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Metabolite Profile and Surface Proteosome of Probiotic Bacteria from Fermented Fruits

by

Abeer Liaqat

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my father Liaqat Hussain. My mother prayers have always enlightened my way throughout my life. It's also dedicated to my brother and all other family members who taught me that the best kind of knowledge to have is that which is learnt for its own sake. They taught me that even the largest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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(Abeer Liaqat)

Abstract

Probiotic bacteria are those microbial stains which have beneficial effects on host GIT. There bacteria have antagonistic activity against pathogenic bacteria present in gut tract. Milk and dairy products are considered as best source of probiotics but not for the one with lactose intolerance and the one who have milk protein allergy. The alternative for lactose intolerant individuals are fermented fruits and vegetable. But there nutritional competency is not reported in depth so far. some probiotic bacteria have special kind of surface proteins also known as proteosurfacesomes. Calcium carbonate test indicates bacteria separated from fermented peach and apricot do not metabolize lactic acid so they are considered best source of probiotic bacteria for lactose intolerant individuals. There proteins help probiotic bacteria to adhere on GIT and show antagonistic activity against pathogens. SDS PAGE indicates presence of proteosurfacesomes in probiotic bacteria separated from fermented peach and apricot. SDS PAGE gives series of light and dark bands. Dark bands of size 100kda, 74kda, 63kda, 44kda, 33kda, 20kda in first sample. 100kda, 74kda, 61kda, 62kda, 42kda, 33kda, 20kda, in second sample. 100kda, 74kda, 61kda, 44kda, 33kda, 20kda, in third sample and 100kda, 74kda, 20kda, 33kda, 63kda in fourth sample. These dark bands indicate that protein of these sizes are present in excessive amount. HPLC indicates presence of metabolites in bacterial samples. Metabolites in fermented fruit sample is more than non-fermented fruits. Amino acid content found in *S. rubrolavendulae, S. fradiae, S. griseofucus and S. albus* is 66.176 ± 8.063, 14.961±1.777, 87.351± 2.740 and 91.209 ± 6.131 respectively but in non-fermented juice amino acid content is 2.352 ± 0. 022. Same case has been observed in sugars, and sugar alcohols as well. Non lactic acid producing strain of probiotic from fermented fruits are equally competent as compared to latic acid producing strains of probiotic and are best source of probiotic for lactose intolerant individual and people's with milk protein allergies.

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Abbreviations

GIT: gastrointestinal tract

HDP: shots defense peptides

HPLC: High-performance liquid chromatography

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

LAB: lactic acid bacteria

MetS: metabolic syndrome

MRS: Man, Rogosa and Sharpe agar

NAFLD: nonalcoholic fatty liver disease

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SLAP: surface layer associated proteins

SLP: surface layer protein

T2DM: insulin resistance, type 2 diabetes

Chapter 1

Introduction

Probiotics are helpful microorganisms or their variants that benefit the hosts' wellbeing. Probiotics are used widely in aquaculture prevention of disease, feed additives, enhancing growth, and antibacterial compound substitutes [1]. Research work is in process in the field of probiotics.

Metchnikoff have come up with the initial definition in the early 1900s [1]. The use of fermented milk products could improve human health. Parker coined the term "probiotic," which he described as "organisms and substances that contribute to intestinal microbial balance." The term "probiotic" derives from the Greek terms "pro" and "bios," which mean "for life" [2]. An important innovations that developed to encounter disease, according to [3], is using probiotic. A live microbial feed supplement that enhances the intestinal microbial balance of the host animal. A live microbial feed supplement that enhances the intestinal microbial balance of the host animal. Probiotic was important because it help to keep the body healthy. Probiotics are live microbes that can be used to enhance the microbial balance and growth efficiency of the host intestinal flora. Because of the recent overdependence on antimicrobial drugs, the synthesis of probiotics in aquaculture management will minimize the prophylactic usage of antimicrobial drugs, posing possible risks to people who eat them [4] [1]. A live microbial adjunct that has a positive effect on the host by changing the host related or local microbial population, by ensuring improved use of the feed or optimizing its nutritive benefits, by

optimizing the host response to disease, or by improving the quality of its ambient environment. The generally accepted definition for probiotics, which he describes as "a live microbial feed supplement that enhances the intestinal microbial balance of the host animal." Fuller's description is a development of the accurate term for probiotic, which identified microorganisms that creates substances that rectorate other microorganisms [1].

Several improvements have been proposed to shorten the concept of probiotics [5]. "A live microbial adjunct that has a positive effect on the host by changing the host related or local microbial population, by ensuring improved use of the feed or optimizing its nutritive benefits, by optimizing the host response to disease, or by improving the quality of its ambient environment," [6]. It should support the host either nutritious or by altering its immediate surroundings [5].

Probiotics can be helpful in a number of ways, and these can work either alone or in conjunction with just a single dose of probiotic. It has been demonstrated that transitory bacteria can even have therapeutic benefits.

- The discovery that active manipulation of the gastrointestinal tract (GIT) can provide antibacterial action,
- Promote growth in immune system, give nutritive advantages, and improve the intestinal epithelial barrier gave rise to the concept of probiotic action [7].

Probiotics are now commonly used in human fostering good health "functional foods," as well as medicinal, intend to provide protection against disease, and growth supplements in animal agriculture and human health [1]. Probiotics can be helpful in a number of ways, and these can work either alone or in conjunction with just a single dose of probiotic. it has been demonstrated that transitory bacteria can even have therapeutic benefits.

Struggle for site of attachment, competition for nutrients, advances in pathogenic enzyme activity, regulation of immune system, and nutritive quality like higher feed digestion and feed usage are only a few examples of pathogen inhibition [1]. A probiotic must adhere to and colonies the GIT, reproduce in large numbers, survive in harsh acidic environment of the gastrointestinal tract, must developed

antimicrobial substances according to common belief [8]. These explanations, however, are deceptive. These point of views or facts are designed on the premise that a probiotic should remain in the gut flora indefinitely. Although microorganism that have these abilities are normal, and Although many probiotic studies focused on microbe's capacity to attach, it has been demonstrated that transitory bacteria can even have therapeutic benefits. Furthermore, unlike bacteria that must be capable to adhere to mucus and create antimicrobial activity, probiotics only need to have single method of operation. Multiple probiotic strains and species have demonstrated that providing synergy bacteria with alternative mechanisms of action to boost protection is achievable [1].

1. Probiotics might have the ability to modify the host's gut defense system, that also includes both of the innate and adaptive immunity immune systems, and this mechanism of action is necessary for treatment of infectious diseases and its prevention and the treatment of inflammation of the digestive tract or parts of all of the above.

2. Probiotics might have had a significant impact on other microbes, both friendly and dangerous, and so this concept is critical for infection detection and treatment, and also gut microbial balance restoration, in many circumstances.

3. Finally, probiotic effects may be dependent on behavior involving microbial products, host products, and food ingredients, which could result in toxins being inactivated and host and food components being detoxified in the gut.

These three ways of probiotic activity possibly related to the stomach or gut microbiota [9]. Thus, with better knowledge of the basic behavior of the gut microbiota, the reality (Probiotics might impact microbial goods, host goods, and dietary recommendations, resulting in toxins being inactivated and host and food constituents being detoxified with in gut). that has obviously been dealing with is another "organ," the so-called "micro-biotic canal" [10]. The gut microbiota,

when established, remains intact for the rest of one's life, while it can be influenced by a variety of variables more like the delivery procedure, cleanliness, and pharmaceutical usage.

In collaboration with the mucosal immune system and mucosal immune system, the gut flora organizes a network of nonimmunological and immunological defenses that would provide pathogen prevention as well as tolerance to symbiotic microbes and nonpathogenic antigens. The importance of symbiotic microbes in the development of a very well mucosal immune system has been proven in bacteria-free animals [11].

As a consequence, inflammatory bowel disease, Atherosclerosis, rheumatoid arthritis, and periodontal disease, and allergies have all been related to an imbalance of intestinal microbiota. This gut ecology is assumed to be supported by probiotics, or helpful bacteria strains [12]. Pathogen suppression via the formation of bacteriocin-like substances, struggle for points of attachment, struggle for resources (particularly iron in marine microorganisms), change of pathogenic enzyme production, immunomodulating activities, and nutritional qualities such as enhanced feeds digestion and feed efficiency are some of the other proposed probiotic mechanisms of action [13]. In order to earn this probiotic designation, the bacteria should meet a variety of biosafety and functional conditions. The microorganism must meet a set of requirements in terms of biosafety and function in order to obtain this probiotic status. A potential probiotic should have the following characteristics.

1. It should not be toxic to the host;
2. It should be transported to the active site and be able to function in that surrounding;
3. Capacity to colonies plus replicate in host system, and
4. There should be no pathogenicity or drug tolerance genes expressed [10].

Bifidobacterium, Lactobacillus, Streptococcus, Carnobacterium spp, Bacillus, Pediococcus Flavobacterium, Pseudomonas, Cytophaga,Alteromonas, Enterococcus,

Nitrosomonas, Nitrobacter, Vibrio spp, and yeast are among the probiotics currently used in the aquaculture industry [13]. Although certain probiotic bacteria are beneficial to fish, others, such as *Vibrio alginolytic* us, are highly pathogenic and may cause havoc in aquaculture systems. As a consequence, prior to administration, it is important to exercise caution when choosing a probiotic. In the aquaculture industry, well-known probiotic strains including *Streptococcus thermophilus, Bifidobacterial, Lactobacilli* are used as diet supplements, and they help to boost the Aquaculture production effectiveness and durability [10].

Lactic acid bacteria (LAB) are widely employed and researched for people and land animals applications, and LAB can be located in the gut of fish [6]. Infact normal inhabitants of the human gastrointestinal tract (GIT) with quality to withstand bile environment and acidic surrounding was main focus of LAB. LAB can convert lactose to lactic acid, lowering the pH in the gastrointestinal tract and naturally preventing the invasion of multiple microbes [14]. *Lactobacilli* and *Bifidobacterial* are the most commonly studied and used laboratories [13].

Bacillus spp., which produce spores, and yeasts are two additional probiotics that already have received a lot of attention. *Bacillus sp.* has indeed been found to exhibit adhesive qualities, release bacteriocins also known as antimicrobial peptides, and stimulate the defense system [5]. The isolates are all powerful probiotics, and commercialized medicines containing them have been found to boost shrimp output to levels comparable to antimicrobials [5].

1. It should not be toxic to the host;
2. It should be transported to the active site and be able to function in that surrounding [10].

Bacillus spp are especially appealing as probiotics as they could be stored in the form of spore and thus kept on the shelf indefinitely [5]. *Saccharomyces cerevisiae* had been extensively researched, with immuno-stimulation behavior plus inhibitory substance production demonstrated [5]. Because of their ease of processing, dairy fermented products have long been regarded as being the most effective

probiotic mediators. All dairy sector goods (milk, yoghurt, cheese, milk proteins, and milk-related sweets) have been pro-biotified, and customers had acknowledged the existence of microbes in the dairy products they purchase [15]. The health drink market is dominated by dairy-based products, with fermented goods contributing for the remaining 43% [15]. Fermented milks, particularly yogurt-style products like buttermilk in Europe and North America and Ymir in Denmark, are the most support the efficient probiotic beverages. Probiotic bacteria found in dairy products have been demonstrated to be quite attractive properties for a multifunctional meal since they help probiotic strains flourish. The favorable impact of dairy composites on probiotics in the digestive tract while compared to certain other matrices., especially from milk proteins, is evident. Proteins are a source of bioactive peptide precursors, which are resistant to digestion. These characteristics help probiotics live in the digestive system under adverse conditions. Milk proteins also operate as just a carrier matrix for probiotic bacteria, enabling them to penetrate specific target areas [2]. A variety of protocols have been proposed and tested to reduce the gastrointestinal system's lethal effects on probiotic microorganisms.

- One of the most successful is the encapsulation technique. In the biotechnology industry, the encapsulation of probiotic living cells, which is based on immobilization technology, it could be utilized to culture catalysts as well as entire cells.
- It's a method of coating bioactive materials with other type of material for protection or mixtures of them, so the content stored in them could release at predefined intervals in influence of particular situation.

The most successful is the encapsulation technique. In the biotechnology industry, the encapsulation of probiotic living cells, which is based on immobilization technology, it could be utilized to culture catalysts as well as entire cells. It's a method of coating bioactive materials with other type of material for protection or mixtures of them, so the content stored in them could release at predefined

intervals in influence of particular situation. The bioactive part is protected by microencapsulation against environmental stresses such as oxygen, excessive acidic, and gastrointestinal disturbances, allowing it to pass through the stomach with minimal damage [16].

The stability of the microencapsulated bioactive part while travelling through the stomach could've been strengthened via utilizing highly hydrophilic wall materials. In recent times, there has been a lot of study done on the protection of probiotic microbes using microencapsulation throughout preserving food [17]. Probiotics may be encapsulated using proteins, polysaccharides, carbohydrates, and their variations, as well as certain liquid food matrices [18].

The probiotic food industry is involved in microencapsulation of probiotic species as perfect way of preserving the efficacy of probiotic bacteria delivered to the GI tract. This outer structure can also be used to extract other proteins. Other proteins, such as surface layer associated proteins (SLAPs) and other non-covalently surface-bound proteins, can be extracted using this outer structure. When contemplating encapsulation, we must keep two things in mind:

1. The size could be in between 1 to 5m diameter,
2. That rules out actually rules out nanotech;
3. As well as the notion that they should being revived.

Some Gram-positive bacteria, particularly probiotic bacteria, have an exterior proteinaceous covering termed a surface-layer that protects them from the environment a para-crystalline layer is used to create this unique structure and is created by the self-assembly of a surface-layer-protein (Slp).

The surface layer is preserved and found in a wide range of prokaryotes. The sequence of the homologous Slp protein, on the other hand, varies greatly between microbial strain and even among microbial strain of the common species. This outer structure can also be used to extract other proteins. Other proteins, such as surface layer associated proteins (SLAPs) and other non-covalently surface-bound

proteins, can be extracted using this outer structure. They can take on a variety of roles. Different authors and experimental methodologies have indicated that probiotic Gram-positives have a taken part in crucial encounter between host and microbe [19].

Depending on the species and strain, they may be involved in tolerating the stress of surrounding, longevity within the host intestinal tract, connectedness to substrate or mucus, or control of IBD. Current predictions include the utilization of one 's properties in the biosynthesis of nanotech, encapsulation and coating, and the development of novel immunization's goal of Gram-positive bacteria's surface chemicals is to modulate the immunization of gut tract first, and afterward the systemic immunization, by initiating a connection between the microbe(probiotic) and host.

1. Several studies have shown that surface-bound proteins take part in the host and bacterial interaction, resulting in positive benefits such as immunological regulation, although the molecular processes are still unknown.

2. Indeed, they contribute to the establishment or maintaining appearance of cell, molecular sieving, catalytic activity, coaggregation [21] [35].

Contribution to adhesion, gut immune cells intonation, antagonistic to stress in surrounding, and host defense peptides (HDPs), among other functions in bacteria Lipoproteins, proteins, lipoteichoic acids, lipopolysaccharides, glycoproteins and flagellins are examples of bacterial surface chemicals that interact with the host PRRs (pathogen recognition receptor) and modulate the immune system. Several recent investigations have highlighted the critical significance of protein attached on surface, they have non-covalent interaction with outer cell wall and are present in some probiotic bacteria but are not required [20].

The proteins on the surface could be part of a Slp lattice, which is the outermost macromolecular monolayer. Hoodwink initially defined it in 1953, and it consists of a Para crystalline bidimensional array made up of a Slp, which was first discovered on the cell surface of *Spirillum sp.* Chaotropic drugs such as SDS, LiCl, are used to extract Slps[19]. Gram-negative, Archaea, Gram positive bacteria all have

S-layers, which are very porous and have a thickness of 5–25 nm. The, hexagonal (p3, p6), tetragonal (p4), oblique (p1, p2) symmetry of the S-layer procrystalline lattice can be used. Non-covalent interactions hold subunits together and connect them to the underlying cell surface, and they have an inherent, entropy-driven capability to cause regularity in solution or even on a decent endorsement in vitro. Subunit proteins seem to be frequently significant in acidified and hydrophobic amino acids but cheap in Sulphur-containing amino acids, with a low total assumed pI value. The genes for S-layer proteins are substantially demonstrated. Various genes of S-layer protein have been identified in a one strain genome, although not all of them are expressed at the same time.

- One of the most successful is the encapsulation technique. In the biotechnology industry, the encapsulation of probiotic living cells, which is based on immobilization technology, it could be utilized to culture catalysts as well as entire cells.
- It's a method of coating bioactive materials with other type of material for protection or mixtures of them, so the content stored in them could release at predefined intervals in influence of particular situation.

Silent genes, antigenic variability based on S-layer expression of genes, alterative gesture of S-layer protein genes in or out of vivo, Superimposed S-layers or S-layers constituted of two separate S-layer proteins have been described, as well as sequential expression throughout growth. Because of the insufficiency of a universal signature sequence and the fairly low sequential similarity between many S-layer protein genes, electron microscopy is nevertheless used to confirm the presence of an S-layer.

Data about role of S-layer of proteins has collected in recent decades, although no single purpose for all S-layer proteins has come out. Cell shape maintenance or determination, as well as functions as a molecular sieve, a binding domain for large molecules, ions, or phages, and a bacterial adhesion mediator [22], have all been identified thus far. S-layers might assist to virulence in pathogenic bacteria through a variety of methods, including adhesion, congregation [23], antigenic

variation, shielding from complement or phagocytosis, and regulation of T-cell or cytokine responses. Furthermore, S-layer proteins may shield the bacterial cell from mechanical and osmotic stressors, antimicrobial peptides, radiation, changes in ambient pH, bacteriophages, bacterial or eukaryotic microbial predators, or bacteriolytic enzymes. Some S-layer proteins, such as the S-layer protein of a marine Synchesis, have the ability to operate as degradative enzymes [24].

S-layers offer a wide range of applications in (Nano) biotechnology due to their self-assembly characteristics and highly organized, regularity in structure down to the nanoscale scale. S-layer applications can be split into two categories. Immunotherapy, heterologous protein production, and surface display applications in diverse living organisms which utilize (genetically modified) S-layered bacterial cells, S-layer (fusion) proteins, or just the expression and/or secretory signals of S-layer protein genes fall into the first group.

For non-life (Nano) technical applications, the second group uses isolated, mainly recombinant S-layer proteins [40] [41].

In the genus *Lactobacillus*, S-layers have been found in several but not all species. Biochemical or functional data have been published about the S-layer proteins of,

1. *L. hilgardii, L. helveticus, L. buchneri and, L. brevis* organisms of the former *L. acidophilus* group, including *L. acidophilus, L . crispatus, L. amylovorus and L. gallinarum.*

2. In addition, strains of *L. kefiranofaciens, L. amylolyticus, L. pasteurii, L. gigeriorum and L. ultunensis* carry predicted S-layer protein genes in their completely or partially sequenced genomes

Furthermore, because did not establish the presence of S-layer proteins on the surface of, *L.paracasei, L. casei subspecies paracasei* , and *L. rhamnosus, L. casei* is now regarded as a non-S-layer producer. Many of the *Lactobacillus* S-layer proteins discovered so far have a 25–32-amino-acid signal peptide preceding them, suggesting release via the broad secretory pathway. The deduced amino acid sequences of matured *Lactobacillus* S-layer proteins differ tremendously, or even

S-layer proteins from the very same strain might have large sequence changes when present. The ability of Slps to self-assemble and produce repeated supramolecular clusters that seem to be unchangeable and resilient to physicochemical invasions prompted the idea of employing them in (Nano)biotechnology [23].

Such monomolecular arrays give well-defined topologies based on the physicochemical characteristics of the glycoprotein that creates the sealed, isoporous lattice and for which bacteria have had a vast diversity. Scholars were inspired to investigate employing re-crystallized Slps to build ultra-filtration membranes having incredibly accurate molecular cutoffs, great intramolecular cross-linking resilience, minimized membrane fouling, and configurable surface properties such as net charges and solubility in water.

Chemical and genetic engineering can also be used to immobilize active molecules like, ligands, enzymes, antibodies, and antigens while still allowing Slps to self-assemble. Because some Slps are recognized to spontaneously make premade nanoparticles, functionalized Slps nanoparticles, particularly metallic and semiconductor nanoparticles, were created on native surface-layers. Slps can also be utilized to fabricate vaccines or even as structural support for working lipid membranes. Due to the inherent adjuvant qualities of various SlpS, composite vaccines containing Slps plus antigens, hatpins, or recombinant allergens produced promising results. in vaccination trials [14].

1.1 Hypothesis

Probiotics from non-traditional sources are commensal microorganisms found in the intestine and yet many fermented foods that have been shown to advance human health by promoting digestive health absorption, boosting gut barrier, influencing immune response, and increasing pathogen antagonism. The proteo-surfaceome, or the complicated combination of proteins found on the bacterial cell surface, plays an important role in the dynamic interplay between bacteria and their hosts. Identification of surface and cytoplasmic proteins from non- conventional sources could be an excellent savior for Lactose Intolerant Individuals.

1.2 Objectives

The study entails following objectives:

1. To isolate and identify probiotic strains from fruit sources.
2. Estimation of strains with good probiotic properties
3. Estimation of proteosurfaceomes by SDS PAGE.
4. Identification of strains with surface proteins by 16s RNA sequencing.

1.3 Problem Statement

Alternative probiotic sources for lactose intolerant individuals are reported to be insufficient and don't possess enough probiotic characteristics as are in dairy probiotics. Non-conventional food sources could be used for identification of probiotic strains with unique surface proteins.

Chapter 2

Review of Literature

2.1 Probiotic Bacteria from Non-Conventional Source

Probiotics are microorganisms that are alive and well [3]. This includes microorganisms present in fermented foods which are consumed by people, along with vitamins and pharmaceuticals. These are commonly mixed in foods to boost their nutritive qualities, as the market for novel products od probiotic continues to rise. Dairy products are commonly treated with probiotics, industries of food has been working to create other matrices for food that are appropriate for this motive in recent years. As a result,

- Making probiotic drinks out of fruit juices may be a good compromise because fruit juices are an good mediator for probiotic bacteria.

As a result, making probiotic drinks out of fruit juices may be a good compromise because fruit juices are a good mediator for probiotic bacteria. When probiotic strains are mixed with fruit juices, they have a number of health benefits from both sources, including antioxidants, vitamins, peptides and amino acids. These items may be classified in new category of nutraceuticals [2].

2.2 Probiotic Microorganism

Verification of a microorganism's attributes, bacterial strain recognition, advantages towards our health , and attributes are needed before it can be considered a probiotic [2]. For quite some time, only a few numbers of strains of microbes were called probiotics classified according to their qualities, which were then used in packaged foods or as supplements [2]. Probiotic microorganisms are often sold as dietary supplements in the form of dried or deep-freeze culture extracts for household and commercial usage. These could be taken in the form of nonfermented or fermented meals, as well as nutraceuticals (capsule, tablet form, powder). These

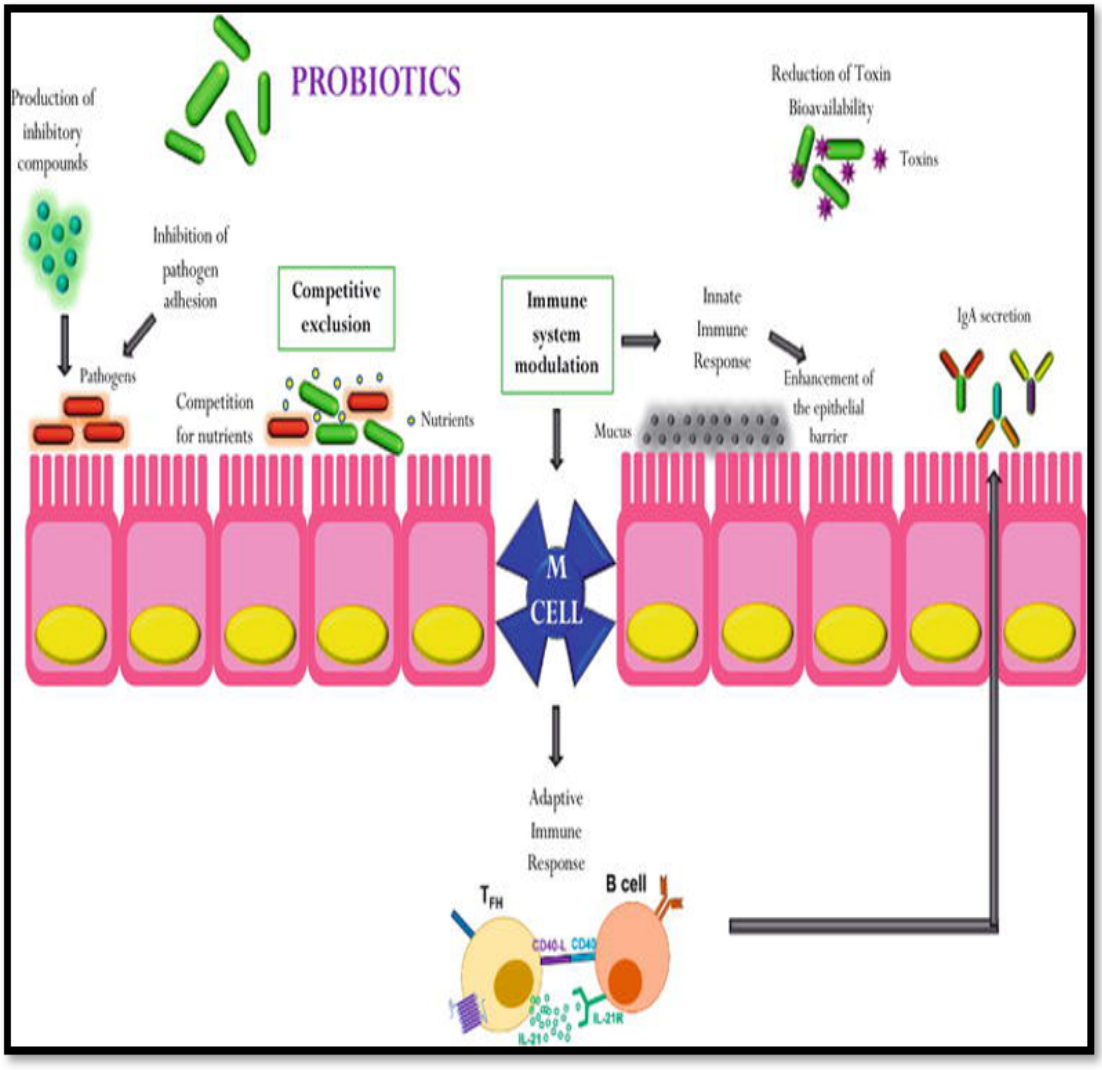


FIGURE 2.1: Mode of action of probiotic bacteria [29].

bacteria must also meet certain criteria in order to be classified as probiotics [2]:

1. Types and definitions in detail.

2. Generation of enterotoxins and cytotoxins, blood cell rupturing or hemolysis, enter invasiveness, serological pathogenicity, pathogen adhesion and the genes resistant to antibiotic are all examples of pathogenic effects [4].

3. Strain hitting its site of action, which is normally the stomach, and surviving physiological tension encountered during ingestion: pH of the stomach and gut, involvement of biliary saline [8].

4. The ability to bind to the epithelium of the intestine.

5. Capacity to colonies a colony.

6. Clinically confirmed health gain.

7. Security is paramount [1].

8. Against pathogenic bacteria, there is a competitive antagonism.

2.3 Advantages of Probiotics

Health practitioners are continually promoting the benefits of food that contain probiotic bacteria with lot of benefits for mankind. Probiotics have been shown to have an important impact on a number of metabolic and immunological functions, as well as on the prevention of infectious disease in children.
GIT cancers, Endotoxemia, Obesity, Metabolic syndrome (MetS), , irritable bowel syndrome (IBS), insulin resistance, type 2 diabetes (T2DM), inflammatory bowel disease (IBD) nonalcoholic fatty liver disease (NAFLD), diet-related maladies and lifestyle have all been connected to disrupted gut microbial equilibrium.
There have been over 900 clinical studies and several review papers published about the benefits of probiotics. Different probiotic strains were used in the trials, which focused on various health benefits and different target populations [23]. airy-related beverages account for around 43% of the functional beverage market and are mostly fermented food products [24]. Fermented milks, notably kefir items,

are the most frequent active probiotic kefir drinks in North America and Western Europe, with Ymir being most popular in Copenhagen.

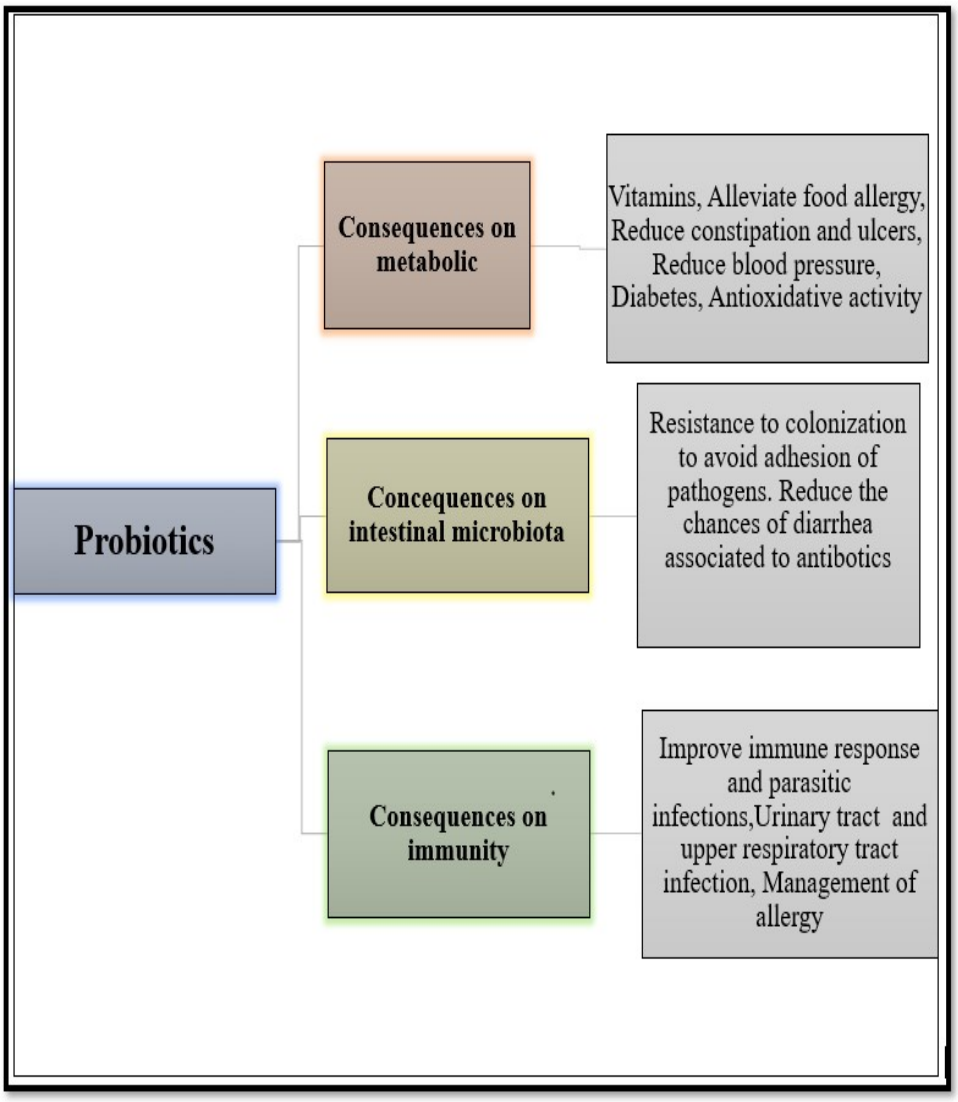


FIGURE 2.2: Major health benefits of probiotic bacteria [29].

2.3.1 Probiotic Products from Dairy Source

Products such as milk and other dairy-related items include probiotics [23]. Lactic acid bacteria (LAB), bifidobacterial, and other microorganisms derived from fermented milks have long been used in this sense. In Mongolia and Africa, spontaneous milk fermentation has a long tradition, and the use of beneficial microorganisms in fermented dairy products has been practiced for many centuries [23].

Dairy-related beverages account for around 43% of the functional beverage market and are mostly fermented food products [24]. Fermented milks, notably kefir items, are the most frequent active probiotic kefir drinks in North America and Western Europe, with Ymir being most popular in Copenhagen. The research has explored the positive impact of dairy matrices on probiotics in the digestive tract when contrasting alternative matrices versus dairy matrices, particularly from milk proteins. Proteins are a source of biologically active peptide precursors, which are resistant to digestion. These factors enable probiotics survive in the digestive tract under unfavorable situations. Proteins in milk are used as a transporter matrix for probiotic bacteria, they are useful in helping probiotic bacteria to find out their target sites [25].

2.3.2 Probiotic from Nondairy Source

Due to higher consumer spending, nondairy probiotic distribution has received more interest in recent times. This demand is driven by an increment in the amount of lactose intolerant people (around 70% in Asia), allergies due to proteins in milk, and the incidence of elevated cholesterol. Vegetarianism is now more common and popular practice by customers in developed countries, as a result, interest for vegan probiotics had increased. These are the main disadvantages of fermented food product from dairy source [26]. Economic and cultural variables may have an impact on the consumption of probiotic dairy products since they are fermented foods. Nondairy probiotic drinks are especially appealing because they are free of allergies in milk or dairy product, have lower level of cholesterol , and are Suitable for vegans [2]. Moreover, Various substrates can give various antioxidants, dietary fiber, mineral, and vitamin combinations.

As a result of the favorable outcome of bifunctional foods, there is a requirement to extend and offer non-dairy probiotic beverage alternatives. Between 2013 and 2018, the market for probiotic from fruits or vegetable meals is expected to expand at a 15% annualized rate. The nutritional and functional business in the United States is gaining traction, however in a distinct fashion than those in Europe, is

demonstrated by the fact that its food sector is more widely defined as nutraceuticals, and consumer demand in botanical nutritional supplements instead of fortified foods is increasing. Due to the disadvantages of dairy probiotics described above, researchers are looking for novel and enhanced probiotic microbe transporters [2]. Nondairy probiotic items, such as fruits, cereals and vegetables are one of the most popular options, and the need of probiotic from non-conventional source is consider as best alternative than from conventional source. (Reddy et al., 2015). Pureed fruit probiotics are becoming more popular as a result of their delicious flavor, Lists of nutrients, and widespread perception as healthful and nutritious foods [2].

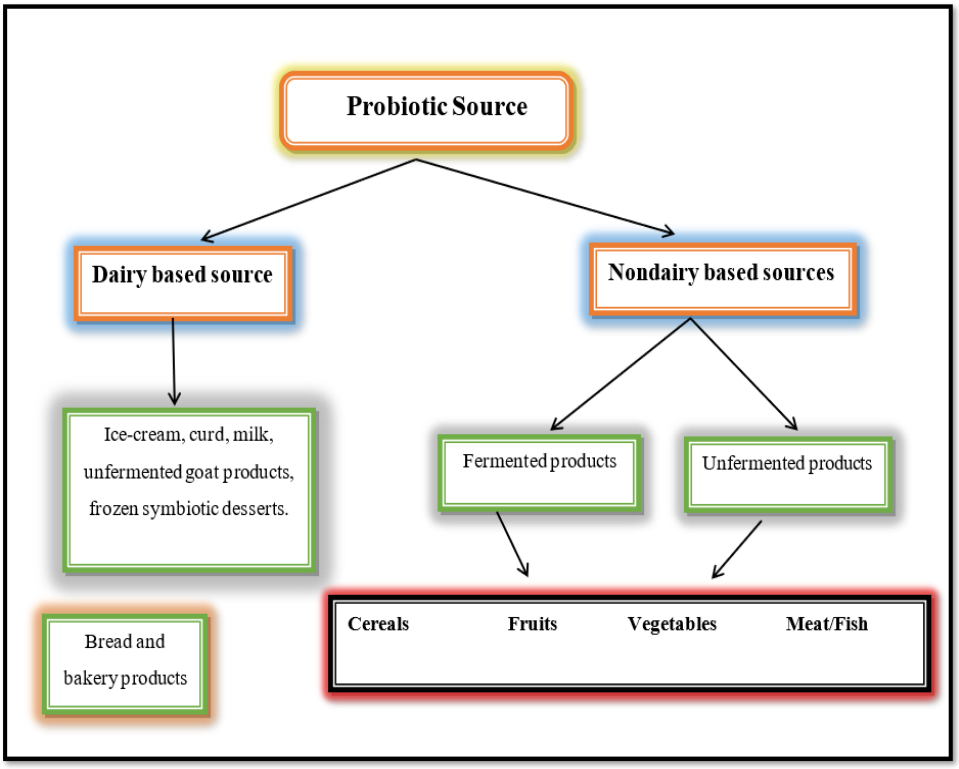


FIGURE 2.3: Source of probiotics [2].

2.3.3 Use of Fruit Juices as a Source of Probiotics

Fruits are one of humanity's most important foods and they're nutritious and have important part in health maintenance. Fruits, both conventional plus organic, supply crucial nutrients such as, vitamins, minerals, carbohydrates and antioxidants,

as well as improving the quality of one's diet. Fermentation is a viable method for developing innovative brands with altered physiochemical and sensorial properties, mainly flavor and dietary nutrients. Fermentation of acetic acid, lactic acid and alcohol is critical for standard of product. Fermented beverages had existed from the beginning of time [24]. Fruit juices contain a high concentration of carbohydrates, minerals, and vitamins, each of which help probiotics survive storage. Fruit juices are also a great option for people who want to eat food with stunted level of cholesterol or intolerant to lactose [25].

According to previous studies, the key parameters determining the vitality of probiotics in fruit juices include alkalinity or acidity, total phenol, organic acid proportion, dietary fiber, protein, and oxygen [18]. Juices contain natural sugars that can aid in the growth of probiotic organisms while still tasting delicious. For example

1. Pomegranate juice,
2. Tomato puree,
3. Orange,
4. Pineapple,
5. Cashew-apple juices.

are all examples of this. These microbes have shown good life expectancies during beverage preservation and can influence physiochemical factors, like varying the viscosity of flavanones and carotenoids in tangerine juice. After fermentation by probiotic Lactobacillus species, the tartness of the end product of these drinks are quite elevated *L. delbrueckii, L.plantarum, L. paracasei, L. casei and L. acidophilus*. Before fermentation, enriching juices with brewer's yeast auto lysate means self-destruction of fruit by action of its own fruit enzymes improves the dietary characteristics of the finished beverage, increasing the possibility of fermentation with the right bacteria and yeast. Biolab and Bio profit are two available commercially probiotic-containing fruit juices [2]. Several unconventional

(fruit juices) had been intended to prevent the drawbacks of non-conventional goods while simultaneously offering pleasing flavor's and soothing characteristics. Apples, peach, apricot, banana, blueberry and other fruits are examples. The majority of cellulose in fruits and vegetables cannot be absorbed in the alimentary canal. Apples were used to evaluate the application of suction impregnation technique for probiotic bacteria, and the results were promising [26]. As a result, non-conventional or fruit source are an important subject to study with a lot of promise for the functional food market [27]. When using fruits and vegetables as a non-dairy probiotic carrier, bear in mind that their higher polyphenolic, organic acid, or dietary fiber content can often minimize their sensory acceptability. For instance, the juice of sea buckthorn berries (Hippophage rhomboids) is known for its high phenolic acid, ascorbic acid, and fatty acid content [28], giving it a tart flavor and low palatability. To address this problem, our lab created a custom formulation for a shelf-stable probiotic-fortified sea buckthorn beverage. Targeted interactions between probiotics, natural or added prebiotics, and other food components during the various unit operations of food processing are needed for the effective transformation of fruit-based matrices into physiologically functional food [29]. By developing a food matrix with synergistic or additive interactions between probiotic strains and ingredients, the product's effectiveness can be increased. When tested against enteropathogenic E, for example, the probiotic-fortified sea buckthorn beverage produced in our lab showed successful pathogen clearance. *Salmonella and E. coli* [28]. Another trend has been to fortify probiotics and minerals with vegetable tissue [29]. The shelf life of the melon pieces was 11 days. Mung bean milk was derived from low-cost pulses like mung bean and used as a probiotic matrix for Lb plantarum [29].

2.4 Fermentation of Fruits and Vegetables

Lactic acid fermentation is anticipated to play an increasingly important role in maintaining fresh vegetables, fruits, and other food items for feeding humanity in developed countries as the global population grows. Several fermented fruits and

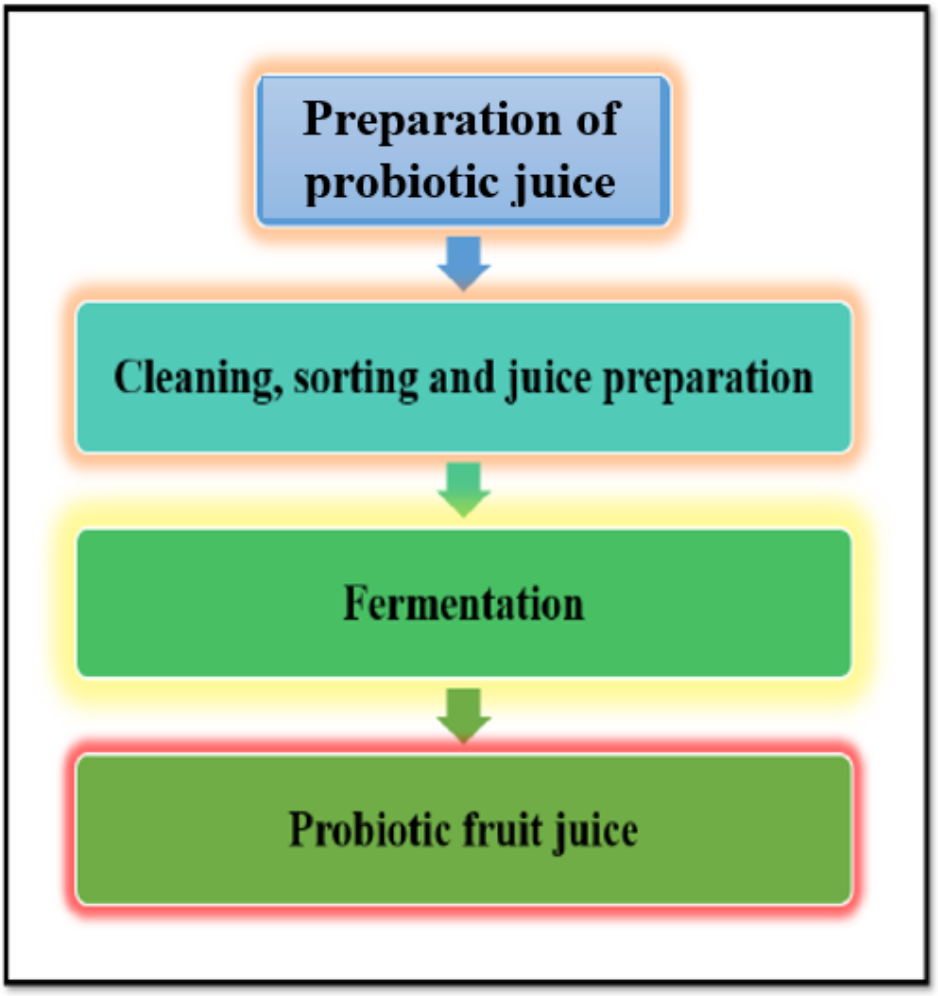


FIGURE 2.4: Preparation of probiotic juice [30]

vegetable products have a strong tradition in human diet dating back to ancient times and are linked to various social aspects of different groups. Fruits and vegetables are one of the most perishable foods due to increased water activity and nutritional value. In equatorial and subtropics countries, where contamination causing bacteria can proliferate is favored, these conditions are even more important. Lacto fermentation extends the storage lifespan of fruits and veggies while improving a lot of desirable commodities such as nutritive value and flavor, as well as lowering toxicity. *L. pentosus, L. acidophilus, L. brevis, L. fermentum ,L. plantarum and L. mesenteroides* are among lactic acid bacteria found in fermented fruits and vegetables [30].

Traditional fermented fruits and vegetables, as a whole, not just take part as dietary supplementation, but also contribute to good wellbeing. It is critical to

have a basic awareness of the relation between diet, good microbes and health of mankind in order to increase integrity of meal and illness prevention [31]. Sugar, salt, and monosodium glutamate, for example, should be used in fermentation of foods in line with agreed-upon norms defined by target market legislation [32].
In recent years, a lot of study has been done on the protection of probiotic microbes via encapsulation during food storage and food processing[36]. Probiotics may be encapsulated using proteins, polysaccharides, carbohydrates, and their variations, as well as certain liquid food matrices[37].The probiotic food industry is interested in microencapsulation of probiotic species as a perfect way to preserve probiotic microorganisms' effectiveness provided to the GIT[38].

2.5 Identification of Microbiota of Ferment Fruits and Vegetables

To isolate and characterize various types of LAB strains of fermented fruits, several modern techniques such as RAPD- (Random Amplified Polymorphic DNA-) PCR, species-specific PCR, multiplex PCR, 16s rDNA sequencing, gradient gel electrophoresis, RFLPs, and cluster analysis of Temporal Temperature Gradient Electrophoresis are used in addition to traditional methods (microscopy, plate count, etc.). RFLP and 16s rDNA were used to isolate and identify lactic acid bacteria from Taiwanese traditional fermented foods such fermented black beans and fermented mustard [33].

A variety of protocols have been proposed and tested to reduce the gastrointestinal system's negative affects on probiotic microorganisms. One of the most successful is the encapsulation technique. In the biotechnology industry, the encapsulation of probiotic living cells, which is based on immobilisation technique, could be utilized for whole cell culture and enzymes.It's a technique for covering biological active materials with other protection substances, or a combination of these, so that confined components could released in moderate levels under certain situations. Microencapsulation shields the bioactive element from challenges of surrounding

such as oxygen, excessive acidity, and gastrointestinal conditions, allowing it to travel through the stomach with minimum dilution. Using water insoluble wall materials, the safety of the microencapsulated bioactive portion when moving via the stomach could be improved[34][35].

In recent years, a lot of study has been done on the protection of probiotic microbes via encapsulation during food storage and food processing[36]. Probiotics may be encapsulated using proteins, polysaccharides, carbohydrates, and their variations, as well as certain liquid food matrices[37][38].

TABLE 2.1: Global commercial probiotic products based on fruit and vegetable matrices

S#	Food Matrix	Commerical Name	Origin	Probiotic
1	Fruit juice	Probiotic juice of garden	USA	*B.coagulans*
2	Fruit soda	Obi, probiotic soda	USA	*B. coagulans*
3	Fruit juice	Biola	Norway	*Lb. rhamnosus*
4	Fruit juice	Valio bioprofit	Finland	*Lb. rhamnosus*
5	Fruit juice	Ria by biogaia	Sweden	*Lb. reuteri*
6	Cold fruits	Welo probiotic	Canada	*B. coagulans*
7	Fruit drink	Probi bravo fruscus	Sweden	*Lb. plantarum*

2.5.1 Challenges for Probiotic Bacteria

2.5.1.1 Stability and Viability

Probiotics beneficial effects are primarily determined by their volume in foods and their capability of staying alive inside the gastrointestinal tract. Probiotic durability is dependent on microbial strain and varies from each other [32]. When the

storage term is up, the number of probiotics in the end result must be at least 106 or 107 CFU/ml, which refers to 109 CFU per part [33]. On improving the storage stability of probiotics, a lot of research has been conducted and useful evidence is collected. Minerals, vitamins, dietary fibers, and antioxidants, in combination to the vital nutrients (vitamin supplements, carbohydrates, minerals and antioxidants), are present in juices and may limit probiotic viability [32]. The majority of these frameworks depend on polymers with varying penetrability, disintegration rates, swelling levels, and erodibility. The probiotic food industry is interested in microencapsulation of probiotic species as the best way to preserve the potency of probiotic microorganisms provided to the gastrointestine[40]. They are

1. Characteristics of diet: pH, acid concentration, Oxygen molecule, moisture content, salt, sugar, and chemicals such as bacteriocins, hydrogen peroxide, synthetic flavoring, and coloring additives, as well as the existence of salinity, sweetness, and chemicals compounds such as hydrogen peroxide, metabolites like bacteriocins, artificial ingredients, and coloring additives.

2. Heat treatment, incubation temperature, cooling time, packing materials and storage methods, oxygen levels, volume are all processing parameters.

3. Microbial framework: probiotic strain, inoculation amount plus percentage. the most key parameters influencing probiotic viability is pH. Beverages have a low pH because they carry a variety of natural acids. As a result, the juices may have a combination of acidic environment and acids' inherent antimicrobial properties. Several important good microbial strains (*Lactobacilli and bifidobacterial*) may live inside fruity drinks and are pH 3.7 to 4.3 resistant. Bifidobacterial have a lower acid tolerance, and a pH of around 4.6 is harmful to their viability [30].

In such situations, pH cannot clarify the patterns observed by certain probiotics, despite the fact that the above probiotics demonstrate amazing viability in low pH fruit juices. In fruit drinks (tangerine, citrus, blackcurrant, pineapple, pomegranate, and strawberries), Bifidobacterium lignum stay alive.

A synergy and opposing combination of numerous variables led to longevity, with phenol chemicals playing a major role. pH has a negative impact in general, but proteins and fibers in diet may shield the cells from stress of surrounding acidic environment; the involvement of malic acids and citric acid and is debatable, prevent probiotics while phenolic compounds may create a significant loss of feasibility[2]. Even though pH is a disadvantage in order for probiotics to remain into liquid drinks, adding lactic acid bacteria to low-pH fruit juices increased bacteria resistance to later stressful acidic conditions, including those absorbed by the gastrointestinal tract [34].

2.5.1.2 Sensory Traits

Another major obstacle to fruit juice probiotification is public approval [35]. Probiotification of fruit juice has been reported to produce flavor's that are characterized as

- as "milk products,"
- "therapeutic,"
- "acidic,"
- "salty,"
- "bitter,"
- "astringent,"
- "artificial,"
- or "earthy." " [35].

However, it's uncertain if all probiotic cultures impart the same taste to the product at about the same intensity levels. The effects of probiotics upon sensorial qualities of fruit drinks differ depending upon type of fruit, strain of probiotic,

storage temperature, prebiotic and protectant additives. According to some studies, probiotics had no effect on the Acceptance of some fruit juices as a whole. Perricone et al found no unfavorable flavor shift in pineapple drink consisting. *Lactobacillus reuteri* found no adverse flavor change in a fresh apple juice fermented by *Lactobacillus casei*and de Souza Neves Ellenberger et al found no adverse flavour change in apple juice [36]. Masking, or the incorporation of nice fragrance and evaporative compounds that "mask" the probiotic presence, is one potential solution for unwanted taste results in probiotic juices.
Finally, Ranadheera et al found certain fruit drinks can naturally camouflage probiotics' "medicinal" flavor [37] [38].

2.5.2 Strategies to Improve Survival of Probiotics

2.5.2.1 Supplementation of Growth Promoters and Protectants

A easy technique to increase probiotic longevity in fruity drinks is to fortify it with growth boosters and protectants or additions that have a survival benefit. Since oligofructose's are accessible as substrates for the metabolism of these microorganisms, they can enhance the viability of probiotic cultures throughout storage and processing of products [31]. As a result, probiotics in fruit drinks may be more stable during storage. Oligofructose's often had sweetness in flavor identical to sucrose's and can be used as alternative to sugar [32].Pimentel et al developed oligofructose-fermented probiotic apple juice with *L.paracasei*. Developed oligofructose-fermented probiotic apple juice with *Lactobacillus paracasei*. They checked the physicochemical characters, probiotic feasibility, and desirability after storage in refrigerator (4°C for 28 days) in glass or plastic containers following fermentation.

2.5.2.2 Mutagenesis

To acquire strains with modified properties or to analyses various microbial systems, ultraviolet radiation or chemicals had been widely utilized. In probiotics

study, this methodology has been useful in promoting the stabilization of B. breve and B. animalism in low pH products [38]. This technique is often used to achieve quality stabilization of sensorial attributes. For example,

- Bifidobacterial metabolic activity throughout food manufacturing or preservation is often unwanted, as large quantities of acetic acid produced can lead in unpleasant flavor's.
- UV mutagenesis was used to create novel type of Bifidobacterial that produce lowest levels of acetic acid;

These strains will allow for the creation of sustainable and organoleptically appropriate products [39].

2.5.2.3 Selective Pressure

Increased sensitivity (stressed factors) is also being utilized to acquire resistant probiotic strains. Strains generated using this approach are often found to have been balanced phenotypically and cross-resistance to various stressors (acid and temperature) [40]. By using a selective pressure technique, Lactobacilli and Bifidobacterial were able to tolerate more heat, oxygen, and acid [41]. While the use of such stress-resistant strains can help improve industrial process stability, caution should be exercised because stress adaptation can change the strain's other characteristics. Using strain resistant to stress in probiotification do not result [32].

2.5.2.4 Genetic Modification of Strain

Probiotic microorganisms may be genetically modified to improve their viability and sustainability. However, this is not possible in all states; for example, in Europe, customers do not embrace GMOs. There are two simple methods that can

be followed:

1. Modifying the expression/production of genes already present on the microorganism using homologous expression.
2. Introduce genes from other microbial organisms by heterologous expression [34].

2.6 Bacterial Surface Chemical

In Modulation of the human defense system, surface chemicals present in Bacteria i.e. Gram-positive have found to have a important role, and then the systemic immune system, in achieving the goal of modulating immune system is by initiating host and bacteria interaction. Proteins, lipoproteins, glycoproteins, lipoteichoic acids and flagellins are helping in modulating immune system and is carried out by the interaction of these chemicals of bacterial surface with host designated receptor for pathogen recognition. The proteins on the surface could be part of a Slp lattice, which is the outermost macromolecular monolayer. Hoodwink initially defined it in 1953, and It is made of a Para crystalline bidimensional array consist of a protein called Slp , first discovered on the cell surface of Spirillum sp. Chaotropic drugs such as guanidine chloride and LiCl are used to extract Slps [42].

2.6.1 Positive Aspects of SLP

Several studies have shown that surface-bound protein take part in the bacteria/host interaction, resulting in positive benefits such as immunological regulation, although the molecular processes are still unknown. Indeed, they contribute to the establishment or maintaining of cell structure, sieving of molecules, enzyme activity, role of adhesion, coaggregation, modulation of gut defense system, defense from environmental extremes, and antimicrobial peptides, among other functions in bacteria [42].

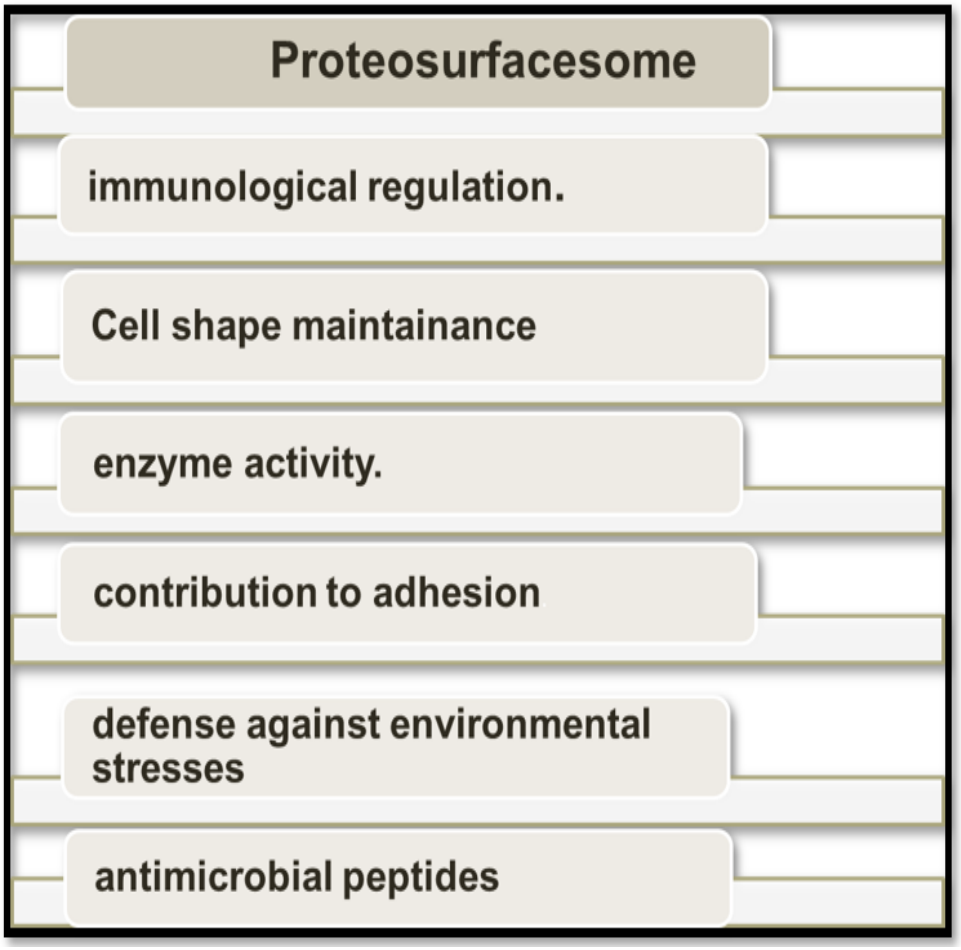


FIGURE 2.5: Importance of surface layer protein. [31]

2.7 Surface Layer Protein (SLP) and Surface Layer Associated Protein (SALP)

Some Gram-positive bacteria, particularly probiotic bacteria, have an exterior proteinaceous covering termed a surface-layer that protects them from the environment. (Slp) are "surface-layer-protein" their self-assembly creates Para crystalline layer. The surface layer is preserved and found in a wide range of prokaryotes. The sequence of the homologous Slp protein, on the other hand, varies greatly between different species of bacteria and between same species strains. This outer structure can also be used to extract other proteins, such as proteins non-covalently bounded on surface of bacterial cell and other (SLAPs) surface layer associated with protein. They can take on a variety of roles. Different authors and experimental methodologies have indicated that probiotic. Gram-positives organisms

have a potential in important interactivity with the host. They can take part in tolerating environmental stress, withstand inside the GIT of host, attachment with cells of host or mucus, or control of inflammation in GIT, depending on species and strain. From including novel vaccines to production of nanoparticles, coating and encapsulation are among the future trends [41].

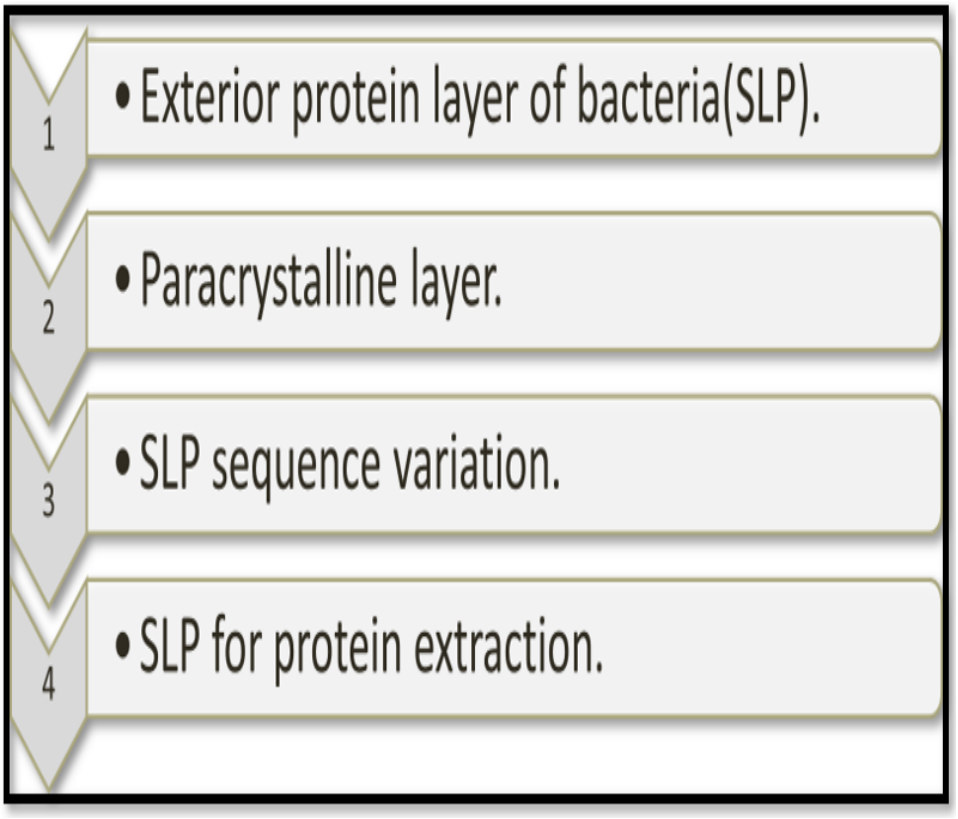


FIGURE 2.6: Properties of SLP. [30]

2.8 Morphology of S- Layer in Bacterial Species

Archaea, Gram-positive, and Gram-negative bacteria all have S-layers, which are very porous and have a thickness of 5–25 nm. The tetragonal (p4), or hexagonal (p6, p3) oblique (p2, p1) symmetry of the S-layer Para crystalline lattice can be used. On-covalent interactions hold the subunits together and connect them to the underlying cell surface, and have an inherent tendency, driven by entropy to create common patterns in solution or solid in vitro support. Subunit proteins are generally elevated in acidic and water repellent amino acids, but low in amino

acid contain Sulphur, and have the lowest expected pI value. The genes for S-layer proteins are substantially expressed. Several S-layer protein genes have been identified in a single strain's genome, although not all of them are indicate at the same time.

1. Alternate expression of S-layer protein genes in or ex vivo, silent genes, antigenic variation depending on S-layer gene expression, Superimposed S-layers or S-layers constituted of two separate S-layer proteins have been described, as well as sequential expression throughout growth.

2. Because of the Low successive similarity between S-layer genes and lack of universal signature sequence, electron microscopy is nevertheless used to confirm the presence of S-layer [43].

2.9 Physiology of S Layer

Data regarding to biological role of Slp has collected in recent decades, although no single function for all S-layer proteins has emerged. The process of determining or maintaining cellular morphology, Functions as a molecular sieve, a binding site for large molecules, ions, or phages, and a modulator of microbial colonization[37] have all been found thus far [44].

1. S-layer protein genes in or ex vivo, silent genes, antigenic variation depending on S-layer gene expression, Superimposed S-layers or S-layers constituted of two separate S-layer proteins have been described, as well as sequential expression throughout growth.

2.10 Pathogenic Bacteria

Surface layers might assist to virulence in pathogenic bacteria through a variety of methods, including adhesion, coaggregation, antigenic variation, shielding

from complement or phagocytosis, and regulation of T-cell or cytokine responses. Furthermore, S-layer proteins may shield the bacterial cell from mechanical and osmotic stressors, antimicrobial peptides, radiation, changes in ambient pH, bacteriophages, bacterial or eukaryotic microbial predators, or bacteriolytic enzymes. Some S-layer proteins, such as the S-layer protein of a marine Synechocystis, have the ability to operate as degradative enzymes [45].

2.11 Properties of Extricable Surface Protein

Certain proteins of surface found to have diverse characteristics which can be separated from probiotic bacterial strain and numerous other species. Biologically supplements i.e. probiotic food supplements and food that is fermented can be oriented by the ability of surface protein such as profuse expression, self-assembly, surface location, resistance to physicochemical assaults, immunomodulation, adhesion, and toxic remediation.Proteins present in S-layer shown to have vast application in the field of nanobiotechnology. Owing to their capacity to construct repeating protein arrays via spontaneous association, proteins present in S-layer shown to have vast application in the field of nanobiotechnology.
This is true for vaccination candidates, epitope surface display, and therapeutic or biotechnological proteins [44]. This brings up new possibilities in the fields of gastrointestinal problems, such as IBS and IBD, infectious diseases, and oral vaccination. Engineered Slps for selective, effective, and that can be targeted at a low cost and other medically essential compounds is a key focus is a key focus.

2.12 Applications of S Layer

S-layers offer a wide range of applications in (Nano)biotechnology due to their self-assembly characteristics and highly ordered, regular structure down to the nanoscale scale. S-layer applications can be split into two categories. Vaccine development, multiple protein production, and exterior showcase applied to different

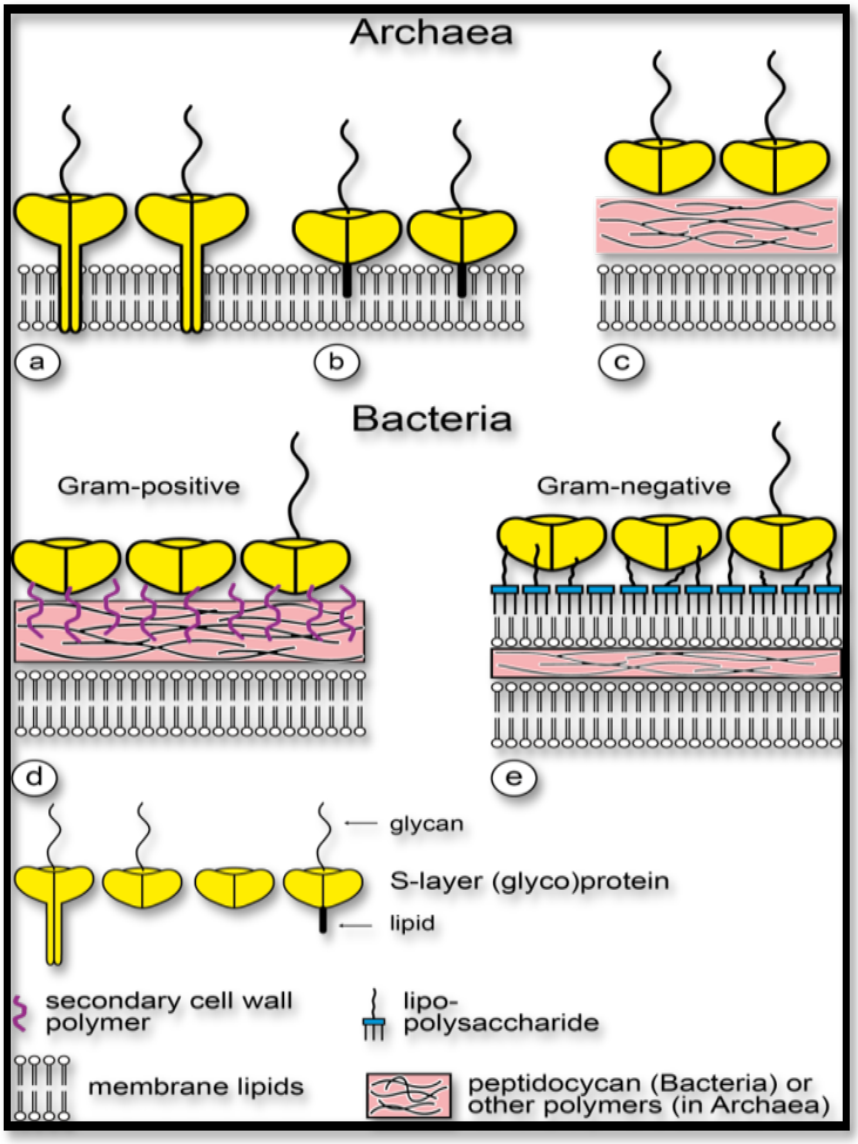


FIGURE 2.7: Composition of s layer protein [41].

biological systems that use (genetically engineered). S-layered bacterial cells, S-layer (fusion) proteins, or only the interpretation and/or secretion signals of S-layer protein genes fall into the first category. For non-life (Nano) technical applications, the second group uses isolated, mainly recombinant S-layer proteins [40] [41].

2.13 S Layer in Lab Bacteria

S-layers have been discovered in numerous *Lactobacillus* species, but not all. S-layer proteins from *L. brevis, L. buchneri, L. helveticus, and L. hilgardii* as well

as organisms from the former L. acidophilus group such as *L. acidophilus, L. amylovorus, L. crispatus, and L. gallinarum* have been studied biochemically or functionally.

1. *L. amylolyticus, L. gigeriorum, L. kefiranofaciens, L. pasteurii, and L. ultunensis* all include putative S-layer protein genes within their genomes.

2. Which are either entirely or substantially sequenced. Because found no S-layer proteins on the surface of *L. casei, Lactobacillus paracasei subspecies paracasei, or L. rhamnosus, L. casei* is currently regarded as a non-producer.

All of the Lactobacillus S-layer proteins discovered so far have a 25–32-amino-acid signal peptide, suggesting release via the basic secretory route. The primary amino acid patterns of mature Lactobacillus S-layer proteins varied greatly, or even S-layer proteins from the very same strain can have large sequence changes when present [42].

2.14 Biotechnological Applications

S layer proteins' ability to self-assemble and create repeated complex aggregates of molecules that are claimed to be irreversible and resistant to physicochemical intrusions led to the idea of employing them in (Nano)biotechnology [43]. Such single molecular arrays give well-defined topologies based on the physicochemical characteristics of the glycoprotein that creates the closed, isoporous lattice and for which bacteria have a vast diversity. Researchers were inspired to investigate employing re-crystallized Slps to build ultrafiltration membranes with extremely accurate molecular cutoffs, great intramolecular cross-linking stability, reduced membrane fouling, and configurable surface properties such as net charges and hydrophilicity. Chemical and genetic engineering can also be used to immobilize functional molecules like catalysts, receptors, antigens, and antibodies while also still allowing Slps to self-assemble. Because some Slps are recognized to spontaneously make premade nanoparticles, functionalized Slps nanoparticles, particularly metallic and

semiconductor nanoparticles, were created on native surface-layers. Slps can also be employed to create vaccines or as structural aid for working lipid membranes. Due to the inherent adjuvant qualities of various SlpS, composite vaccines containing Slps plus antigens, hatpins, or recombinant allergens produced best outcome in vaccine testing [43].

A variety of protocols have been proposed and tested to reduce the gastrointestinal system's negative effects on probiotic microorganisms. One of the most successful is the encapsulation technique. In the biotechnology industry, the encapsulation of probiotic living cells, which is based on immobilization technique, could be utilized for whole cell culture and enzymes. It's a technique for covering biological active materials with other protection substances, or a combination of these, so that confined components could release in moderate levels under certain situations. Microencapsulation shields the bioactive element from challenges of surrounding such as oxygen, excessive acidity, and gastrointestinal conditions, allowing it to travel through the stomach with minimum dilution [43]. Using water insoluble wall materials, the safety of the microencapsulated bioactive portion when moving via the stomach could be improved [63]. In recent years, a lot of study has been done on the protection of probiotic microbes via encapsulation during food storage and food processing [33].Probiotics may be encapsulated using proteins, polysaccharides, carbohydrates, and their variations, as well as certain liquid food matrices [34]. The probiotic food industry is interested in microencapsulation of probiotic species as a perfect way to preserve probiotic microorganisms' effectiveness provided to the GIT [67].

The following are the main reasons for using this approach to protect probiotics:

• Improving probiotic cultures' viability and stability during processing, storage, and transit through the gastrointestinal tract [68].

• Delivering probiotic bacteria to the gastrointestinal tract in a controlled and productive manner [69].

• Cultures are handled more quickly [70].

• Microcapsules have only mild effects on the sensory properties of the substance [56].

Chapter 3

Research Methodology

3.1 Methodology Flowchart

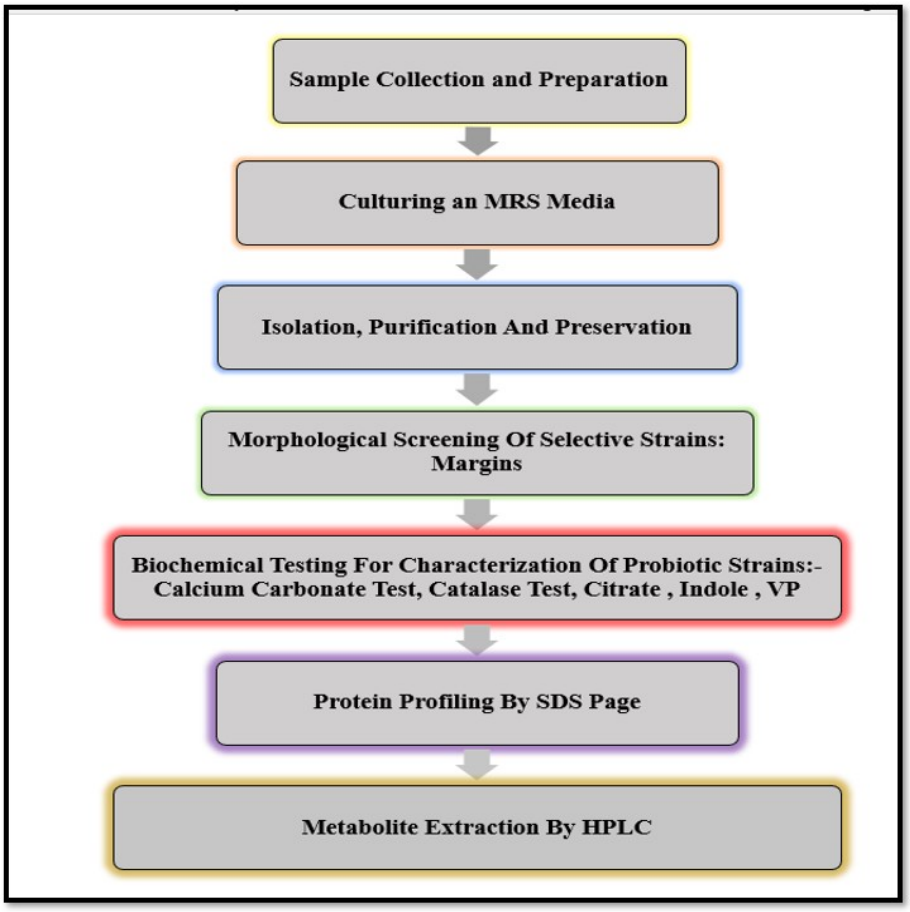


FIGURE 3.1: Methodology of Project

3.2 Sample Preparation

Sample preparation was done by peach and apricot fermentation for 7 days. Fermentation encourages the formation of probiotic bacteria, which are helpful microorganisms. Probiotics have been demonstrated to help with immunological function, digestion, and cardiovascular health. About 50g of peach and 32g of apricot was fermented. About 10g and 6.4g of of salt was added in peach and apricot for fermentation [44].

3.3 Serial Dilution of Fermented Sample

The goal of the serial dilution approach is to estimate the concentration of an unknown sample (number of colonies, organisms, bacteria, or viruses) by counting the number of colonies produced from serial dilutions of the sample and then backtracking the recorded counts to the unknown concentration. For a ten-fold dilution, 1 ml of sample is added to 9 ml of diluent. After the first tube, each tube is the dilution of the previous dilution tube. Now, for total dilution factor. The formula is

Total dilution factor for the second tube = dilution of first tube × dilution of the second tub [45].

3.4 Preservation of Purified Strain

Glycerol stock of 100ml was prepared for the preservation of purified strain. 50% of glycerol was prepared by dissolving 50ml of glycerol and 50ml of distilled water. it was autoclaved at 121 degree centigrade for 15-20 minutes. 2.5ml Eppendorf tubes were taken and autoclaved at 121% for 15 minutes. The Eppendorf tubes were numbered in the laminar flow hood. 1ml of glycerol solution was filled in these Eppendorf tubes with the help of 1000µL pipette. Suspension was made with loop full of bacteria picked from each differential media and added into Eppendorf

tubes containing glycerol stock. Eppendorf tubes with bacteria and glycerol were kept at -4^{o}C [47].

3.5 MRS Agar Media

MRS agar was created primarily for the development of lactobacilli from various sources with the goal of establishing a defined medium that could be used instead of tomato juice agar. It is used to cultivate the entire lactic acid bacteria family. Serial dilutions are poured on media plates with the help of pipette. It is prepared by suspending 67.7g of MRS agar in 1000 ml of distilled water [46].

3.6 Streaking of Bacterial Colonies

Streaking of bacterial colony is important to get separate colonies of bacteria.it is important in recognition of bacterial strain and to remove contamination as well [47].

3.7 Inoculation of Purified Bacteria in MRS Broth

Each Bacterial culture is inoculated in mrs broth of 100 ml and incubated for 24-48 hours at 37 °C. MRS Broth is a culture and enumeration media. It promotes the lush growth of Lactobacilli from the mouth, dairy products, meals, excrement, and other places. After that mrs broth in centrifuge in falcon tubes at 10,000 rpm for 20 minutes at 4 degree Celsius [50]. 16s RNA was done to identify the strain.

3.8 Biochemical Characterization

Different types of biochemical tests were performed for the biochemical characterization of two prevalent selected strains [48].

3.8.1 Indole Spot Test

The capability of certain bacteria to degrade the amino acid tryptophan to indole, which aggregates in the medium, is demonstrated in this test take a filter paper and moisturize it with a drop of kovac reagent.
Color on that area will change. If there is no change in color it means it is indole negative if color turn pink in means result is positive [48].

3.8.2 Urease Test

This test is basically use for the utilization of urea by the bacterial samples. For this test, the Urea Agar Base [UAB] was weighed 2.5g. Then added it in the conical flask with 100ml of distilled water in it. After proper mixing, the conical flask was properly covered and prevented from the contamination; it was autoclaved for 15 to 20 minutes at 121°C.

- The autoclaved was done.
- The media was poured into the six test tubes.
- Streaking of isolated cultures was done on the test tubes containing Urea Agar Base [UAB].
- The plates were incubated in the incubator at 37°C for 48-72 hours. The bacterial strains with pink color are urease positive and other that don't turn the color into pink was urease negative [49].

3.8.3 Catalase Test

Catalase is an enzyme, enzyme that decomposes hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the byproduct of aerobic carbohydrate metabolism. If this oxidative product remains in the body of bacteria, it becomes

lethal for their survival. The function of the catalase is that it decomposes hydrogen peroxide into water and oxygen. The reagents that were present in the catalase test contain 3% hydrogen peroxide. A loop full of bacteria from pure culture were taken and placed on the slide. In addition, two drops of 3% hydrogen peroxide was added on the slide to check the production of hydrogen peroxide in the bacteria [49].

3.8.4 Calcium Carbonate Test

Calcium carbonate test was performed to check production of lactic acid. For this test MRS agar supplemented with 1% calcium carbonate was poured onto petri plates and purified colonies were spread on media using sterile loop. Plates are incubated at 32°C for 2-4 days [49].

3.9 Ammonium Persulphate

Dissolve 1g of ammonium per sulphate in 5 ml of water than bring it volume upto 10ml with distilled water. Always make fresh and then use [54].

3.10 Resolving Gel Buffer

Add 20ml 1.5M tris HCl of ph 8.8,1.6ml of 10% SDS,1.6ml distilled water and store it at 4°C [55] .

3.11 4X Stacking Gel Buffer

Stacking gel is a type of the polyacrylamide gels which is used to separate the protein as well as the amino acid from the sample. Add 20ml of 0.5 M tris HCl pH. 6.8,1.6ml 10% SDS,18.4ml dis water. Store it at 4 °C [55].

3.12 1X Electrophoresis Buffer

Add 3g of tris base,14.4 ml glycine,1 g SDS in 500 ml of water. Brought the volume up to 1000ml.there is no need to adjust pH. Store at 4°C [56].

3.13 4X Sample Loading Buffer

Add 10 ml 4x stacking gel buffer, 18ml 0f 10% SDS, 2ml sigma,20ml glycerol and 5mg of bromophenol blue. Store it at 4°C [57].

3.14 Distain solution

Take 675ml of distilled water. Add 250ml of methanol and 75ml acetic acid. Distain the gel over night to remove background [58].

3.15 Staining Solution

Add 227ml methanol in 227ml of distilled water. Add 46ml acetic acid. Add 1.3g of commissive brilliant blue. Staining solution is use to stain the gel to visualize bands of proteins [58].

3.16 Resolving Gel (12%)

First of all make resolving gel the further procedure.to make resolving gel [58].

- Distilled water 3.4ml
- 4X resolving gel buffer 2.5 ml
- 30% acrylamide 4ml

- 10% APS 100 microliter
- Temed 6.8 microliter

3.17 SDS PAGE

3.17.1 30% Acrylamide monomers

Dissolve 29g of acrylamide and 0.8g of bis(N,N-methylene-bis-acrylamide) in 70ml of distilled water and then bring volume to 100ml.store bottle at 4 degree Celsius for up to 3 months. Bottle must be covered in aluminum foil [51].

3.17.2 10% SDS

Dissolve about 10g of SDS in 50ml of distilled water. Brought the volume up to 100ml and store at 4°C [53].

3.17.3 1.5 M Tris-HCl, pH 8.8

Dissolve tris 18g of tris base in 50ml distilled water. After that add conc HCl drop wise to bring pH. up to 8.8 and then brought the volume to 100ml and stored [51].

$$x = MolecularMass \times Volume \times Molecularweight \div 1000$$

$$x = 1.5 \times 100 \times 121.4 \div 1000$$

$$x = 18.2g$$

3.17.4 0.5 M tris HCl, pH 6.8

Dissolve about 3g of tris base in 40ml of distilled water. Add conc HCl drop wise to adjust pH. up to 6.8. brought volume up to 50ml with distilled water and store

it at 4 degree Celsius. Calculation of molar concentration [52].

$$x = MolecularMass \times Volume \times Molecularweight \div 1000$$

$$x = 0.5 \times 50 \times 121.4 \div 1000$$

$$x = 3.035g$$

3.18 Stacking Gel (5%))

After resolving gel solution solidifies than add stacking gel solution to make it [58]:

- Distilled water 5.8 ml
- 4X stacking gel buffer 2.5 ml
- 30% acrylamide 1.626ml
- 10% APS 75 microliter
- Temed 10 microliter

3.19 Sample Preparation

To extract surface proteins from bacterial pellet, about 50 microliter chaotropic agent has been used that is SDS. Vortex bacterial pellet for 2 minutes.after that centrifuge it. Bacterial pellet and supernatant got separated. Now supernatant has been separated which is now rich in surface layer protein. 50 microliter of sample loading buffer has been added in supernatant.

- Probiotics have been demonstrated to help with immunological function, digestion, and cardiovascular health.
- About 50g of peach and 32g of apricot was fermented.

- About 10g and 6.4g of of salt was added in peach and apricot for fermentation [44].

FIGURE 3.2: SDS PAGE apparatus.

3.20 Apparatus Setting

Adjust two glass plates with integrated spacer in casting frame was done. Than adjust the whole casting frame in casting strand. To check leakage add distilled water. no water came out of glass plates which means there is no leakage. resolving gel between two glass plates was added and kept for about 30 minutes or until it solidifies. Then add resolving gel and keep it for about 30 minutes or until it solidifies. Add stacking gel solution and put plastic combs in it to make valves. Wait until it solidifies. Remove combs and put the glass plates with gel

in electrophoresis apparatus fill it with electrophoresis buffer. Set the electrodes. Negative on top while positive on bottom. Adjust voltage at 60V when bands start moving out of valves increase voltage that is 90V.All the band move from cathode towards anode on gel. Than take the gel out of glass plates carefully. Add staining solution. Stain the gel overnight than detain it. Blue bands will appear on gel which indicates proteins of different sizes [59].

3.21 HPLC Method

Only substances that are dissolved in solvents can be evaluated using HPLC. HPLC isolates compounds that are dispersed in a liquid sample, enabling both qualitative and quantitative analysis of which constituents are present and how much of each constituent is present. The solvent used to separate constituents in a liquid sample for HPLC analysis is known as the mobile phase. The mobile phase is carried to a separation column, also known as the stationary phase, and then to the detector at a constant flow rate controlled by the solvent delivery pump. Once a specific amount of sample is placed in the column, the compounds in the sample are separated. The mobile phase is the liquid that dissolves the target material. The stationary phase is the part of a column that engages with the targeted compound [58].

- The higher the affinity (e.g., van der Waals force) between the component and the mobile phase in the column, the faster the component and mobile phase in the column move through the column together.
- On the other side, the higher the affinity for the stationary phase, the slower it travels through the column.
- The chromatogram is a two-dimensional graph with a vertical axis that represents concentration and a horizontal axis that represents analysis time.

When no chemicals are eluted from a column, a line parallel to the horizontal axis is drawn.Instead of being in the shape of a triangle, the plot obtained looks

more like a bell. A "peak" is the name for this shape [59]. Sample was prepared by adding chloroform in samples.this solution was left over night.Chloroform was evaporated and residues were left in the beaker.1 ml hplc grade distilled water was added in residue and sample was run for HPLC [59].

Chapter 4

Results and Discussions

4.1 Sample Preparation

Sample preparation is done by peach and apricot fermentation for 7 days. Fermentation encourages the formation of probiotic bacteria, which are helpful microorganisms. About 50g of peach and 32g of apricot was fermented. About 10g and 6.4g of of salt was added in peach and apricot for fermentation.

(a) (b)

FIGURE 4.1: (a) Fermentation of 50g of peach for 7 days., (b) Fermentation of 32g of apricot for 7 days.

4.2 Serial Dilution of Fermented Sample

Serial dilution of sample was done to get separate number of bacterial strains which was unknown. Tenfold dilution was done in which 1ml sample is diluted in distilled water which was 9ml.after that 1ml.

4.3 MRS Agar Media

MRS agar was created primarily for the development of lactobacilli from various sources with the goal of establishing a defined medium that could be used instead of tomato juice agar. It is used to cultivate the entire lactic acid bacteria family. Serial dilutions are poured on media plates with the help of pipette.it is prepared by suspending 67.7g of mrs agar in 1000 ml of distilled water. MRS media is autoclaved for 15 min at 121 degree Celsius.

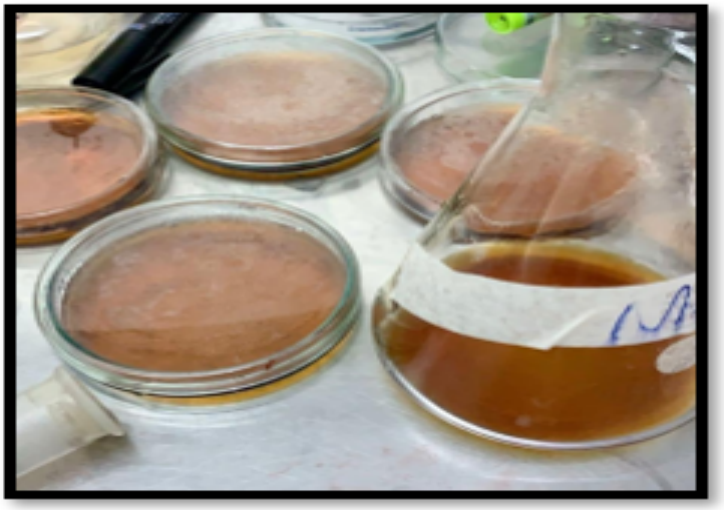


FIGURE 4.2: Pouring of MRS media in petri plates.

4.4 Streaking of Bacterial Colonies

In microbiology, agar streak plates are a must-have instrument. They allow bacteria and fungi to grow in separate colonies on a semi-solid surface. These colonies

can be used to recognize the organism, purify the strain to remove contaminants, and create a genetic clone that is 100% pure. Streaking is done by streaking bacterial strains from parent plate onto MRS agar.

4.5 Preservation of Purified Strain

Glycerol stock of 100ml was prepared for the preservation of purified strain. 50% of glycerol was prepared by dissolving 50ml of glycerol and 50ml of distilled water. it was autoclaved at 121°C for 15-20 minutes. 2.5ml Eppendorf tubes were taken and autoclaved at 121% for 15 minutes. The Eppendorf tubes were numbered in the laminar flow hood. 1ml of glycerol solution was filled in these Eppendorf tubes with the help of 1000µL pipette. Suspension was made with loop full of bacteria picked from each differential media and added into Eppendorf tubes containing glycerol stock. Eppendorf tubes with bacteria and glycerol were kept at -4°C.

- Probiotics have been demonstrated to help with immunological function, digestion, and cardiovascular health.
- About 50g of peach and 32g of apricot was fermented.
- About 10g and 6.4g of of salt was added in peach and apricot for fermentation [44].
- The calcium carbonate was added in peach and apricot.

4.6 Morphological Characterization

The culture that was obtained on the differential media was streaked further to isolate the bacteria. Bacterial species or genus were categorized based on the color characteristics and morphology on differential media. Following results including their sample size, shape and color, elevation,and margin predicted strain name and figure was shown in table 4.1.

TABLE 4.1: Bacteria isolates on different media

S#	Strain Name	Size	Shape	Colony Color	Elevation	Margin	Figures
1	St 1	Medium	Round	White	Raised	Entire	
2	St 2	Medium	Round	White	Raised	Entire	
3	St 3	Medium	Round	White	Raised	Entire	

4	St 4	Medium	Round	Milky white	Convex	Entire	
5	St 5	Medium	Round	Whie	Raised	Entire	
6	St 6	Medium	Round	White	Raised	Entire	

4.7 Preservation of Purified Strain

Glycerol stock of 100ml was prepared for the preservation of purified strain. 50% of glycerol was prepared by dissolving 50ml of glycerol and 50ml of distilled water. it was autoclaved at 121°C for 15-20 minutes. 2.5ml Eppendorf tubes were taken and autoclaved at 121% for 15 minutes. The Eppendorf tubes were numbered in the laminar flow hood. 1ml of glycerol solution was filled in these Eppendorf tubes with the help of 1000µl pipette. Suspension was made with loop full of bacteria picked from each differential media and added into Eppendorf tubes containing glycerol stock. Eppendorf tubes with bacteria and glycerol were kept at -4°C.

4.8 Biochemical Characterization

- Different types of biochemical tests were performed for the biochemical characterization of two prevalent selected strains [70].
- 20 strains were selected for biochemical test and 7 strains were selected as they have probiotic properties.

4.8.1 Urease Test

This test is basically use for the utilization of urea by the bacterial samples. For this test, the Urea Agar Base [UAB] was weighed 2.5g. Then added it in the conical flask with 100ml of distilled water in it. After proper mixing, the conical flask was properly covered and prevented from the contamination; it was autoclaved for 15 to 20 minutes at 121°C for the best results. In this the urea was very reactive with the other thing such a in the environment their was a dust particals which have microorganism that may be lead to contamination. The media was poured into the 7 test tubes. The plates were stored in the refrigerator for future use for one day. Streaking of isolated cultures was done on the test tube containing Urea Agar Base . The plates were incubated in the incubator at 37°C for 48-72 hours.

The bacterial strains with pink color are urease positive and other that don't turn the color into pink are urease negative.

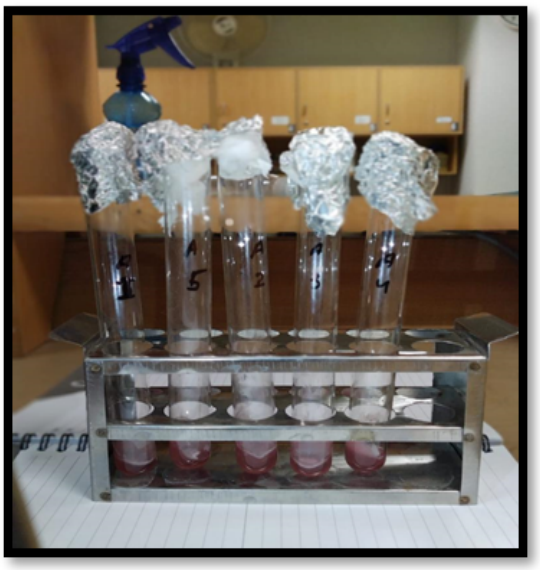


FIGURE 4.3: Urease test.

TABLE 4.2: Results of urease test

S#	Strains	Result
1	St 1	-ve
2	St 2	-ve
3	St 3	-ve
4	St 4	-ve
5	St 5	-ve
6	St 6	-ve
7	St 7	-ve

4.8.2 Catalase Test

The reagents that are present in the catalase test contain 3% hydrogen peroxide. A loop full of bacteria from pure culture were taken and placed on the slide. In addition, two drops of 3% hydrogen peroxide was added on the slide to check the production of hydrogen peroxide in the bacteria. All bacterial strains were tested

negative for catalase test which indicates that these strains do not have catalase enzyme.

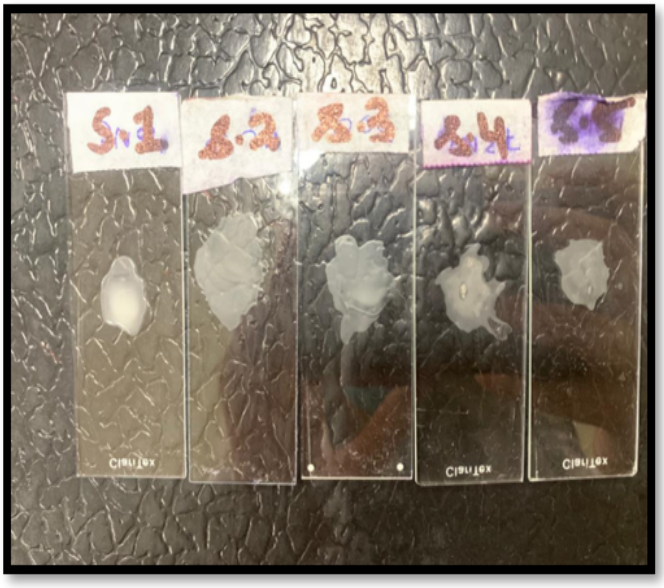


FIGURE 4.4: No bubble formation in catalase test

TABLE 4.3: Catalase test results

S#	Strains	Result
1	St 1	-ve
2	St 2	-ve
3	St 3	-ve
4	St 4	-ve
5	St 5	-ve
6	St 6	-ve
7	St 7	-ve

4.8.3 Calcium Carbonate Test

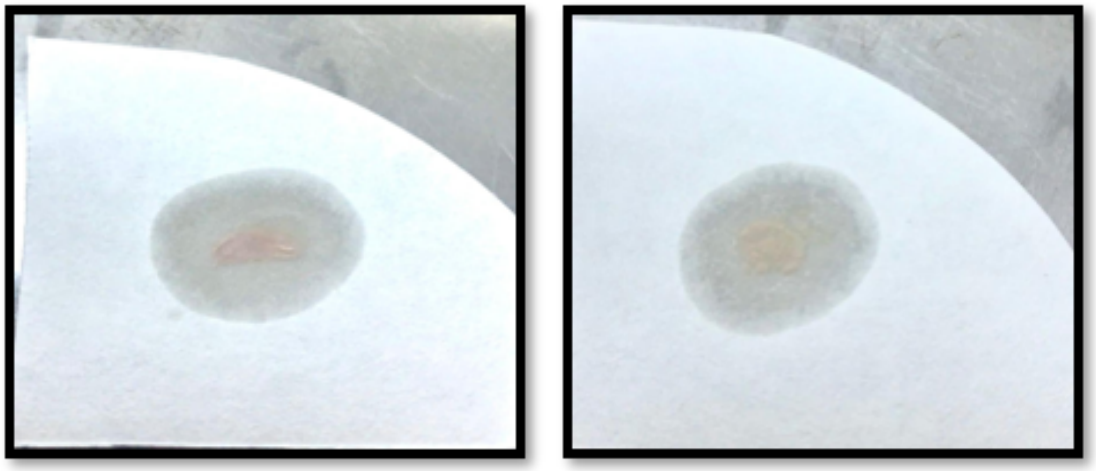
Calcium carbonate test was perform to analyze production of lactic acid. For this test MRS agar supplemented with 1% calcium carbonate was poured onto petri plates and purified colonies were spread onto media using sterile loop. Plates were incubated at 32°C for 2-4 days. St1 st2 and st7 show clear zones where a st3, st4,

st5 and st.6 shows did not make any clear zone which indicates these stains are non-lactic acid producers.

FIGURE 4.5: Calcium carbonate test results

4.8.4 Indole Test

The capability of certain bacteria to degrade the amino acid tryptophan to indole, which aggregates in the medium, is demonstrated in this test.



(a) (b)

FIGURE 4.6: (a) Indole negative spot test. (b) Indole positive test.

St 1 till 3 and st 5 till st 7 were tested positive for indole test and st 4 was tested positive for indole test.

TABLE 4.4: Indole test results

S#	Strains	Result
1	St 1	+ve
2	St 2	+ve
3	St 3	+ve
4	St 4	-ve
5	St 5	+ve
6	St 6	+ve
7	St 7	+ve

4.8.5 Inoculation of Pure Culture in MRS Broth

In 1000 mL distilled water, dissolve 55.15 g of broth. If required, heat the medium to completely dissolve it. As needed, distribute in tubes, bottles, or flasks.

4.9 SDS Page Result

SDS page was done to to estimate surface protein in bacterial sample.four samples were used for SDS PAGE method.there are series of light anf dark bands has been observed on gel which indicates minimum and maximum amount of proteins of different size in four of bacterial sample respectively.results indicates persence of proteosurfacesomes in probiotic bacterial strains. There was molecular protien ladder in the first lane ranges fron ∼245-∼11.this marker or ladder was used to estimate protein size of sample in the form of band. Sample 1 was run in lane 2 of gel. Different types of dark and light bands were observed. Dark band were Labelled as slp A, slp B,slp C and slp D and so on. SDS PAGE gives series of light and dark bands.

- Dark bands of size 100kda, 74kda, 63kda, 44kda, 33kda, 20kda in first sample,

- 100kda, 74kda, 61kda, 62kda, 42kda, 33kda, 20kda, in second sample,
- 100kda,74kda, 61kda, 44kda, 33kda, 20kda,
- In third sample and 100kda,74kda, 20kda, 33kda, 63kda in fourth sample.
- These dark bands indicate that protein of these sizes is present in excessive amount.

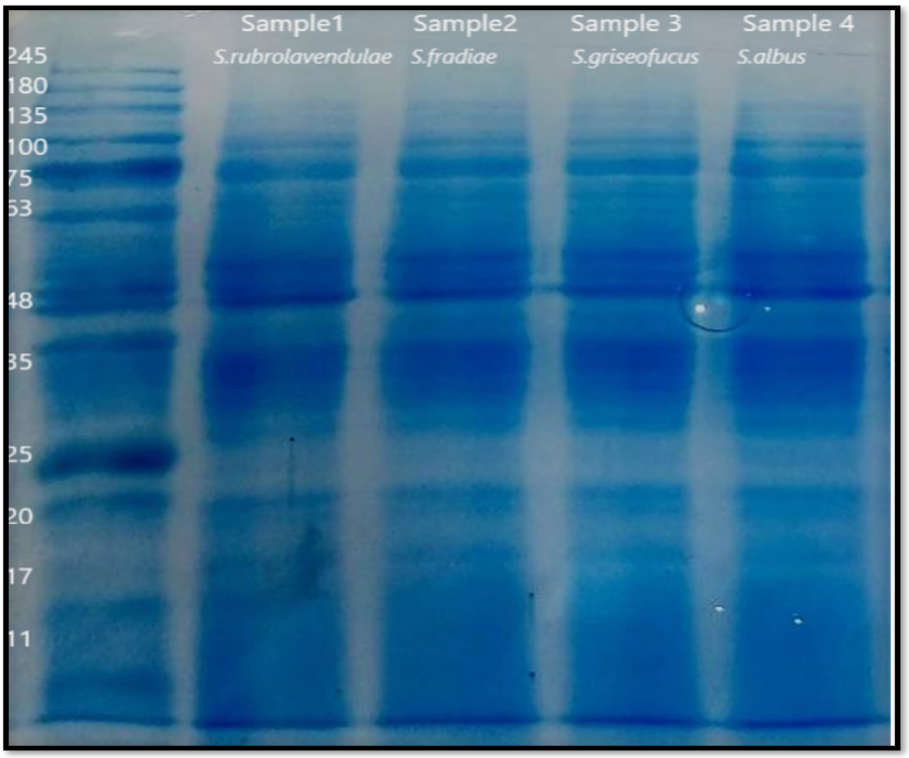


FIGURE 4.7: The bands are shown on the gel surface protein was extracted by rupturing bacterial strains using 50 microliter of SDS by using vortex and than centrifugation reults in supernatant rich in surface layer protein.

4.10 Identification of Strains

16srRNA analysis showed following identities of strains. .

4.10.1 Strain Result

1. The different strain are identify such as the St 1 *L. plantarum* (HY7715)

2. St 2*L. helveticus* (HY7801)

3. St 3*S. rubrolavendulae* M56

4. St 4 *Streptomyces fradiae*

5. St 5*Streptomyces griseofucus* B15

6. St 6*Streptomyces albus*

7. St 7*B. lactis*(HY8002)

8. *S. rubrolavendulae, S. fradiae, S. griseofucus and S. albus* were selected for fermentation of fruits for comparison of fermented vs unfermented food metabolite profiling.

4.11 HPLC

4.11.1 Metabolite Extraction and Estimation

Standardization by HPLC Every 2 hours, aliquots of the fermentation fluid were sampled to assess metabolite concentrations. Thermally processed samples were heated to 95°C for 20 minutes before being stored at 18°C. HPLC chromatograph (Agilent Technologies 1200 Series) with UV-VIS detector and HPLC column Acclaim OA 5 m, 4 X 250 mm. The mobile phase was sodium sulphate (100mM) solution (pH 2.65 adjusted with MSA) eluted at 0.6 ml/min using an isocratic elution method. The wavelength for detecting total metabolites was set at 210 nm. The peak area recorded at a certain retention time was used to calculate

metabolites.Micronutrients, phenolic compounds, carotenoids, and fibre have all acquired recognition as a result of their appeal.

4.11.2 Results and Discussion of HPLC

If probiotic bacteria create more than 80% lactic acid from glucose, they may be homofermentative. It creates twice the quantity of lactic acid and ATP for every molecule of glucose (1Mol) (2 mol each). In hetero fermentation, however, they barely create about half of the lactic acid. Equivalent amounts of lactic acid (1M), ethanol (1M), and carbon dioxide are generated for every molecule of glucose consumed (1M).

Traditional probiotic fermented foods, such as yoghurt and cheese, include both homo and heterotactic acid fermenting probiotic bacteria. *L. acidophilus, E. faecalis, B. lactis, E. faecium, Lactococcus lactis, and S. thermophilus* were the microbes accountable for these responses. However, other types of bacteria, such as *Bacillus and Saccharomyces* strains that do not create lactic acid, are also considered probiotics.

- In comparison to dairy probiotics, fruits, and raw vegetables, fruits and vegetable extracts (fermented) are regarded a potential alternate.
- Micronutrients, phenolic compounds, carotenoids, and fibre have all acquired recognition as a result of their appeal.
- Hydrolysis, biochemical metabolism, and microbial activity produce metabolic products in fermented fruits.

For technical, nutritional, and microbiological reasons, quantitative identification of these products is critical in fermented goods. Only substances that are dissolved in solvents. HPLC isolates compounds that are dispersed in a liquid sample. The HPLC technology was employed for metabolite extraction due to its simplicity and speed of analysis. The metabolic characteristics of fermented fruit extracts and

vegetable extracts. Metabolite profile of non-fermented extracts was not enriched at all as shown in table below 4.5 4.6, 4.7, 4.8.

TABLE 4.5: Relative presence of in *S. rubrolavendulae*the fermented and non-fermented fruit extracts

Sr.No	Compounds	Retention Time	Control	***S. rubrolavendulae***
1	Amino Acid	17.37	2.35 ± 0.02	66.17 ± 8.06
2	Fatty Acid	371	8.64 ± 0.10	3.781 ± 1.278
3	Organic Acid	8.12	2.14 ±0.02	0.124 ±0.008
4	Sugar	26.34	520.5 ± 22.6	663.881 ± 8.851
5	Sugar Alcohol	29.22	13.09 ± 0.60	1.057 ± 0.055

TABLE 4.6: Relative presence of in *S. fradiae*the fermented and non-fermented fruit extracts

Sr.No	Compounds	Retention Time	Control	***S. fradiae***
1	Amino Acid	17.37	2.35 ± 0.02	14.96±1.77
2	Fatty Acid	371	8.64 ± 0.10	3.80± 0.02
3	Organic Acid	8.12	2.14 ±0.02	0.33 ±0.12
4	Sugar	26.34	520.5 ± 22.6	550.4±28.5
5	Sugar Alcohol	29.22	13.09 ± 0.60	1.21 ± 0.04

TABLE 4.7: Relative presence of in *S. griseofucus*the fermented and non-fermented fruit extracts

Sr.No	Compounds	Retention Time	Control	***S. griseofucus***
1	Amino Acid	17.37	2.35 ± 0.02	87.35± 2.74
2	Fatty Acid	371	8.64 ± 0.10	3.84 ± 0.43
3	Organic Acid	8.12	2.14 ±0.02	0.44 ± 0.01
4	Sugar	26.34	520.5 ± 22.6	712.8±9.70

Amino acid detected in HPLC was BCAAs (Isoleucine, Leucine, Valine) Histidin, Lysine, Methionine, Phenylalanine, Threonine and Tryptophan. Fatty acids detected in HPLC stearic acid and lauric acid. Organic acids detected in HPLC was lactic acid, citric acid and gluconic acid. Sugars includes fructose, glucose and lactose. Sugar alcohols were lactitol, sorbitol and maltitol. production in four of selected bacterial strain were enough as compared to unfermented fruit juice.

TABLE 4.8: Relative presence of in *S. albus*the fermented and non-fermented fruit extracts

Sr.No	Compounds	Retention Time	Control	***S. albus***
1	Amino Acid	17.37	2.35 ± 0.02	91.20 ± 6.13
2	Fatty Acid	371	8.64 ± 0.10	4.37±1.14
3	Organic Acid	8.12	2.14 ±0.02	0.12 ±0.01
4	Sugar Alcohol	29.22	13.09 ± 0.60	1.84± 0.53

4.12 Discussion

The term probiotic bacteria refers to those bacteria which play an essential part in our body, like helping in digestion. These bacteria play an extremely important part in our body as they are responsible for fighting off pathogenic bacteria that enter the gut. This creates a barrier which protects the elementary canal from infections and keeps it healthy. These bacteria not only fight off living pathogens, but also assist in fighting against non- living pathogens by detoxification; they break down toxins into simpler non-harmful substances [60]. This protects our body from toxins and bacteria keeping us healthy. It is not enough to say that these bacteria "help" our digestive as that would greatly downplay their importance. These bacteria modulate our own immune system, activating the gut defense system, fighting against infectious diseases.

1. These bacteria do not harm our body in anyway and are completely comfortable in our digestive track;
2. They are able to pass through the elementary canal, as they are resistant to gastric acid and bile acid,
3. Are able to colonize the intestinal epithelial without damaging it,
4. For they able to adhere to the epithelial cells and are able to survive only on the nutrients present in a healthy human's die.

These bacteria are completely non-pathogenic and non-carcinogenic. The importance of these probiotic bacteria cannot be emphasized enough as they are a

crucial part of our digestive track. These bacteria have to be attained via external means and need to be replenished after certain things such as taking antibacterial medicine. Dairy products are known to contain vast amounts of these probiotic bacteria, unfortunately not all people are able to eat dairy products such as lactose intolerant people. For these people searching for an alternate source of probiotic bacteria is crucial. Non-conventional sources of probiotic are considered best for those of lactose intolerant individual [66].

- Fermentation of peach and apricot was done to isolate probiotic bacteria. Serial dilution of the fermented sample has been done and concentration of probiotic bacteria has been estimated by serial dilution of fermented peach and apricot sample.
- MRS agar was used to grow bacterial colonies of serial diluted sample as it is selective which only allows the growth of probiotic bacteria. Further streaking of isolated bacterial colony gives purified bacterial strain.
- Morphological characterization has been done. All stains seem same except strain 4.

Biochemical test gives catalase negative test. Calcium carbonate test was done to check whether there bacterial strains produce lactic acid or not [67].
Test indicates that these strains did not metabolize lactic acid as they do not metabolize lactic acid they are consider as a good source of probiotics for lactose intolerant individual which was our target. Inoculation of pure strains in MRS broth has been done to get biomass of bacterial isolates and its supernatant which helps in identification of metabolites secreted by our strain [68].
Centrifugation of MRS broth inoculated with purified bacterial strain gives supernatant and bacterial culture. Afterward our target was to estimate number and size of proteins in bacterial culture via SDS PAGE and to estimate metabolite in our bacterial strain by testing supernatant via HPLC. SDS PAGE which is known as sodium dodecyl sulphate- polyacrylamide gel electrophoresis was done to estimate proteins of different sizes. It separates protein with molecular weight of

about 5 and 250kDa. Separation of surface layer protein was our target as it is important in growth and longevity of bacterial strain maintain integrity of bacterial cell and help bacterial strain to build commensal relationship with host and attach active sites of GIT and make sure to keep the GIT away from pathogenic bacteria or have antagonistic activity. HPLC which stands for high performance liquid chromatography.

This technique is used in analytical chemistry and pharma industry to analyze drug product. This technique was used to analyze metabolites secreted by our bacterial strain. In SDS-PAGE protein marker was used to find out exact size of protein in sample. There were series of light and dark band has been observed. Dark band of any respective size indicates excessive amount of that specific protein in probiotic bacterial surface and light band indicates minimum amount of specific protein on surface of bacteria. SDS PAGE of probiotic bacteria gives sizes of different proteins present on surface of bacteria which indicates that sample st.1, st.2, st.3 and st.4 have proteosurfacesomes which help them in adhesion to host GIT, have antagonistic activity against pathogens, help in colonization of probiotic bacteria [70].

Chapter 5

Conclusions and Recommendations

The term Probiotic bacteria refers to those bacteria which play an essential part in our body, like helping in digestion. These bacteria play an extremely important part in our body as they are responsible for fighting off pathogenic bacteria that enter the gut. This creates a barrier which protects the elementary canal from infections and keeps it healthy. These bacteria not only fight off living pathogens, but also assist in fighting against non- living pathogens by detoxification; they break down toxins into simpler non-harmful substances. milk and dairy products are considered as best source of probiotics but not for the one with lactose intolerance and the one who have milk protein allergy. The alternative for lactose intolerant individuals is fermented fruits and vegetable. But their nutritional competency is not reported in depth so far.to isolate probiotic bacteria from fruit that is apricot and peach has been done. After fermentation for 7 days. Serial dilution of sample was done to get isolated colonies of probiotic strain. MRS agar was used for this purpose as it is selective media specialized for the growth of probiotic bacteria. Bacterial colonies were subculture on MRS to get purified strain. Bacterial stain were isolated and purified from fermented sample.

The strains isolated from fermented peach and apricot are 6 and 1 respectively that have probiotic properties. Their probiotic characteristic were identified by

set of biochemical tests that is catalase test, urease test, indole test and most important test is calcium carbonate test.

There were two categories of probiotic strain. These were lactic acid producing strain and non-lactic acid producing strain. The main target of research work was to isolate the non-lactic acid producing probiotic bacteria as they do not metabolize lactic acid into lactose which was one of the most favorable source of probiotic for lactose intolerant individual. calcium carbonate test confirms that St3, St4, S5 and St6 strains were non-lactic acid producing probiotic strain and St1, St2 and St7 were lactic acid producing strain. So 4 non lactic acid producing strains have been selected for further experimentation.

There were specialized type of proteins in probiotic bacteria on the surface which were known as surface layer proteins or proteosurfacesomes. These proteins help in adhesion of bacteria on GIT and have antagonistic activity. To estimate size of these extractable surface protein SDS PAGE was done. There were four bacterial strain selected for SDS PAGE.

Lactic acid producing strains strains were also identified but main target was to isolate non lactic acid producers.bacterial strains were ruptured using 10% SDS than centrifugation results in supernatant rich in surface layer protein has also been identified but main target was to isolate non-lactic acid producers. SDS PAGE gives series of light and dark bands.

- Dark bands of size 100kda, 74kda, 63kda, 44kda, 33kda, 20kda in first sample,
- 100kda, 74kda, 61kda, 62kda, 42kda, 33kda, 20kda, in second sample,
- 100kda,74kda, 61kda, 44kda, 33kda, 20kda,
- In third sample and 100kda,74kda, 20kda, 33kda, 63kda in fourth sample.
- These dark bands indicate that protein of these sizes is present in excessive amount.

Amount of metabolites produced from fermented source and amount of metabolites from selectively fermented fruit juice was evaluated by HPLC method. Four

probiotic bacteria separated from fermented fruit were isolated and fruit juice was selectively ferment with four bacterial strains for 24 hrs was compared with non-fermented fruit juices.it was analyzed that metabolites in selectively fermented source produce enough metabolites than non-fermented one. Amino acid detected in HPLC was BCAAs (Isoleucine, Leucine, Valine) Histidin, Lysine, Methionine, Phenylalanine, Threonine and Tryptophan. Fatty acids detected in HPLC stearic acid and lauric acid. Organic acids detected in HPLC was lactic acid, citric acid and gluconic acid. Sugars includes fructose, glucose and lactose. Sugar alcohols were lactitol, sorbitol and maltitol. production in four of selected bacterial strain were enough as compared to unfermented fruit juice. Purification and identification of protein bands from SDS PAGE by metagenomics analysis and testing antagonistic activity of protein against pathogenic bacteria, Vaccine and drug targeting , novel vaccines production, NMR to find out structure of metabolites and Identification by FTIR and conformation of production materials(incoming and outgoing) are among future trends.

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An Appendix

Table 5.1

S. fradiae	***S. griseofucus***	***S. albus***
43	19	15
40	13	13
37	15	19
19	12	10
12	9	7
12	18	6
18	7	18
20	25	20
23	33	8

Table 5.2

S. fradiae	***S. griseofucus***	***S. albus***
0	1	0
0	0	0
1	0	0
0	2	0
0	0	1
0	0	2
2	1	0
0	0	0