

PRODUCTION AND CHARACTERIZATION OF PROBIOTICS FROM THE FERMENTED FOOD BACTERIAL SOURCE AND ITS APPLICATION

*Submitted in partial fulfilment of the requirements for the award of Master of
Science in Medical Biotechnology and Clinical Research*

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Mr. JEBARAJ D (42101006)** who carried out Project entitled **"PRODUCTION AND CHARACTERIZATION OF PROBIOTICS FROM THE FERMENTED FOOD BACTERIAL SOURCE AND ITS APPLICATION"** under the supervision of **Dr. A. DAYANANDAN, Professor (Research)** from September 2023 to April 2024.

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DECLARATION

I, **Mr. JEBARAJ D (42101006)** hereby declare that the Project Report entitled **"PRODUCTION AND CHARACTERIZATION OF PROBIOTICS FROM THE FERMENTED FOOD BACTERIAL SOURCE AND ITS APPLICATION"** done by me under the guidance of **Dr. A. Dayanandan, Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

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ABSTRACT

Probiotics, live microorganisms providing health benefits to the host when administered in adequate amounts, have garnered significant attention for their potential in promoting gastrointestinal health and overall well-being. This study focuses on the production and characterization of probiotics sourced from fermented foods, exploring their diverse microbial communities and beneficial properties. Fermented foods, rich in probiotic bacteria, serve as promising reservoirs for the isolation and cultivation of potential probiotic strains. The production process involves isolation, identification, and evaluation of microbial strains for their probiotic potential, emphasizing criteria such as survival under gastrointestinal conditions, adhesion to intestinal epithelial cells, and antagonistic activity against pathogens. Characterization techniques, including molecular biology, biochemical assays, and genomic analysis, provide insights into the physiological, genetic, and functional attributes of probiotic strains. Furthermore, this study investigates the applications of fermented food-derived probiotics in various sectors, including food and beverage industries, pharmaceuticals, and agriculture. Their incorporation into functional foods, such as yogurt, cheese, and fermented beverages, enhances nutritional value and confers health benefits to consumers. Additionally, probiotics exhibit potential therapeutic applications in preventing and treating gastrointestinal disorders, modulating immune responses, and improving metabolic health. Moreover, their role in enhancing soil fertility, promoting plant growth, and mitigating plant diseases underscores their significance in agricultural practices. In conclusion, the production and characterization of probiotics from fermented food bacterial sources offer promising avenues for enhancing human health and agricultural sustainability. Understanding their mechanisms of action and exploring innovative applications pave the way for the development of novel probiotic products with diverse health-promoting properties.

Key Words: Probiotics, Microorganisms, Pathogens, Fermented Food Bacterial source.

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ABBREVIATIONS

GABA	Gamma Aminobutyric Acid
IBS	Irritable Bowel Syndrome
IBD	Inflammatory Bowel Disease
CFUs	Colony-Forming Units
LAB	Lactic Acid Bacteria
ISAPP	International Scientific Association for Probiotics and Prebiotics
FDA	Food and Drug Administration
EFSA	European Food Safety Authority
PAMPs	Pathogen Associated Molecular Patterns
PRRs	Pattern Recognition Receptors
H ₂ O ₂	Hydrogen Peroxide
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography

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CHAPTER-1

INTRODUCTION

1.1 Probiotics

Probiotics are live microorganisms, mainly bacteria and yeasts, which are believed to confer health benefits when consumed in adequate amounts. They are commonly found in fermented foods like yogurt, kefir, kimchi, sauerkraut, and kombucha, as well as in dietary supplements (*Kechagia et al., 2013*). The primary rationale behind consuming probiotics is to replenish and maintain the balance of beneficial bacteria in the gut microbiome. These microorganisms play a crucial role in various aspects of human health, including digestion, immune function, and even mental health (*Dimidi et al., 2019*).

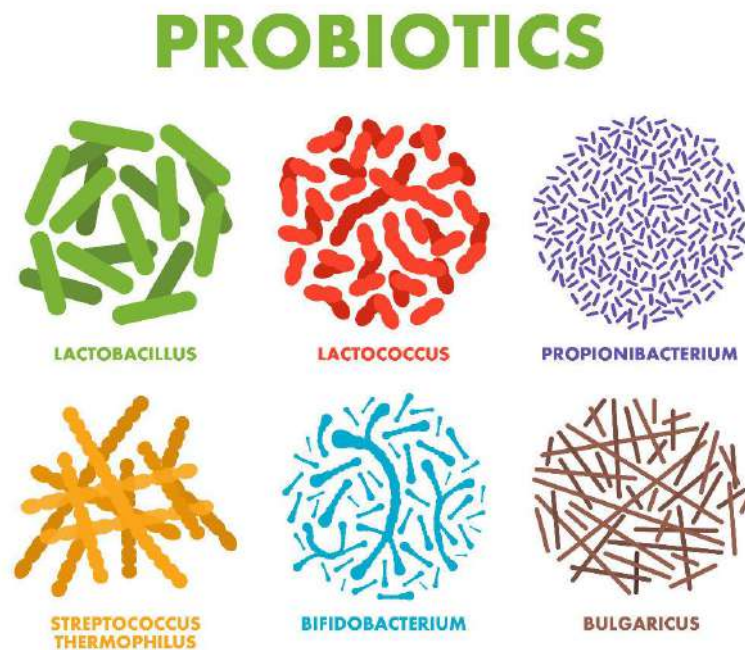


Fig:1.1 Probiotics

1.2 Mechanisms of action

Numerous microorganisms, including bacteria, viruses, fungi, archaea, and protozoa, populate the human gastrointestinal system. These bacteria, sometimes referred to as the gut microbiota, microbiome, or intestinal microflora, have the ability to impact human health and disease through their

activity and makeup (Thursby and Juge, 2017). Probiotics typically affect the intestinal microbiota in the gastrointestinal tract where they may have an impact. Depending on the probiotic strain, the gastrointestinal tract area, and the baseline microbiota, probiotics can transiently colonize the human gut mucosa in highly customized ways (Gadaleta et al., 2022). Probiotics also affect health through pathways that are strain-, species-, and nonspecific. The nonspecific mechanisms of routinely used probiotic supplements differ greatly between strains, species, and even taxa. These mechanisms include the generation of bioactive metabolites (such as short-chain fatty acids), the decrease of luminal pH in the colon, and the inhibition of the growth of pathogenic microorganisms in the gastrointestinal tract (by fostering colonization resistance, improving intestinal transit, or helping to normalize a perturbed microbiota) (Jandhyala et al., 2015). Vitamin synthesis, gut barrier reinforcement, bile salt metabolism, enzymatic activity, and toxin neutralization are examples of species-specific systems. Few strains of a particular species employ strain-specific processes, which are uncommon and include cytokine generation, immunomodulation, and effects on the neurological and endocrine systems. Probiotics may affect human health and disease in a variety of ways via each of these methods. Recommendations for using probiotics in the clinic or in research studies must be species- and strain-specific since the effects of probiotics can be unique to certain probiotic species and strains. Moreover, combining data from various probiotic trials can lead to inaccurate conclusions on the safety and effectiveness of these supplements (Rowland et al., 2018).

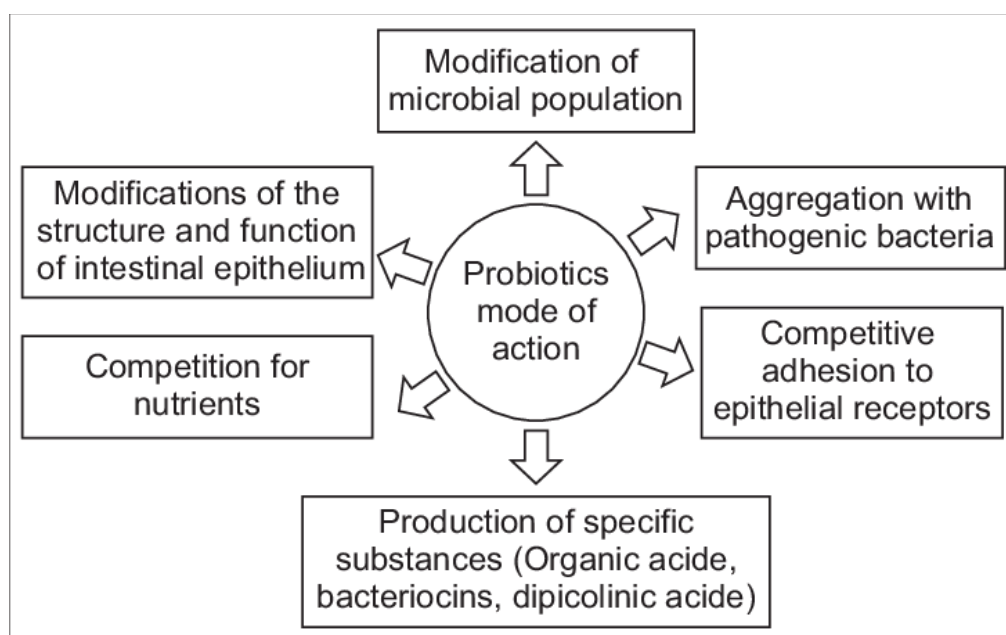


Fig:1.2 Mechanism of action

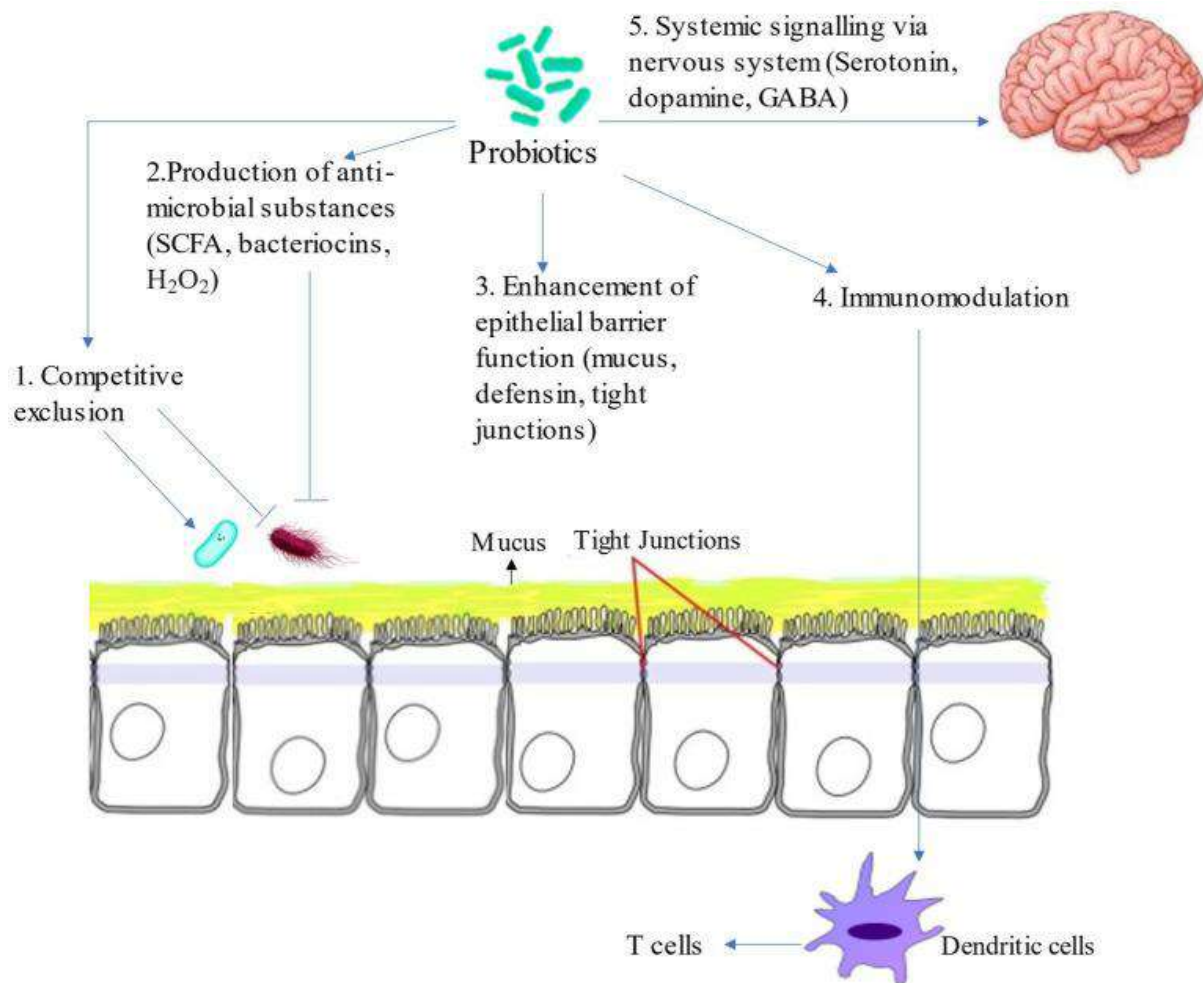


Fig:1.3 The way that probiotics work. 1. Probiotics work by preventing infections from surviving and adhering to the intestinal mucosa by competing with them for nutrition and binding receptors. 2. Probiotics create antimicrobial compounds that stop the growth of infections. 3. Probiotics improve mucus formation and tight junction protein expression, which stop pathogens from moving from the intestine into the bloodstream and support the operation of the epithelial barrier. 4. Probiotics control the host's immunity by altering the development and function of dendritic cells, which in turn boosts the activity of T cells, which are crucial for maintaining immunological homeostasis. 5. Probiotics also control the synthesis of dopamine, gamma aminobutyric acid (GABA), and serotonin, among other neurotransmitters (Latif et al., 2023).

1.3 Potential benefits of Probiotics

Probiotics can help alleviate symptoms of digestive disorders such as diarrhea, irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD). Probiotics may strengthen the immune system by promoting the production of antibodies and enhancing the activity of immune cells (Sharma *et al.*, 2023). Probiotics help maintain a healthy balance of bacteria in the gut, which is essential for overall digestive health and well-being. There is some evidence to suggest that probiotics could reduce the risk of developing allergies or alleviate symptoms in individuals with existing allergies. Emerging research suggests that the gut microbiome may influence mental health, and probiotics may play a role in improving mood and reducing symptoms of anxiety and depression (Sharma *et al.*, 2023).

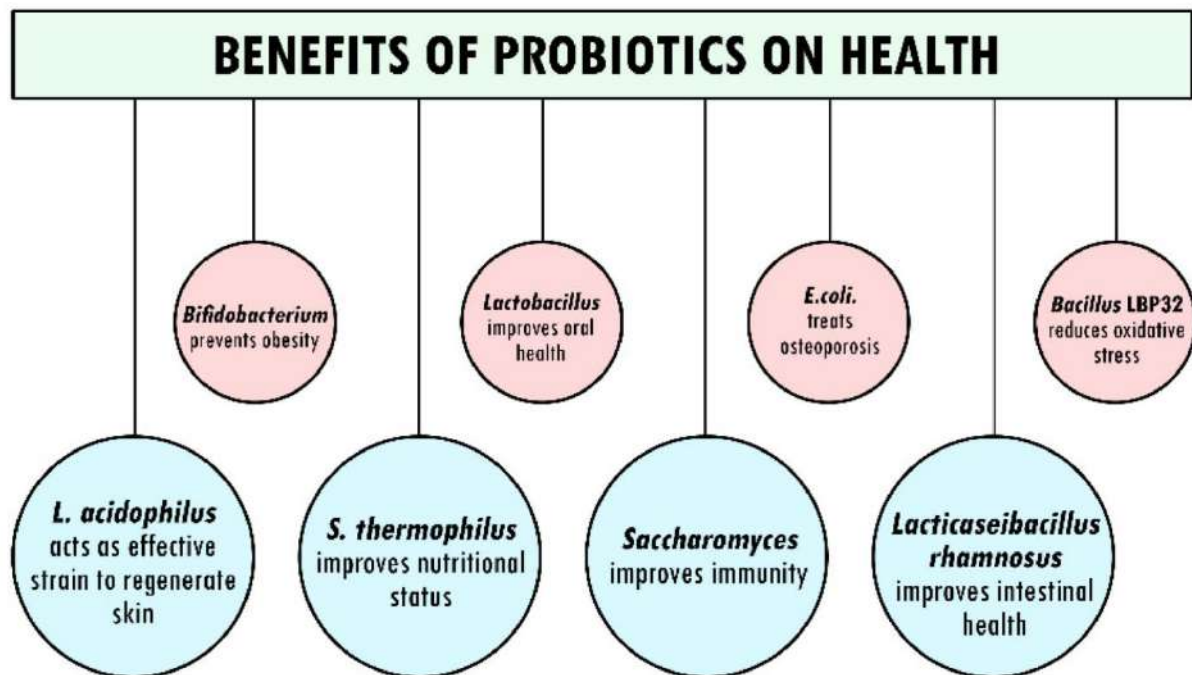


Fig:1.4 Benefits of probiotics.

1.4 Choosing Probiotics

Strain: Different strains of probiotics may have different effects on health, so choose a product that contains strains supported by research for your specific health needs.

CFUs (Colony-Forming Units): This indicates the number of viable microorganisms present in the supplement. Look for products with a high CFU count, typically in the billions (*Kechagia et al., 2013*).

Quality and Purity: Choose reputable brands that ensure the quality and purity of their products through third-party testing and certification.

Storage and Handling: Probiotics are sensitive to heat and moisture, so store them according to the manufacturer's instructions to maintain their potency (*Jackson et al., 2019*).

Precautions and Side Effects: While probiotics are generally considered safe for most people, some individuals may experience mild side effects such as bloating, gas, or digestive discomfort. People with compromised immune systems or underlying health conditions should consult with a healthcare professional before taking probiotic supplements (*Fenster et al., 2019*).

1.5 Fermented food as probiotics

A significant part of diets is made up of fermented foods and beverages that come from both plants and animals. Natural ingredients in these meals include organic acids, ethanol, and antimicrobial compounds that can suppress pathogenic bacteria and spoilage organisms in fermented foods. Lactic acid bacteria (LAB) are the bacteria that proliferate during fermentation and are what give these foods their characteristic flavour. Also referred to as probiotics, these bacteria are beneficial to both human and animal health, particularly in the digestive tract, and they may readily adapt to the spontaneous fermentation process. Given that probiotics typically strengthen the immune system against harmful bacteria, the purpose of this study is to characterize the microorganisms found in fermented foods that may be developed into probiotics to enhance human health (*Soemarie et al., 2021*).

1.6 Probiotics as microbial source

Probiotics are live bacteria that give the host health benefits when given in sufficient doses. The majority of scientific papers utilize this definition of probiotics, which is also accepted by the International Scientific Association for Probiotics and Prebiotics (ISAPP). Nevertheless, the U.S. Food and Drug

Administration (FDA) and the European Food Safety Authority (EFSA) currently reject this definition, arguing that the health claim included in it is not quantifiable because commercial markets have outpaced science's capacity to support the evidence (*Salminen et al., 2021*). Consequently, no probiotic in the United States has been approved to make any kind of verified health claim. Nevertheless, health claims, such as the use of probiotics to treat and cure diseases, are measurable and can be proven with similar type of studies as are conducted for drugs (*Fijan, 2014*).

1.7 Probiotics as bacterial source

The potential of lactic acid bacteria species that were isolated from different food sources as probiotics was investigated. *Lactobacillus bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, and *Pediococcus cerevisiae* were the most common species found in the isolated strains (*Monika et al., 2017*). Bacteriocins that they created stopped the growth of *Bacillus cereus*, *Escherichia coli*, and *Streptococcus aureus*. To investigate the suitability of these raw materials for the manufacture of probiotic juice, *L. bulgaricus* and *L. plantarum* were chosen to ferment vegetable substrates, such as carrots, cucumbers, and tomatoes, as well as cereals, such as rice and maize (*Mokoena et al., 2016*).

1.8 Production of Probiotics

The first step is to select the appropriate strains of bacteria or yeast that have proven health benefits. These strains are often selected based on their ability to survive the digestive tract and confer health benefits to the host. The selected strains are then cultured in a suitable medium that provides the necessary nutrients for their growth. This medium may include ingredients like sugars, proteins, vitamins, and minerals (*Terpou et al., 2019*). The cultured strains are then fermented under controlled conditions, such as temperature, pH, and oxygen levels. Fermentation allows the bacteria or yeast to multiply and produce the desired metabolites, such as lactic acid in the case of lactic acid bacteria. Once the fermentation process is complete, the probiotic cells are harvested from the culture medium. This can be done through methods like

centrifugation or filtration (*Dimidi et al., 2019*). The harvested probiotic cells are concentrated to increase their potency. This may involve removing excess culture medium and concentrating the cells into a more compact form. The concentrated probiotic cells are then formulated into the desired product, which could be capsules, tablets, powders, or liquids. Formulation may involve mixing the probiotics with excipients or other ingredients to improve stability and shelf-life (*Fenster et al., 2019*). Throughout the production process, quality control measures are implemented to ensure the potency, purity, and safety of the final product. This includes testing for microbial contamination, viability of the probiotic strains, and compliance with regulatory standards. The final probiotic product is packaged in suitable containers that protect it from moisture, light, and oxygen, which can degrade the viability of the probiotic cells. Proper storage conditions are also important to maintain the potency of the product until it reaches the consumer. The finished probiotic products are then distributed to retailers or directly to consumers through various channels. Continuous monitoring of the production process and gathering feedback from consumers helps to improve the quality and effectiveness of probiotic products over time (*Iannitti and Palmieri, 2010*).

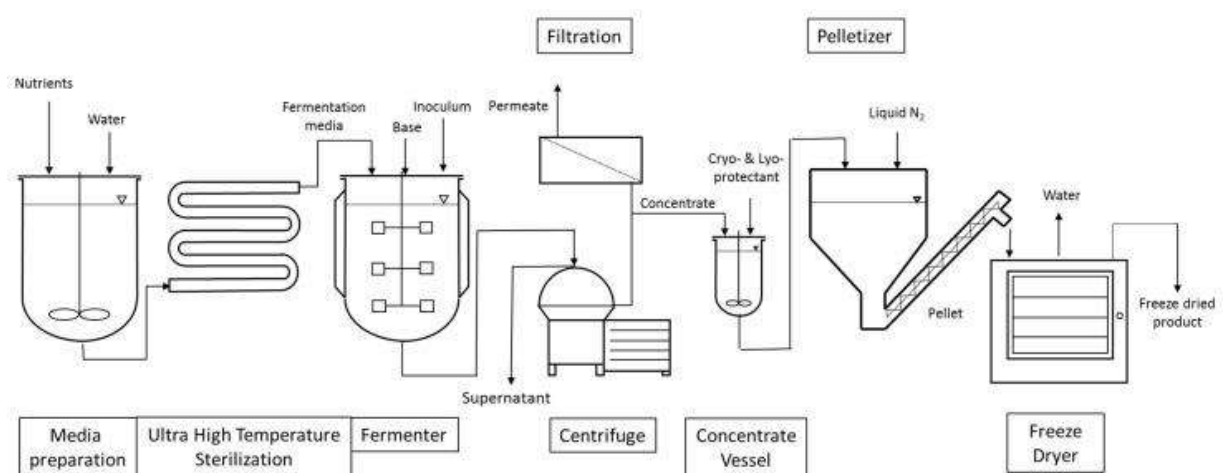


Fig:1.5 Schematic representation of the production of probiotics for dietary supplements and dairy starter culture strains (*Fenster et al., 2019*).

1.9 Characterization of Probiotics

Probiotic microorganisms belong to various taxonomic groups such as bacteria (e.g., *Lactobacillus*, *Bifidobacterium*) and yeasts (e.g., *Saccharomyces*). Accurate identification of probiotic strains is crucial to understand their characteristics and potential health benefits (*Fijan, 2014*). Genetic analysis helps in determining the genetic makeup of probiotic strains, including their genomic sequences, genes responsible for probiotic functions, and any potential virulence factors. This includes studying the physiological characteristics of probiotic strains such as their growth requirements, metabolic activities, tolerance to various environmental conditions (e.g., temperature, pH), and ability to survive passage through the gastrointestinal tract (*Zawistowska-Rojek et al., 2022*). Probiotics exert health benefits through various mechanisms such as competitive exclusion of pathogens, production of antimicrobial substances, modulation of the immune system, and enhancement of gut barrier function. Characterization involves evaluating these functional properties through in vitro and in vivo studies. Safety evaluation is essential to ensure that probiotic strains are safe for human consumption (*Pramanik et al., 2023*). This includes assessing their pathogenic potential, antibiotic resistance profile, and potential for adverse effects in vulnerable populations. Probiotics must remain viable and stable throughout their shelf life and passage through the gastrointestinal tract to exert their beneficial effects (*Latif et al., 2023*). Characterization involves studying factors affecting their stability, such as storage conditions, formulation, and encapsulation techniques. Preclinical and clinical studies are conducted to evaluate the efficacy of probiotics in promoting human health and managing various health conditions. Characterization involves analyzing the outcomes of these studies to understand the therapeutic potential of probiotic strains (*Wang et al., 2022*).

1.10 Application of probiotics

Probiotics play a crucial role in maintaining a healthy balance of gut bacteria. They can help alleviate symptoms of digestive disorders such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and diarrhea caused by infections or antibiotic use. Probiotics work by replenishing beneficial bacteria in

the gut and inhibiting the growth of harmful bacteria (*Verna and Lucak, 2010*). There is evidence to suggest that probiotics can boost the immune system by enhancing the production of antibodies and promoting the activity of immune cells. This can help reduce the risk of infections and may be particularly beneficial during times of stress or illness. Probiotics are often used to prevent and treat vaginal infections such as yeast infections and bacterial vaginosis (*Hemarajata and Versalovic, 2013*). By restoring the natural balance of bacteria in the vagina, probiotics can help maintain vaginal health and reduce the risk of recurrent infections. Some research suggests that probiotics may have a beneficial effect on certain skin conditions, including acne, eczema, and rosacea. Probiotic supplements and topical preparations containing probiotics have been shown to reduce inflammation, improve skin barrier function, and inhibit the growth of acne-causing bacteria (*Mei and Li, 2022*). Probiotics may help support oral health by reducing the growth of cavity-causing bacteria, preventing bad breath, and alleviating symptoms of gum disease. Some studies have shown that probiotic strains such as *Lactobacillus reuteri* and *Lactobacillus salivarius* can inhibit the growth of harmful bacteria in the mouth and promote the growth of beneficial bacteria (*Bustamante et al., 2020*). Emerging research suggests that there may be a connection between gut health and mental health, and probiotics may play a role in this relationship. Some studies have shown that probiotic supplementation can improve symptoms of depression, anxiety, and stress by modulating the gut-brain axis and reducing inflammation (*Madabushi et al., 2023*). While more research is needed, some studies have suggested that probiotics may help support weight loss and prevent obesity. Probiotics may influence energy metabolism, fat storage, and appetite regulation, although the mechanisms are not yet fully understood (*Appleton, 2018*).

CHAPTER 2

REVIEW OF LITERATURE

2.1 PROBIOTICS

Since humans have been consuming fermented foods for about 10,000 years, there has been a significant increase in their diversity in the 21st century. The discovery of bioactive peptides and microbial metabolites in fermented foods that can improve human health has solidified the interest in the health benefits of fermented foods. Generally, every fermented meal has its own unique population of bacteria (Leeuwendaal et al., 2022).

Yulistia Budianti Soemarie, et al., 2021 had reported that Lactic Acid Bacteria (LAB) were essential to food fermentation because they could create substances that were good for both human and animal health. Additionally, by preserving the equilibrium of the gut microbiota, LAB generated from single or mixed cultures derived from fermented foods were appropriate probiotics for enhancing the digestive system. These microbes could also strengthen the immune system's defences against harmful bacteria. Probiotics from a number of species, including *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Pediococcus*, and numerous yeasts, were utilised (Soemarie et al., 2021).

2.1 MECHANISM OF ACTIONS

The control of the host immune response was one of the primary mechanisms that probiotics worked. Consequently, the innate and adaptive components of the immune system were separated. B and T cells were essential for the adaptive immune response because they bound to particular antigens. On the other hand, the majority of infections contain common features known as pathogen-associated molecular patterns (PAMPs) that elicited responses from the innate system. Pattern recognition receptors (PRRs) were responsible for the initial response to infections by binding to PAMPs.

The mechanisms of action of probiotics involved colonization and normalization of perturbed intestinal microbial communities in both children and adults; competitive exclusion of pathogens and bacteriocin production; modulation of enzymatic activities related to metabolization of a number of carcinogens and other toxic substances; and production of volatile fatty acids, namely, SCFAs

and BCFAs, which played a role in the maintenance of energy homeostasis and regulation of functionality in peripheral tissues (Plaza-Diaz et al., 2019).

Although the precise way in which probiotics function is still undetermined, they have a variety of methods of action. These include the formation of bacteriocin and short chain fatty acids, the reduction of gut pH, nutritional competition, and the promotion of immune modulation and mucosal barrier function. Probiotics, specifically the latter, have been the focus of multiple studies, and there is strong evidence that they affect various aspects of the innate and acquired immune response. These effects include phagocytosis and IgA secretion, T-cell response modification, Th1 response enhancement, and Th2 response attenuation (McNaught and MacFie, 2001).

2.3 FERMENTED FOOD AS PROBIOTICS:

(Natasha K. Leeuwendaal, et al., 2022) After being consumed, nutrients and microbes from fermented foods may live and interact with the gut microbiome. The species and strain level of this microbiome was subsequently determined by metagenomics. It would then be possible to ascertain if fermented food strains colonise the gut temporarily or permanently, as well as how fermented foods affect natural gut microorganisms. This review took into account the main food fermentation pathways and the microorganisms that are involved, as well as the possible health benefits and the potential for these foods to affect the gut microbiome once consumed, either through compounds produced during fermentation or through interactions with the food's microorganisms that can survive in the gastrointestinal tract. Bacterial EPS, which can lower cholesterol, and the well-known ACE inhibitory peptides are two examples of the bioactive peptides that have been shown to be released during fermentation. Although several studies have shown the health advantages of fermented foods have been published by Marco et al. (2017), controlled human clinical trials remain the gold standard for confirming benefits linked with foods or medications (Marco et al., 2017).

(Antonia Terpou, et al., 2019) To increase and maintain microbial cell viability, a number of strategies are used, including strain selection, immobilisation techniques, the creation of synbiotics, etc. Among these, the immobilisation of

cells in diverse carriers—such as composite carrier matrix systems—has drawn attention lately as a means of shielding probiotics from various forms of environmental stress (such as pH and heat treatments). Similarly, for the probiotics to be effectively delivered in the large intestine, the cells need to be able to endure the stresses of the upper gastrointestinal tract as well as food processing and storage. Because of this, choosing the right probiotics and delivering them efficiently continue to be technological challenges, with a particular emphasis on maintaining the viability of the probiotic culture in the formulated product. Another method of using functional foods to promote the growth of probiotics is demonstrated by the development of synbiotic combos. This review's objective was to provide an overview of the innovative methods and tactics used to increase the viability of probiotics (Terpou et al., 2019).

2.4 BENEFITS OF PROBIOTICS:

Probiotics consumption has been demonstrated to enhance intestinal health, minimise the symptoms of lactose intolerance, and lower the risk of developing a number of other diseases. Several well-studied strains of Bifidobacteria and Lactobacilli are also available for human use. (Salminen et al., 2005) They have effectively changed throughout time, with the more recent ones accumulating more convincing proof that probiotic bacteria can benefit human health. These findings align with consumers' growing understanding of the connection between nutrition and health, which has fostered the growth of the functional food concept a term used to characterise foods or food ingredients that have positive health effects in addition to their nutritional value. The market for functional foods is growing, particularly in Japan, where it originated. There are also opportunities for expansion in Europe and the US. Probiotics account for the majority of the goods in most of these nations (*Ziemer and Gibson, 1998*).

Scientific data was available to support the use of probiotics in diet to achieve health advantages. For some conditions, this data appeared sufficient for therapy and prevention; for others, it was only promising or even contentious. The most well-researched side effects included allergic reactions, diarrhoea linked to antibiotics, lactose intolerance, and infectious diarrhoea. Growing evidence has emerged regarding their possible function in additional conditions.

Concurrently, there was a surge in consumer awareness of the issue, and these products gained popularity, making up one of the biggest markets for functional foods. Yoghurt and other dairy products remained the most significant means of supplying consumers with probiotic bacteria, but the non-dairy market was also changing at a rapid pace due to advancements in food technology and rising consumer demand. As a result, a positive feedback loop was established: as the selection of new items with enhanced sensory appeal expanded, customer acceptability rose and the food sector made more investments by creating new goods and procedures that addressed to this expanding market (*Kechagia et al., 2013*).

There is evidence that eating foods high in probiotic microorganisms may help lower the incidence of cancer. Studies have shown that certain members of *Lactobacillus* and *Bifidobacterium* spp. reduce the levels of carcinogenetic enzymes produced by colonic flora by improving the host's immune system, producing antimutagenic organic acids, and restoring intestinal permeability and microflora balance. The precise mechanisms underlying these effects are still being investigated. (*Hirayama and Rafter, 1999*) Additionally, research indicates that foods containing probiotic bacteria may help manage blood pressure and lower serum cholesterol, which may help avoid coronary heart disease. The generation of end fermentation products, which influence the systemic levels of blood lipids and mediate an antihypertensive impact, direct cholesterol assimilation, and interference with gut-derived cholesterol absorption are some of the proposed processes. Nevertheless, more investigation is required in long-term human studies, so these probiotic effects remain up for discussion.

Finally, probiotic strains added to dairy products have been demonstrated to enhance the therapeutic result for women suffering from bacterial vaginosis; this is likely due to the probiotics' ability to maintain a healthy vaginal lactobacilli microbiome (*Falagas et al., 2007*).

2.5 PRODUCTION OF PROBIOTICS:

Following the demonstration by human intervention studies that microorganisms met the criteria for classification as probiotics, the next step was to determine whether the strains could be successfully cultivated on an industrial scale and

added to consumer products. To avoid researching a strain that cannot be commercialised, this portion of the probiotic industry should ideally have occurred concurrently with clinical studies. Large-scale strain culture and industrial processing have substantially different requirements for strains than does laboratory-scale strain culture; medium requirements also range greatly because of cost and other considerations. A quality control programme had to be set up to guarantee the consistent quality of everything, from ingredients to finished product, and a quality assurance programme had to be in place in order to guarantee the strains' consistently excellent quality. All of this necessitated meticulous recording of steps taken and outcomes. The strain(s) needed to be added to consumer goods once high-quality probiotic bulk was created. The shelf life, storage conditions, and product mix of these items varied. Regardless, at the end of the product's shelf life, the user should receive a minimally effective dose. Because probiotics are live microbes, this was all difficult. Though much may be accomplished and the customer could receive the researched health advantages, much would depend on selecting the appropriate strains, culture conditions, and product manufacture (Fenster et al., 2019).

Probiotics are harmless bacteria that have some positive effects on the host when given to humans in the right amounts and at the right times. Probiotics work through a variety of mechanisms, including the colonisation and normalisation of disturbed intestinal microbial communities in both adults and children, the competitive exclusion of pathogens and the production of bacteriocin, the modulation of enzymatic activities related to the metabolism of various carcinogens and other toxic substances, and the production of volatile fatty acids, specifically short-chain fatty acids (SCFAs and BCFAs), which are important for maintaining energy homeostasis and regulating peripheral tissue functionality. Probiotics also improve mucin synthesis and intestinal cell adhesion, as well as alter immune system and gut-associated lymphoid tissue activity (Plaza-Diaz et al., 2019).

2.6 APPLICATION OF PROBIOTICS:

Consumer interest in probiotic foods increased as a result of rising public awareness of diet-related issues and mounting evidence of the health advantages of probiotics. Probiotic foods came in a wide variety of forms, such as yoghurt, powdered milk, frozen fermented dairy desserts, cheese and cheese products, ice creams, infant meals, cereals, and fruit juices (Grasso et al., 2022).

Probiotics' sensitivity to elevated temperature treatments during processing and the GI stressors experienced by humans were the greatest barrier to their use in the food sector. To address the problems, though, scientists and the food industry were searching for fresh approaches. Food manufacturers were already highly interested in creating new probiotic-infused goods as a result of the probiotic products' exponential surge in sales. In the dairy, beverage, baking, and edible film sectors, probiotics were widely used (Reque and Brandelli, 2021).

Furthermore, a growing body of research has been conducted using the animal model; therefore, it is necessary to extrapolate these findings to people. Presently, unique chemicals with potential health benefits are being delivered by genetically altered commensal lactic acid bacteria. However, the majority of research on recombinant microorganisms is focused on vaccinations. Genetically engineered bacteria, however, can be employed to investigate novel approaches for delivering bioactive compounds to mucosal tissues. To determine probiotics' effects on the immune system and to disclose their limitations, safety, and efficacy, more reliable and repeatable clinical research are needed (Latif et al., 2023).

In addition to stomach issues, as demonstrated, probiotics can aid with a variety of acute and chronic infectious ailments. Numerous elements can compromise the body's defences, leading to viral, inflammatory, neoplastic, and degenerative illnesses. The typical flora in the stomach may change as a result of other treatments such as immunosuppressive therapy, radiotherapy, and antibiotics. Probiotics and other functional food products aim to enhance cellular health and

hasten the activation of cells' defence mechanisms. Probiotic impact on human sickness, diseases, mood, behaviour, and performance have been the subject of several research. The authors suggest that pharmacological therapy, dietary modification, and the use of complementary therapies such as probiotics be included in the treatment regimen. Probiotics are a prospective therapeutic and preventive option for a range of disorders, including inflammatory disease, because of their proven immune-modulatory effects on the host. Probiotic use in the form of powder, capsules, and liquids aids in the restoration of the gut's healthy microorganisms, which strengthens immunity in humans (Tegegne and Kebede, 2022).

CHAPTER – 3

AIM AND OBJECTIVES

3.1 Aim

To produce and characterize the probiotic organisms from fermented food sources.

3.2 Objectives

- Isolate bacterial strains from fermented foods with probiotic potential.
- Optimize the production process for probiotics, including fermentation conditions and media composition.
- Characterize the isolated probiotic strains for key properties such as survival in simulated gastrointestinal conditions, adhesion to intestinal epithelial cells, and antimicrobial activity against pathogenic bacteria.
- Evaluate the safety profile of the isolated probiotics through toxicity assays.
- Investigate the potential applications of the isolated probiotics in functional foods, dietary supplements, or pharmaceutical formulations.
- Assess the efficacy of probiotic products in improving human health outcomes through in vitro and/or in vivo studies, focusing on parameters such as gut microbiota modulation and hypercholesterolaemia modulation.

CHAPTER-4

MATERIALS AND METHODS

4.1 Pure Culture Techniques

A mixed culture, which comprises multiple species, can be converted into a pure culture by either thinning the sample multiple times prior to inoculating the new medium or by putting a small sample into a fresh, sterile growth medium in a way that distributes the individual cells around the medium surface (Mauerhofer et al., 2019).

4.1.1 Streak Plate Method

A small sample of the mixed culture is streaked onto the surface of an agar plate using an inoculating loop. The loop is sterilized between streaks to dilute the bacterial population and encourage the growth of individual colonies. After incubation, individual colonies derived from single bacterial cells are visible and can be isolated for further study (Sanders, 2012).

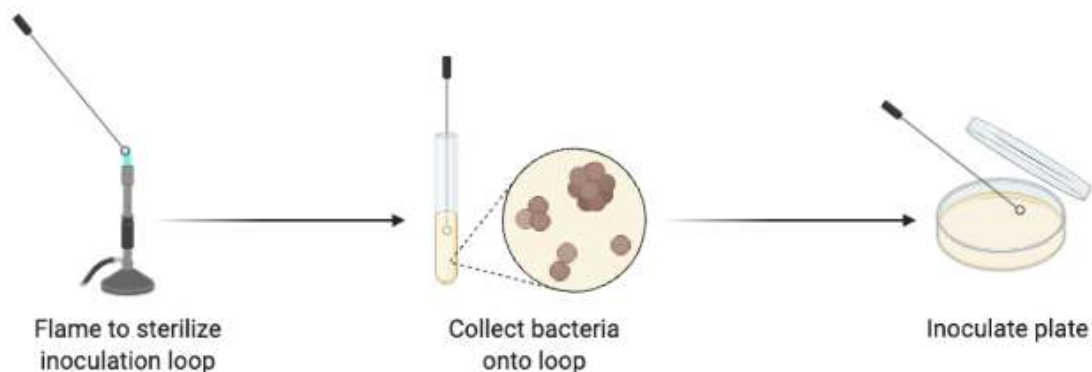


Fig:4.1 Streak plate method

4.1.2 Serial Dilution and Spread Plate Method

The mixed culture is serially diluted in a series of tubes containing sterile diluent (e.g., saline solution). Aliquots from each dilution tube are spread onto the surface of agar plates using a sterile spreader. After incubation, plates with well-isolated colonies are selected, and individual colonies are picked for further cultivation (Thomas et al., 2015).

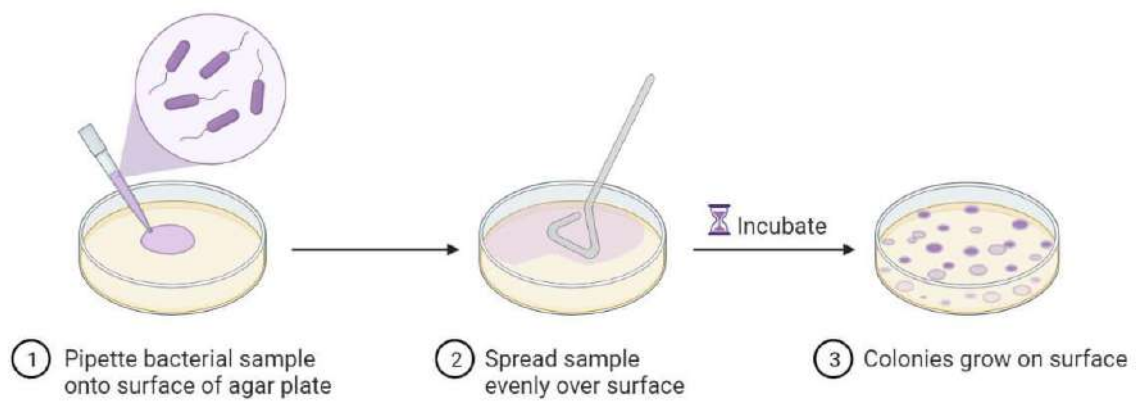


Fig:4.2 Spread plate method

4.1.3 Pour Plate Method

The mixed culture is diluted in a series of tubes containing molten agar medium. Diluted samples are poured into sterile petri dishes and allowed to solidify. After incubation, colonies grow both on the surface and within the agar medium. Individual colonies can be picked from within the agar for isolation (Mauerhofer et al., 2019).

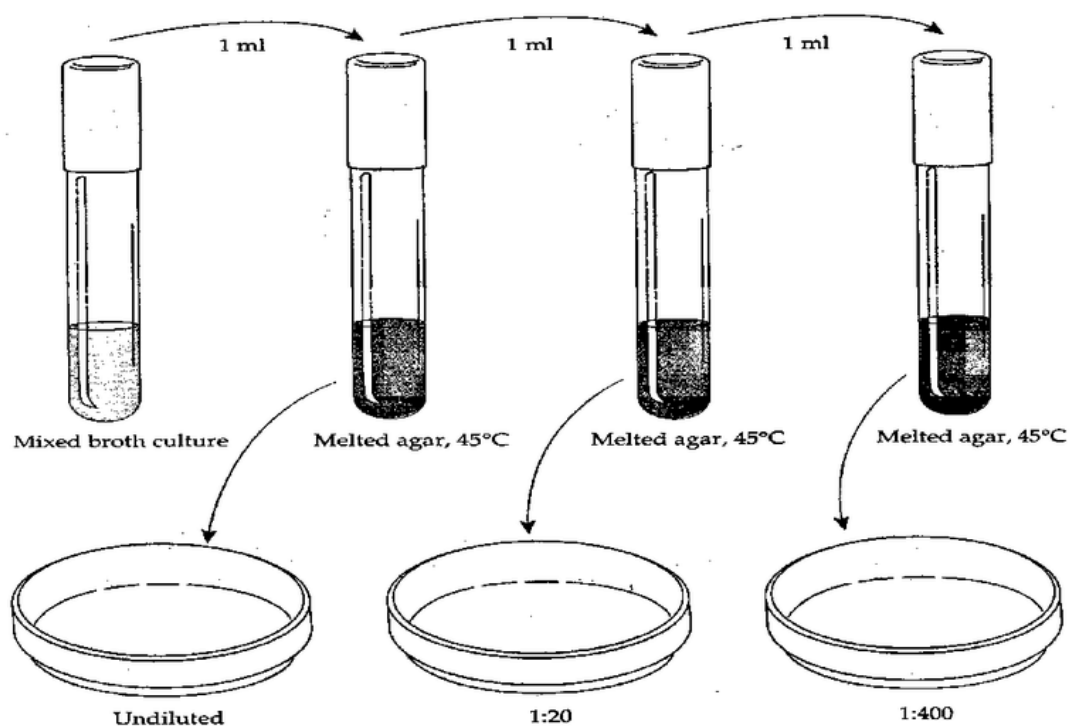


Fig:4.3 pour plate method

4.1.4 Subculture Technique

Individual colonies from streak plates or spread plates are transferred to fresh agar plates or liquid media to establish pure cultures. This technique ensures the continued growth and maintenance of pure cultures over successive generations (Jiang et al., 2016).

4.2 Identification

4.2.1 selective media

Selective media contain specific ingredients that inhibit the growth of unwanted microorganisms while allowing the growth of target organisms. Differential media contain indicators that allow for the differentiation of different types of microorganisms based on their metabolic activities or other characteristics (Bonnet et al., 2020). Selective media are used to encourage the growth of specific types of microorganisms while inhibiting the growth of others. We have to use

4.2.2 Gram Staining

Materials

Bacterial culture, Microscope slides, Bunsen burner or other heat source, Crystal violet (primary stain), Gram's iodine solution (mordant), Ethanol or acetone (decolorizer), Safranin (counterstain), Distilled water, Oil immersion microscope lens, Bibulous paper or paper towels, Immersion oil (for oil immersion microscopy) (Tripathi and Sapra, 2024).

Reagents and chemicals

Crystal Violet: This is the first stain applied in the process. It stains both Gram-positive and Gram-negative bacteria purple or blue.

Iodine: This solution is applied after the crystal violet and helps to fix the stain within the bacterial cells. It forms a crystal violet-iodine complex.

Decolorizer: Typically, ethanol or acetone is used as a decolorizer. It is applied after the iodine and removes the stain from the Gram-negative bacteria while

leaving the stain trapped in the thicker peptidoglycan layer of Gram-positive bacteria (Budin et al., 2012).

Safranin: This is the final stain applied in the process. It stains the Gram-negative bacteria pink or red, contrasting with the purple or blue of the Gram-positive bacteria.

Methods

The initial staining of the slide with crystal violet dye is the first stage in the gram staining process. The following procedure, referred to as "fixing the dye," entails utilizing iodine to create a crystal violet-iodine complex in order to inhibit the dye's simple removal. The dye is then removed using a decolorizer, which is often a solvent mixture of ethanol and acetone. The ability of the bacterial cell wall to hold onto the crystal violet dye after being treated with a solvent is the fundamental idea behind gram staining (*Tripathi and Sapra, 2024*). Gram-negative bacteria have a higher lipid content, while gram-positive bacteria have a higher peptidoglycan content. All bacteria initially absorb crystal violet dye; however, gram-negative species' lipid coating dissolves when a solvent is used. Gram negatives lose the main stain as the lipid layer dissolves. Contrarily, solvent causes the pores in gram-positive cell walls to close, dehydrating them and blocking the diffusion of the violet-iodine combination, which keeps the bacteria marked (*Tripathi and Sapra, 2024*). A crucial aspect of gram staining is the duration of decolorization, since an extended exposure to a decolorizing agent can eliminate all stains from both kinds of bacteria. Gram-negative bacteria that have been decolorized are given a pink hue using a basic fuchsin stain as the last stage in the staining process, which facilitates their identification.

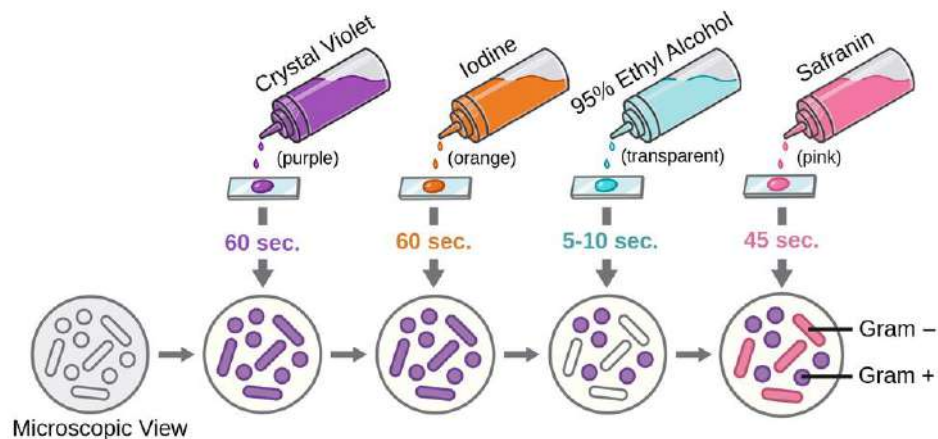


Fig:4.4 work flow of Gram staining method

4.3 Biochemical Test

Materials

Bacterial culture, Biochemical test kits or individual reagents, Sterile inoculation loops or swabs, Incubator set to appropriate temperature test tubes or wells in a microtiter plate, Pipettes, Distilled water (*Bhutia et al., 2020*).

Methods

Prepare the bacterial culture by inoculating a suitable growth medium and incubating until the culture reaches the logarithmic growth phase. Select the appropriate biochemical test based on the characteristics of the microorganism being identified. Inoculate the test medium or well with the bacterial culture using a sterile inoculation loop or swab. Incubate the inoculated test medium or plate at the appropriate temperature for the specified time (*Shen and Sergi, 2024*). After incubation, observe the medium or plate for any visible changes, such as color changes, gas production, or precipitation. Record the results of the biochemical test based on the observed reactions. Compare the results to known patterns or databases to identify the microorganism (*Khushboo et al., 2023*).

4.3.1 Catalase test

Materials

Bacterial culture, Sterile inoculation loop or swab, 3% hydrogen peroxide solution, Sterile test tubes or wells in a microtiter plate, Distilled water, Bibulous paper or paper towels.

Reagents and chemicals

Hydrogen Peroxide (3% solution): This is the substrate for the catalase enzyme. It is usually used at a concentration of 3%.

Sterile Swab or Inoculation Loop: This is used to transfer the bacterial culture to the test area.

Test Reagent: Typically, distilled water is used as a control, and hydrogen peroxide is used as the test reagent.

Methods

All organisms that can survive in oxygen contain the common enzyme catalase, which catalyzes the breakdown of hydrogen peroxide to release oxygen and water. In pathogenic organisms, catalase is a vital enzyme because it shields the organism from reactive oxygen species-induced oxidative damage. The concentration of the enzyme, which counteracts hydrogen peroxide's bactericidal effects, has been linked to the pathogenicity of bacteria. The catalase test has been widely utilized because it makes it possible to distinguish between species that are catalase-negative, like streptococci, and those that are catalase-positive, such as staphylococci (*Nandi et al., 2019*). For the presumptive characterization of most bacteria, the catalase test is helpful. 15% H₂O₂ is utilized in anaerobic conditions while 3% H₂O₂ is used in aerobic conditions. A microscope slide is placed inside a petri dish. The use of a petri dish is optional and is used to limit catalase aerosols, which might carry viable bacterial cells. A small amount of organism is collected from a well-isolated 18- to 24-hour colony with a sterile inoculating loop or wooden applicator stick and placed onto the microscope slide (*Mishra and Imlay, 2012*). However, no agar

must be picked up with the colony, especially when the culture is picked up from blood agar. A drop of 3% H₂O₂ onto the organism on the microscope slide by using a dropper or Pasteur pipette. The formation of bubbles is observed against a dark background to enhance readability (Iwase et al., 2013).

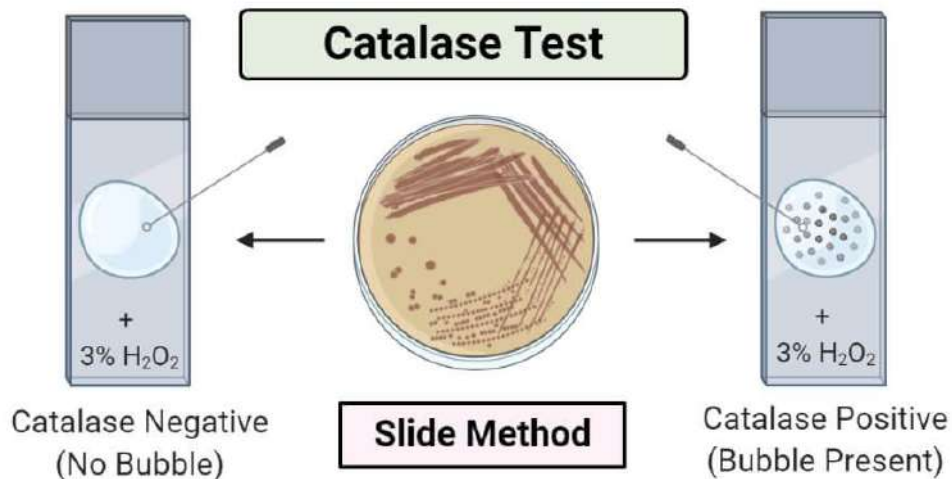


Fig:4.5 Catalase test

4.5 Screening

The first step in screening involves collecting samples from the environment, patients, food products, or other sources where microorganisms may be present. The collected samples are enriched in a suitable growth medium to encourage the growth of target microorganisms while inhibiting the growth of unwanted organisms. This step helps increase the concentration of the target microorganisms in the sample. Depending on the specific objectives and characteristics of the microorganisms of interest, different screening methods may be employed. These methods can include biochemical tests, molecular techniques, serological assays, or phenotypic assays. During screening, the sample is subjected to the chosen screening method to detect the presence or absence of the target microorganisms or specific traits associated with them. Positive samples may undergo further confirmation or characterization, while negative samples may be discarded or subjected to additional screening methods. Positive screening results may be confirmed using additional tests or techniques to ensure the accuracy of the findings (Habibzadeh et al., 2021). This step helps reduce the risk of false-positive results. Once screening is

complete and confirmed, the results are analyzed to draw conclusions about the presence, abundance, or characteristics of the target microorganisms in the original sample. This information can be used for various purposes, such as diagnosis, surveillance, research, or quality control.

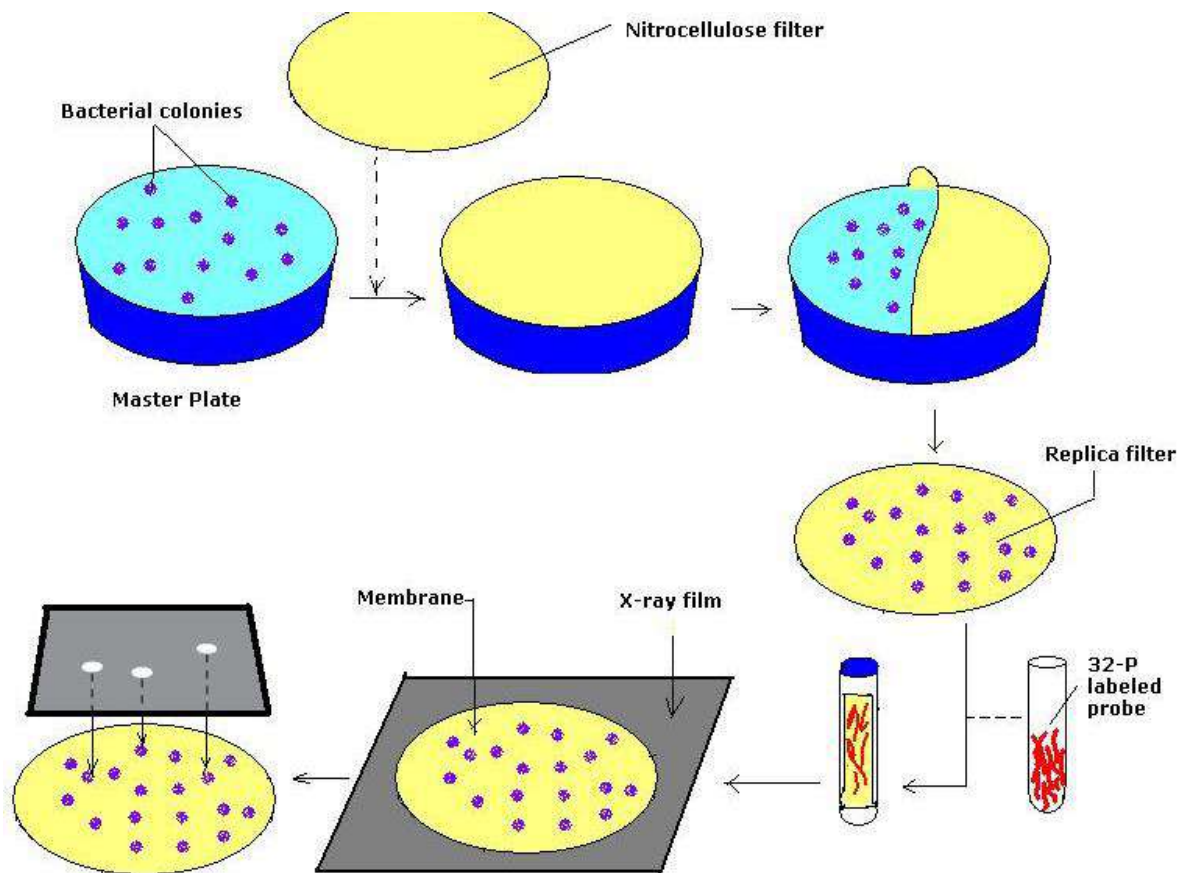


Fig:4.6 Screening

4.5.1 ACID TOLERANCE TEST

Materials

Bacterial culture, Sterile tubes or wells in a microtiter plate, Acid solution (e.g., dilute hydrochloric acid or acetic acid), Buffered diluent (e.g., phosphate-buffered saline, pH 7.2), Sterile pipettes, Incubator set to appropriate temperature

Reagents and Chemicals

Acid Solution: Typically, a dilute hydrochloric acid (HCl) solution is used to create an acidic environment. The concentration of the acid solution may vary

depending on the specific experimental conditions and the bacteria being tested. A commonly used concentration is around 0.3% to 0.5%.

Bacterial Culture: A pure culture of the bacteria being tested is required. This culture should be in the logarithmic growth phase and prepared according to standard microbiological techniques.

Buffered Diluent: To prepare bacterial suspensions for the acid tolerance test, a buffered diluent may be used to maintain the pH of the bacterial suspension. Common buffered diluents include phosphate-buffered saline (PBS) or other appropriate buffering solutions.

Incubator: An incubator set to the appropriate temperature for the growth of the bacteria being tested is necessary to maintain constant conditions during the test.

Sterile Petri dishes or Microtiter Plates: These are used to hold the bacterial suspensions and acid solutions during the test.

Sterile Pipettes: To accurately transfer bacterial suspensions and acid solutions.

Method

Twenty-eight *Lactobacillus* isolates, representing three distinct groups within the *Lactobacillus* genus *Lactobacillus plantarum*, *Lactobacillus Casei*, and *Lactobacillus delbruki* were obtained from cheese samples that had previously been identified through morphological and biochemical assays, including glucose fermentation, arginine hydrolase, and catalase testing. These isolates were all cultured for 24 hours at 35 °C in MRS broth. After adjusting each active culture's pH to 3.0 or 2.0 using 5 N HCl, 0.1 mL aliquots were kept at 37 °C for three hours. The strains were maintained in MRS broth at 37 °C for the entire night (*Hassanzadazar et al., 2012*). Samples were collected hourly for three hours, and pour plate counts of all samples using 10-fold serial dilutions made in 0.1% peptone water were used to determine the viable count of bacteria. Using a spectrophotometer set to measure absorbance at 600 nm, bacterial growth was simultaneously observed. Every experiment was carried out twice. Overnight, the strains were cultivated in MRS broth at 37 °C (*Karami et al., 2017*). Separately, a saturated bile solution was made by dissolving powdered

bile extract (Oxoid). After filter-sterilizing the bile solution using a 4-micron filter, it was added to two of the cultures to reach a final concentration of 0.3%; the control sample was the second culture that contained 0% bile. After three hours at 37 °C, the cultures were incubated every hour for a further three hours (Thomas *et al.*, 2015).

Pour plate counts of all the samples using 10-fold serial dilutions made in 0.1% peptone water were used to assess the viable counts of *Lactobacillus* strains. A spectrophotometer (*Nova Spec II, Pharmacia*) was used to measure absorbance at 600 nm in order to track the development of bacteria simultaneously. Every experiment was conducted twice. The well-diffusion method was used to assess the inhibitory effect of *Lactobacillus* strains on a subset of clinical reference bacteria. The agar well diffusion assay involved inoculating (Brain Heart Infusion) BHI agar growth media at 37 °C with an overnight culture of the indicator strain (*Escherichia coli*, *Bacillus cereus*, *Salmonella enteritidis*, and *Listeria monocytogenes*; roughly 10^6 cells mL⁻¹ of indicator strains were overlaid onto Agar plates were cut into 5mm diameter wells, and each well was filled with 50µL of *Lactobacillus* culture supernatant fluid, which likely included antibacterial activity. *Lactobacillus*'s inhibitory zone was examined following a 24-hour incubation period at 37 °C.

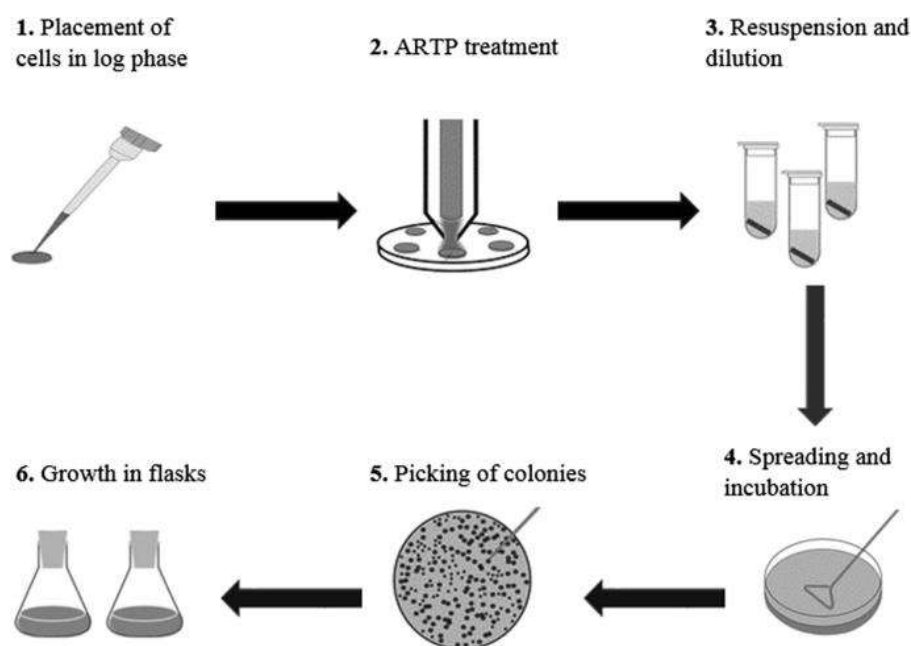


Fig:4.7 Acid Tolerance Test

4.6 Bile salt hydrolysed assay

Materials

Bacterial culture known or suspected to produce bile salt hydrolase (BSH), Sodium glycocholate or sodium taurocholate (bile salt substrate), Sterile tubes or wells in a microtiter plate, phosphate buffer, Spectrophotometer or other measuring equipment, Pipettes, Distilled water.

Reagents and Chemicals

Bile Salts: Bile salts are the substrate for the BSH enzyme. Sodium glycocholate and sodium taurocholate are commonly used bile salts in BSH assays.

Bacterial Culture: A pure culture of the bacteria being tested, known or suspected to produce BSH, is required. This culture should be in the logarithmic growth phase and prepared according to standard microbiological techniques.

Reaction Buffer: A suitable buffer is needed to maintain the optimal pH for the BSH enzyme activity. Common buffers include phosphate buffers or Tris-HCl buffers.

Enzyme Inhibitor (optional): In some assays, an enzyme inhibitor may be included to selectively inhibit the activity of other enzymes present in the bacterial culture. For example, phenylmethyl isulfonyl fluoride (PMSF) is commonly used as a serine protease inhibitor.

Substrate Detection Reagents: Reagents needed to detect the hydrolysis of bile salts by the BSH enzyme. This may include chemical indicators or assays specific to the products of bile salt hydrolysis, such as ninhydrin or high-performance liquid chromatography (HPLC) for detecting amino acids released from bile salts.

Spectrophotometer or other measuring equipment: This is necessary for quantifying the hydrolysis of bile salts by measuring changes in absorbance or other properties of the reaction mixture.

Methods

Prepare the bacterial culture by inoculating a suitable growth medium and incubating until the culture reaches the logarithmic growth phase. Label sterile tubes or wells in a microtiter plate with the sample name or number. Prepare the reaction buffer by diluting the buffer component with distilled water to the appropriate concentration and pH. The pH should be adjusted to the optimal range for the BSH enzyme activity, typically around pH 6.8 (*Mauerhofer et al., 2019*). Prepare the bile salt substrate solution by dissolving sodium glycocholate or sodium taurocholate in the reaction buffer to achieve the desired final concentration, usually around 0.5%. Inoculate the test medium or well with the bacterial culture using a sterile pipette to transfer a standardized volume of the culture (*Ruhluel et al., 2022*). Add the bile salt substrate solution to the test medium or well to achieve the desired final concentration. Mix gently to ensure uniform distribution of the substrate. Incubate the inoculated test tubes or microtiter plate in the appropriate incubator set to the desired temperature for the specified time, typically 1-2 hours. After the incubation period, remove the test tubes or microtiter plate from the incubator and measure the absorbance of the reaction mixture at the appropriate wavelength using a spectrophotometer (*Yasmin et al., 2020*). This baseline measurement represents the initial concentration of bile salts. Optionally, incubate the reaction mixture for a longer period (e.g., overnight) to allow for further hydrolysis of the bile salts. Measure the absorbance of the reaction mixture again at the same wavelength and compare it to the baseline measurement. The decrease in absorbance indicates hydrolysis of the bile salts by the BSH enzyme.

Calculate the percentage of bile salt hydrolysis using the formula

$$\% \text{BSH activity} = \frac{\text{Absorbance initial} - \text{Absorbance final}}{\text{Absorbance initial}} \times 100\%$$

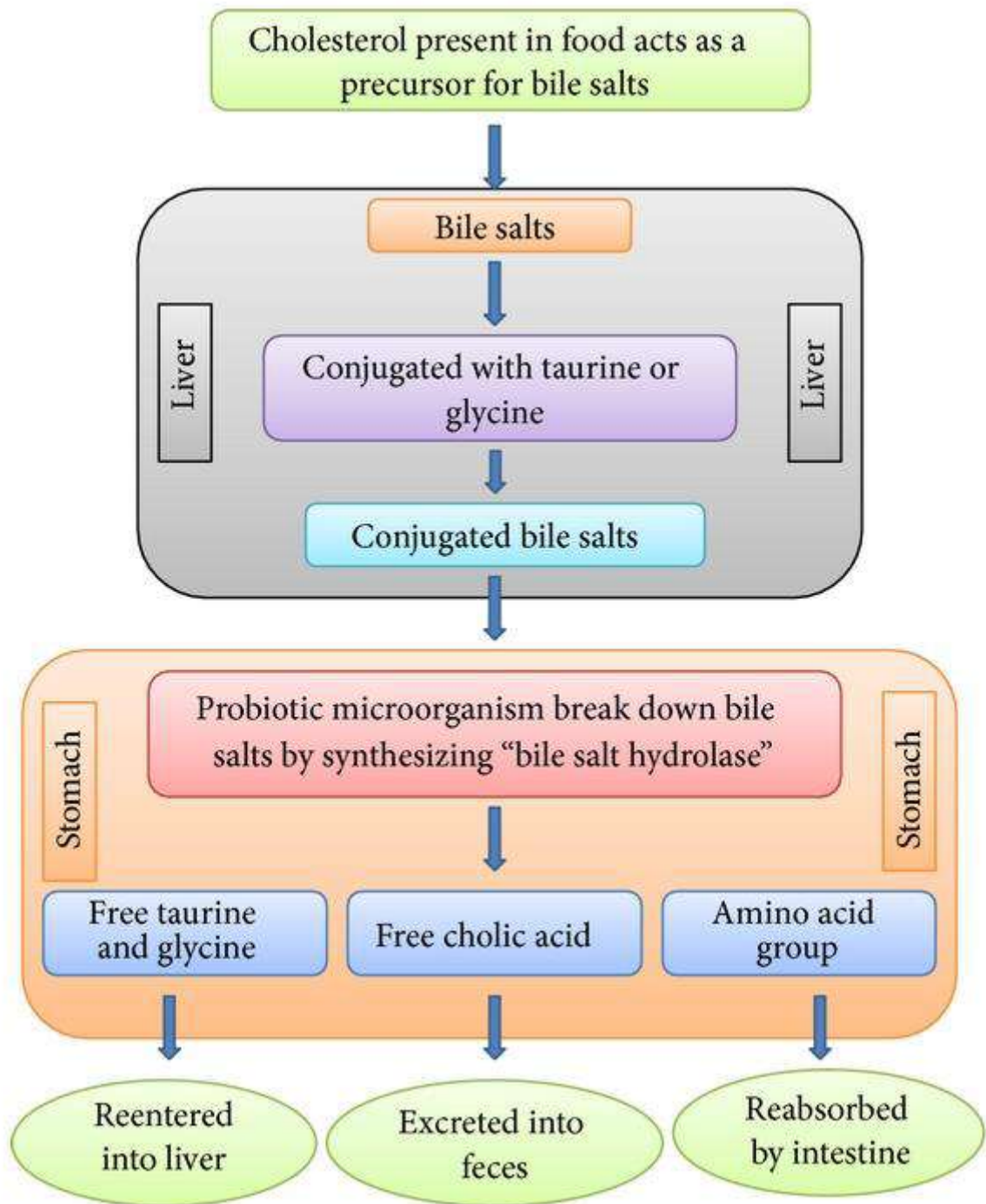


Fig:4.8 Bile salt hydrolysed

CHAPTER – 5

RESULT AND DISCUSSION

5.1 Pure culture Techniques

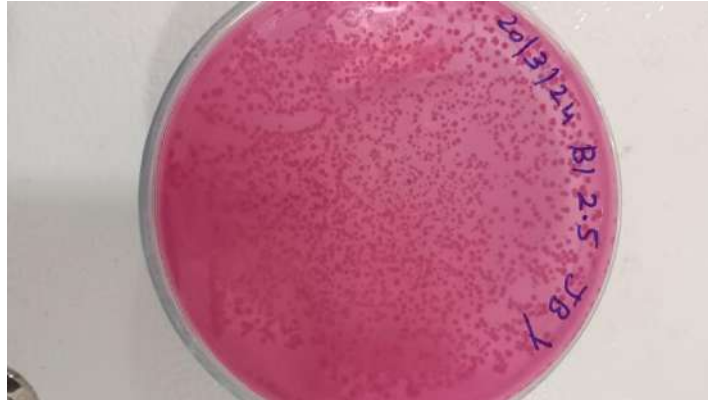


Fig:5.1 Pour plate method

In this case, the growth medium (LB) contains X-Gal, so those cells expressing a functional Lactobacillus enzyme form blue colonies while those cells with a mutation in the lacZ gene and thus unable to express a functional Lactobacillus enzyme form pink colonies.

5.2 Sample collection



Fig:5.2 Sample identification

5.3 Gram Staining

A Gram stain is coloured purple. When the stain combines with bacteria in a sample, the bacteria will either stay purple or turn pink or red. If the bacteria is pink in colour they are gram positive.

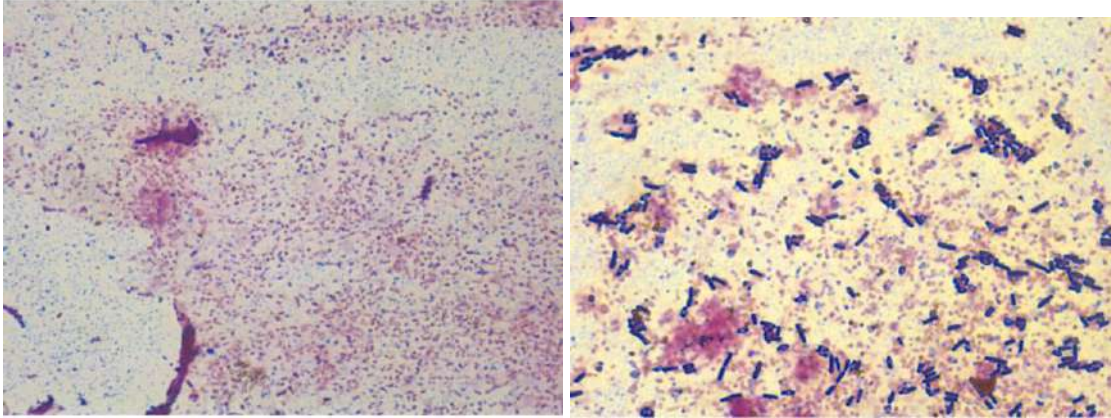


Fig:5.3 Gram Staining

In our studies the gram stain is coloured purple, The bacteria is pink in colour so, it is gram positive.

5.4 Catalase Test

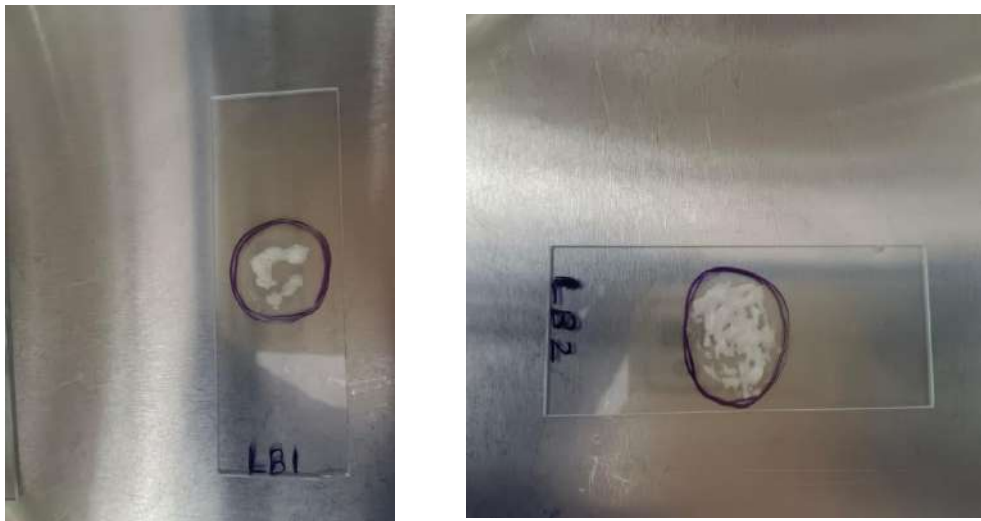


Fig:5.4 Catalase test sample



Fig:5.5 Catalase test

5.5 Acid Tolerance Test



Fig:5.6 Acid Tolerance test

The acid tolerance test can help diagnose conditions related to gastric acid secretion, such as gastritis, peptic ulcers, GERD, or Zollinger-Ellison syndrome. It can also be used to monitor the effectiveness of treatment for these conditions, such as acid-suppressing medications or lifestyle changes. The

acid tolerance test is a diagnostic test used to assess the ability of the stomach to produce and tolerate acid.

5.6 Bile Salt Hydrolase assay

Bile salt hydrolase activity towards glycine- and taurine conjugated bile substrates could be recorded in cell-free extracts (CFEs) of all *Lactobacillus* strains at varying degree. In general, cell-free extracts (CFEs) of lactobacilli had higher Bsh activity units than their resting cell suspensions. All *Lactobacillus* strains exhibited highest Bsh activity towards glycocholate compared with other bile conjugates, ranging from 213.11 to 517.88 U/ml. Strains Lp1, Lp9 and Lp11 produced more than 455 Bsh activity units towards glycocholate, whilst reference strains *L. johnsonii* NCDO1693 and *L. plantarum* NCDO82 could also express 331.20 (± 20.65), 312.68 (± 14.82) enzymatic units, respectively.

Cell-free extracts (CFEs) of Lp1 and Lp2 strains had comparatively high GDC-hydrolase activity units, that is, 228.63 (± 6.88) and 206.20 (± 6.03), respectively, whilst reference strain *L. johnsonii* NCDO 1693 expressed 164.30 (± 12.26) enzymatic units. In addition, strains Lp1, Lp2 and Lp11 had more than 243.28 and 269.56 units of TC- and TDChydrolase activity, respectively, compared with other lactobacilli. Lp8 exhibited the lowest total-Bsh activity towards glycocholate and taurodeoxycholate, whilst Lp7 showed minimal total-Bsh activity towards glycodeoxycholate and taurocholate. Specific- and total-Bsh activity did not correlate well amongst all lactobacilli.

In general, Lp2 had higher total GC-hydrolase activity than other lactobacilli, whilst highest specific GC-hydrolase activity was demonstrated by Lp9, which might be due to varying protein contents in their CFEs. Furthermore, Lp9 had highest total-Bsh activity towards glycodeoxycholate, taurocholate and taurodeoxycholate and exhibited highest specific-Bsh activity as well, whilst in respect to the above bile substrates, Lp7 and Lp8 had lowest total- and specific-Bsh activity. Overall, it was observed that all lactobacilli produced higher total-Bsh activity and specific-Bsh activity towards glycocholate than other bile conjugates.

CHAPTER-6

SUMMARY AND CONCLUSION

In conclusion, the production and characterization of probiotics from fermented food bacterial sources offer significant potential in enhancing human health and well-being. Through meticulous cultivation and isolation techniques, beneficial bacteria can be extracted and utilized to develop probiotic supplements and functional foods with tailored health benefits. The comprehensive characterization of these probiotics ensures their safety, efficacy, and stability during production and storage, paving the way for their successful application in various sectors, including pharmaceuticals, nutraceuticals, and the food industry. Moreover, the utilization of probiotics derived from fermented foods aligns with the growing consumer demand for natural, sustainable, and culturally relevant health-promoting products. Their incorporation into diverse food matrices not only enhances nutritional value but also enriches flavor profiles, catering to consumer preferences.

However, further research is warranted to explore the full spectrum of potential applications and to better understand the mechanisms underlying their beneficial effects on human health. Additionally, regulatory frameworks must be established to ensure standardized production processes and labeling practices, thereby promoting consumer confidence and facilitating market accessibility. Overall, the production and characterization of probiotics from fermented food bacterial sources hold immense promise in advancing both preventive and therapeutic healthcare strategies, contributing to the promotion of holistic well-being in populations worldwide.

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