THE SURVIVAL STUDY OF *Lactobacillus reuteri* IN THE PRESENCE OF SELECTED SUPPOSITORY BASES AND *Escherichia coli*

BY

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SUPERVISED BY

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FACULTY OF PHARMACY,

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PRESENTED TO THE DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY AND BIOTECHNOLOGY, FACULTY OF PHARMACY,

UNIVERSITY OF BENIN.

A RESEARCH WORK SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DOCTOR OF PHARMACY DEGREE BY THE FACULTY OF PHARMACY UNIVERSITY OF BENIN

NOVEMEBER, 2025.

DECLARATION

I, **DIVINE UDELE** with matriculation number **PHA2003777** hereby declare that the project work entitled **THE SURVIVAL STUDY OF *Lactobacillus reuteri* IN THE PRESENCE OF SELECTED SUPPOSITORY BASES AND *Escherichia coli*** is the original work carried out by me under the supervision of Dr E. Oloton in the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Benin, Benin-city, Nigeria.

_____ _____

DIVINE UDELE **DATE**

CERTIFICATION

This is to certify this is an original research work carried out by **DIVINE UDELE** in the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Benin, in partial fulfillment of the requirements for the award of Doctor of Pharmacy degree.

_____ _____

DIVINE UDELE **DATE**

(PROJECT STUDENT)

_____ _____

DR E. OLOTON **DATE**

(PROJECT SUPERVISOR)

_____ _____

DR MRS. UPE BABAIWA **DATE**

(HEAD OF DEPARTMENT)

DEDICATION

This project work is dedicated to God Almighty for the gift of life, the zeal, wisdom and strength throughout my academic pursuit.

ACKNOWLEGEMENT

My profound gratitude goes to God Almighty for His divine protection and provision throughout my studies.

I sincerely appreciate the guidance and support of my supervisor, Dr. E. Oloton whose expertise and encouragement made this research work a success. My sincere appreciation also goes to the Head of Department; Dr Mrs. Upe Babaiwa, the lecturers and staffs of the department of Pharmaceutical Microbiology and Biotechnology for their contribution to my academic pursuit.

I am also grateful to my project colleagues for their maximum support during the course of this work.

My sincere appreciation to my parents, siblings and friends for their care, support and prayers throughout the period of study.

Finally, my gratitude to the Dean; Prof Igbe Ighodaro, the lecturers and staff in the Faculty of Pharmacy for their support and academic mentorship.

All of these contributions has shaped my career journey and personality and I am truly grateful.

ABSTRACT

Probiotic organisms coexist with a diverse range of pathogenic microbes within mucosal environments, where survival is influenced by ecological competition and surrounding physicochemical conditions. *Lactobacillus reuteri* is known to exert antagonistic effects against *Escherichia coli*, yet its viability can be altered by the medium in which it is delivered. This study evaluated the survival of *L. reuteri* in selected suppository bases in the presence of *E. coli*. *L. reuteri* was incorporated into glycerogelatin, polyethylene glycol (PEG), and theobroma bases, and co-incubated with *E. coli.* Viable counts were monitored over time and survival was analysed using linear regression, while differences in survival relative to the control were evaluated using paired t-test.

Theobroma supported the highest survival of *L. reuteri* (slope = 0.26; R^2 = 0.795), followed by polyethylene glycol (slope = 0.25; R^2 = 0.799), indicating that these environments better maintained microbial viability under competitive stress. In contrast, glycerogelatin significantly reduced *L. reuteri* survival (slope = 0.06; R^2 = 0.229), with the reduction being highly significant ($P < 0.001$), suggesting strong susceptibility to inhibitory effects.

The result of the finding indicate that the survival of *L. reuteri* in the presence of *E. coli* is markedly influenced by the surrounding base, and theobroma base with a lipid and polymeric nature provide more favorable conditions for probiotic persistence than glycerogelatin which is a hydrophilic gelatinous systems.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF STUDY

Probiotics are live bacteria that, when consumed in enough amounts, improve the host's health by restoring or maintaining a balanced microbiota.(FAO/WHO, 2002). Over recent decades, their applications have expanded beyond functional foods into pharmaceutical dosage forms due to increasing evidence of their therapeutic roles in managing infections of the gastrointestinal, vaginal, and urinary tracts (Spinler *et al.*, 2017; Jastrząb *et al.*, 2021). *Lactobacillus reuteri* remains one of the most widely researched probiotic species because of its strong affinity for mucosal surfaces, potent antimicrobial activity, and its ability to inhibit pathogens like *Escherichia coli* through the secretion of reuterin and organic acids.

Although probiotics offer significant health benefits, their clinical performance is often hampered by low viability during processing, storage, and delivery. Factors such as changes in temperature, oxygen exposure, pH fluctuations, and interactions with formulation components can lead to decreased cell survival before administration (Rokka &Rantamäki, 2010; Liu *et al.*, 2020). Consequently, enhancing probiotic stability through suitable dosage forms that maintain viability during storage and at the site of action has become a major focus within pharmaceutical microbiology and biotechnology.

Suppositories represent a promising but relatively underexplored option for probiotic delivery. They provide direct access to mucosal tissues such as the vaginal or rectal regions, bypass gastrointestinal degradation, and promote localized therapeutic effects (Saarela *et al.*, 2000). However, the survival of probiotic in suppository bases largely depends on the physicochemical

nature of the base material. Lipid bases like theobroma can help protect microorganisms from oxidative and osmotic stress, while hydrophilic and polymeric carriers such as polyethylene glycol (PEG) and glycerogelatin may differently regulate moisture transfer and hydration levels (Kanjan *et al.*, 2022; Shori, 2023).

Also, the coexistence of probiotics with pathogenic bacteria especially *Escherchia coli*, a frequent mucosal pathogen can further influence probiotic viability through nutrient competition, acid generation, or bacteriocin secretion (Ouwehand &Vesterlund, 2004). A thorough understanding of these microbial interactions is critical for developing stable probiotic formulations that preserve both viability and biological activity during administration.

Hence, this study investigates the survival profile of *Lactobacillus reuteri* incorporated into various suppository bases in the presence of *Escherichia coli.* The findings are expected to offer insights that will aid in designing stable, safe, and effective probiotic suppositories for pharmaceutical use.

1.2 AIM OF STUDY

The survival of *Lactobacillus reuteri* in selected suppository bases in the presence of *Escherichia coli.*

1.3 OBJECTIVES OF THE STUDY

1. To evaluate the survival of *Lactobacillus reuteri* in the presence of selected suppository bases and *Escherichia coli.*

2. To identify the suppository base that best maintains the survival of *Lactobacillus reuteri* in the presence *Escherichia coli.*

1.4 JUSTIFICATION OF THE STUDY

The therapeutic efficacy of probiotics relies not only on their biological properties but also on their ability to maintain viability and metabolic activity within both formulation matrices and physiological environments. Most commercially available probiotic products are intended for oral use; however, such formulations are susceptible to degradation by gastric acid, bile salts, and poor storage stability (Shori, 2023). Suppository formulations, on the other hand, present a viable alternative offering localized delivery to the mucosal surface, minimizing degradation, and enabling targeted action (Saarela et al., 2000; Kanjan *et al.*, 2022).

Nevertheless, the physicochemical characteristics of suppository excipients critically influence probiotic stability and performance. Lipid-based matrices such as theobroma provide a protective environment against oxidation and osmotic shock, whereas polymeric systems like polyethylene glycol promote controlled hydration and moderate osmotic conditions (Rokka &Rantamäki, 2010; Liu *et al.*, 2020). Conversely, glycerogelatin bases, with their high water content and protein composition, may induce osmotic stress or cause cell lysis, leading to decreased probiotic viability (Shori, 2023).

Furthermore, probiotics seldom exist in isolation in natural habitats; they typically coexist with pathogenic organisms such as *E. coli,* which may compete for nutrients or release inhibitory substances affecting probiotic persistence (Spinler *et al.*, 2017; Ouwehand &Vesterlund, 2004). Despite the biological importance of this coexistence, only a few studies have explored the combined effects of suppository base properties and microbial antagonism on probiotic stability.

This study is therefore justified as it aims to assess the survival of *Lactobacillus reuteri* formulated in different suppository bases and co-cultured with *Escherichia coli.* The outcomes will contribute

to the scientific understanding of formulation microbe interactions, inform the rational design of probiotic suppositories, and support the development of stable, effective probiotic dosage forms in the fields of pharmaceutical microbiology and biotechnology.

1.5 WHAT ARE PROBIOTICS

In 1965, Lilly and Stillwell coined the word "probiotic" to describe a microorganism. Rather than adopting the term "antibiotic," they coined the term "probiotic," a special blend of Greek (bios = life) and Latin (pro = for, in favour of). This phrase was used in the 1960s to refer to substances derived from protozoa that could aid in the development of other microbes. The origins of probiotics can be traced back to the ancient Greek and Roman cultures, who produced fermented milk and utilised it for health purposes. Probiotic are live organisms that improve or restore the proper balance of intestinal bacteria, hence improving health.

According to the Food and Agriculture Organization of the United Nations and the World Health Organization, probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts.

Potential probiotics include a number of bacteria, such as those from the genera Bacillus, Lactococcus, Enterococcus, Streptococcus, Propionibacterium, and Pediococcus. Lactobacillus and Bifidobacterium, the most widely utilised strains, have been associated with a number of health advantages. These advantages include reducing lactose intolerance and avoiding and treating diarrhoeal illnesses brought on by bacteria, viruses, or medical interventions like radiation and antibiotics (Parvez et al., 2006). They also have anti-mutagenic qualities (Chalova et al., 2008), anti-carcinogenic effects (Liong, 2008), immunomodulation (Forsythe and Bienenstock, 2010), and even lower blood cholesterol levels (Ooi and Liong, 2010).

It is crucial to confirm that certain safety and functioning requirements are fulfilled before utilising any microbial strain as a probiotic. According to de Melo Pereira et al. (2018), these requirements include genetic stability, resistance to bile and acid, the capacity to stick to the intestinal lining, anti-genotoxic qualities, non-pathogenicity, lactic acid production, resilience to harsh processing conditions, and a shorter generation time.

The intestinal mucosa's epithelial barrier is strengthened by probiotics. They affect the immune system, encourage microbial adherence while blocking pathogen adhesion, and create molecular substances that stop dangerous microorganisms from growing (Bermudez-Brito et al., 2012). Bacteriocins are biological substances made up of active protein moieties. These bacteriocins can be produced by nearly all types of lactobacilli and bifidobacteria.

1.6 ORGANISMS KNOWN TO BE PROBIOTICS

The bulk of common probiotics are composed of lactobacilli, which have recently been split into 25 genera. These genera include Bifidobacterium species like Bifidobacterium bifidum, Bifidobacterium animalis subsp. infantis, and Bifidobacterium longum, and Lactobacillus species like the Lactobacillus gasseri, Lactobacillus crispatus, Lacticaseibacillus rhamnosus, along with lactic acid bacteria casei, Lactiplantibacillus plantarum, lactic acidophilus, and Limosilactobacillus reuteri. Apart from the well-known probiotics, certain bacterial strains can also be considered probiotics. These include Bacillus subtilis, Bacillus coagulans, Enterococcus faecium, Streptococcus thermophilus, Lactococcus lactis, Pediococcus mesenteroides, and Clostridium butyricum. Furthermore, certain yeast strains—like Saccharomyces cerevisiae var. boulardii—are acknowledged as probiotics. A broad category of Gram-positive, non-spore-forming bacteria, lactic acid bacteria are essential to many fermentation processes. They use either

the homofermentative or heterofermentative route to convert carbohydrates into lactic acid. The genera that include Lactococcus, Streptococcus species, Leuconostoc, Pediococcus, Enterococcus, Oenococcus, and Weissella, among others, are the main members of this category. Numerous strains of the Lactobacillus genus and other lactic acid bacteria are recognised for their antibacterial characteristics (Fijan, 2014).

Table 1.1 - Microorganisms known to be probiotics (Bhunia *et al;* 2023)

Genus	Species	Uses
Lactobacillus	*L. rhamnosus*	Prevention of antibiotic-associated diarrhea (AAD), reduction of infant colic crying time, prevention/reduction of atopic dermatitis, reduction of viral-associated pulmonary damage
	L. acidophilus	Inflammatory bowel disease (IBD) symptoms (pain, bloating), traveller's diarrhoea treatment, fewer hospital admissions due to acute diarrhoea, antifungal activity, and bacterial vaginosis treatment as well as prevention.
	L. plantarum	Decrease of intestinal gas and abdominal pain associated with IBS; inhibition of endotoxin

		formation; antifungal action; and alleviation of symptoms of eczema.
	L. casei	Reducing diarrhoea caused by C. difficile, treating functional constipation, restoring vaginal flora, alleviating IBS symptoms, and shortening the frequency of diarrhoea
	L. reuteri	Controlling baby colic, lowering acute gastroenteritis, shortening the duration of diarrhoea, and lowering low-density lipoprotein cholesterol
	L. crispatus	Prevention as well as management of bacterial vaginosis, reduction of recurrent UTIs (Urinary Tract Infections)
Bifidobacterium	*B. animalis* subsp. *lactis*	Management of IBS symptoms (constipation, pain, bloating), support for healthy immune response, decrease in AAD and necrotising enterocolitis in premature babies

	B. infantis	Reduction of IBS symptoms (pain, bloating, gas), reduction of necrotizing enterocolitis in preterm infants, initial protective effect against rotavirus infection
	B. bifidum	Reduction of IBS symptoms (abdominal pain, overall), admission for acute diarrhoea, including reduction in total cholesterol levels and intestinal inflammation in premature babies.
Saccharomyces	*S. boulardii*	Most frequently used as medication to treat and prevent diarrhoea, particularly infectious forms like rotavirus illness in children.
	S. cerevisiae	Extensively utilised as an effective and affordable supplement against gastrointestinal tract ailments like inflammation of the bowel.
Bacillus	*B. licheniformis*	Prevention of AAD (Antibiotic-associated diarrhea)
	B. Coagulans	Support for digestive health, improvement in IBS symptoms (abdominal pain, bowel movements)

1.7 BENEFICIAL PROPERTIES OF PROBIOTICS

1.7.1 Probiotics for Urinary Tract Infection

An imbalance in the vaginal microbiota can lead to urinary tract infections (UTIs). They might appear as upper or lower UTIs and are prevalent in both young and old women. Antibiotic resistance is a risk, even if preventive antibiotics may occasionally be helpful in treating recurrent UTIs. Studies have demonstrated that lactic acid bacteria, which are frequently detected in many women's vaginal samples, can successfully reduce the pH of the vagina. Lactobacillus species, such as Lactobacillus reuteri, Lactobacillus vaginalis, and Lactobacillus rhamnosus, are the basis for more than 50 efficient probiotics used to treat UTIs (Van et al., 2023).

1.7.2 Probiotics in the treatment of Vaginal Infections

Serious health problems such as infertility, preterm birth, pelvic inflammatory disease (PID), premature rupture of membranes, and miscarriage can result from vaginal infections (Chen et al., 2021a; Dong et al., 2022). An imbalance in vaginal flora is frequently linked to common illnesses, including bacterial vaginosis, vulvovaginal yeast infection, aerobic vaginitis, viral infections, and other sex-related illnesses (STIs). This imbalance, which can harm the vaginal mucosa and epithelial cells, is typified by a high presence of pathogens and a low abundance of lactobacilli.

For the past century, the use of antibiotics has been limited due to the introduction and rapid spread of antibiotic-resistant infections, especially those caused by bacteria that are resistant to multiple drugs. Probiotic lactobacilli have become popular as an additional or replacement treatment for vaginal infections. Gardnerella vaginalis cells may stretch or burst due to the acetic and lactic acid compounds that lactobacilli produce (Huang et al., 2022). Furthermore, these two compounds can interfere with Gardnerella vaginalis's sodium/ potassium-ATPase function, resulting in aberrant

ATP metabolism and ultimately preventing both the bacterium's development and reproduction (Huang et al., 2022).

A. Probiotic lactobacilli in Bacterial Vaginosis (BV)

Among women of reproductive age, bacterial vaginosis (BV) is one of the most prevalent vaginal infections. Gardnerella vaginalis, Prevotella, Atopobium, and Mobiluncus are among the anaerobic bacteria that overgrow and replace helpful lactobacilli (Chen et al., 2021b). Probiotic lactobacilli have been given intravaginally or orally as part of BV treatment to preserve and restore a healthy vaginal microbiome (Basavaprabhu et al., 2020). Lactobacilli are useful in treating BV infections, according to recent in vitro research. There is antagonistic action against BV pathogens like *Prevotella bivia, Atopobium vaginae,* and *Gardnerlla vaginalis* in a number of Lactobacillus strains that have been identified from dairy products and the vaginal flora of healthy women nationwide (Happel *et al; 2021;* Kumherova *et al; 2021*). In particular, the shape of G. *vaginalis* can be affected from rupturing due the acetic and lactic acids produced by lactobacilli.

B. Probiotic lactobacilli in Vulvovaginal candidiasis (VVC)

Vulvovaginal candidiasis which is thought to be the second most common vaginal infection after bacterial vaginosis, is primarily caused by the opportunist fungus Candida. Studies as shown that fluconazole are frequently used to treat VVC; however, it only works to prevent Candida growth, not to completely eradicate it. The development of fluconazole resistance may result from this. In the treatment of VVC, probiotic lactobacilli have demonstrated significant potential as an adjuvant or alternative therapy. (Sun *et al.,* 2023).

Lactobacilli can prevent Candida from changing from yeast to hyphal form, according to research done in vitro using vaginal yeast and lactobacilli co-cultures. Because they have a greater affinity

for adhesion sites on epithelial cell receptors, this activity helps decrease the number of Candida in the vagina (Spaggiari et al., 2022). For instance, by modifying the expression of Candida proteins, such as agglutinin-like sequence protein (AlS3) and the hypha-associated proteins (HGC1, Ece1, Hwp1, and Hyr1), lactic acid secreted by lactobacilli can prevent Candida overgrowth and its change from the naturally occurring yeast form to the infectious hypha form. Additionally, lactobacilli can limit the ability of these yeasts to produce biofilms by preventing the growth of infectious hypha forms of Candida species (Liu, 2023).

C. Probiotic lactobacilli use in other Sexually Transmitted Diseases (STIs)

Although they are rarely fatal, sexually transmitted diseases (STIs) such chlamydia, mycoplasma, trichomoniasis, ureaplasma, and gonorrhoea can have a substantial impact on female patients. Studies have demonstrated that Lactobacillus gasseri's cell surface aggregation-producing component can, in a dose-related way, stop Trichomonas vaginalis from attaching to human vaginal ectocervical cells (Malfa et al., 2023). Furthermore, research has shown that the three main intracellular parasites in the female vagina—Chlamydia, Mycoplasma, and Ureaplasma—are prevented from invading the vaginal tissue by lactate, bacteriocins, and the acidic environment produced by lactobacilli (Garza et al., 2021; Chen et al., 2022).

D. Probiotic use in mixed vaginal infections

Mixed vaginal infections are defined as the simultaneous presence of a minimum of two different vaginal pathogens, with both of which induce symptoms while also contributing to an unhealthy vaginal environment. According to in vitro research, almost all probiotic Lactobacillus species are capable of fighting off different vaginal infections because of the crucial function lactobacilli play in the vaginal micro biome. Four different pathogens that cause both BV and AV were investigated

in co-culture tests using *Lactobacillus acidophilus* GLA-14 and Lactobacillus rhamnosus HN001 are two strains of Lactobacillus that are available widely. Lactic acid, organic acid, and H2O2 are also produced by *L. Rhamnosus* CA15 demonstrated increased colonisation tolerance and a broad spectrum of antagonistic action against urinary tract infections, including *E. faecalis, E. coli, C. albicans, G. vaginalis* in addition to *S. agalactiae* (Pino et al., 2022a). According to Kumherova et al. (2021) and Liu (2023), a number of Lactobacillus strains that were isolated from the vaginal flora of healthy women also demonstrated inhibitory activity against the microbes that may cause BV, VVC, and AV.

1.7.3 Probiotics for Obesity

Obesity is becoming to a greater extent of a concern these days, and the main causes are genetic variability and imbalances in energy intake and expenditure. Adipocyte tissues contain the hormones adiponectin and leptin, which are primarily to blame for obesity. *Lactobacillus gasseri* BNR17 prevents these substances from growing. Probiotics aid in weight loss by activating the adrenergic nerve system, which produces a thermogenic reaction. Some probiotics like *Lactobacillus acidophilus, and Bifidobacterium longum,* lower triglyceride, LDL, and HDL levels (Sarita *et al.*, 2025; George *et al;* 2018)

1.7.4 Probiotics for Respiratory Diseases

Common respiratory conditions include bronchitis, sinusitis, pharyngitis, rhino sinusitis, and otitis. Because of their anti-inflammatory and anti-microbial qualities, probiotics can help prevent a variety of respiratory conditions. *Lactobacillus rhamnosus,* for example, can help people with a condition known as cystic fibrosis control pneumonia. Several probiotics are frequently utilised for the purpose, including *Bifidobacterium longum, Lactobacillus fermentum, and Lactobacillus casei.* (Succol *et al.*, 2024)

1.7.5 The Effects of Probiotics on Dental Health

One of the main advantages of probiotics in the oral cavity is the reduction of inflammation. By eradicating these infections, probiotics can help shield teeth and gums from dangerous germs. Probiotics are typically thought to have no adverse side effects when used as a natural therapy. The antifungal qualities of Lactobacillus acidophilus and Bifidobacterium lactis are widely recognised. According to recent studies, using probiotics can enhance oral health by reducing a number of ailments such caries in the teeth, periodontal infections, oral illnesses, and foul breath (halitosis).

1.7.6 Probiotics for Cardiovascular Diseases

One of the main enzymes responsible for hypertension is the angiotensin-converting enzyme (ACE). It has been reported that Saccharomyces cerevisiae and Lactobacillus helvetics produce peptides that can block ACE activity (Sarita et al., 2025).

1.7.7 Probiotics for Inflammatory Diseases

A long-term inflammatory condition that affects the digestive system is called inflammatory bowel disease (IBD). It comprises Crohn's disease (CD), ulcerated colitis (UC), and idiopathic colitis (IC), each of which can be identified by the particular regions of the digestive system that are inflamed (Bjarnason et al., 2019). IBD is believed to result from an abnormal immune response, while the precise reason is still unknown. Inflammatory bowel disease (IBD) has been linked to a number of factors, including poor eating habits and psychological stress. Some study suggests that gut pathogens could possibly be involved in its development. Furthermore, a number of studies have demonstrated that the gut microbiota of people with IBD differs from that of healthy people, suggesting that preserving a balanced microbiome may be essential to preventing the illness

(Shadnoush et al., 2015). In recent years, probiotics have received substantial interest as a possible therapeutic method to alter gut microbiota and increase intestinal health in individuals with IBD. For example, they are being used to help achieve treatment as well as control complications of ulcerative colitis (Furrie et al., 2005; Yang & Yu, 2018).

However, newer evidence indicates that probiotic therapy may be more beneficial as an adjunct treatment for UC rather than for CD. Consequently, there is still limited understanding of probiotics' overall effectiveness in Crohn's disease, making it difficult to establish general treatment recommendations.

Weight loss, constipation, fever, exhaustion, and stomach pain are some of the symptoms of Crohn's disease (CD), a chronic inflammatory gastrointestinal disorder. Although the precise mechanism is yet unknown, a mix of hereditary, microbiological, and ecological factors affect its initiation (Furrie et al., 2005). Although there is no known cure for CD, its symptoms can be controlled with a variety of drugs, such as corticosteroids and immunosuppressants, which help lower intestinal inflammation and regulate immunological activity (Baumgart et al., 2007). Probiotics have also been investigated as an alternative to traditional therapies. Early probiotic therapy may be more beneficial than postoperative supplementation in CD patients, according to some research. More recent research, however, indicates that multi-strain probiotic treatment did not considerably change inflammatory markers in CD. Further research is required to further clarify the importance of probiotics in the treatment of Crohn's disease because of these conflicting clinical outcomes.

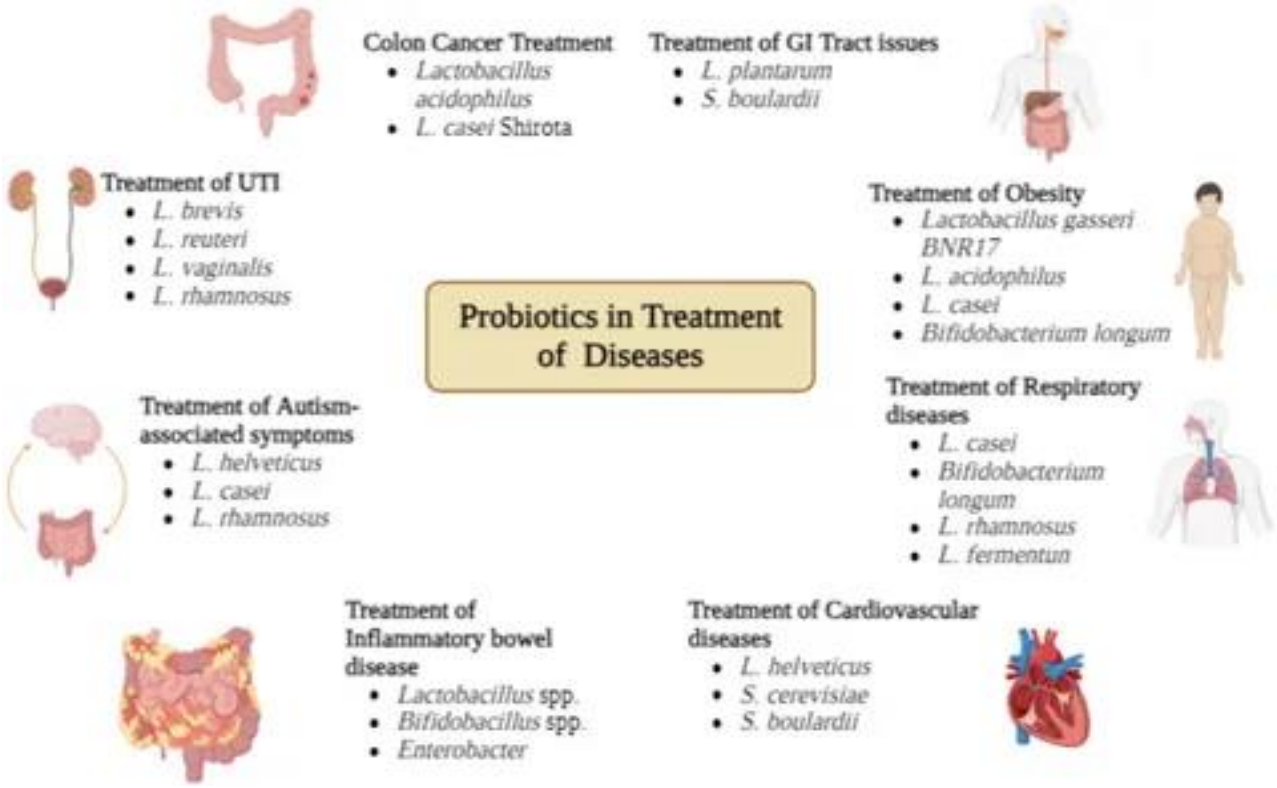

Figure 1.1 - Probiotics in the treatment of diseases (Sarita *et al.*, 2025)

1.8 Safety Considerations and Potential Side Effects

Probiotics have a number of possible health advantages, but a thorough understanding requires discussing their safety record and any adverse effects. But there are typical, frequently transient reactions in healthy people and crucially emphasizes the particular factors and possible dangers for vulnerable groups.

1.8.1 General Safety Profile in Healthy Individuals

Probiotics are commonly used and generally regarded as safe for the majority of healthy people. Their broad and consistent use over an extended period of time, along with strong safety profiles recorded in multiple clinical trials, supports their general safety for the general public. Although

the majority of healthy people do not suffer from side effects, the most frequently reported reactions

A. Digestive symptoms

Some people who take probiotics for the first time experience bloating, gas, or diarrhea. Accordingly, alterations in gut microbiota may cause bacteria to produce more gas than normal, which can cause bloating (Marteau and Seksik, 2004). Probiotics based on yeast may cause indigestion and enhanced thirst. These minor digestive issues typically disappear after a few weeks of consistent use as the body adjusts to the new microbial input. To lessen the likelihood of these first side effects, it is sometimes recommended to start with a modest dose of probiotics and gradually increase to the full dosage over a few weeks. If symptoms persist for more than a few weeks, it is advised to stop using the medicine and consult a physician.

B. Histamine reactions

Our immune system creates chemicals known as histamines to fight allergies. Histamines aid our bodies in eliminating allergens through allergic reactions such as irritated eyes, nasal congestion and skin irritation. Biogenic amines (including histamine and tyramine) are naturally present in several fermented foods that are high in probiotics, like kimchi, sauerkraut, and yoghurt. When meals containing protein ferment or age, these compounds are created and people who are amine-sensitive may experience headaches after eating these items.

A combination of histamine-producing, histamine-free, and histamine- inhibiting bacteria can be found in most probiotic supplements. Because of this, they usually have little effect on the body's total histamine levels. However, people who are sensitive to histamine may still experience

adverse reactions. If typical allergic reactions occur after taking probiotics, this could be a sign of an imbalance in gut flora or an excess of histamine (Ö Özdemir, 2010).

1.8.2 Specific Considerations for Vulnerable Populations

Although typically safe for healthy people, there are known dangers, especially for vulnerable groups. Although they are uncommon, adverse effects can include immune system stimulation, gastrointestinal side effects, skin issues, and systemic infections like sepsis and endocarditis. Serious complications are much more likely to occur in people who are critically ill, have central venous catheters, have recently undergone surgery, or have weakened or immune systems that are compromised (such as those on drugs that suppress the immune patients with cancer, diabetes, leaky gut, or recipients of organ transplants). In some groups, probiotic bacteria may occasionally operate as opportunistic pathogens, leading to potentially deadly illnesses. A recent case of bacteremia (bacteria in the circulation) was discovered when Lactobacillus sp. was administered to a patient who had chronic inflammatory bowel disorder and mucosal rupture (Pace et al., 2020).

Another vulnerable population is infants, especially neonates, premature babies, and low-birth weight babies. Probiotics may help reduce necrotizing enterocolitis in this demographic, according to some research, but there haven't been many safety evaluations of trials involving this group. A press statement from the Food and Drug Administration in 2023 raised concerns on the possible dangers of giving probiotics to premature babies.

In order to be used in patient populations, probiotics must pass stringent safety testing, which includes whole genome sequencing to look for genes linked to antibiotic resistance and virulence, and they must also meet quality requirements suitable for the users for whom they are designed. Better regulation, third-party verification, and more stringent adverse event reporting are necessary

to assure product safety, particularly for at-risk groups, as seen by the variety of strains and formulations and the lack of uniform safety reporting.

1.9 Mode of Action of Probiotics

Probiotics have come a long way, but there hasn't been a significant breakthrough in our knowledge of how they work. The human body may benefit from probiotics through the following primary mechanisms:

1.9.1 Competitive pathogen exclusion

When one species of bacteria fights more fiercely than other species for receptor sites in the digestive tract, this is known as competitive exclusion. It is mainly unknown what precise routes and important regulatory mechanisms underlie these probiotic benefits. Among the primary processes for competitive exclusion of infections are reduction of luminal pH, competition for nutrient, and synthesis of bacteriocin or compounds similar to bacteriocin. The majority of research has been on lowering human infections like E. coli and Salmonella typhi. Probiotics make it harder for infections to survive in the gut by competing with them for resources and receptor-binding sites (Plaza-Diaz *et al.,* 2019).

1.9.2 Improvement in intestinal barrier functions

Probiotics increase the intestinal barrier function by promoting the production of mucin proteins (Chang et al., 2021), modulating the expression of the tight junction proteins, including occluding and claudin 1, and controlling how the immune system reacts in the gut (Bu et al., 2022).

1.9.3 Immunomodulation in the host's body

By influencing dendritic cells, macrophages, and B and T lymphocytes, probiotics are essential for controlling both innate and adaptive immune responses. Additionally, they connect with intestinal

epithelial cells, draw macrophages and mononuclear cells, and increase the production of anti-inflammatory cytokines (Petruzziello et al., 2023).

1.9.4 Production of neurotransmitters

Through the connection between the gut and the brain, probiotics can cause the production of neurotransmitters in the gut. Certain probiotic strains have the ability to alter serotonin, dopamine, and gamma-aminobutyric acid (GABA) levels, which have an impact on mood, behaviour, gastrointestinal motility, and psychological pathways (Gangaraju et al., 2022).

1.9.5 Production of antimicrobial substance

Organic acids, hydrogen peroxide, bacteriocins, and short chain fatty acids (SCFA) reduce harmful bacteria in the stomach (Ahire et al., 2021).

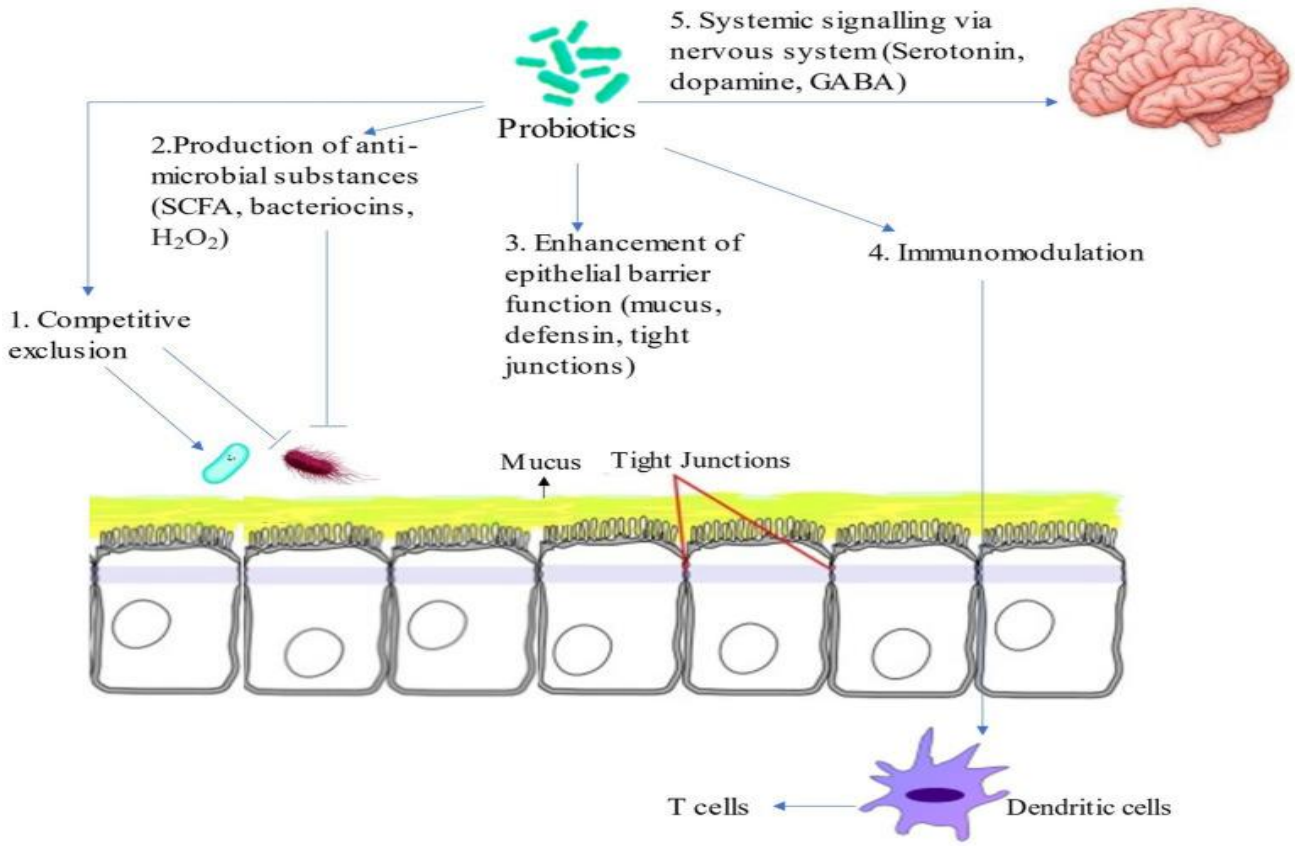

Figure 1.2 - Mechanism of action of probiotics (Plaza-Diaz *et al.*, 2019)

1.10 Antimicrobial Properties of Probiotics

The antimicrobial or antagonistic activity of probiotics is a crucial property that aids in the production of antimicrobial compounds, the competitive removal of pathogens, and the enhancement of intestinal barrier function for improved health, among other benefits.

This ability to combat microorganisms is especially significant for probiotics, as it represents one of their key functional properties. Probiotics produce a variety of antimicrobial metabolites, including organic acids, diacetyl, acetoin, hydrogen peroxide, short-chain fatty acids, propionic acid, carbon dioxide (CO2), and bacteriocins. These activities help maintain microbiological safety

by controlling the growth of other microorganisms and inhibiting pathogenic bacteria (Hassan et al., 2014).

1.10.1 Antimicrobials from probiotics

Lactic acid.

The generation of metabolites such organic acids (lactic and acetic acid) has been linked to the antibacterial action of probiotic microorganisms. Compared to lactic acid, acetic acid possesses more antibacterial action. Lactic acid and acetic acid are examples of lipid-based acids that can pass through microbial cell membranes in their undissociated form. At higher intracellular pH, these acids dissociate to produce hydrogen ions, which disrupt vital pathogen cell metabolic processes like substrate translocation and oxidative phosphorylation. Intracellular pH decrease and membrane potential breakdown are among the harmful consequences of lactic and acetic acid.

Hydrogen peroxide (H_2O_2)

All lactic acid bacteria produce hydrogen peroxide (H2O2) through electron transport via flavin enzymes. When H2O2 is present, superoxide anions can form destructive hydroxyl radicals. These radicals lead to the peroxidation of membrane lipids, increasing membrane permeability. The resulting bactericidal effect of the oxygen metabolites occurs due to their strong oxidizing impact on bacterial cells, as well as their ability to damage nucleic acids and cell proteins (Malik et al., 2021). H2O2 exhibits antimicrobial activity against yeasts, as well as Gram-positive and Gram-negative bacteria.

Carbon dioxide

Heterofermentative lactic acid bacteria create carbon dioxide, which has antibacterial properties against the majority of generic groups of microorganisms. It is a significant byproduct of the fermentation of hexose by heterofermentative lactic acid bacteria. Many lactic acid bacteria can produce CO2 from citrate and malate. Additionally, they can produce CO2 by converting arginine through the arginine deaminase pathway. Lastly, CO2 can also be produced via decarboxylation of amino acids (tyrosine, histidine). By substituting existing molecular oxygen, CO2 produces an anaerobic atmosphere. It has detrimental effects on cell membranes and can lower pH (Denkova et al., 2017).

Diacetyl

Many lactic acid bacteria create diacetyl, acetaldehyde, and acetoin, which are also effective against yeasts and both Gram-positive and Gram-negative bacteria. The last byproduct of pyruvate metabolism by lactic acid bacteria that ferment citrate is diacetyl (2, 3-butanedione). It has antibacterial activity against a variety of spoilage microorganisms and food-borne diseases. Diacetyl works much better against moulds, yeasts, and Gram-negative bacteria than it does against Gram-positive microbes.

Reutrin and Reutericyclin

Two substances produced from the chosen isolates of Lactobacillus reuteri that are effective against Gram-positive bacteria are reutrin and reutericyclin. Reuterin is a combination of monomeric and cyclic dimeric forms of β-hydroxy propionaldehyde with a wide range of inhibitory activities, including Gram-positive bacteria, fungi, and protozoa. Reutericyclin is a derivative of tetrameric acid. Methicillin-resistant Staphylococcus aureus (MRSA), Streptococcus

sp., and Clostridium diffuicle are among the pathogens linked to tropical infections that reutrincyclin combats (Hurdle et al., 2011).

1.11 Methods of determination of antimicrobial properties of probiotics

1.11.1 In vitro methods for determining the antimicrobial activity of probiotics against pathogens

A. Spot-on lawn Method

The method has the following steps:

1) Selective or differential media are produced along with various nutrients. Before being poured into Petri dishes, the selected pathogen is either mixed with the melted and cooled agar or spread onto the hardened agar medium at different initial concentrations.

2) The medium inoculated with the selected pathogenic bacteria is then spotted with varying dilutions of the tested probiotic microbe, acellular supernatant (which contains bacteriocins and organic acids), or neutralised acellular supernatant (where the organic acids are neutralised).

3) The antibacterial activity is given as either the inhibition zone or arbitrary units (AU/mL) following incubation. Either the diameter or the zone of the inhibitory are recorded. Calculated as (1000/a) x D in AU/mL, where the volume (µL) of the spotted sample and D is the dilution factor, arbitrary units are the reciprocal of the maximum dilution at which the pathogen's development is prevented (Fijan, 2016).

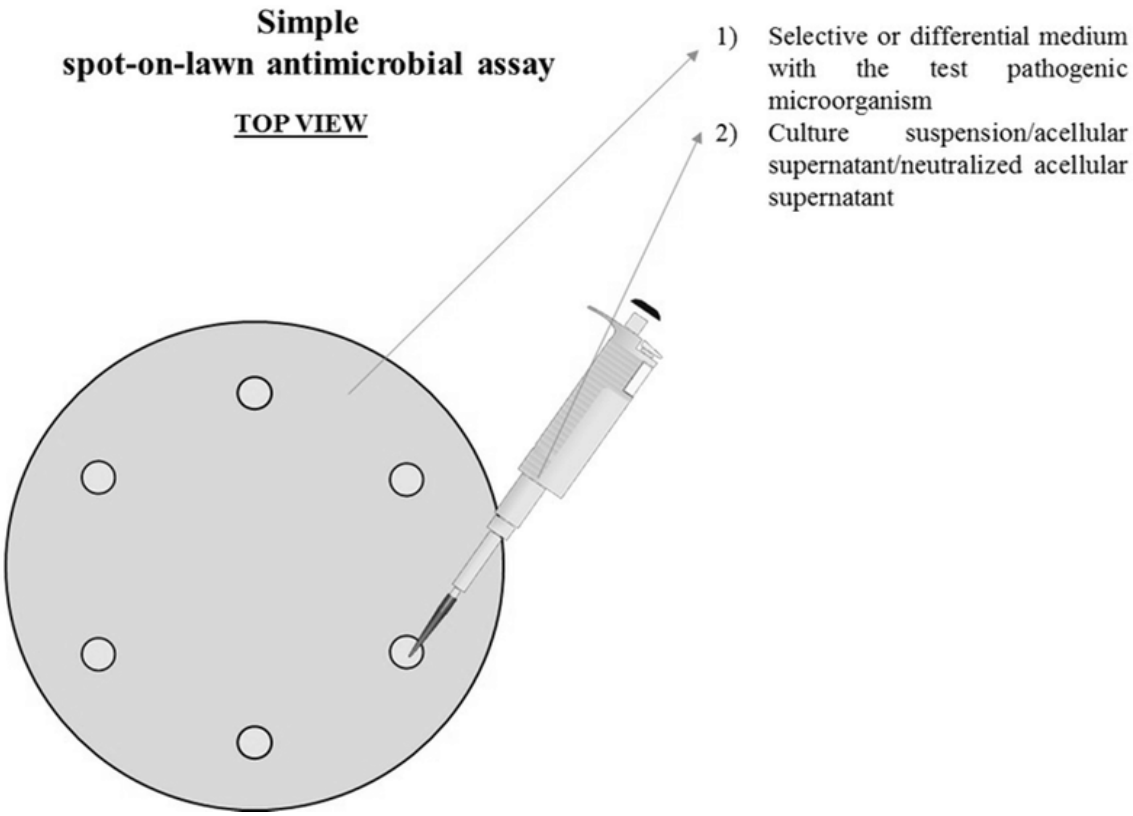

Figure 1.3: Simple spot-on-lawn antimicrobial assay (Fijan, 2016)

B. Agar spot-on-lawn Method

The following steps are included in the method:

1. Fill Petri dishes with prepared selective or differential media.

2. Apply different dilutions of the probiotic microbe under investigation, acellular supernatant (containing organic acids and bacteriocins), or neutralised acellular supernatant (containing neutralised organic acids) to the medium. To create the spots, incubate the Petri dishes.

3. Cover the spotted Petri dishes with a suspension of the pathogenic microbe mixed with a particular soft agar (0.7%).

4. Measure the inhibition zones after incubating the Petri dishes either anaerobically or aerobically. A clear zone surrounding the area that is larger than 1 mm is regarded as a successful outcome (Fijan, 2016).

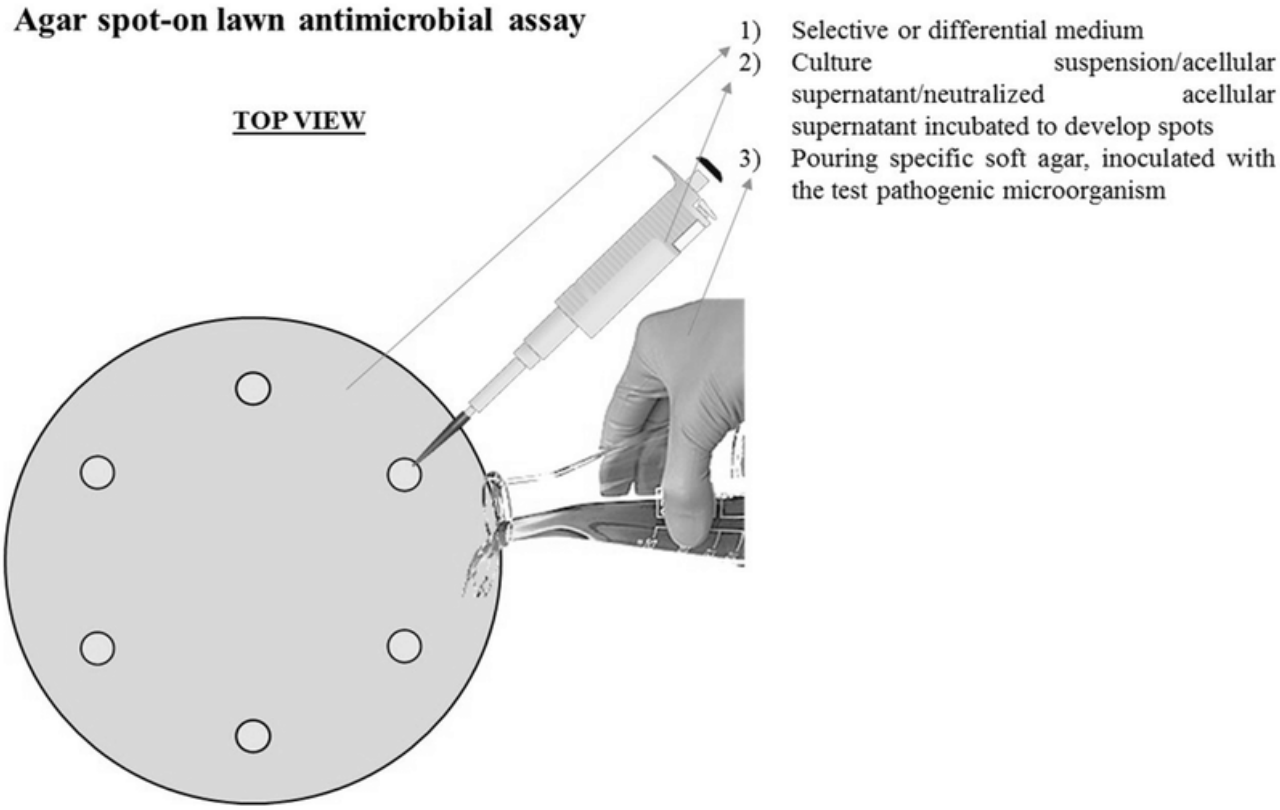

Figure 1.4: Agar spot-on-lawn antimicrobial assay (Fijan, 2016)

C. Cross streak Method

The process involves using a loop to streak each probiotic strain in three parallel lines onto an appropriate agar medium. After allowing the lines to dry, the test pathogenic strain is streaked in the same manner parallel to the original strains. When inhibition occurs, the probiotic strain under test prevents the second streaked (pathogenic) microbe from growing (Fijan, 2016).

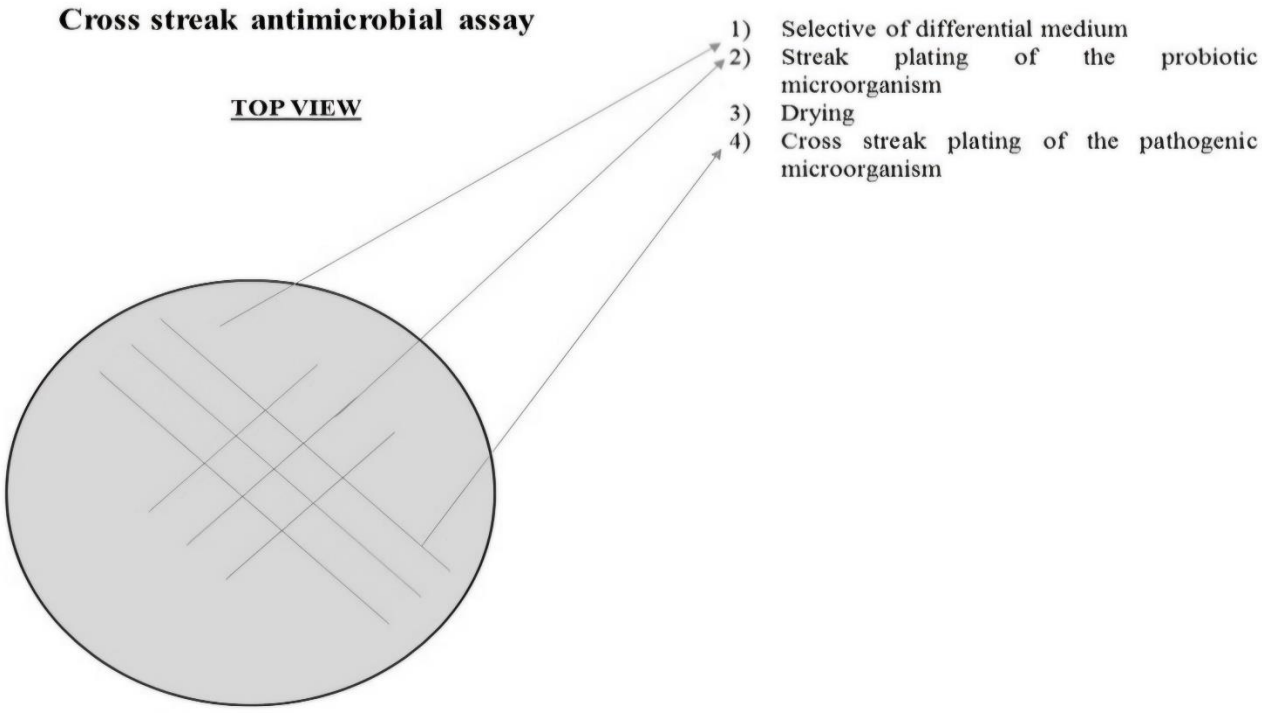

Figure 1.5: Cross streak antimicrobial assay (Fijan, 2016)

D. The Agar Well-Diffusion Method

The inhibitory effects of acellular supernatants are frequently ascertained using this technique. The following steps make up the assay:

1. Prepare various selective, differential, or nutrient-rich mediums. Use either pour plating or spread plating to introduce the selected pathogenic bacterium into the Petri dishes.

2. Make wells in each Petri dish that are 6 or 7 mm in diameter.

3. Transfer aliquots of different acellular supernatant dilutions into the wells using a pipette. Measure the antimicrobial activity after incubation; it can be expressed in arbitrary units (AU/mL) (Fijan, 2016).

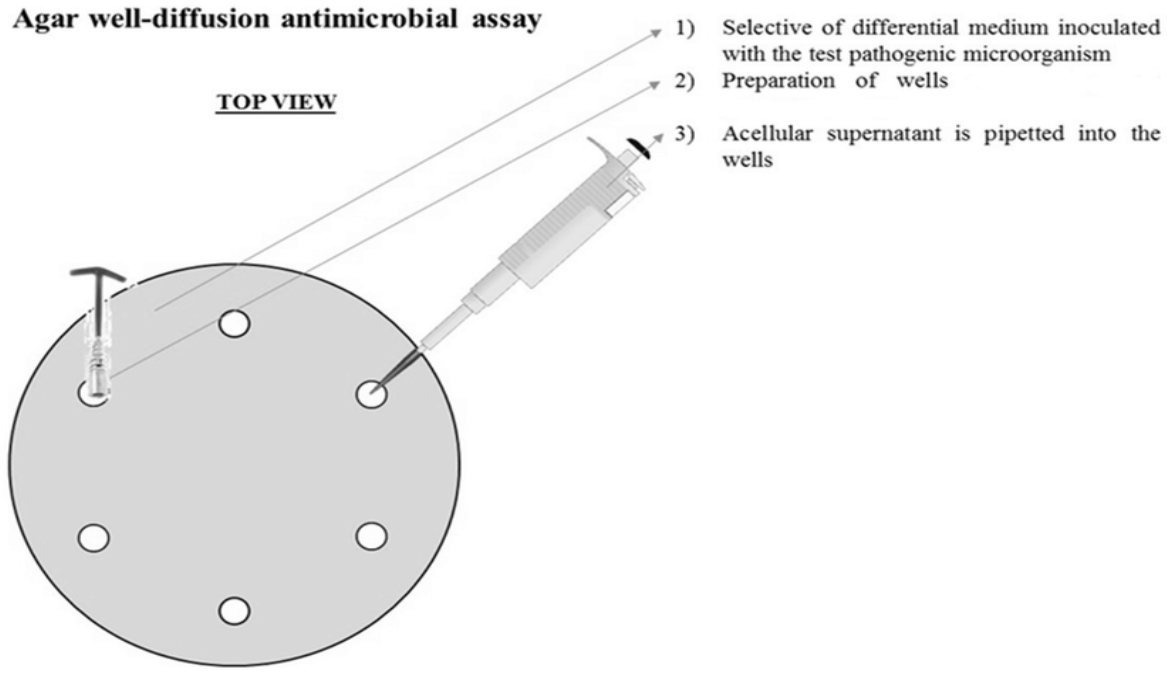

Figure 1.6: Agar well-diffusion antimicrobial assay (Fijan, 2016)

E. Paper Disc Method

The method consists of the following steps:

1) Prepare different nutrients using selective or differential media. Inoculate the Petri dishes with the chosen pathogenic microorganism using either spread-plating or pour-plating techniques.

2) Soak paper discs (6 mm in diameter) in aliquots of the acellular supernatant, and then place them on the hardened agar medium. After incubation, evaluate the inhibition zone by examining the clear area surrounding the paper disc (Fijan, 2016).

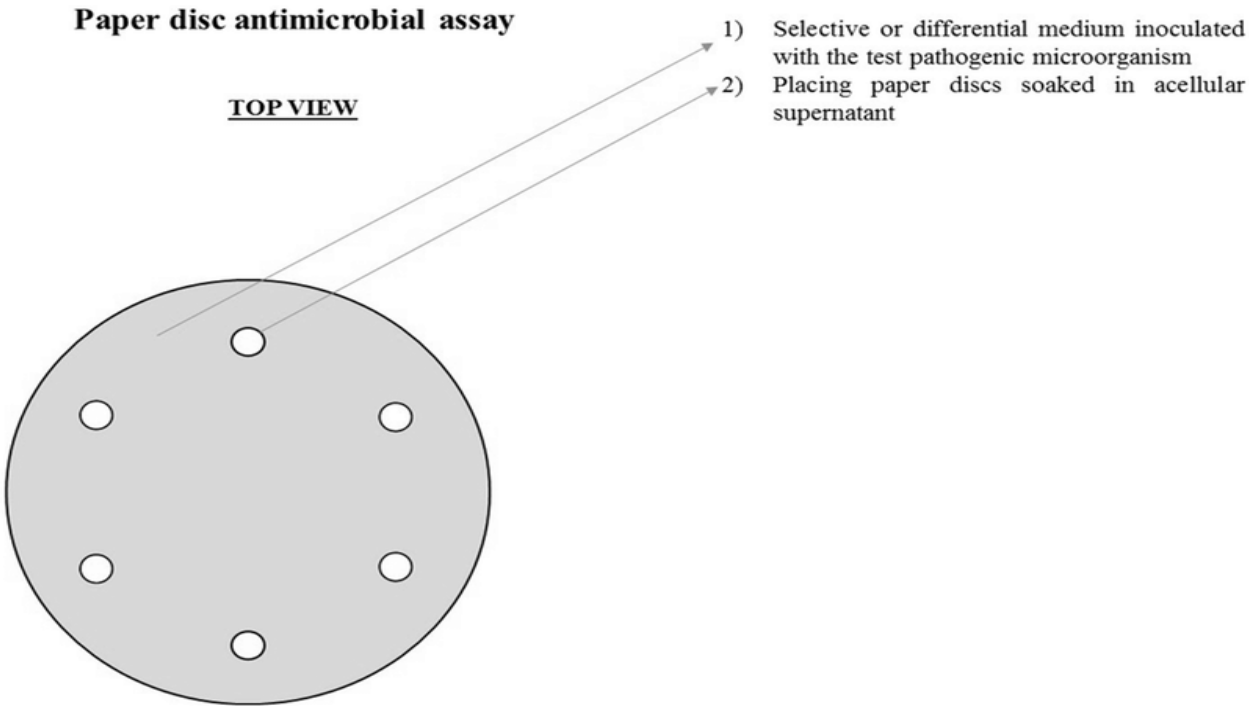

Figure 1.7: Paper disc antimicrobial assay (Fijan, 2016)

F. Simple co-culturing Method

This method involves the following steps:

1. **Preparation of Incubation Media:** Prepare the appropriate media for incubation.

2. **Inoculation:** Add aliquots of both a pathogenic microorganism and a probiotic microorganism into the incubation media. Mix the samples thoroughly and incubate them.

3. **Viable Cell Counting:** After incubation, determine the number of viable cells of both the pathogenic and probiotic microorganisms on the appropriate agar medium. The results are typically expressed as log CFU/ml (Fijan, 2016).

1.11.2 In vivo methods for determining the antimicrobial activity of probiotics against pathogenic microorganisms

A. In vivo examination using human

Randomised double-blind, placebo-controlled studies are used in in vivo human studies to determine the effectiveness of probiotic products. Investigations on humans with enough participants to reach statistically significant levels are required (Fijan, 2016). Numerous carefully planned, double-blind, placebo-controlled clinical trials have been carried out to show that certain lactobacilli or bifidobacteria strains have these advantageous qualities in people with particular illnesses (Servin, 2024). There is research suggesting these probiotics have adhesive qualities in the gut and that they are beneficial to health in cases of *Helicobacter pylor*i infections and diarrhoea.

B. In vivo examination with animal models

Every animal model used in an in vivo antimicrobial experiment has at least two groups under carefully monitored conditions. The first group (treated infected group) is given both the pathogen and the chosen probiotic, whereas the second group (untreated infected group) is given just the pathogen. Following a predetermined amount of time, the animals' faeces and various cells—such as the spleen, lymph nodes, blood, liver, colon, cecum, etc.—are examined in order to identify and evaluate any variations. Mice, rats, chicks, rabbits, pigs, fish, and even worms were employed in these investigations.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Micro-organisms:

Lactobacillus reuteri (L. reuteri), Escherichia coli (E. coli)

Chemicals and Reagents:

Glycerogelatin, Polyethylene glycol, Theobroma (Cocoa butter), Crystal violet, Gram iodine, Acetone-alcohol, Safranin, Sodium dihydrogen phosphate (GH Tech), Disodium hydrogen phosphate (GH Tech), Sodium chloride (GH Tech), Phenol red, hydrogen peroxide (H_2O_2), broth containing: Raffinose, Lactose, Maltose, D-fructose, D-glucose, D-mannitol, L-rhamnose, D-xylose, and L-arabinose.

Growth Media:

Muller Hinton Broth (MHB) (Titan Biotech Limited), de Man, Rogosa and Sharpe (MRS) Broth (Chaitanya Agro Biotech PVT LTD), MRS Agar (Chaitanya Agro Biotech PVT LTD), Nutrient Broth (Titan Biotech Limited), Eosin Methylene Blue Agar (Titan Biotech Limited).

Equipment:

Autoclave, Microscope, Refrigerator, Digital weighing balance, bunsen burner, pressure pot

Glass wares: Beakers, Measuring cylinder, volumetric flask, Petri dishes, Universal bottles, Bijou bottles, Media bottles, Glass sides, cover slips

Other Materials Used:

Masking tape, Micropipette, Forceps, Marker, Cotton wool, Methylated spirit, Disinfectant, Surgical blade, Spatula, Candle, Teal, Plastic vacuum tubes.

2.2 METHODS

2.2.1 Gram Staining

A loopful of Lactobacillus reuteri was used to create a smear mixture on a spotless, grease-free slide. After being heat-fixed, the slide was let to cool. Crystal violet was poured and stored for roughly 1 minute to saturate the slide with stain. It was then given a water rinse. The slide was then flooded with Gram's iodine for a minute before being rinsed with water. After decolorising the slide for 30 seconds with acetone-alcohol, it was rinsed with water. Safranin was used for one minute of counterstaining before being rinsed with water. After letting the slide dry, the microscope's oil immersion objective was used to view it. Gram positive bacteria became blue purple after gram staining while gram negative bacteria retained the colour of the secondary dye. This procedure was carried out for *Escherichia coli.*

2.2.2 Biochemical Test

A. Tube catalase test

A 24 hours culture of the test organism *(L. reuteri or E.coli)* from an MRS plate was immersed in a 3% hydrogen peroxide solution in a test tube using a glass rod and observed. The results were documented as positive if active bubbling occurred and negative in the absence of bubbling.

B. Carbohydrate fermentation test

A carbohydrate fermentation test was conducted using the following sugars: Raffinose, Lactose, Maltose, D-fructose, D-glucose, D-mannitol, L-rhamnose, D-xylose, and L-arabinose. A series of test tubes each containing a carbohydrate broth with a specific sugar is prepared. The pH indicator; phenol red is also added. Each of the tube is inoculated with a loop full of *L. reuteri* and the tubes are incubated at 37°C for 24 hours. The result is documented as positive if colour changes from red to yellow and negative if the medium remains red. (Reiner, 2012). The experiment was repeated for *E.coli* and the result documented.

2.2.3 Preparation of Microbiological Broth and Agar

A. Preparation of MRS broth and MRS agar

Following the manufacturer's instruction, 55.15g of microbiological media MRS Broth was prepared with 1000ml of distilled water 10ml of the broth was pipetted into universal bottles, autoclaved and sterilized for 15 minutes at 121°C

For MRS agar, as directed by the manufacturer, 67.15g of microbiological media was dissolved in 1000ml of distilled water and poured into a media bottle. It was heated for about 5 minutes with continuous stirring to allow dissolving of all particles and until it turns gel-like. About 10ml was dispensed into each of the petri dishes and allowed to set. Thereafter, it was oven-dried in the hot chamber which was sterilized for 30 minutes at 60°C.

B. Preparation of Muller Hinton Broth

As directed by the manufacturer, 38g of Muller Hinton Broth (MHB) was dissolved in 1000 mL of distilled water and then 10ml was pipetted into universal bottles, autoclaved, and sterilized for 15 minutes at 121°C and allowed to cool. Thereafter, a loopful of *E. coli* was inoculated into each of the bottles.

C. Preparation of Eosin Methylene Blue (EMB) Agar

Following the manufacturer's information, 36 g of Eosin Methylene Blue (EMB) Agar was dissolved in 1000 mL of distilled water and poured into a media bottle. It was then heated for about 5 minutes with continuous stirring to allow dissolving of all particles and until it became gel-like. About 10 mL was dispensed in each of the Petri dishes and allowed to set. It was oven-dried in the hot chamber, which had been sterilized for 30 minutes at 60°C.

2.2.4 Preparation of Phosphate Buffer Saline (Diluent)

10.76g of sodium dihydrogen phosphate, 17.37g of disodium hydrogen phosphate, and 17.0g of sodium chloride were weighed and mixed to prepare a phosphate buffer solution in 400 ml of distilled water and then 600ml of distilled water was added. The solution was constantly agitated until all salts are totally dispersed. The solution was transferred into a 2L volumetric flask and about 900ml of distilled water was added to make up to the volume of 2L mark. The solution was sterilized in an autoclave at 121°C for 15 minutes.

2.2.5 Cultivation of *Lactobacillus reuteri*

A universal bottle containing *L. reuteri* culture was utilized in MRS Broth and Muller Hinton Broth in a ratio of 1:1. 100µl of broth from the tube was subjected to a 1:10 serial dilution using 0.9ml of phosphate buffer saline in twelve vacuum tubes as diluent. Using the drop plate method,

20µl of each dilution was plated on an MRS Agar. The experiment was carried out at an interval of 4 hours over 48 hours. The innoculated plate was allowed to dry at room temperature and incubated for 48 hours at 37°C and the result was recorded.

2.2.6 Co-Cultivation Assay In The Presence of Suppository Base

An equal concentration of *L.reuteri* and *E.coli was* inoculated in MRS broth and MH (6ml) in a ratio of 1:1 and 3ml of the suppository base- glycerogelatin was added. 100µl was pipetted from the bottle and subjected to a 1:10 dilution using 0.9ml of phosphate buffer saline in twelve red cover tubes as the diluent. Using the drop plate method, 20µl of each dilution was plated on both the MRS Agar and EMB Agar which was divided into twelve portions. The experiment was carried out at an interval of 4 hours for 48 hours. The innoculated plate was allowed to dry at room temperature and incubated for 48 hours at 37°C and the result was recorded.

The experiment was repeated using polyethlene glycol and theobroma as the suppository base in an equal concentration of *L.reuteri* and *E.coli.*

2.3 METHOD OF DATA ANALYSIS

Data obtained from the experiment was analyzed using SPSS statistics 27. Mean values of viable counts of *Lactobacillus reuteri* recorded at different time intervals were used to generate a regression graphs showing the survival in each suppository base and *Escherichia coli.* The slope of each regression line indicates the rate of survival with time, but the coefficient of determination (R2) indicates the effectiveness of the linear connection between time and survival

To ascertain whether the survival variations between the test and the reference conditions were statistically significant, a paired t-test was conducted. The test compared the mean survival values of *L.reuteri* under each treatment condition with the control group at corresponding time points.

Statistical significance was defined as a P-value less than 0.05 ($P < 0.05$), whereas $P < 0.001$ indicated a highly significant difference.

Results were presented in tables and graphs for easy interpretation.

CHAPTER THREE

3.0 RESULTS

Table 3.1: GRAM STAINING AND BIOCHEMICAL TEST FOR *Lactbacillus. reuteri*

Test	**Specific interactions**	***Lactobacillus reuteri*.**
Gram staining	Primary and Secondary Dyes	GPB
Catalase test	Hydrogen peroxide	Negative
Biochemical Characteristics		
	Sucrose	Positive
	Raffinose	Positive
	Lactose	Positive
	Maltose	Positive
	D-Fructose	Positive
	D-Glucose	Positive

	D-Mannitol	Negative
	L-Rhamnose	Negative
	D-Xylose	Negative
	L-Arabinose	Negative

Table 3.2: GRAM STAINING AND BIOCHEMICAL TEST FOR *Escherchia coli*

Test	**Specific interactions**	***Escherchia coli***
Gram staining	Primary and Secondary Dyes	GNB
Catalase test	Hydrogen peroxide	Positive
Biochemical Characteristics		
	Sucrose	Negative

	Raffinose	Negative
	Lactose	Positive
	Maltose	Positive
	D-Fructose	Positive
	D-Glucose	Positive
	D-Mannitol	Positive
	L-Rhamnose	Positive
	D-Xylose	Positive
	L-Arabinose	Positive

KEY:

GNB: Gram Negative Bacteria

GNP: Gram Positive Bacteria

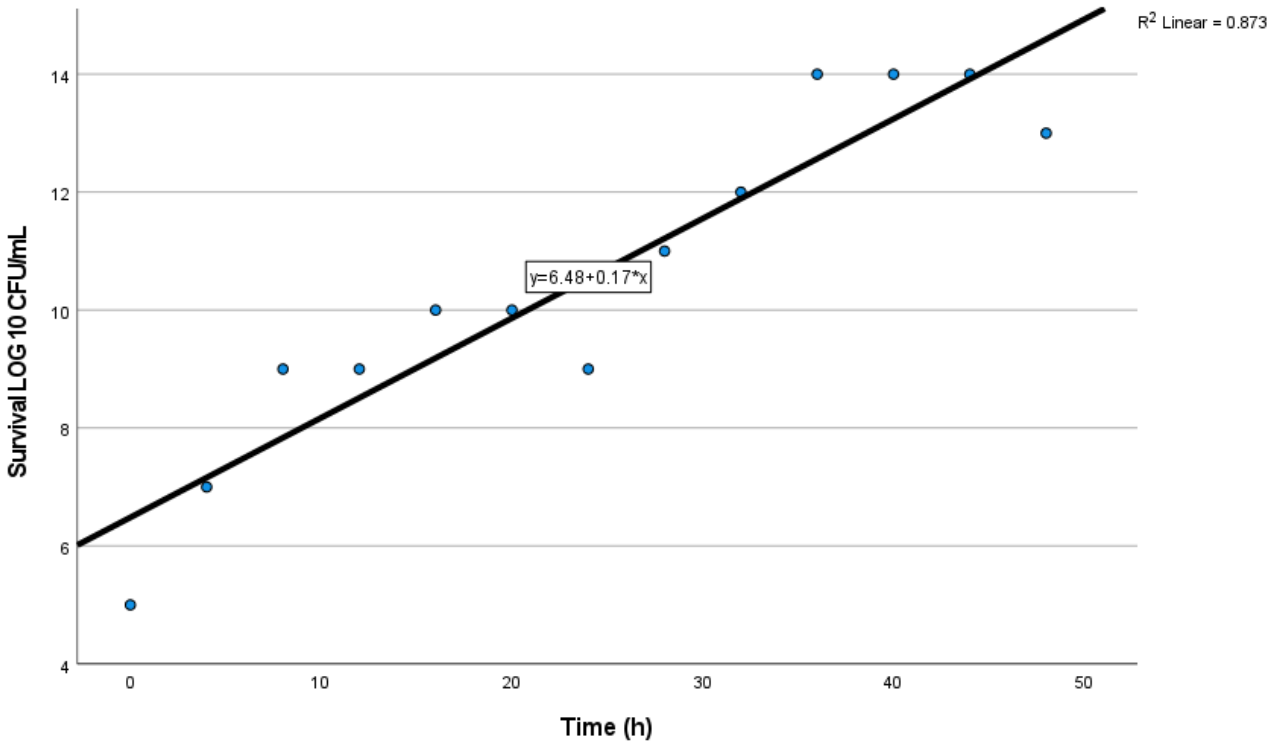

Figure 3.1: Growth Pattern for *Lactobacillus reuteri*

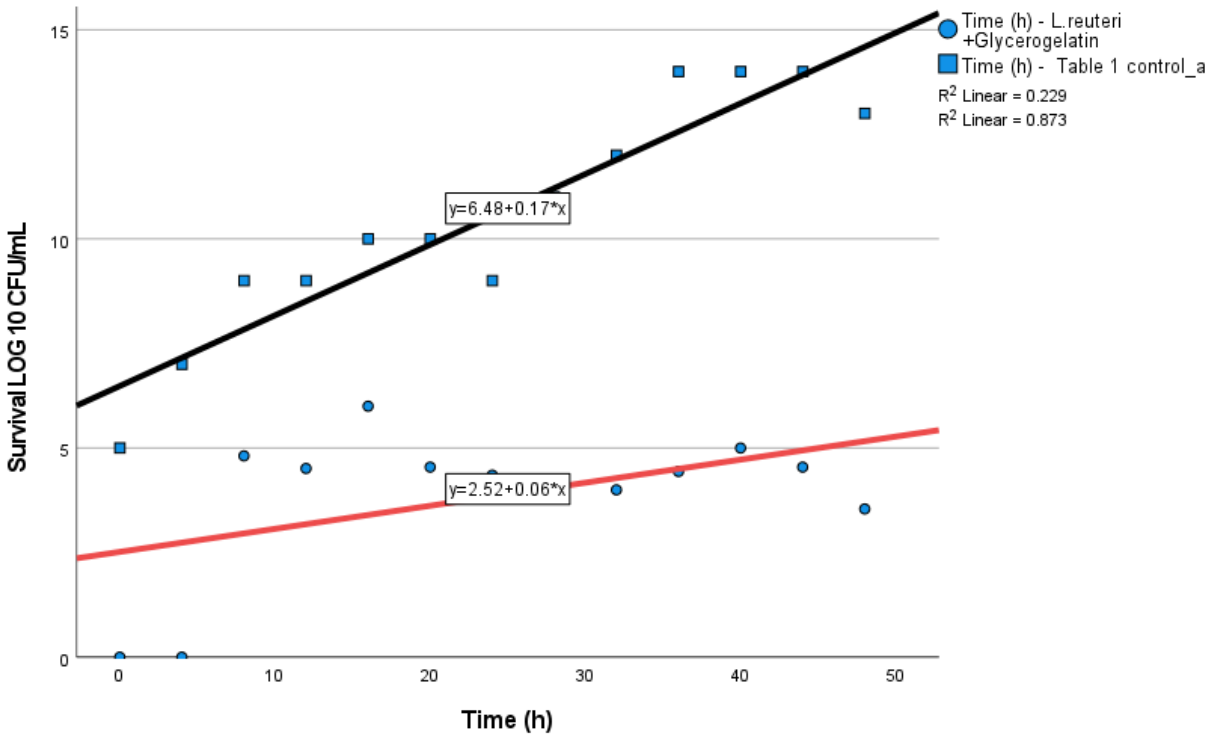

Figure 3.2: Growth Pattern for *Lactobacillus reuteri* in the presence of Glycerogelatin and *Escherchia coli*

P-value < 0.001

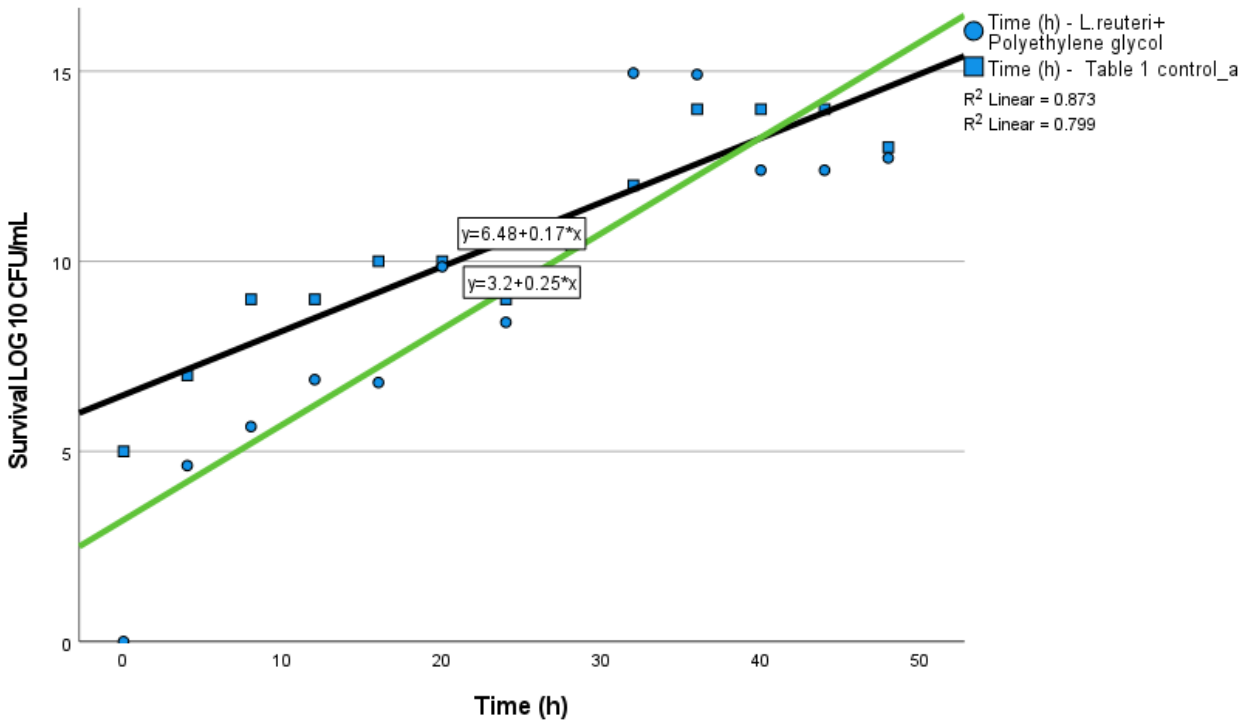

Figure 3.3: Growth Pattern for *Lactobacillus reuteri* in the Presence of Polyethylene glycol and *Escherchia coli*

P-value = 0.039

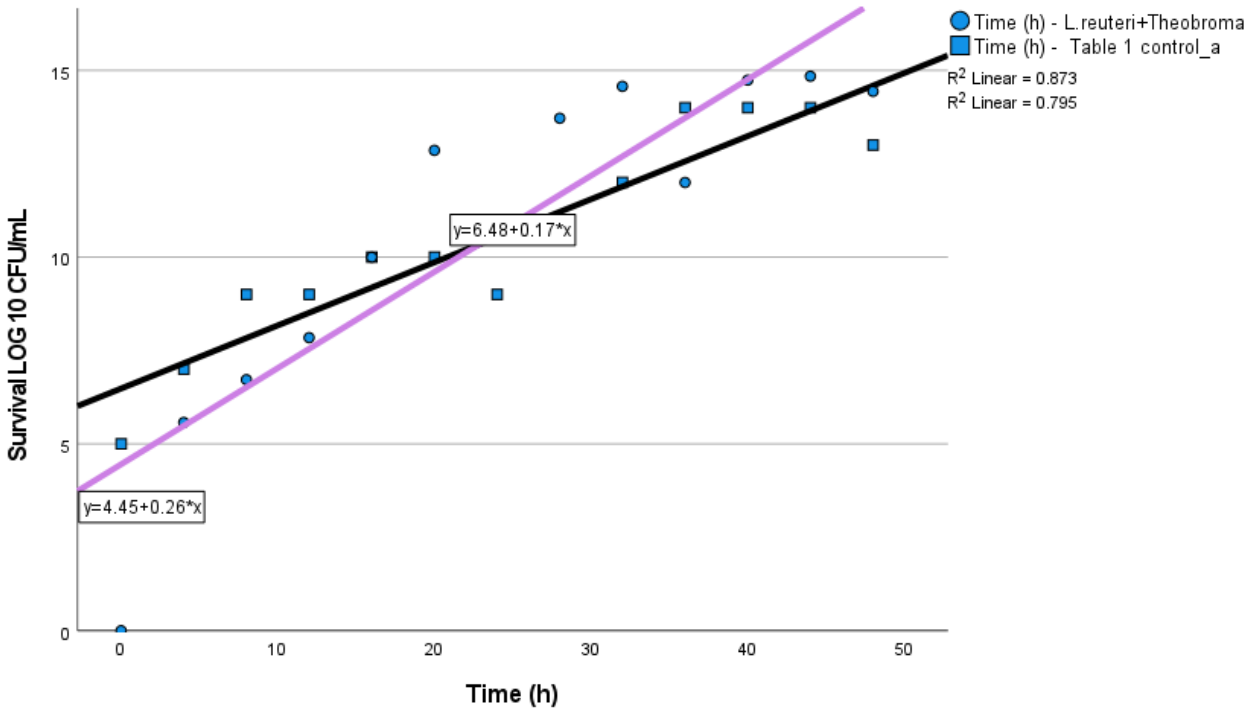

Figure 3.4: Growth Pattern for *Lactobacillus reuteri* in the Presence of Theobroma and *Escherichia coli*

P-value = 0.883

CHAPTER FOUR

4.0 DISCUSSION

4.1 GRAM STAINING

The gram staining test was done to ensure the right organism was isolated for the experiment. *Lactobacillus reuteri* was found gram positive as it became blue-purple after gram staining similar to the finding of a study that lactobacillus bacteria are generally gram-positive rods or coccobacilli occurring in chains (Akweya *et al;* 2022)

Escherichia coli retained the colour of the secondary dye- pink confirming to be a gram negative bacteria and similar to the findings of the research by (Abu-Sini *et al;* 2023).

4.2 BIOCHEMICAL TEST

4.2.1 Tube Catalase Test

In the tube of the isolated *L. reuteri,* no bubbles were observed indicating that the bacteria is catalase-negative (Table 3.1). This result correlates with study that lactic acid bacteria are catalase negative (Mokoena, 2017) and a study confirming *L. reuteri* as a catalase negative bacteria (Ali et al; 2023) which cannot mediate the decomposition of hydrogen peroxide (H_2O_2) to produce Oxygen (O_2) and water (H_2O)

The isolated *E. coli* was found catalase positive due to the production of bubbles in 3% hydrogen peroxide (Table 3.2). Gas bubbles indicate the presence of the catalase enzyme, which breaks down hydrogen peroxide into oxygen and water (the catalase positive characteristics of the bacterium). (Hossain *et al*; 2021)

This observations corresponds with the result of a study on the identification of coliform bacteria and multi-drug resistant *E. coli* from water intended for drug compounding in community pharmacies in Jordan. (Abu-Sini et al; 2023)

4.2.2 Carbohydrate Fermentation Test

The medium's colour change from red to yellow indicates that the bacteria are capable of fermenting the carbohydrates. Table 3.1 displays the glucose fermentation profile of L. reuteri. To find the sugar fermentation pattern, ten different sugars were utilised. Six out of ten times, the bacteria fermented. L-Arabinose, D-Mannitol, D-Xylose, and L-Rhamnose did not ferment. The results of the study by Ali et al. (2023), which found that not all carbohydrates could be fermented from the probiotic, were consistent with this fermentation pattern. The incapacity of the enzyme generated by the isolated probiotic bacterium to break down the other four sugars could be the reason for their incompatibility.

Eight of the ten sugars were found to be fermented by E. coli. This pattern of fermentation is comparable to the research conducted by Abu-Sini et al. (2023). The inability of the organism's enzyme to break down these sugars in the medium or the availability of enough sucrose and raffinose could be the cause of the two sugars' incompatibility.

4.3 CO-CULTIVATION ASSAY

This research examined the survival of *Lactobacillus reuteri* in the presence of selected suppository bases: glycerogelatin, polyethylene glycol (PEG), and theobroma co-incubated with *Escherichia coli*. Linear-regression analysis was used to determine changes in bacterial survival over time, expressed as slope and correlation coefficient (R^2). These quantitative parameters describe the rate and consistency of *L. reuteri* viability within each formulation.

In the control group (Figure 3.1), *L. reuteri* exhibited a slope of **0.17** and a strong linear correlation (**R^2 = 0.8732),** confirming predictable and steady growth under optimal conditions.

However, in the glycerogelatin formulation containing *E. coli* (Figure 3.2), the slope declined sharply to **0.06** with a poor linear relationship (**R^2 = 0.229)**, demonstrating a substantial 65% reduction in growth rate and inconsistency in viability over time. This pattern indicates that the combined presence of *E. coli* and glycerogelatin exerted an inhibitory effect on *L. reuteri*, reducing both its growth rate and survival predictability. The result of the paired *t*-test **(p <0.001)** confirms that the difference in survival between the control and test conditions was statistically significant, meaning there is less than 0.1% probability that this difference happened randomly. The sharp decline in slope demonstrates that the combined effect of glycerogelatin and *E. coli* considerably slowed the rate of viable cell accumulation, while the low R^2 shows that the survival pattern was inconsistent over time.

The hydrophilic and proteinaceous nature of glycerogelatin likely altered moisture and osmotic balance, producing stress conditions that disrupted membrane stability and enzyme function. (Saarela *et al.,* 2000) and (Shori, 2023) both reported that high water activity and protein interaction accelerate probiotic degradation. Moreover, *E. coli* may have intensified inhibition through nutrient competition, acid production, and bacteriocin-like activity (Ouwehand & Vesterlund, 2004; Poppi *et al.,* 2021).

In the formulation containing *E. coli* and polyethylene glycol (PEG) (Figure 3.3), *L. reuteri* demonstrated a slope of **0.25** and a strong correlation (**R^2 = 0.799**), higher than the control. This reflects enhanced linear growth and improved predictability of viability. PEG's semi-hydrophilic polymeric structure provides controlled water retention and near-neutral pH, supporting bacterial

stability while limiting osmotic stress. Despite the statistical significance of the difference from control (P = 0.039), the slope suggests that PEG promoted a more favourable survival rate. (Liu *et al.,* 2020) showed that polymer matrices like PEG act as semi-permeable barriers that maintain osmotic equilibrium and protect probiotics from environmental fluctuations. Similarly, recent findings by (Kanjan *et al.,* 2022*)* demonstrated that PEG-based carriers sustain Lactobacillus viability through improved hydration and surface interaction.

In the theobroma (Figure 3.4), *L. reuteri* recorded a slope of **0.26** and an $\mathbf{R^2}$ of **0.795,** values closely comparable to those for PEG and the control, indicating that *L. reuteri* survival followed a near-perfect linear relationship with time, even in the presence of *E. coli*. The statistical analysis **(p = 0.883)** further confirmed that there was no significant difference in growth between the control and theobroma formulation. This suggests that theobroma provided a highly compatible and protective environment for *L. reuteri* viability. The hydrophobic and inert properties of lipid-based systems limit oxygen penetration, moisture uptake, and oxidative stress, all of which enhance probiotic stability. (Rokka and Rantamäki, 2010*)* demonstrated that lipid systems form physical barriers that enhance probiotic survival, while (Song *et al.,* 2022; Azeem *et al.,* 2023) reported that solid-lipid carriers preserve microbial viability during processing and storage. The slightly higher slope for theobroma suggests that its lipid environment allowed efficient nutrient retention and stress resistance, producing stable linear growth despite microbial challenge, confirming its suitability as a base for probiotic suppositories.

Overall, the pattern of slopes and correlations follows the order glycerogelatin < control < PEG ≈ theobroma, indicating that as matrix hydrophobicity increases, *L. reuteri* survival becomes more linear and consistent. These results reinforce the concept that formulation environment and

microbial coexistence determine probiotic kinetics (Rokka & Rantamäki, 2010; Teymoori *et al.*, 2024).

4.3 LIMITATIONS OF THE STUDY

This study was limited by the inclusion of one probiotic organism *(Lactobacillus reuteri)* and a single pathogenic *(Escherichia coli)* organism. A broader inclusion of other probiotic and pathogenic strains could give a more thorough explanation of the interactions between microbes in suppository systems.

The experimental setup was in vitro, so it may not completely reproduce physiological conditions such as rectal or vaginal pH, temperature, mucous membrane interaction, and host immunity.

CHAPTER FIVE

5.0 RECOMMENDATION AND CONCLUSION

5.1 RECOMMENDATION

Based on these results, lipid-based suppository formulations are especially recommended for probiotic delivery systems using PEG and theobroma for their superior protective properties. Future research should focus on the development of hybrid lipid-polymer formulations and the incorporation of microencapsulation or solid lipid nanoparticle technology to enhance strength of bacteria (Azeem et al., 2023; Teymoori *et al.*, 2024). Expanding future studies to include multiple probiotic and pathogenic species and performing in-vivo validation would provide more comprehensive insights.

5.2 CONCLUSION

The survival of *Lactobacillus reuteri* were strongly influenced by the suppository base and the presence of *Escherichia coli*. Glycerogelatin exhibited the lowest slope (0.06) and weakest correlation ($R^2 = 0.229$) with a 65%, decrease in survival indicating severe inhibition of probiotic growth ($p < 0.001$). PEG produced a slope of 0.25 and correlation ($R^2 = 0.799$) with a 47% increase in survival, while theobroma yielded a slope of 0.26 and correlation ($R^2 = 0.795$) with a 53% increase in survival, both comparable to or slightly exceeding the control. These findings confirm that lipid and polymer-based matrices provide more stable and predictable environments for probiotic survival. Hence, theobroma and PEG are suitable suppository bases for probiotic delivery, whereas hydrophilic systems like glycerogelatin should be modified or avoided to ensure optimal microbial viability and therapeutic efficacy.

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