile acid metabolism from *Lactobacillus plantarum* of food origin. *Scientific Reports*, 10, 1165.

QI, Y., HUANG, L., ZENG, Y., LI, W., ZHOU, D., XIE, J., XIE, J., TU, Q., DENG, D. & YIN, J. 2021. *Pediococcus pentosaceus*: screening and application as probiotics in food processing. *Frontiers in Microbiology*, 12, 762467.

RAMAN, J., KIM, J.-S., CHOI, K. R., EUN, H., YANG, D., KO, Y.-J. & KIM, S.-J. 2022. Application of lactic acid bacteria (LAB) in sustainable agriculture: advantages and limitations. *International Journal of Molecular Sciences*, 23, 7784.

RAMOS, S., SILVA, V., DAPKEVICIUS, M. L. E., IGREJAS, G. & POETA, P. 2020. Enterococci, from harmless bacteria to a pathogen. *Microorganisms*, 8, 1118.

RASHAD HAMEED, S. & ABDUL SATTAR SALMAN, J. 2023. Co-aggregative effect of probiotics bacteria against diarrheal causative bacteria. *Archives of Razi Institute*, 78, 831-841

REUBEN, R. C., ROY, P. C., SARKAR, S. L., ALAM, R.-U. AND JAHID, I. K. 2019. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BioMedCentral Microbiology*, 19, 1-20.

ROCHA-RAMÍREZ, L. M., HERNÁNDEZ-CHIÑAS, U., MORENO-GUERRERO, S. S., RAMÍREZ-PACHECO, A. & ESLAVA, C. A. 2021. Probiotic properties and immunomodulatory activity of *Lactobacillus* Strains isolated from dairy products. *Microorganisms*, 9, 825.

RODRIGUES, I. AND CHOCT, M. 2018. The foregut and its manipulation via feeding practices in the chicken. *Poultry Science*, 97, 3188-3206.

RUBIN, D. C. AND SHAKER, A. 2015. Small intestine: anatomy and structural anomalies. *Yamada's Textbook of Gastroenterology*, Article ID: 73-92.

RYAN, K. A., JAYARAMAN, T., DALY, P., CANCHAYA, C., CURRAN, S., FANG, F., QUIGLEY, E. M. & O'TOOLE, P. W. 2008. Isolation of lactobacilli with probiotic properties from the human stomach. *Letters in Applied Microbiology*, 47, 269-274.

Rychlik, I. 2020. Composition and function of chicken gut microbiota. *Animals*, 10, 103.

SAARELA, M., ALAKOMI, H. L., MÄTTÖ, J., AHONEN, A. M. & TYNKKYNEN, S. 2011. Acid tolerant mutants of *Bifidobacterium animalis* subsp. *lactis* with improved stability in fruit juice. *LWT - Food Science and Technology*, 44, 1012-1018.

SALEM, H. M., SAAD, A. M., SOLIMAN, S. M., SELIM, S., MOSA, W. F. A., AHMED, A. E., JAOUNI, S. K. A., ALMUHAYAWI, M. S., EL-HACK, M. E. A., EL-TARABILY, K. A. AND EL-SAADONY, M. T. 2023. Ameliorative avian gut environment and bird productivity through the application of safe antibiotics alternatives: A comprehensive review. *Poultry Science*, 102, 032-5791.

SOUTH AFRICAN POULTRY ASSOCIATION (SAPA), 2019. Available: https://www.agriseta.co.za/wp-content/uploads/2021/02/Poultry.pdf (Accessed 04 February 2023).

SATHYABAMA, S., VIJAYABHARATHI, R., BRUNTHA DEVI, P., RANJITH KUMAR, M. AND PRIYADARISINI, V. B. 2012. Screening for probiotic properties of strains isolated from feces of various human groups. *Journal of Microbiology*, 50, 603-612.

SHAHBAZ, F., MUCCEE, F., SHAHAB, A., SAFI, S. Z., ALOMAR, S. Y. & QADEER, A. 2024. Isolation and *in vitro* assessment of chicken gut microbes for probiotic potential. *Frontiers in Microbiology*, 15, 1278439.

SHANG, Y., KUMAR, S., OAKLEY, B. AND KIM, W. K. 2018. Chicken gut microbiota: importance and detection technology. *Frontiers in Veterinary Science*, 5, 254.

SHARMA, H., FIDAN, H., ÖZOGUL, F. AND ROCHA, J. M. 2022. Recent development in the preservation effect of lactic acid bacteria and essential oils on chicken and seafood products. *Frontiers in Microbiology*, 13, 1092248.

SHI, Y., ZHAI, M., LI, J. & LI, B. 2020. Evaluation of safety and probiotic properties of a strain of *Enterococcus faecium* isolated from chicken bile. *Journal of Food Science and Technology*, 57, 578-587.

SHOKRYAZDAN, P., FASELEH JAHROMI, M., LIANG, J. B. AND HO, Y. W. 2017. Probiotics: from isolation to application. *Journal of American Collegel Nutrition*, 36, 666-676.

SHOKRYAZDAN, P., KALAVATHY, R., SIEO, C., ALITHEEN, N., LIANG, J., JAHROMI, M. AND HO, Y. 2014. Isolation and characterization of *Lactobacillus* strains as potential probiotics for chickens. *Pertanika Journal of Tropical Agricultural Science*, 37, 141-157.

SILVA, D. R., SARDI, J. D. C. O., DE SOUZA PITANGUI, N., ROQUE, S. M., DA SILVA, A. C. B. & ROSALEN, P. L. 2020. Probiotics as an alternative antimicrobial therapy: Current reality and future directions. *Journal of Functional Foods*, 73, 104080.

SIRISOPAPONG, M., SHIMOSATO, T., OKRATHOK, S. AND KHEMPAKA, S. 2023. Assessment of lactic acid bacteria isolated from the chicken digestive tract for potential use as poultry probiotics. *Animal Bioscience*, 36, 1209-1220.

SON, S.-H., JEON, H.-L., YANG, S.-J., LEE, N.-K. AND PAIK, H.-D. 2017. *In vitro* characterization of *Lactobacillus brevis* KU15006, an isolate from kimchi, reveals anti-adhesion activity against foodborne pathogens and antidiabetic properties. *Microbial Pathogenesis*, 112, 135-141.

SRIFANI, A., MIRNAWATI, M., MARLIDA, Y., RIZAL, Y., NURMIATI, N. & LEE, K. W. 2024. Identification of novel probiotic lactic acid bacteria from soymilk waste using the 16s rRNA gene for potential use in poultry. *Veterinary World*, 1001-1011.

STEFAŃSKA, I., KWIECIEŃ, E., JÓŹWIAK-PIASECKA, K., GARBOWSKA, M., BINEK, M. AND RZEWUSKA, M. 2021. Antimicrobial susceptibility of lactic acid bacteria strains of potential use as feed additives: The basic safety and usefulness criterion. *Frontiers in Veterinary Science*, 8, 687071.

SURESHKUMAR, S., JUNG, S. K., KIM, D., OH, K. B., YANG, H., LEE, H. C., JO, Y. J., LEE, H. S., LEE, S. AND BYUN, S. J. 2020. Administration of *L. salivarius* expressing 3D8 scFv as a feed additive improved the growth performance, immune homeostasis, and gut microbiota of chickens. *Animal Science Journal*, 91, 13399.

ŠUŠKOVIĆ, J., KOS, B., BEGANOVIĆ, J., LEBOŠ PAVUNC, A., HABJANIČ, K. AND MATOŠIĆ, S. 2010. Antimicrobial activity–the most important property of probiotic and starter lactic acid bacteria. *Food Technology and Biotechnology*, 48, 296-307.

TAHERI, H., MORAVEJ, H., TABANDEH, F., ZAGHARI, M. AND SHIVAZAD, M. J. P. S. 2009. Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. *Poultry Science*, 88, 1586-1593.

TAWAB, F., ELKADR, M., SULTAN, A., HAMED, O., SAMIR, A. & AHMED, M. 2023. Probiotic potentials of lactic acid bacteria isolated from Egyptian fermented food. *Scientific Reports*, 13, 16601.

TAYE, Y., DEGU, T., FESSEHA, H. & MATHEWOS, M. 2021. Isolation and identification of lactic acid bacteria from cow milk and milk products. *The Scientific World Journal*, 4697445.

THAMES, H. T. & SUKUMARAN, A. T. 2020. A Review of *Salmonella* and *Campylobacter* in broiler meat: emerging challenges and food safety measures. *Foods*, 9, 776.

TIAN, C., WANG, L., LIU, M., LIU, J., QIU, M. & CHEN, Y. 2024. Isolation and identification of chicken-derived lactic acid bacteria: *in vitro* probiotic properties and antagonistic effects against *Salmonella pullorum*, *Staphylococcus aureus*, and *Escherichia coli*. *Microorganisms*, 12, 795.

TIWARI, P., TIWARI, P., PANDEY, S. AND PANDEY, P. 2012. Promising future of probiotics for human health: Current scenario. *Chronicles of Young Scientists*, 3, 17-28.

TODOROV, S. D., FURTADO, D. N., SAAD, S. M. I., TOME, E. AND FRANCO, B. D. G. D. M. 2011. Potential beneficial properties of bacteriocin-producing lactic acid bacteria isolated from smoked salmon. *Journal of Applied Microbiology*, 110, 971-986.

TOROK, V. A., OPHEL-KELLER, K., LOO, M. AND HUGHES, R. J. 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Applied and Environmental Microbiology*, 74, 783-791.

TORTUERO, F. 1973. Influence of the implantation of *Lactobacillus Acidophilus* in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. *Poultry Science*, 52, 197-203.

TSEGA, K. T., MAINA, K. AND BERHANE, N. 2019. Characterization of lactic acid bacteria isolated from the gastrointestinal tract of ethiopian rhode island red chicken and their potential use as probiotics. *Research Square*, Article ID.

TULUMOGLU, S., YUKSEKDAG, Z. N., BEYATLI, Y., SIMSEK, O., CINAR, B. & YAŞAR, E. 2013. Probiotic properties of lactobacilli species isolated from children's feces. *Anaerobe*, 24, 36-42.

TUO, Y., YU, H., AI, L., WU, Z., GUO, B. AND CHEN, W. 2013. Aggregation and adhesion properties of 22 *Lactobacillus* strains. *Journal of Dairy Science*, 96, 4252-4257.

VANTSAWA, P. A., MARYAH, U. T. & BULUS, T. 2017. Isolation and identification of lactic acid bacteria with probiotic potential from fermented cow milk (nono) in Unguwar Rimi Kaduna State Nigeria. *American Journal of Molecular Biology*, 7, 99-106.

VANCAMELBEKE, M. & VERMEIRE, S. 2017. The intestinal barrier: a fundamental role in health and disease. *Expert Rev Gastroenterol Hepatol,* 11**,** 821-834.

UEZEN, J. D., FICOSECO, C. A., FÁTIMA NADER-MACÍAS, M. E. AND VIGNOLO, G. M. 2023. Identification and characterization of potential probiotic lactic acid bacteria isolated from pig feces at various production stages. *Canadian Journal of Veterinary Research*, 87, 127-145.

VASIEE, A., FALAH, F. & MORTAZAVI, S. A. 2022. Evaluation of probiotic potential of autochthonous lactobacilli strains isolated from Zabuli yellow kashk, an Iranian dairy product. *Journal of Applied Microbiology*, 133, 3201-3214.

VEERAPAGU, M., JEYA, K. R., KHALIFA, A., SANKARANARAYANAN, A. Dwivedi, M. K., AMARESAN, N., SANKARANARAYANAN, A. AND BEGUM, R 2022. Determination of Antibiotic Resistance. *Springer*, 71-84.

VIECO-SAIZ, N., BELGUESMIA, Y., RASPOET, R., AUCLAIR, E., GANCEL, F., KEMPF, I. AND DRIDER, D. 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Frontier Microbiology*, 10, 57.

WANG, C., CUI, Y. AND QU, X. 2018. Mechanisms and improvement of acid resistance in lactic acid bacteria. *Archives of Microbiology*, 200, 195-201.

WANG, L., LIN, Z., ALI, M., ZHU, X., ZHANG, Y., LI, S., LI, K., KEBZHAI, F. & LI, J. 2023. Effects of lactic acid bacteria isolated from Tibetan chickens on the growth performance and gut microbiota of broiler. *Frontiers in Microbiology*, 14, 1171074.

WANG, P., CHEN, S., LIAO, C., JIA, Y., LI, J., SHANG, K., CHEN, J., CAO, P., LI, W., LI, Y., YU, Z. & DING, K. 2022. Probiotic properties of chicken-derived highly adherent lactic acid bacteria and inhibition of enteropathogenic bacteria in Caco-2 cells. *Microorganisms*, 10, 2515.

WANG, X., WANG, W., LV, H., ZHANG, H., LIU, Y., ZHANG, M., WANG, Y. AND TAN, Z. 2021. Probiotic potential and wide-spectrum antimicrobial activity of lactic acid bacteria isolated from infant feces. *Probiotics Antimicrobial Proteins*, 13, 90-101.

WHITEHEAD, K., VERSALOVIC, J., ROOS, S. & BRITTON, R. A. 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. *Applied and Environmental Microbiology*, 74, 1812-1819.

WON, G., CHOI, S.-I., PARK, N., KIM, J.-E., KANG, C.-H. & KIM, G.-H. 2021. *In vitro* antidiabetic, antioxidant activity, and probiotic activities of *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* Strains. *Current Microbiology*, 78, 3181-3191.

WORLD HEALTH ORGANIZATION 2017. Antimicrobial resistance: The food chain. Available:https://www.who.int/news-room/questions-and-answers/item/antimicrobial-resistance-in-the-food-chain. (Accessed 18 May 2024).

WU-WU, J. W. F., GUADAMUZ-MAYORGA, C., OVIEDO-CERDAS, D. & ZAMORA, W. J. 2023. Antibiotic Resistance and Food Safety: Perspectives on New Technologies and Molecules for Microbial Control in the Food Industry. *Antibiotics (Basel)*, 12.

XU, Y. B., LI, D. L., DING, X. Q., WANG, Y. Y., LIANG, S., XIE, L. Y., ZHANG, Y. F., FU, A. K., YU, W. Q. AND ZHAN, X. A. 2023. Probiotic characterization and comparison of broiler-derived *lactobacillus* strains based on technique for order preference by similarity to ideal solution analysis. *Poultry Science*, 102, 102564.

YADAV, R. & SHUKLA, P. 2017. An overview of advanced technologies for selection of probiotics and their expediency: a review. *Critical Reviews in Food science and Nutrition*, 57, 3233-3242.

YAO, M., XIE, J., DU, H., MCCLEMENTS, D. J., XIAO, H. & LI, L. 2020. Progress in microencapsulation of probiotics: A review. *Comprehensive Reviews in Food Science and Food Safety*, 19, 857-874.

YANG, X., HUANG, J., ZHANG, Y., LIU, S., CHEN, L., XIAO, C., ZENG, H., WEI, X., GU, Q., LI, Y., WANG, J., DING, Y., ZHANG, J. & WU, Q. 2020. Prevalence, abundance, serovars and antimicrobial resistance of *Salmonella* isolated from retail raw poultry meat in China. *Science of the Total Environment*, 713, 136385.

YANG, X., HONG, J., WANG, L., CAI, C., MO, H., WANG, J., FANG, X. & LIAO, Z. 2024. Effect of lactic acid bacteria fermentation on plant-based products. *Fermentation*, 10, 48.

YEGANI, M. AND KORVER, D. 2010. Manipulation of poultry gut microflora with probiotics. *Feed International*, 31, 20-21.

YENIZEY, M. Á.-C. AND EDITH, P.-A. 2018. Antibiotic Resistance in Lactic Acid Bacteria. *IntechOpen*, Chapter, 4. Available: https://doi.org/10.5772/intechopen.80624 (Accessed 2024-03-14).

YU, Y., XU, Y., LI, L., CHEN, S., AN, K., YU, Y. & XU, Z.-L. 2023. Isolation of lactic acid bacteria from Chinese pickle and evaluation of fermentation characteristics. *Food Science and Technology*, 180, 114627.

ZAWISTOWSKA-ROJEK, A., KOŚMIDER, A., STĘPIEŃ, K. AND TYSKI, S. 2022. Adhesion and aggregation properties of *Lactobacillaceae* strains as protection ways against enteropathogenic bacteria. *Archives of Microbiology*, 204, 285.

ZHENG, J., LIAO, Y., LI, Y., LI, D., SUN, Y. AND XIAO, Z., 2024. Description of *Microbacterium dauci,* a plant growth hormone indoleacetic acid-producing and nitrogen-fixing bacterium isolated from carrot rhizosphere soil. *Archives of Microbiology*, 206, 102.

ZOMMITI, M., CONNIL, N., HAMIDA, J. B. & FERCHICHI, M. 2017. Probiotic characteristics of *Lactobacillus curvatus* DN317, a strain isolated from chicken ceca. *Probiotics and Antimicrobial Proteins*, 9, 415-424.

ZHOU, N., ZHANG, J. X., FAN, M. T., WANG, J., GUO, G. AND WEI, X. Y. 2012. Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. *Journal of Dairy Science*, 95, 4775-4783.

8 APPENDICES

8.1 APPENDIX 8.1: Antibiotics and their standard zone of inhibition

Antibiotic class	Antibiotics	Concentration (µg)	Standard zone of inhibition (mm)		
			Resistant (R)	Intermediate (I)	Susceptible (S)
Aminoglycosides	Gentamicin	10 µg	≤12	13-14	≥15
	Streptomycin	10µg	≤11	12-14	≥15
Amphenicols	Chloramphenicol	30 µg	≤12	13-17	≥18
Cephalosporins	Ceftriaxone	30 µg	≤13	14-20	≥21
Fluoroquinolones	Ciprofloxacin	5 µg	≤15	16-20	≥21
Glycopeptides	Vancomycin	30 µg	≤14	15-16	≥17
Macrolides	Erythromycin	15 µg	≤13	14-22	≥23
Penicillin	Ampicillin	10 µg	≤11	12-13	≥14
	Penicillin	10 µg	≤19	20-27	≥28
	Oxacillin	1 µg	≤10	11-12	≥13
Tetracyclines	Tetracycline	30µg	≤14	15-18	≥19
Aminocoumarin	Novobiocin	5 µg	≤17	18-21	≥22

8.2 APPENDIX 8.2: Antibiotic susceptibility profiles of LAB isolated from the crops and small intestine of broiler chickens. Zones of inhibition are shown as mean ± SEM.

	C4	C5	C7	C13	C24	SI2	SI4	SI6	SI7	SI8	SI9	SI22	SI23	SI38	SI42	*L. casei* (ATCC 393)
Gentamicin	0±0	11±1	0±0	14±1	15,5±2,5	14±0	18±4	11±1	13±1	15,5±0,5	11,5±1,5	14±2	13±1	14±1	12±0	19±0
Streptomycin	0±0	0±0	0±0	0±0	0±0	0±0	17±5	0±0	17±2	16,5±1,5	14,5±0,5	0±0	0±0	0±0	0±0	0±0
Chloramphenicol	31±0	28,5±1,5	29,5±0,5	26±0	32±4	33±3	0±0	30±1	33±1	32,5±2,5	32±2	29,5±1,5	25,5±4,5	25,5±5,5	34±1	0±0
Ceftriaxone	25,5±0,5	27,5±2,5	18±3	17±1	28±5	31±1	27,5±1,5	30,5±1,5	32±3	24±1	23,5±1,5	0±0	20,5±2,5	22,5±2,5	29,5±0,5	0±0
Ciprofloxacin	17,5±2,5	0±0	7,5±7,5	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	13±1
Vancomycin	21±1	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Erythromycin	0±0	27,5±0,5	22,5±2,5	29±1	27±0	35±0	32±0	31±1	32,5±1,5	31,5±0,5	27,5±2,5	0±0	24,5±1,5	24,5±2,5	34,5±0,5	23,5±1,5
Ampicillin	27,5±2,5	32±3	26±6	19±1	24,5±2,5	27±0	30,5±5,5	31±1	27±1	32,5±1,5	30±0	0±0	21,5±1,5	22±4	26±1	23,5±0,5
Penicillin	20,5±1,5	21±2	22±1	22,5±0,5	27±1	34±2	24±0	22±1	23,5±1,5	19±1	20,5±0,5	0±0	23,5±3,5	22,5±4,5	32,5±0,5	14,5±1,5
Oxacillin	16,5±3,5	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	5,5±5,5	0±0	0±0
Tetracycline	0±0	21±1	22±2	20,5±1,5	17±3	32±1	33±1	25±1	33±1	24±1	17,5±1,5	0±0	21±9	23±5	31,5±2,5	0±0
Novobiocin	13,5±3,5	0±0	18,5±4,5	18±3	23,5±1,5	26±1	12,5±2,5	16±1	0±0	13,5±0,5	11,5±0,5	0±0	14,5±3,5	17,5±0,5	24±1	12±2