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Metabolite Profile and Surface Proteasomes of Probiotic Bacteria from Fermented Vegetable

by

Mishal Javed Satti

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my father Raj Javed Akhtar Satti. 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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Abstract

Probiotic bacteria are those microbial stains which have beneficial effects on host gastrointestinal tract. 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 cabbage 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 gastrointestinal tract and show antagonistic activity against pathogens. SDS PAGE indicates presence of proteosurfacesomes in probiotic bacteria separated from fermented cabbage. SDS PAGE gives series of light and dark bands. SDS PAGE gives series of light and dark bands. Dark bands of size 225kda, 182kda, 170kda, 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in first sample. 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in second sample. 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in third sample, 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in forth sample. These dark bands indicate that protein of these sizes are present in excessive amount. HPLC highlighted the presence of metabolites and showed that the amino acid in *E. hirae, S. thermophiles* and *S. rubrolavendulae, S. fradiae* were ±69.186, ±14.1961, ±97.351 and ±81.289 respectfully where as in non-fermented vegetables they were ±2.052. Same case has been observed in other metabolites that are sugars and sugar alcohols and organic acids. Non lactic acid producing strain of probiotic

from fermented vegetables are equally competent as compared to lactic 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 bacteria are microorganisms or their derivatives that are crucial for the healthy development of their host. These probiotics range from acting as aquaculture disease control agents, feed additives, growth enhancers, to acting as, antimicrobial compound replacement [1]. While vast amounts of research have been conducted in the field of probiotics over the last 30 years, Metchnikoff may have come up with the original definition in the early 1900s.

Metchnikoff (1907) suggested that the use of fermented milk products could improve human health. Parker (1974) coined the term "probiotic," which he described as "organisms and substances that help in the microbial balance of the intestine." The term Probiotic is derived from the Greek words "pro" and "bios," that translates to "for life". The most important innovations developed in response to disease control concerns, according to Browdy (1998), is the utilization of probiotics. Probiotics are living microorganisms that can be employed to enhance the microbial balance and development efficiency of the host's intestinal microbiota [2].By enhancing the host's disease response, or enhancing the efficiency of the host's surrounding environment.

Because of the recent overdependence on antimicrobial drugs, the production of probiotics in aquaculture management will minimize the prophylactic usage of antimicrobial drugs, posing possible risks to people who eat them [3] . Fuller (1989) proposed the generally agreed upon concept of probiotics, which he describes as

"a live microbial feed supplement that enhances the intestinal microbial balance of the host animal." Fuller's description was a development of the original probiotic term, which was referring to protozoans creating chemicals that induce growth of other protozoans [4]. To shorten the concept of probiotics, several improvements have been suggested [5]. According to Verschuere et al. (2000), a live microbial adjunct is "a live microbial adjunct that has a beneficial impact on the host by changing the host associated or ambient microbial community, by ensuring that the host's associated or ambient microbial community is preserved, by ensuring that the host's associated or ambient microbial community is maintained, by ensuring that the host's associated or ambient microbial community is maintained, by enhancing the host's disease response, or enhancing the efficiency of the host's surrounding environment." It supports the host by aiding in the acquirement of nutrients or by altering its environment, according to [6] Nonviable microbial components function in a useful manner, according to existing probiotic applications and scientific evidence on modes of action, and this advantage is in no way limited to the gastrointestinal tract [6].

- The discovery that active manipulation of the gastrointestinal tract (GIT) can provide antibacterial action [7].
- Promote growth in immune system, give nutritive advantages,

The principle of probiotic activity stems from the understanding that active modulation of the gastrointestinal tract (GIT) can evoke antimicrobial activity, aid immune system growth, provide nutritional advantages, and support the intestinal mucosal barrier [8]. Probiotics are now commonly used in human "functional foods," which promote healthy growth and even as medicinal, prophylactics and growth enhancers for animal agriculture and health of mankind [9]. Probiotics can be helpful in a number of ways, and these can work alone or together with a single probiotic. They Inhibit pathogens through the antagonistic compound synthesis , competing for site of adhesion with pathogens, competing for nutrients, improvements in pathogen enzymatic behavior, stimulation of immune system,

and nutritional benefits such as improvement in digestion and utilization are just a few examples [10]. A probiotic microorganism needs to adhere to and colonize the GIT, reproduce quickly, develop antimicrobial substances, and be acid resistant to survive in GIT, according to common belief. These explanations, however, are deceptive. This concept is based on the premise that a probiotic need to become a long-term resident of the intestine. Although this capacity is ubiquitous in microorganisms, and much probiotics study focused on bacteria's ability to attach to the GIT, transitory bacteria have also been found to have beneficial effects [11]. Furthermore, unlike bacteria that must bind to GIT and produce antimicrobial substances, probiotics only need to have one mechanism of action. Multi-strain and multispecies probiotics have shown that supplying synergistic bacteria with complementary modes of action can improve defense [12]. Probiotic acts can be divided into three categories:

1. Probiotics may be able to stimulate the host's gastrointestinal defense system, which includes both the inherent and adaptive immune systems, and this mechanism of action may be important for the avoidance and control of chronic diseases, as well as the treatment of inflammatory response of the elementary canal or its parts. Probiotic bacteria may have a direct effect on other microorganisms, both commensal and pathogenic, and this idea is crucial for the prevention and treatment of infections, as well as the restoration of microbial balance in the elementary canal. [13]

2. Lastly, probiotic effects can be dependent on behavior involving microbial products, host products, and food ingredients, which may lead to the detoxification of food components in the elementary canal. [14]

All three modes of probiotic action are possibly related to the stomach and/or gut microbiota, according to the above hypotheses [15]. As a consequence, the reality (probiotics may affect microbial products, host products, and food ingredients, resulting in detoxification and inactivation toxins of host and food components in the elementary canal) that has obviously been dealt with is another "organ,"

the "micro biotic canal," with a greater understanding and awareness of the basic activity of the gut microbiotic [1] .The gut microbiota, once developed, is relatively stable throughout life, though it can be affected by several factors like the process of delivery , hygienic practices, and use of antibiotics. The gut microbiota, in conjunction with the epithelium and mucosal immune system, runs an immunological and nonimmunological defense system that provides both protection from pathogens and immunity to commensal bacteria and harmless antigens. In germ-free animals, the significance of commensal bacteria in the production of a well-functioning mucosal immune system was clearly expressed.

As a consequence, rheumatoid arthritis, atherosclerosis , inflammatory bowel disease , periodontal disease, and allergies have all been related to an imbalance of intestinal microbiota. Probiotics, or beneficial microbial strains for the host, are believed to be supportive to the intestinal ecosystem.

Furthermore, some probiotic strains stimulate the secretion of several antimicrobial substances by intestinal Paneth cells via cell-autonomous MyD 88- dependent toll-like receptor activation and regulate permeability changes associated with infections, stress, and inflammatory conditions. Colonization of the digestive system in marine animals such as shrimp and fish begins shortly after development and is accomplished within few hours, modifying gene expression in the elementary canal and inhibiting entrance by other bacteria introduced later in the environment.

1. This is due to increased immune system growth and maturation, as well as competitive exclusion mechanisms. Probiotic ingestion has been shown to change the microbiota's composition, assisting in the restoration of an unbalanced microbiota (due to antibiotics or other risk factors) to its normal balanced and crucial composition [17].

Antimicrobial substance production, adhesion receptors or competing for nutrients, enhancement of immune response, and inhibition of the expression of virulent genes are all mechanisms involved in this physiological process [18]. It is not clear how or by what mechanism these probiotics achieve this. Understanding the

mechanisms between probiotics and gut microbiota, as well as how the immune systems of marine animals reacts to gut microbiota in general, will be a breakthrough in identifying the molecular targets of probiotics and biomarkers of their impact and to provide more solid evidence of their benefits in the treatment of immune-mediated disorders and physiologic conditions.

Other potential probiotic mechanisms of action include inhibitory activity against pathogen via the producers of bacteriocin-like compounds, competing for adhesion locations and nutrients, improvements in pathogen enzyme activity, immune system stimulation, and nutritive value such as better food digestion and utilization [19]. The microbes must meet a few requirements in terms of biosafety and function for it to be considered probiotic. A potential probiotic's beneficial characteristics include:

- (i) must not be harmful to the host,
- (ii) should have proper transportation to its active site and be comfortable in that area,
- (iii) they should have the ability to colonize and spread in the host, and
- (iv) they must not have any virulence genes or antibiotic resistance genes expressed [20].

Lactobacillus, Bifidobacterium, Pediococcus, Streptococcus, Carnobacterium spp., Bacillus, Cytophaga, Nitrobacter, Pseudomonas, Alteromonas, Flavobacterium, Aeromonas, Enterococcus, Nitrosomonas, Vibrio spp., and yeast are among the probiotics currently used in aquaculture. [20] Although certain probiotic bacteria are beneficial to fish, others, such as *Vibrio alginolyticus*, may be highly pathogenic, causing damage to aquaculture systems. As a consequence, before administering a probiotic, it is best to choose carefully. Lactic acid bacteria (LAB) are commonly utilized and studied for human and terrestrial animal uses, and

LAB are often found in the intestine of healthy fish [21]. The fact that they live in the human gastrointestinal (GIT) track as normal residents that are able to withstand the acidic and alkaline environment was the main focus of LAB. LAB also converts lactose to lactic acid, which lowers the pH of the gastrointestinal tract and naturally prevents the invasion of many bacteria. *Lactobacilli and bifidobacteria* are the most commonly studied and used LABs [22].

Bacillus spp., which form spores, and yeasts are two other probiotics that have been studied extensively. *Bacillus sp.* has been shown to be able to bind to surfaces, develop bacteriocins (antimicrobial peptides), and activate the immune system [23]. Usually the strains are effective as probiotics, and consumer products with such strains added to them have been shown to boost shrimp performance to levels comparable to those achieved with antimicrobials [24]. *Bacillus spp.* are particularly appealing as probiotics because they can be stored indefinitely in spore form [25]. The yeast Saccharomyces cerevisiae have been studied in great detail, with immune-stimulatory behavior and inhibitory substance development demonstrated.

Health practitioners are increasingly promoting the impact of probiotics on human health. 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.

1. Insulin resistance, type 2 diabetes (T2DM), obesity, endotoxemia , gastrointestinal track cancers, inflammatory bowel disease (IBD), nonalcoholic fatty liver disease (NAFLD), irritable bowel syndrome (IBS), and other metabolic, lifestyle, and diet-related diseases have all been connected to a disturbed gut microbial equilibrium

. There have been over 900 clinical studies and several review papers published on the positive effects of probiotics. Different probiotic strains were used in the trials, which focused on various advantages to health and different target populations [26] [27]. Since they are simple to produce, fermented dairy products are believed to be the strongest probiotic carriers. Probiotification has been applied

to all dairy products, and consumers have recognized the existence of probiotics in the dairy products they eat [28]. Dairy-based products account for roughly 43% of the functional beverage industry, with fermented products accounting for the remainder [29]. The most common functional probiotic beverages are fermented milks, and yogurt-style products. Probiotics present in dairy products have been demonstrated to be especially promising feature as a functional food, as they provide exceptional conditions for viability of probiotic bacteria [30].

When comparing other matrices to dairy matrices, the literature has discussed the protective effect dairy products have on probiotic bacteria present in the GIT, especially from milk proteins. Proteins are a source of bioactive peptide precursors, which are resistant to digestion. Milk also has a physicochemical composition that is high in protein and low in lipids, providing a protective matrix for probiotics. Probiotics have a better chance of surviving in the digestive system because of these characteristics. Proteins in milk act as a carrier matrix for probiotic bacteria, implying that they are effective in transporting probiotic bacteria to their target sites. Food inventions occur as a result of market demand or scientific and technological advancements.

A method for developing probiotic products that works should pay attention to the following [30]:

a Industry dynamics and consumer demands [31]

b The physicochemical properties of the chosen food matrix; and [32]

c The possible reaction between the probiotic strain and the matrix's food ingredients during food processing and storage [33].

A variety of protocols have been proposed and tested to reduce the gastrointestinal system 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, can be utilized for the immobilization of both enzymes and even the entire cell culture. Microencapsulation can be characterized as the bundling of solids, fluids, or vaporous

materials with thin polymeric coatings, resulting in microcapsules. The polymer acts as a protective barrier which isolates the core and protects it from the harmful effects of its poor presentation. This barrier breaks down by a specific improvement, releasing the core at its target site or at the ideal time [34].
Microencapsulation is widely used in the microencapsulation of essential oils, colorings, flavorings, carbohydrates, and microorganisms in fields such as the pharmaceutical, agricultural, restorative, and nourishment industries, among others [35].
Probiotics are live microorganisms that are typically considered safe for human consumption and prove advantageous to the host health when consumed in appropriate quantities. Gram-positive bacteria such as LAB, and propionibacteria make up the majority of probiotic microorganisms [36]. Probiotic usage may also be considered for yeasts and Gram-negative bacteria. Probiotics have potential to be a preventive measure and cure for rotavirus diarrhea, IBD, and the enhancement of intestinal comfort, Helicobacter pylori infection, and metabolic illnesses.
LAB are Gram-positive bacteria that are widely used for fermentation of a large variety of foods. They contain a large number of species of probiotic bacteria, including the following:

- *L. plantarum,*
- *L. rhamnosus,*
- *L. casei,*
- *L. helveticus,*
- *L. salivarius,*
- *L. reuteri,*
- *L. johnsonii,*
- *L acidophilus, and L. acidophil* [37].

Sometimes emergent probiotics, such as *Propionibacterium freudenreichii* strains, are utilized as a starter in the ripening of Emmentaler cheese production and

even as a vitamin providers. Not long ago propionibacteria were discovered to have a number of positive properties, including the ability to reduce colon cancer cell proliferation and inflammation. Several biological mechanisms underlying the positive effects of probiotics are being investigated.

Composition reegulation of gut microbiota, promotion of the absorption in the epithelial barrier and in protection of host from pathogens, and production of immunological responses are all part of the plan. Furthermore, Gram-positive bacteria's surface chemicals have a role in modulating the immune system of the gut first, and later the systemic immune system, by acting as a mediator between the bacteria and host, whether commensals or probiotics. Proteins, lipoproteins, lipoteichoic acids, and flagellins are examples of bacterial surface chemicals that interact with the host PRRs and modulate the immune system. Various recent investigations have highlighted the critical significance of surface-bound proteins, which are present on the cell wall but are not bonded to it covalently and are present in some probiotic bacteria but are not required. The proteins on the surface could be part of a Slp lattice, which is the outermost macromolecular monolayer. Houwink initially defined it in 1953, and it consists of a Para crystalline bidimensional array made up of a Slp, which was discovered on the surface of the *Spirillum sp* cell [38]. Chaotropic drugs like guanidine chloride and lithium chloride are used to extract Slps. Alternate proteins, either connected with the S-layer lattice or bound non-covalently to the cell wall, may be extracted by these agents. CWBDs, and SLH domains are examples of these proteins [39].

1.1 Hypothesis

Probiotic bacteria are beneficial microorganisms found in the GIT and in various fermented foods and are crucial for human health, promoting digestion and absorption of dietary nutrients, strengthening of intestinal barrier, modulation of immune responses, and producing compounds antagonistic toward pathogens. The proteosurfaceome, i.e., the complex set of proteins present on the bacterial surface, takes a very crucial part in the to-way communication between bacteria and its

host. Identification of surface layer protein, surface layer associated proteins and cytoplasmic protein from vegan source could be beneficial for diabetic individuals or hypoallergic individual.

1.2 Problem Statement

Fermented vegetable are considered to be health friendly preserved food traditionally. Identification of proteins i.e., bacteriocins present in probiotic's from vegetable could help vegans in selection of dietary supplements with strong antibacterial properties.

1.3 Aims and Objectives

1. To isolate and identify probiotic strains from fermented vegetable.
2. Estimation of strains for probiotic properties.
3. Estimation of proteosurfaceome (SLP), surface layer by SDS-PAGE.
4. Identification of strain with s-layer protein.

Chapter 2

Review of Literature

2.1 Probiotics

The word "probiotic" traces back its roots to the Greek word "o-o," which translates to "life." They were introduced in the early twentieth century, in 1900 by Elie Metchnikoff, a Russian zoologist who earned a Nobel Prize for his researched on the long lives of farmers in Bulgaria and proposed a correlation to the daily consumption of fermented milk products which had vast quantities of live non-pathogenic bacteria for example, *L bulgaricus*, alters the human intestinal flora in favor of microbial species that are beneficial to the host organism [40].

Lactic acid bacteria (LAB) are Gram-positive bacteria that do not form spore, give a negative result for the catalase test, are able to tolerate acid, and strictly fermentative bacteria that produce lactic acid as the primary end product during sugar fermentation.Probiotics have sparked widespread curiosity and relevance for various medical ailments, and millions of people eat probiotics on a regular basis for alleged health benefits. LABs that have probiotic potential has been shown to be beneficial for the health and physiology. Several studies have shown that surface-bound proteins play a part in the bacteria/host interaction , resulting in positive benefits such as immunological regulation, although the molecular [41]. *Lactococcus and Streptococcus*, for example, are homofermentative, meaning they

produce two lactates from a single cell. Others, such as *Leuconostoc, are heterofermentative* and turn glucose molecule into lactic acid carbon dioxide, lactate, and ethanol LAB are present in all probiotic bacteria, but not all LAB are probiotic. Discovered this phenomenon in their research (2011). LAB is an essential component for the gastrointestinal track to have a healthy microhabitat for the host and plays a vital role in host metabolism. Probiotics have sparked widespread curiosity and relevance for various medical ailments, and millions of people eat probiotics on a regular basis for alleged health benefits. Probiotic bacteria need to be able to function inside host's digestive tract, which includes the extremely acidic environment of the ventriculus and the extremely alkaline concentrated bile in the small bowel [42].

2.2 Probiotics as microorganism's

Before a microbe can be described as probiotic, its features, strain recognition, health benefits, and other attributes must be validated [43]. For a lengthy period, only a small count of bacteria strains were classified as probiotics on the basis of their relevant properties, which were then brought in in use as nutritional supplements and in diet. [44].

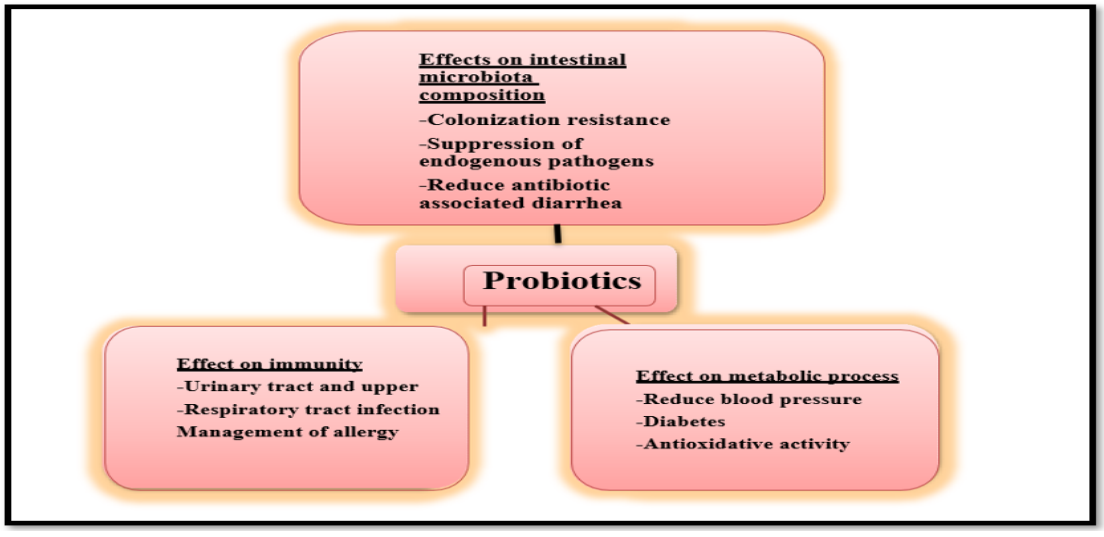


FIGURE 2.1: Health benefits of probiotics microorganisms [29].

Probiotic microorganisms are commonly available as food additives in the form of concentrates of culture in dried or frozen form for its use in industry or at home. These can be consumed as fermented or non-fermented foods, or even as supplements in one's diet in form of powders, capsules or even as tablet. These bacteria must also meet certain basic requirements set out by the European Union in order to be labeled as probiotics:

1. Detailed description and classification [45]
2. A lack of pathogenic effects (such as the development of enterotoxins and cytotoxins, enter invasiveness, pathogen adhesion, hemolysis, serological pathogenicity, and the existence of antibiotic-resistant genes) [46].
3. Strain reaching its action site, which is normally the stomach, and surviving the tension encountered during ingestion: pH levels in the stomach and gut are acidic, and biliary salts are present [47].
4. The ability to bind to the epithelium of the intestine.
5. To be able to make colonies in the colon [48].
6. There needs to the proof of its impact on host's health [49].
7. Safe to use [50].
8. Competitive antagonism against pathogenic bacteria [50].

Health practitioners are continually promoting the benefits of probiotics for human health. Probiotics have been shown to have an important impact on a variety of metabolic and immunological functions, as well as on the prevention of infectious disease in children.
Obesity, endotoxemia, insulin resistance, type 2 diabetes (T2DM), metabolic syndrome (MetS), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), nonalcoholic fatty liver disease (NAFLD), GIT cancers, and morbid obesity have all been connected to disrupted gut microbial equilibrium.

The beneficial effects of probiotics have been studied in over 900 human studies and multiple review papers. Different probiotic strains were used in the trials, which focused on various health benefits and different target populations [51].

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 [23].

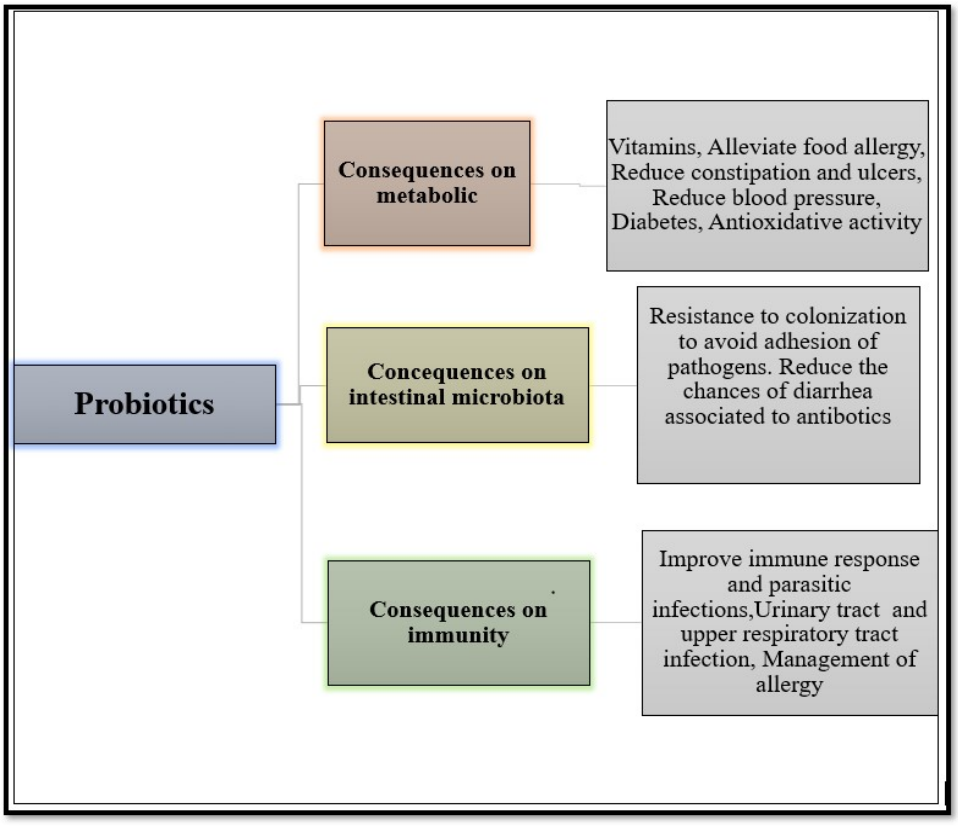


FIGURE 2.2: Major health benefits of probiotic bacteria [29].

2.4 Probiotics from Dairy Source

Fermented dairy products are believed to be the strongest probiotic carriers due to their ease of production. Probiotification has been used in all dairy industry products ranging from milk to cheeses, and consumers are aware of the existence of probiotics in the dairy products they eat. 43% of the functional beverage market consists of milk products with fermented products accounting for the majority.
Fermented milks, especially yogurt-style products, are consumed more often than any other functional probiotic beverage, Kefir in West Europe is a good example. There is great potential in milk products as features in functional foods, as the conditions provided by milk products for probiotic bacteria are great for the viability of probiotics.
Dairy matrices in contrast to other matrices, the literature has discussed the protective effect of milk on probiotics in the digestive track, especially from milk proteins. Proteins are a source of bioactive peptide precursors, which are resistant to digestion.
Milk also has a physicochemical composition that is high in protein and low in lipids, providing a protective matrix for probiotics. These characteristics help probiotics live in the digestive system under adverse conditions. Milk proteins are used as a carrier matrix for probiotic bacteria, meaning that they are effective in allowing probiotic bacteria to enter their target sites [53].

2.5 Probiotics from Non-Dairy Source

Due to rise in demand, alternate nondairy sources of probiotic delivery have received more attention in recent years. This demand is a result of a rise in the number of people with lactose intolerance (around 70% in Asia), allergies to some of the proteins present in mild, and the increase in people suffering from high cholesterol.

Vegetarianism has become common in developed countries, which has greatly increased demand for probiotics from nondairy sources. These are the main disadvantages of fermented dairy items since most probiotic dairy products are fermented foods, economic and cultural factors may have an impact on their consumption. Nondairy probiotic drinks are especially appealing [53].

The substances that cause allergies which are present in milk, have low levels of cholesterol, and are suitable for vegans . Moreover, many substrates are able to provide different antioxidant, dietary fiber, mineral, and vitamin combinations. Due to popularity of bifunctional foods, there is a willingness to broaden and include non-dairy probiotic beverage options. Between 2013 and 2018, the demand for non-dairy functional/probiotic foods is expected to expand at a 15% annual pace.

In the US, the functional food industry, is taking a much different path than in Europe, is illustrated by the fact that its functional food sector is broadly referred to as nutraceuticals, while the consumer, instead of fortified foods, is more interested in vegetarian dietary supplements. Due to the drawbacks of dairy probiotics described above, researchers are looking for new, nondairy carriers for probiotic bacteria. As the demand for nondairy probiotic products is growing, it has become apparent that the best options consist of foods such as fruits, vegetables, and cereals [31]. Fruits, vegetables, and cereals have been proven to be ideal substrates for probiotic bacteria because of their structural characteristics and composition (nutrients such as minerals, vitamins, dietary fibers, and antioxidants, as well as a decent amount of sugars). Fruit juice-based probiotics are becoming more common as a result of their delectable flavor, nutrient profiles, and the fact that they are generally accepted as nourishing and reviving foods [55].

2.6 Vegetables as a Source of Probiotics

Vegetables and fruits are one of humanity's most essential foods because not only are they nutritional but are also crucial for health maintenance. Both processed and fresh, fruits provide an excellent nutritional source of carbohydrates, minerals,

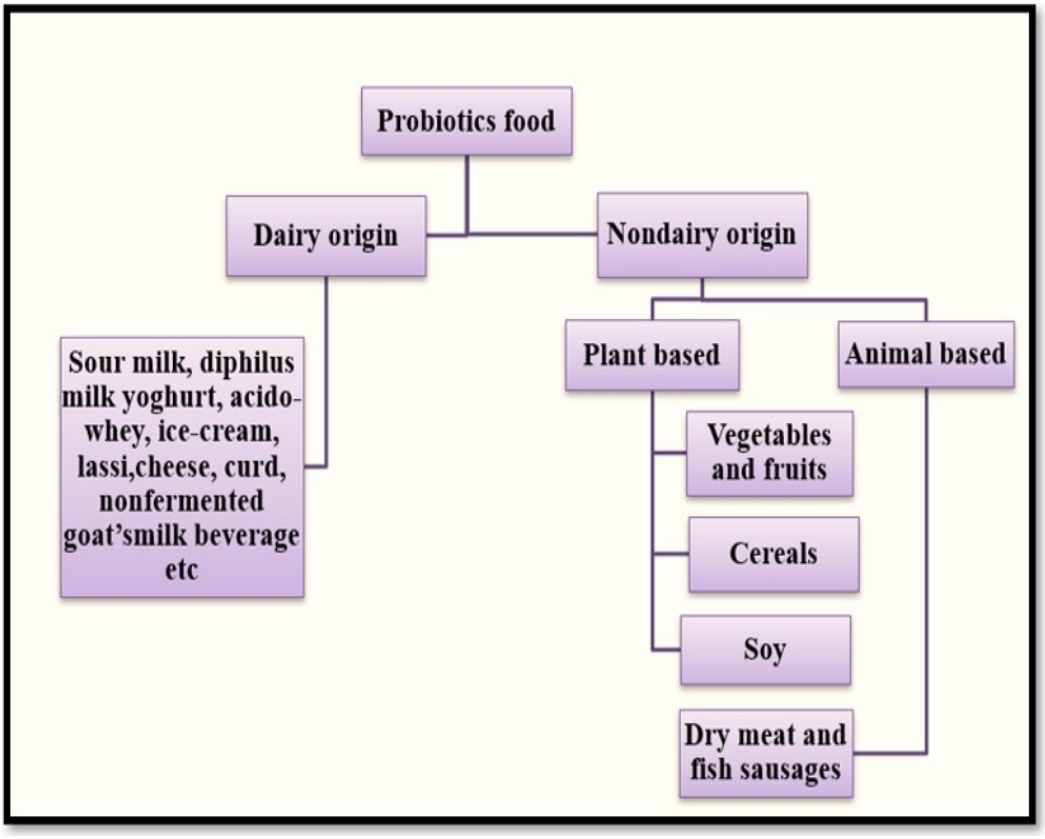


FIGURE 2.3: Types of probiotics[29].

and antioxidants, as well as improving the consistency of one's diet. Fermentation is an excellent method for the development of new products with slightly altered physicochemical and sensory properties, especially flavor and nutritional components. Fermentation of alcohol, acetic acid, and lactic acid is important for product consistency. Fermented beverages have always been around [56].

Vegetable and Fruit juices contain a high concentration of carbohydrates, minerals, and vitamins, All of which help probiotics survive storage. Vegetable and fruit juices are also a great alternative for those who choose to eat foods with low cholesterol or have lactose intolerance. pH, organic acid levels, dietary fiber, protein, total phenol, and oxygen are the main variables that effects the survivability of probiotics bacteria in vegetables, according to previous research. Vegetables and Fruit juice has been proposed as a suitable means of transporting probiotics since it has a limited residence period in the ventriculus and therefore the bacteria are not exposed to its the extremely acidic conditions [57].

Found that the protein and dietary fiber present in fruit juices were favorable for

probiotic survival during storage in vegetables and fruit juices. The cell count of L casei in the milk of a was (8.590.04logCFU/mL) and pineapple juice (8.200.01 logCFU/mL) is comparable, implying that fruits, while naturally nutrient rich and being delectable, are the best media for probiotic development . Fruits and vegetables have a lot of advantages as they contain plenty of antioxidant compounds such as anthocyanin, flavanols, epicatechins, flavanones, carotenoids, soluble and insoluble fiber, isothiocyanates, phenolic acids, and sulfides, as well as vitamins C and E, are present in fruits and vegetables, resulting in their consumption. Fruit and vegetable manufacturers have profited from the portrayal of fruits and vegetables as "safe" and "good for you." Fruit and vegetable juices are gradually being integrated with nutraceuticals and probiotic strains as a means of increasing their appeal.

When using fruits and vegetables as a non-dairy probiotic carrier, it is crucial to keep in mind that their higher polyphenolic, organic acid, or dietary fiber content can often give them sensorial unacceptability. For instance, the juice of sea buckthorn berries (Hippophae rhamnoides) contain a large amount of phenolic acid, ascorbic acid, and fatty acid content [58], giving it a very tangy taste and low palatability. In order to address this dilemma, our lab created a custom formulation for a shelf-stable probiotic-fortified sea buckthorn beverage. For the effective transformation of fruit-based matrices into physiologically functional foods it is necessary for the targeted interactions between probiotics, natural or added prebiotics, and other food components during the various unit operations of food processing to occur. 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 sea buckthorn beverage fortified by probiotics produced in the lab was observed to be successful at clearance of pathogens. Salmonella and E. coli [59].

1. A lack of pathogenic effects (such as the development of enterotoxins and cytotoxins, enter invasiveness, pathogen adhesion, hemolysis, serological, and the existence of antibiotic-resistant genes) [46].

Another trend has been to fortify probiotics and minerals with vegetable tissue [59]. Recently it was proven that tissue from pumpkins can be used to fortify iron and *L. casei*. Despite the fact that the probiotic lasted 14 days, it is yet to be decided if this product is acceptable by consumers. Fruit and vegetables, both cut and whole, such as apples and olives, have been identified too. Jaboska-ry et al. (2016) published a fascinating analysis on the fruiting body of the Lb. plantarum fermented button mushroom (*Agaricus bisporus*). Probiocation of many slightly processed fruits in cut form has been done, such as fresh-cut cantaloupe, which had undergone inoculations with riboflavin-producing Lb. plantarum B2 and L. fermentum PBCC 11.5. Shelf life of the melon pieces was 11 days. Mung bean milk was derived from cheaply cost pulses like mung bean and used as a probiotic matrix for Lb. plantarum. Since the most of fruit and vegetable matrices result in low probiotic strain viability, techniques like micro-encapsulation and spray drying are getting investigated as potential solutions. Notheless, the economic efficacy of these approaches must be carefully assessed.

Lactic acid bacteria present in fermented fruits and vegetables include *Lactobacillus plantarum, L pentosus, L brevis, L acidophilus, L fermentum, L fallax, and L mesenteroides*. Traditional fermented fruits and vegetables, as a whole, contribute to good health in addition to serving as dietary supplements. For the improvement in standard of foods and for disease prevention, it is crucial to understand the relationship between food, beneficial microorganisms, and human health. Sugar, salt, and monosodium glutamate, for example, should be used in fermented foods in compliance with agreed-upon guidelines established by target market legislation.

2.7 Challenges for Probiotic Bacteria

2.7.1 Stability and Viability

The health benefits of probiotic are largely measured by their quantity in which they are present foods and their ability to survive inside the GIT. Viability of probiotics has shown to be dependent on the strain and varies from strain to strain

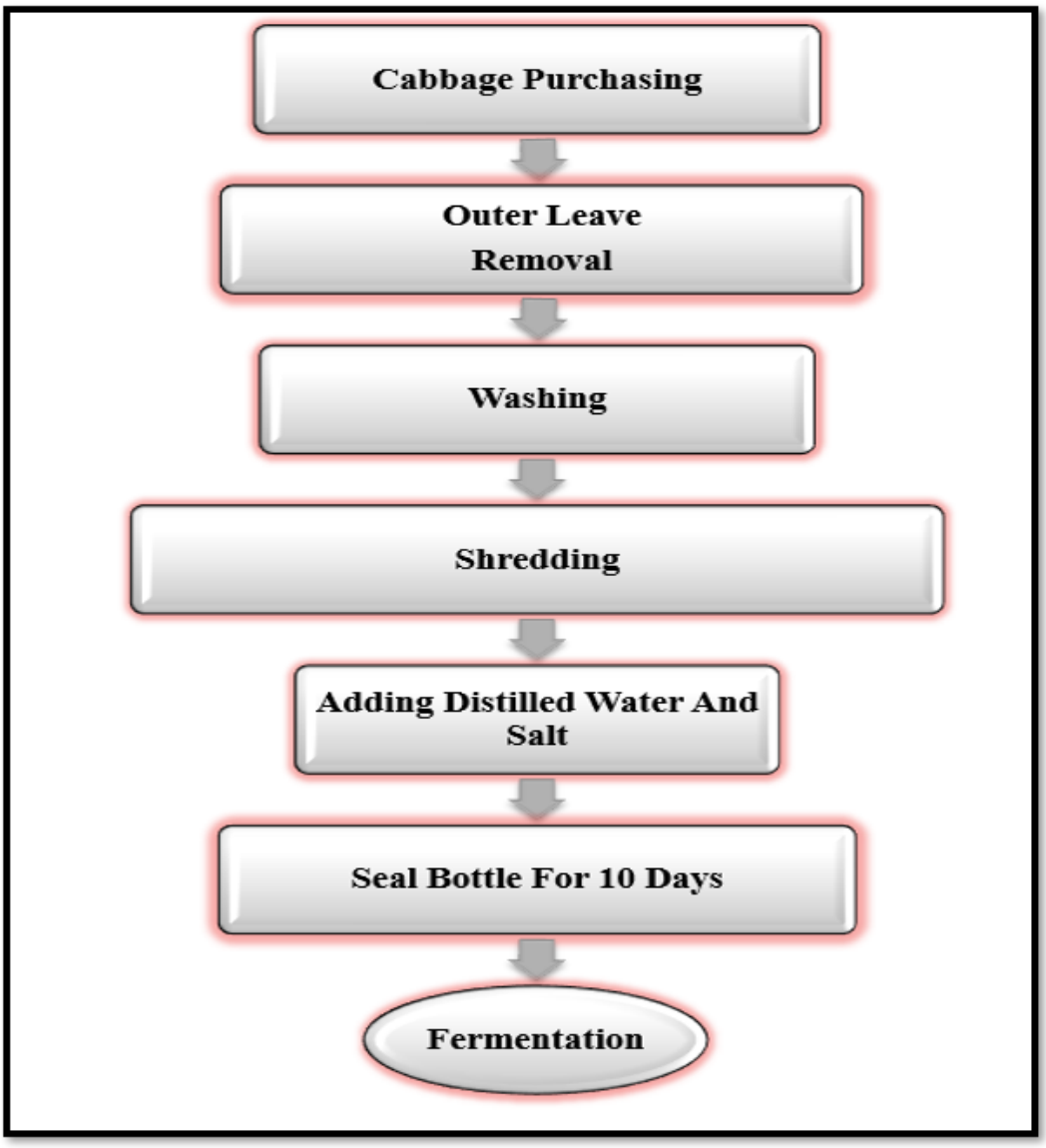


FIGURE 2.4: The process of fermentation of Cabbage [29].

[53]. At the end of storage, the number of probiotics in the final product needs to be a minimum of 106 or 107 CFU/mL, or 109 CFU per part. Vast amounts of research has been conducted on improving the stability of probiotics in storage, a significant amount of research has been done and useful data is available. Certain variables, like the conditions the probiotics are exposed to before storage, make it difficult to interpret this data, and most studies lack the necessary kinetic data. Kinetic data, together with storage temperatures and aw, will boost interlaboratory comparisons and predictions of survivability of probiotics in multiple storage conditions. Using the same line of reasoning, it is insufficient to just ensure that the cell count of viable cells reaches the minimum requirement at the end of storage (typically

106–107 cells/g), additionally it is important to minimize the loss of viability during shelf life. This would help in the prevention of overdosing on probiotic bacteria in the product at the start and lower costs of production. There are many strong substances in juices that are able to restrict survivability of probiotics in juices, while also containing essential nutrients (minerals, vitamins, dietary fibers, antioxidants) [60].

1. Food characteristics: pH, titratable acidity, molecular oxygen, water activity, salt, sugar, and chemicals such as hydrogen peroxide, bacteriocins, artificial flavoring, and coloring agents.

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

3. Microbiological parameters: probiotic strains, inoculation rate, and proportion.

One of the most significant factors influencing probiotic viability is ph. There a lot of organic acids present in juices, which is why they have a low pH. As a result, the juices could have a combination of acidic conditions and acids' inherent antimicrobial properties. Some of the major probiotics (Lactobacilli and bifidobacteria, Lactobacilli) can live in fruit juices and are immune to pH 3.7 to 4.3. Bifidobacteria have a lower acid tolerance, and a pH of about 4.6 is hazardous to their survival [61]. For cases like this, the patterns observed in some probiotics are not elaborated by pH, despite the fact that these probiotics have shown good viability in fruit juices with low pH. Bifidobacterium longum survival in model solutions and fruit juices (orange, grapefruit, blackcurrant, pineapple, pomegranate, and strawberry) was investigated by. Bifidobacteria decreased by not more than 0.8 logCFU/mL after 6 weeks of being stored in orange, grapefruit, blackcurrant, and pineapple juices, at 4 degree Celsius, and orange and pineapple juices have been observed to support the largest cell count. [61].

2.7.2 Use of Vegetables Juices as a Probiotics

Vegetable are one of humanity's most important foods and they're nutritious and have important part in health maintenance. Vegetables, 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]. Vegetables 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].

- the probiotic-fortified sea buckthorn beverage produced in our lab showed successful pathogen clearance. *Salmonella and E. coli* [28].

According to previous studies, the key parameters determining the vitality of probiotics in 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.
Pomegranate juice, tomato puree, orange, pineapple and 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 vegetable juices [2]. Several unconventional had

been intended to prevent the drawbacks of non-conventional goods while simultaneously offering pleasing flavor's and soothing characteristics.

The majority of cellulose in fruits and vegetables cannot be absorbed in the ali-

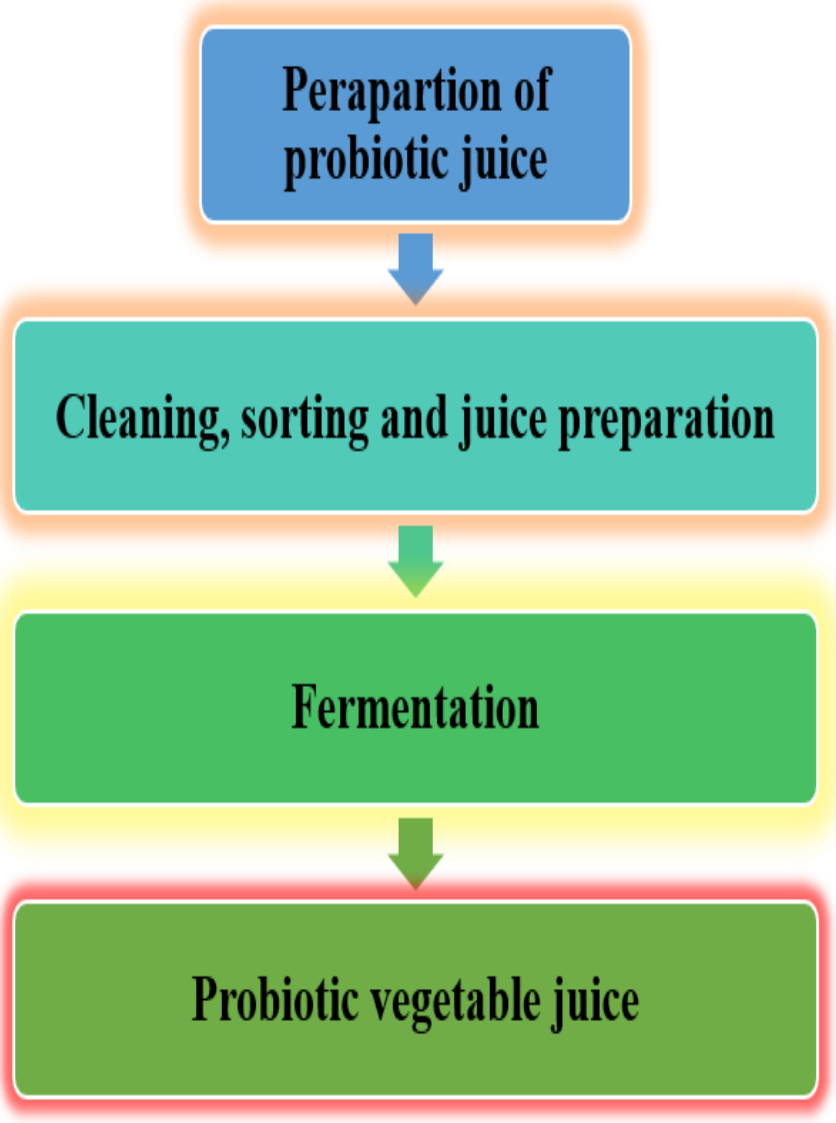


FIGURE 2.5: Preparation of probiotic juice [30].

mentary canal. 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]

2.8 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 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] [32].

2.9 Sensory Trait

Consumer approval is another major hurdle for fruit juice probiotification. Probiotification of fruit juice has been reported to produce flavors that are described as "dairy," "medicinal," "acidic," "salty," "bitter," "astringent," "artificial," or "earthy." . However, it's uncertain if all probiotic strains have the same effect on the formula's taste probiotics effect the palatability of the juices various ways depending on what kind of fruit was used, the type of probiotic organism used, the temperature at which they are stored, and prebiotic and protectant supplementation. According to some studies, probiotics had little impact on the overall acceptance of such fruit juices. Found no undesirable change in the taste of pineapple juice containing *Lactobacillus reuteri*; found that *L. casei* had no adverse effect on the flavor of fresh apple juice fermented with it; and [49] found no adverse flavor change in apple juice. Masking, or the addition volatile substances and pleasant fragrances that can "mask" the existence of probiotics, is one potential remedy for undesirable taste outcomes in probiotic juices.

- the probiotic-fortified sea buckthorn beverage produced in our lab showed successful pathogen clearance. *Salmonella and E. coli* [28].

According to, adding juices from tropical fruits like pineapple, or passion fruit (10 percent, v/v) to the final product can boost the aroma and flavor. Finally, found that the "medicinal" taste is naturally "masked" by certain fruit juices. However, the low pH, high oxygen content, and the particularity fruits possess inherently, adding probiotic cultures to fruit juices poses several technological difficulties [62]. The use of growth promoters and protectants (oligosaccharides, cellulose, and dietary fiber) for the fortification of the juices or the use of ingredients that can exert a protective effect may be a simple way to probiotic bacteria more stable in

fruit juices. *Lactobacillus rhamnosus*, for example, may be preserved during refrigerated storage by oat flour (which contains 20% -glucan). Since oligofructoses are available as substrates for these microorganisms' metabolism, the viability of probiotic cultures during processing and storage is increased by them [63]. As a result, probiotics in fruit juices may be more stable in storage. Oligofructoses often taste sweet like sucrose therefore could be useful substituents to sugar [64]. Developed oligofructose-fermented probiotic apple juice with *L paracasei*. They assessed the physicochemical properties, the viability of the probiotic, and its acceptability after refrigerated storage (4°C for 28 days) in plastic or glass packages after fermentation. Adding oligofructose had no impact on the products' physicochemical properties or storage stability, according to the findings.
Fermented beet and carrot juices with yeast auto lysate before lactic acid fermentation with *L acidophilus*; this increased *L. acidophilus* growth, decreased time required for fermentation, and made the juices rich in amino acids. Auto lysate supplementation produced vitamins, minerals, and antioxidants, as well as a beneficial impact on the survival of probiotics.

2.10 Mutagenesis

To acquire strains with modified characteristics or to analyze various microbial processes, UV light or chemical substances have been widely employed. This technique has been used to successfully increase the stability of Bifidobacterium breve and Bifidobacterium animal is in acidic products in probiotics study.
This technique is also utilized for the improvement of the product's sensorial attribute stability. For example, the metabolic activity of Bifidobacterial during food manufacturing or storage tends to be undesirable, as large quantities of acetic acid produced results in an unpalatable taste [65].

1. Homologous expression change the expression/production already existing genes present in the microbe..

2. Genes from other microbial species are added through heterologous expression.

2.11 Selective Pressure

In order to obtain resistant probiotic strains, selective pressure (stress factor) can also be utilized. Stable phenotypes and cross-resistance to other stresses are common in strains obtained using this technique (acid and temperature). With increased heat, oxygen, or acid tolerance tolerance, both Lactobacilli and Bifidobacterial were enhanced. While these stress- resistant strains can help improve stability in industrial processes, caution must be exercised because stress adaptation can change the strain's other properties [63]. According to [64], using stress-resistant strains in probiotification doesn't lead to any major improvements in regards to actions of starter cultures or the sensory properties of fermented milks [66].

2.12 Genetic Modification of the Strains

Probiotic microorganisms may be genetically modified to improve their stability and survivability. Unfortunately, this is not possible in all countries; for instance, in Europe, consumers do not embrace GMOs. Probiotics are live microorganisms that are typically considered safe for human consumption and provide advantages to the host's health when consumed in appropriate quantities. Gram-positive bacteria such as LAB, bifidobacteria, enterococci, and propionibacteria make up the majority of probiotic microorganisms. Yeasts and Gram-negative bacteria should also be considered for use in probiotics. Probiotics have potential applications in the prevention and treatment of rotavirus diarrhea, allergy and eczema, IBD, and the enhancement of intestinal comfort, lactose intolerance, Helicobacter pylori infection, and metabolic illnesses. LAB are Gram-positive bacteria that are commonly utilized in the fermentation of a broad range of foods. They contain a large

of probiotic species, including the following: *Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus salivarius, Lactobacillus reuteri* [66]. There are two fundamental methods that can be taken:

1. Homologous expression change the expression/production already existing genes present in the microbe. *Lactobacillus brevis* are included.

2. Genes from other microbial species are added through heterologous expression. They contain a large of probiotic species.

2.13 S-Layer Proteins

Surface (S) layers are structures that envelope cells, which are protein in nature. They are present in both Gram-positive bacteria, Gram- negative bacteria, and even in organisms from the Archaea domain. They usually makeup the cell's outermost layer when present, with capsules covering them only on rare occasions. S-layers are made up of many homogenous glycoprotein subunits with molecular weights in the range of 40 to 200 kDa that create a two- dimensional, regular, and highly porous array with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry. Non-covalent interactions hold the subunits together and connect them to the underlying cell surface, and they possess an inherent, inclination driven by entropy to the formation of regular shapes in a solution or on a solid support in vitro.
The subunit proteins tend to have a large number of acidic and hydrophobic amino acids, whereas the number of Sulphur-containing amino acids is very low, and have a low total expected pI value. The genes for S-layer proteins are substantially expressed.

- A single strain's genome has several S-layer protein genes, but all of these are not expressed simultaneously. Antigenic variation depending on the expression of S-layer genes, silent genes.

- The probiotic-fortified sea buckthorn beverage produced in our lab showed successful pathogen clearance. *Salmonella and E. coli* [28].

Alternative expression of S-layer protein genes in or ex vivo, sequential expression throughout growth, and, on rare occasions, superimposed Slayers or S-layers made up of two separate S-layer proteins have all been described. Due to very little similarity in the overall sequence of S-layer protein genes and the absence of any universal signature sequence, electron microscopy is nevertheless used to confirm the presence of an S-layer [68]. . Bacteriophages, bacterial or eukaryotic microbial predators, or bacteriolytic enzymes are examples of antimicrobial peptides, radiation changes in environmental pH. In marine Synechococcus strain the S-layer assists in the cells motility, and certain S-layer proteins re able to operate as degradative enzymes.

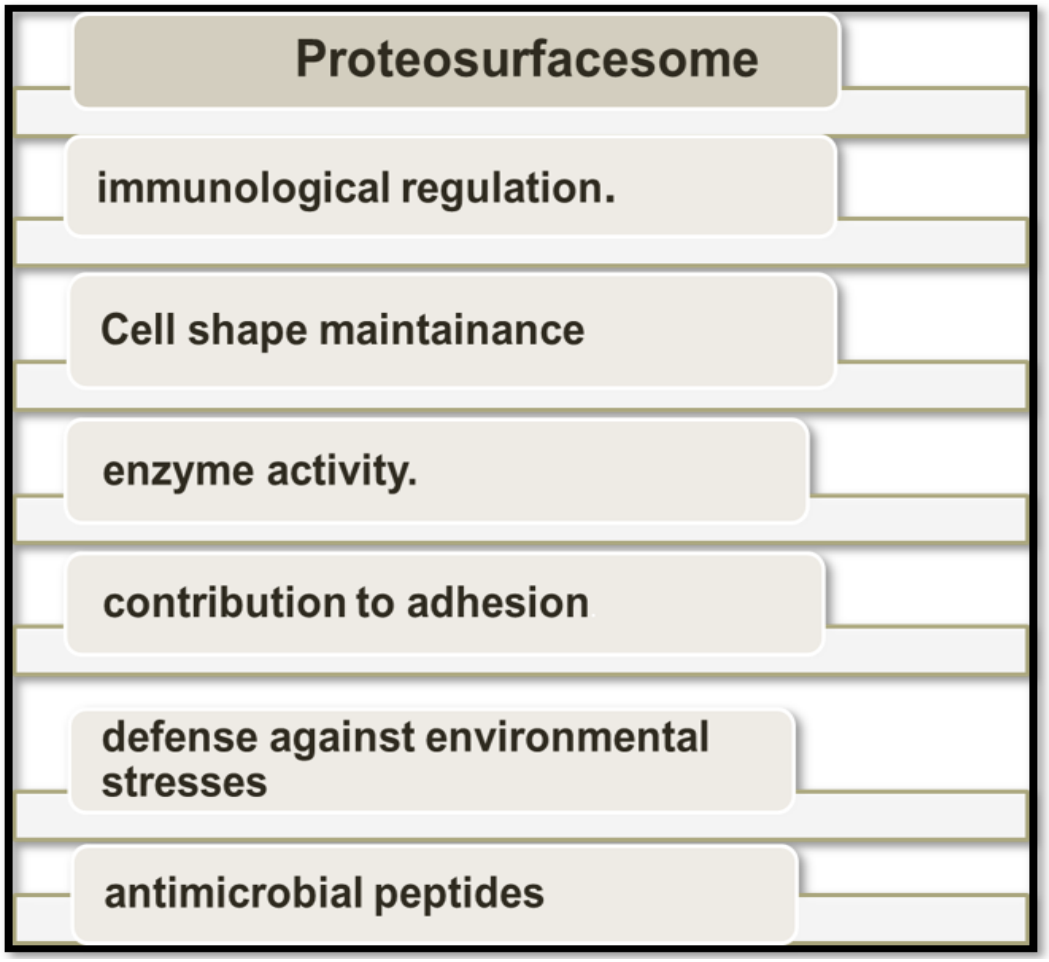


FIGURE 2.6: Importance of surface layer protein.

Information regarding the role of S-layer proteins play biologically has been collected in recent decades, although universal function for S-layer proteins has emerged. The S-layer assist in determining and maintaining of the shape of the cell, as well as functioning as a sieve on a molecular level, a binding site for big molecules, and a modulator of bacterial adhesion, have all been identified thus far. In pathogenic bacteria S-layers may contribute to severity of the disease through a variety of methods, including adhesion, coaggregation, antigenic variation, complement or phagocytosis protection, and modulation of T-cell or cytokine responses. Furthermore, it is probable that S-layer proteins shields the bacterial cell against a variety of external stimuli, including mechanical and osmotic pressures.
Bacteriophages, bacterial or eukaryotic microbial predators, or bacteriolytic enzymes are examples of antimicrobial peptides, radiation changes in environmental pH, bacteriophages, bacterial or eukaryotic microbial predators, or bacteriolytic enzymes. In marine *Synechococcus* strain the S-layer assists in the cells motility, and certain S-layer proteins re able to operate as degradative enzymes. S-layer applications can be split into two categories [69].

2.14 Occurrence, Location, and Structure

To investigate the ultrastructure and location in several bacteria and Archaea of S-layer electron microscopy of thin-sectioned, freeze-etched, freeze-dried and shadowed, negatively stained or frozen hydrated preparations are used. AFM (atomic force microscopy) has lately become a popular tool for analyzing S-layer lattices. In Archaea, more often than not, the S-layers are the only wall component they have after the plasma membrane.
Very few Archaea have a stiff wall layer between the S-layer and cytoplasmic membrane (for example, pseudomurein in methanogenic Archaea). S-layers cling to the hard peptidoglycan-containing layer in Gram- positive bacteria, but to the lipopolysaccharide of the outer membrane in Gram-negative bacteria. Radiation changes in environmental [68]. Freeze-etching of whole cells is the most important preparation technique in electron microscopy for the identification of S-layers on

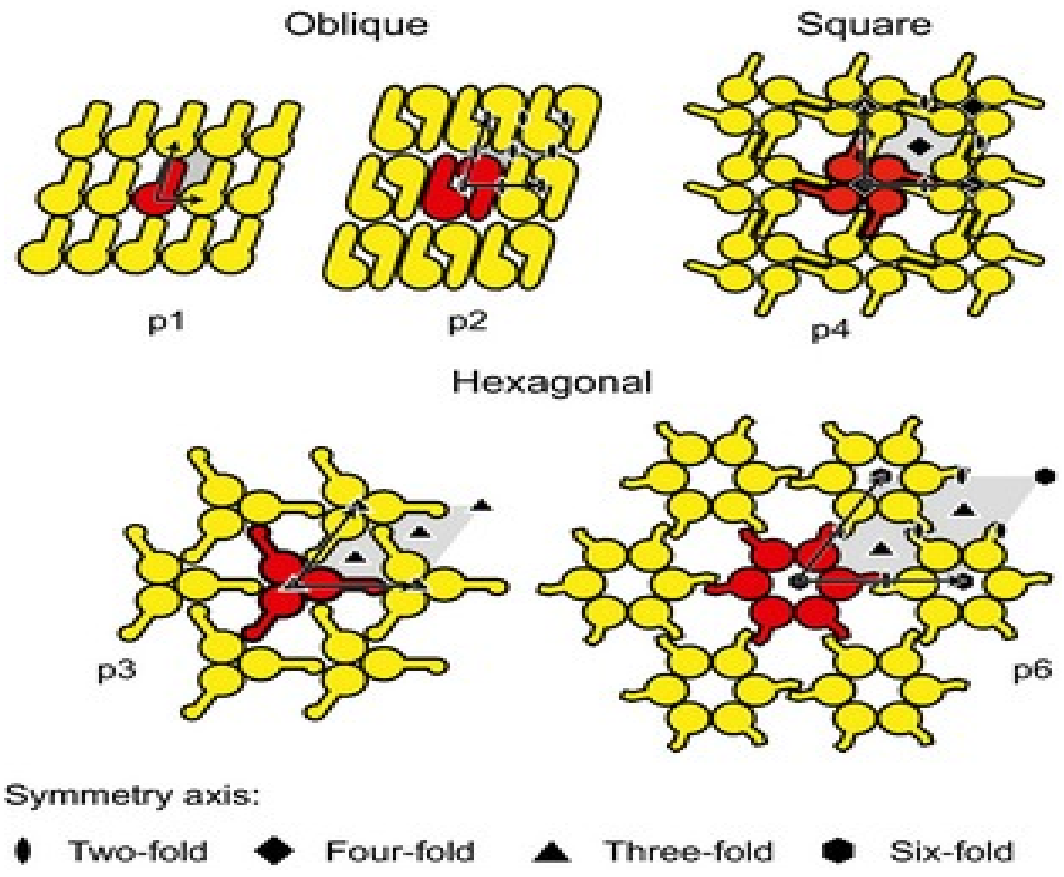


FIGURE 2.7: Structure of S-layer [29].

a specific organism. S-layers completely encompass the cell during all stages of cell growth and spore coats, as well as the surface of eukaryotic algae's cell walls, fungal spores, and prokaryotic organisms' gas vacuoles. The techniques used for the determination of the two-dimensional spatial arrangement of S-layer include electron crystallography, scanning probe microscopy, and X-ray and neutron scattering.

S-layer lattices typically have oblique (p1, p2), square (p4), or hexagonal (p3, p6) space group symmetry, with morphological unit center-to-center spacing's of 4–35 nm. Archaea is characterized by hexagonal symmetry. The morphological units are made up of one, two, three, four, or six monomers, depending on the type of lattice. Bacterial S-layers are typically 5–10 nm thick, while the archaeal S-layers are usually substantially thicker, with domains shaped like pillars linked to the plasma membrane [67].

2.15 Properties of *Lactobacillus* S-Layer Proteins

Many Lactobacillus species, but not all, have been found to possess S-layers.S-layer proteins from *L brevis, L buchneri, L helveticus, and L hilgardii*, as well as microorganisms from the former Lactobacillus acidophilus group, such as *Lactobacillus Acidophilus, Lactobacillus amylovorus, Lactobacillus acidophilus*, the function and biochemical properties of *L crispatus and L gallinarum* have been studied. *L amylolyticus, L gigeriorum, L kefiranofaciens, L pasteurii, and L ultunensis* all include putative genes in their genomes which code for S-layer proteins, which are either entirely or partially sequenced. Even the genes for *Lactobacillus kefir and Lactobacillus parakefir* have not been sequenced, they are known to have an S-layer. S-layers on L fermentum and *L delbrueckii* subspecies bulgaricus were previously demonstrated by electron microscopy, although the identification of the strain's species has been questioned.

- The fact that in public databases the *L.fermentum* subspecies did not show any S-layer proteins, and that the entire genome sequencing of *L. delbrueckii* subspecies bulgaricus revealed no S-layer protein gene, suggests that these species are now non-S-layer producers.
- Similarly, even though a normal layer was detected on *Lactobacillus casei* in a previous study, this species lacks an S-layer protein-encoding gene, and the isolate would now be reassigned to another species.

Furthermore, as S-layer proteins are present on the surface of *L casei, L paracasei subspecies paracasei, and L rhamnosus* has yet to be proven, *L. case* is now thought to be a non-S-layer producer.
Even though it is easy to differentiate between strains are by pulse field gel electrophoresis of chromosomal DNA, the S-layer protein SlpA of L. acidophilus NCFM in sequence is identical to SA of L. acidophilus ATCC 4356. Compared to other probiotic lactic acid bacteria, L. acidophilus NCFM is much more diverse

in mobile genetic components. Despite the lack of resemblance to any currently known integrative and conjugative elements, horizontal gene transfer in the acquisition of the slpA gene in L. acidophilus is impossible to rule out. A much more plausible explanation for the occurrence of an identical slp gene in two genetically distinct strains is that they have a common ancestor as the arrangement of their genome is very similar [65].

Lactobacilli S-layer proteins in comparison to other S-layer proteins are lesser in size (25–71 kDa) and have a high predicted total pI value (9.4–10.4). *Lactobacillus* S-layer proteins have oblique or hexagonal lattice symmetry when known. Only *it-Lactobacillus buchneri* has had its glycan structure defined, although *Lactobacillus* kefir has had its glycosylated S-layer proteins reported. Due to the prediction algorithms being based on structures of quite different types of proteins, Predictions of the secondary structure for S-layer proteins so far have proven to be fruitless. The amino acid sequences of the unprocessed versions of six *Lactobacillus* S-layer proteins were predicted to have an average of 14 percent -helices, 39 percent extended strands, and 47 percent random coils. For a few Lactobacillus species, in order to find the secondary structure physical measurements were carried out. Helixes were found to consist of 0–21% of the structures, -sheet account for 23–50%, and various structural contents, including -turns and random coils, of 37–63% were found in the S-layer proteins of *L. kefir* and *L. brevis*, according to a Fourier transform infrared spectroscopy analysis. In SlpA of L. brevis ATCC 8287, for example, the ratio of -helix, -sheet, and other structures were 0, 50, and 50%, respectively. There was a minimum of four -helical structures varied in their sizes, rather than – sheets, in the N-terminal regions. [69].

2.16 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

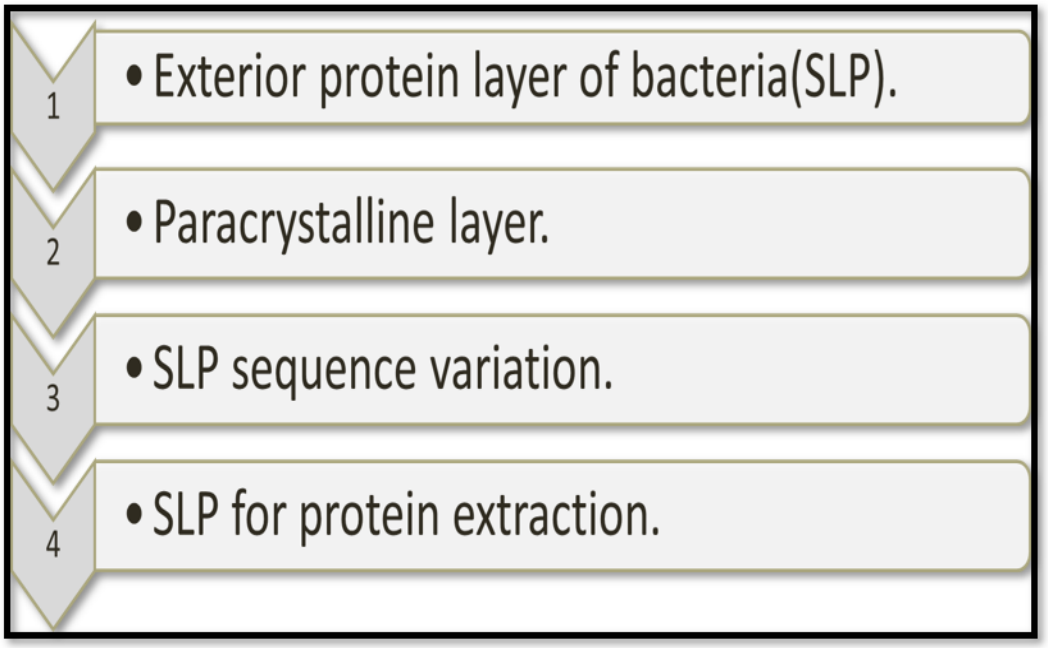


FIGURE 2.8: Properties of SLP.

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.

When it comes to encapsulation, two factors must be kept in mind: their diameter (usually around 1 and 5 meters in diameter), which immediately rules out nanotech; as well as the notion that they should be preserved. The following are the key reasons for using this approach to protect probiotics// Improving probiotic cultures' feasibility and sustainability during production, storage, and movement via the gastrointestinal tract; ensuring a regulated and effective discharge of probiotic bacteria in the GIT; and making the cultures easier to handle.

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 gastrointestinal [67]. When it comes to encapsulation, two things must be kept in mind: their size (typically

between 1 and 5m diameter), which rules out nanotechnologies right away; and the fact that they must be kept alive. 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

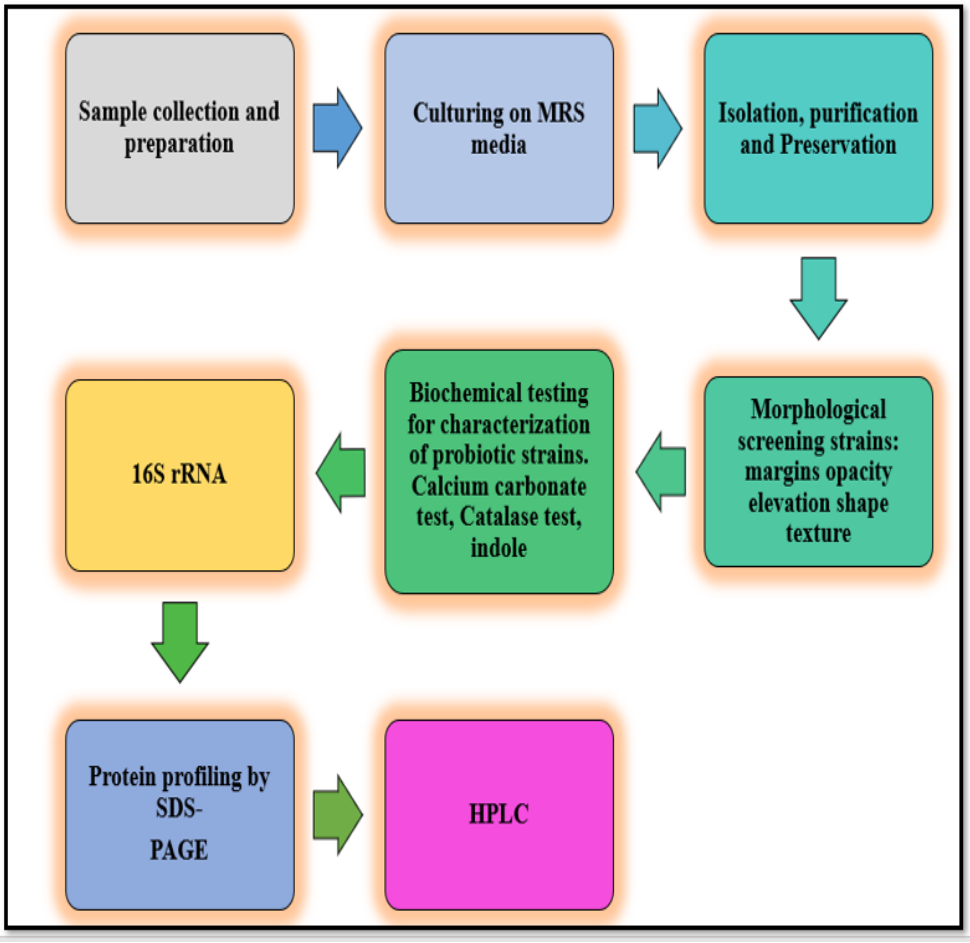
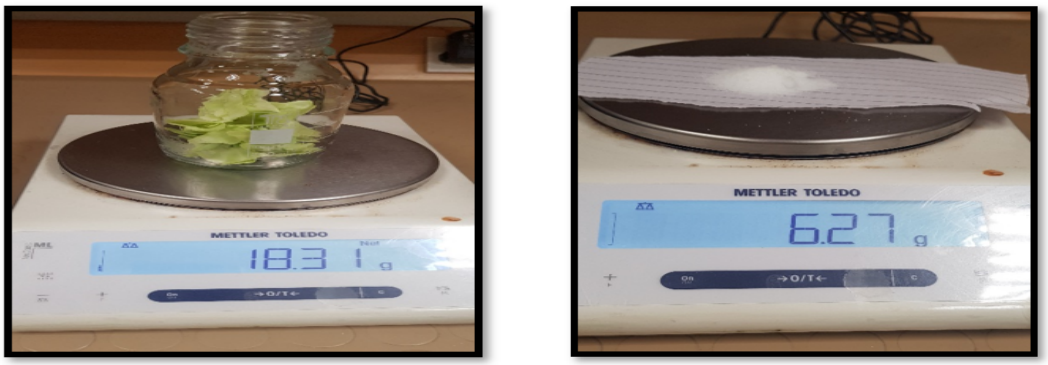


FIGURE 3.1: Methodology of Project

3.2 Preparation of Sample

Sample preparation was done by cabbage fermentation for 10 days. 6.3 g of table salt was used and brine solution of table salt was made added in 18.3 g of cabbage. The cabbage and brine solution were poured into an airtight jar and fermentation was done at 25°C for 10 days. [70].



(a) (b)

FIGURE 3.2: (a) Weight of cabbage taken for fermentation, (b) Weight of salt used in fermentation

3.3 Serial Dilution

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. [70].

3.4 Preparation of MRS 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 probiotic 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 [70].

3.5 Purification of Culture Media

The media culture was then subjected to purification. This was done by taking an inoculation loop and sterilizing it over a spirit lamp. The inoculation loop was use to pick up bacteria from the parent plate and the inoculum was gently streaked onto the purified plate of agar previously prepared with the above-mentioned method. Streaking helps form distinct colonies of bacteria by spreading. Bacteria over a larger area allowing them to form separate colonies. The purified plate were placed back into the incubation chamber to allow the bacteria to grow. This process was repeated until distinct colonies of bacteria are seen growing in the MRS media [72].

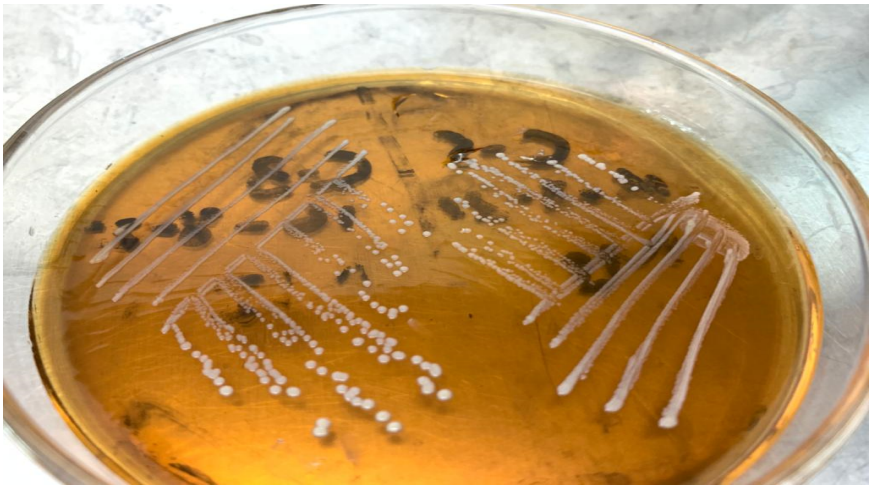


FIGURE 3.3: Agar plate with purified colonies

3.6 Spreading of Serial Diluted Sample

100 micro liter of serially diluted sample was spread on MRS media for the growth of bacterial colonies.this process was done in controlled environment[71].

3.7 Incubation

The labeled and ready petri dishes were moved from the laminar flow cabinet to an incubation chamber. Petri dishes were incubated at 37°C for 24 hours to allow bacteria to grow in their suitable environment.

3.8 Biochemical Characterization

Biochemical characterization of bacteria was performed by conducting biochemical tests on bacteria. For the identification of different bacteria species based on their biochemical differences biochemical tests was conducted [70].

- Urease test
- Catalase test
- Spot indole test
- Calcium carbonate test
- MSR broth

3.8.1 Urease Test

This test was used 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 media was poured into the six plates.
The plates were stored in the refrigerator for future use for one day. Streaking of isolated cultures was done on the plates 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 doesn't turn the color into pink is urease negative [69].

3.8.2 Catalase Test

The enzyme, Catalase is used for the decomposition of hydrogen peroxide; a byproduct of aerobic carbohydrate metabolism, into water and oxygen. If this oxidative product remains in the body of bacteria, it becomes lethal for their survival. The reagents that are present in the catalase test contain 3% hydrogen peroxide. A loop full of bacteria from pure culture was taken and placed on the slide. In addition, two drops of 3% H2O2 was added on the slide to check the production of hydrogen peroxide in the bacteria [60].

3.8.3 Spot Indole Test

Pro-Lab's Spot Indole Reagent is to be used in the qualitative method to determine the ability of an organism to split indole from the tryptophan molecule.

- A piece of filter paper was taken, and saturated with the 1% p-dimethylamino cinnamaldehyde reagent. Then a small portion of a bacterial colony was removed from the surface of the agar gel using a bacteriologic loop and then rubbed the extracted colony sample on the filter paper saturated earlier.

It was observed for a period of 1 to 3 minutes for any color change. Development of a blue color would show positive test and no color development or slightly pink color would mean a negative result. For our test we observed no change in color showing that our result was negative [50].

3.8.4 Calcium Carbonate Test

6.72g of MRS agar powder was taken and 1g of calcium carbonate was added into the agar to make 100ml of MRS agar with calcium carbonate. Media was dissolved in 70ml water and raising the volume upto 100ml. It was autoclaved at 121°C for 15 minutes. Then poured into petri dishes while under the Laminar flow cabinet.

The agar was allowed to solidify before it was inoculated with bacteria from the purified plates. Wells were make in media and bacterial strain was inoculated in these wells. If clear zones are formed bacterial strains are lactic acid producers and if clear zones are not formed they are non lactic acid producers in this case results will be negative [52].

3.8.5 MRS Broth

MRS broth is a special media for the cultivation of lactobacilli bacteria. We make MRS broth by adding 50g of powder of every 1000ml of broth we intend to make, for our purpose we need 100ml of broth so we took the necessary amount of powder. 1ml of MRS supplement is added for every 100ml of media. This mixture is left to autoclave for 15 minutes at 121 degree Celsius. We inoculate the resulting broth with our purified petri plates. The inoculated broth is left to incubate for 48 hours at 37°C inside an incubator [52].

3.9 16S rRNA Sequencing

To high throughput the earliest technique to study the microbial ecology is the use of 16S rRNA sequence that seems to be the most conserved one. It is cost effective approach in community for the survey of bacteria. In order to determine the non-lactic acid probiotic bacteria the preserved strains were send for 16S sequencing, the samples were sequenced from Microgen.

3.10 SDS PAGE

3.10.1 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°C for up to 3

months. Bottle must be covered in aluminum foil [51].

3.10.2 10% SDS

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

3.10.3 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 it at 4°C [51].

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

$$x = 1.5 \times 100 \times 121.4 \div 1000 \ = 18.2g$$

3.10.4 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 [52].

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

$$x = 3.035g$$

3.11 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].

3.12 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] .

3.13 4X Stacking Gel Buffer

Add 20ml of 0.5 M tris HCl pH. 6.8,1.6ml 10% SDS,18.4ml dis water. Stored at 4 °C [55].

3.14 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].

3.15 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].

3.16 Staining Solution

Add 227ml methanol in 227ml of distilled water. Add 46ml acetic acid and 1.3g of commissive brilliant blue [58].

3.17 Distain solution

Take 675ml of distilled water. Add 250ml of methanol and 75ml acetic acid [58].

3.18 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

FIGURE 3.4: Different chemical used in making the resolving gel

3.19 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

FIGURE 3.5: Different chemical used in making the stacking gel

3.20 Apparatus Setting

Adjust two glass plates with integrated spacer in casting frame. Than adjust the whole casting frame in casting strand. Make sure there is no leakage and to check leakage add distilled water. If distilled water didn't come out from anywhere it means there is no leakage. 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 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.

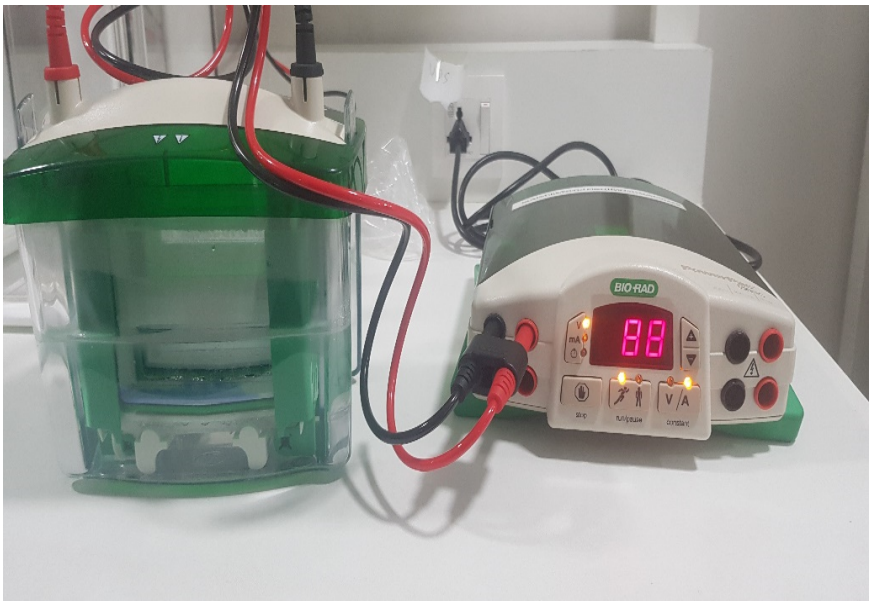


FIGURE 3.6: Apparatus of SDS PAGE.

3.22 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.

FIGURE 3.7: Apparatus of HPLC.

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. Apparatus of HPLC used is Waters e2695. [72].

Chapter 4

Results and Discussions

We started with a cabbage and had it undergo fermentation. The completion of the fermentation for marked by presence of bubbles inside the solution and the solution turning turbid after 10 days. This gave us a fermented solution which we diluted serially to make various concentrations of the solution. We then prepared MRS media as mentioned above to start growing bacteria. We added the serially diluted fermented solution into the agar under a laminar cabinet to avoid contamination, and then incubated our agar plates at 37°C for 24 hours.

Bacterial growth was seen in MRS media which proves that the bacteria present are "probiotic". These probiotic bacteria presents were purified by inoculation onto a new petri dish.

We streaked the bacteria onto the dish 4 times to give distinct colonies of bacteria. The distinct colonies were seen to be of white color 75% of the time and otherwise they were seen to be yellow. The colonies were always circular and moist with a smooth margin.

4.1 MRS Broth

We make MRS broth with the above-mentioned method and inoculate it with our bacteria from the purified petri dish. We placed our tightly sealed flask into an

incubator to let it incubate at 37 degree Celsius for 24 hours. After 24 hours bacterial growth was observed inside the broth.

4.2 Centrifugation

The resulting bacteria containing broth is poured into a falcon tubes and is placed into a centrifuge. This separated the pellet of the solution with supernatant.

4.3 Identification of Strain

16S rRNA analysis showed following identities of strains.

TABLE 4.1: Result of strains

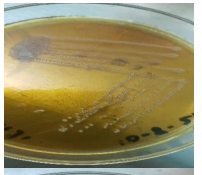
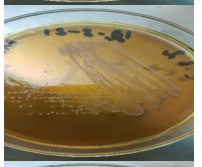
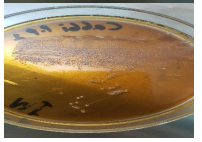
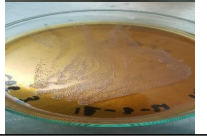
Strain	**Result**
C1	*Enterococcus hirae*
C2	*Streptococcus thermophiles*
C 3	*St. rubrolavendulae*
C 4	*Streptomyces fradiae*
C 5	*Pediococcus acidilactici*

E. hirae, S. rubrolavendulae, S. fradiae and P. acidilactici were selected for fermentation of vegetables for comparison of fermented vs unfermented food metabolite profiling .

4.4 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. As shown in table 4.2.

TABLE 4.2: Bacteria isolates on different media

S#	Strain Id	Margin	Colony Colour	Opacity	Colony Shape	Texture	Figures
1	C 1	Smooth	White	Opaque	Circular	Moist	
2	C 2	Smooth	White	Opaque	Moist	Entire	
3	C 3	Smooth	Yellow	Iridescent	Moist	Entire	
4	C 4	Entire	Milky	Translucent	Moist	Entire	
5	C 5	Entire	Milky	Translucent	Moist	Entire	

4.5 Biochemical Test

4.5.1 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. Take a filter paper and moisturize it with a drop of kovac reagent than take 24-hour pure bacterial colony and spread it on the drop. 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.

TABLE 4.3: Results Indol Test4

S#	Strains	Result
1	C1	-ve
2	C 2	-ve
3	C 3	-ve
4	C 4	-ve
5	C 5	-ve

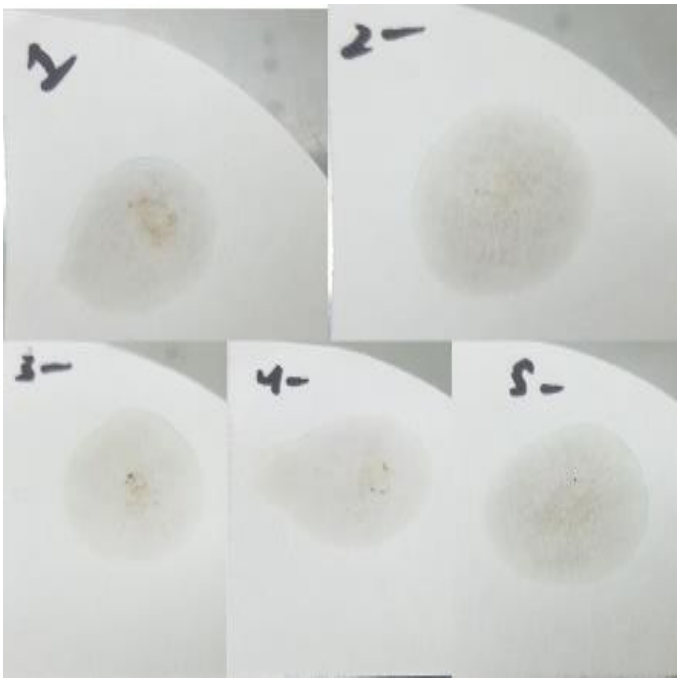


FIGURE 4.1: Result of Indol Test

4.5.2 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 for the best results. 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 [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 are urease negative.

TABLE 4.4: Results of urease test

S#	Strains	Result
1	C 1	-ve
2	C 2	-ve
3	C 3	-ve
4	C 4	-ve
5	C 5	-ve

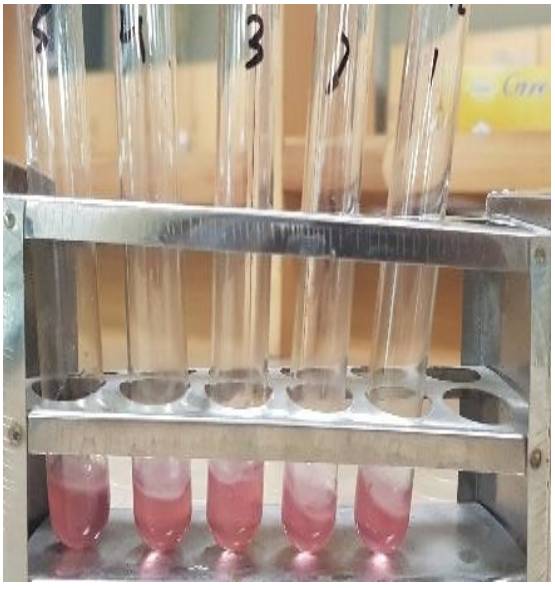


FIGURE 4.2: Urease test.

4.6 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 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% H2O2 was added on the slide to check the production of hydrogen peroxide in the bacteria.

Table 4.5: Catalase test results

S#	Strains	Result
1	C 1	-ve
2	C 2	-ve
3	C 3	-ve
4	C 4	-ve
5	C 5	-ve

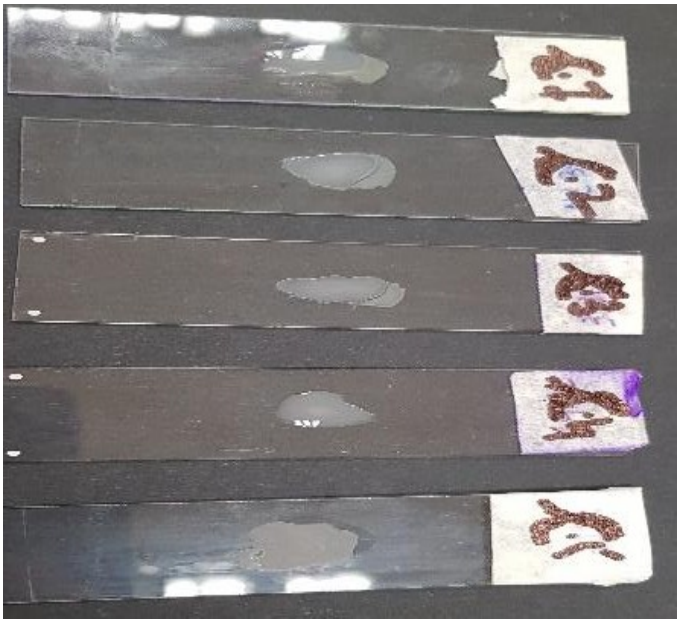


Figure 4.3: Bubble formation in catalase test

4.6.1 Calcium Carbonate Test

Calcium carbonate test is 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 are spread onto media using sterile loop. Plates were incubated at 32°C for 2-4 days. C1, C2 and C3 and C4 show clear zones where as C5 and C6 shows do not make any clear zone which indicates these stains are non-lactic acid producers.

TABLE 4.6: Calcium carbonate test results

S#	Strains	Result
1	C 1	-ve
2	C 2	-ve
3	C 3	-ve
4	C 4	-ve
5	C 5	-ve

FIGURE 4.4: Calcium carbonate test results

4.7 SDS Page

SDS PAGE is done to estimate surface protein in bacterial sample. Four samples has been used for SDS PAGE method. There are series of light and 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 presence of proteosurfacesomes in probiotic bacterial strains. There is molecular protein ladder in the first lane ranges from 245- 11.this marker or ladder has been used to estimate protein size of sample in the form of band. Sample 1 is run in lane 2 of gel. Different types of dark and light bands has been observed. Dark band are Labelled as C1, C2, C3 and C4 and so on.

SDS PAGE gives series of light and dark bands. Dark bands of size 225kda, 182kda, 170kda, 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in first sample. 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda,19kda in second sample. 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in third sample. 75kda, 62kda, 61kda, 55kda, 54kda, 51kd, 47kda, 46kda, 54kda, 50kda, 47kda, 46kda, 45kda, 44kda, 40kda, 37kda, 35kda, 33kda, 29kda, 27kda, 25kda, 23kda, 20kda, 19kda in forth sample. These dark bands indicate that protein of these sizes are present in excessive amount.

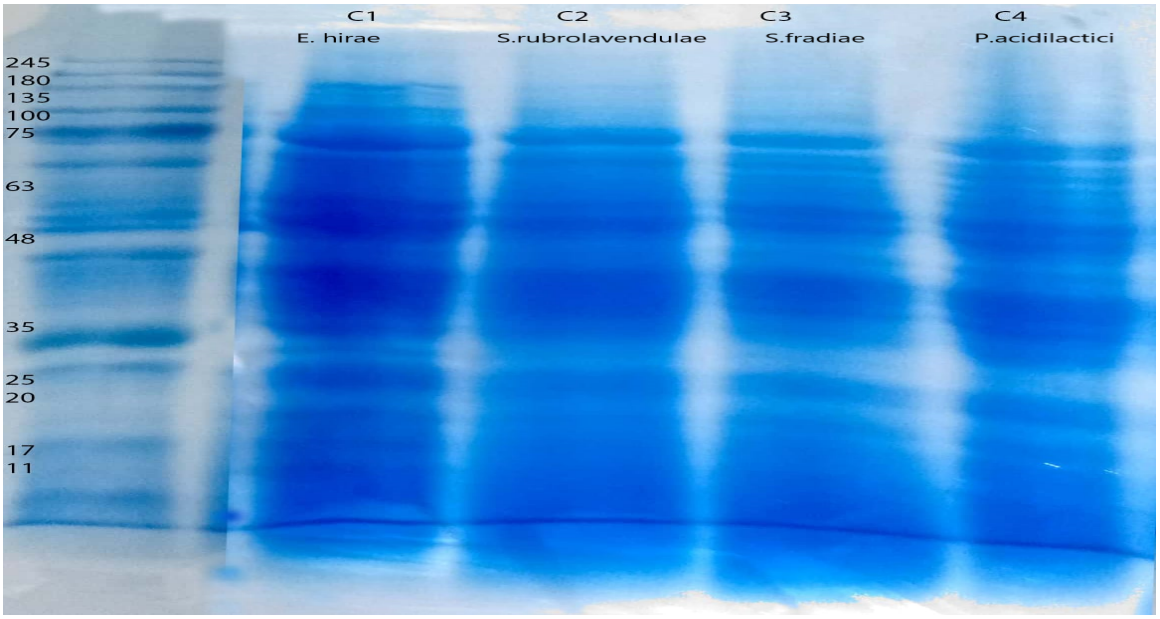


FIGURE 4.5: The bands are shown on the gel are representing S1, S2, S3, and S4 as *E. hirae, S. rubrolavendulae, S. fradiae and P. acidilactici* respesctively.

4.8 HPLC

4.8.1 Metabolite Extraction and Estimation

HPLC Characterization Aliquots of the fermentation liquid were taken every 2 h to determine concentration of metabolites. Samples were thermal treated at 95 °C for 20 minutes, and store at 18 °C. HPLC (Agilent Technologies 1200 Series) chromatograph coupled with UV-VIS detector and an HPLC column Acclaim OA 5 , 4 X 250 mm. The mobile phase was sodium sulfate (100mM) solution (pH 2.65 adjusted with MSA) using an isocratic elution with a flow rate of 0.6 ml/min. The detection of total metabolites was set at lambda =210 nm.

4.9 Results and Discussion of HPLC

Probiotic bacteria are considered to be homofermentative if they produce more than 80% lactic acid of the amount of glucose used. For every mole of glucose used it produces twice as much lactic acid with 2 moles of ATP per mole of glucose. Heterofermentative are bacteria that produce less than 50% lactic acid. For every glucose molecule equal amounts of lactic acid (1M), ethanol (1M), and CO 2 (1M) are produced. Traditional fermented food contain both homelactic and hetrolactic fermentative types of bacteria. Bacteria responsible for these reactions involve *L acidophilus, B. lactis, E. faecalis, E. faecium, L. lactis, and S. thermophilus.*

Regardless of that, there are other categories of bacteria i.e., *Bacillus and Saccharomyces strains*, which do not produce lactic acid, but are still considered probiotics. Fermented fruits and vegetable extracts are considered to have great potential as alternative sources of probiotics which would replace dairy probiotics, fruits and raw vegetables. Recently there has been a rise in popularity of fermented fruits and vegetable as sources of micronutrients, phenolic compounds, carotenoids, and fiber. Metabolic products are present in fermented fruits due to hydrolysis, biochemical metabolism, and microbial activity. Technical, nutritional, and microbial

reasons make the Quantitative determination of these products very important in fermented products. The HPLC technique was used as it is a fast and simple way of metabolite separation.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 vegetable. Metabolomics were used for the investigation of the metabolic changes that occurred in fermented fruit extract.

HPLC can rapidly discriminate the metabolic profiles of fermented fruit extracts and vegetable extracts in different isolated strains. This shows that there are unique characteristics that influence the types of metabolites produced. Metabolites like, malic acid, branched-chain amino acids, and gamma-aminobutyric acid were observed in different amounts during isolation.

10 types of amino acids, many fatty acids, metabolites and organic acids were seen in different fermented samples. Metabolite profile of non-fermented extracts was not enriched at all as shown in Table 4.7,4.8,4.9,4.10

TABLE 4.7: Relative presence of in *E. hirae*the fermented and non-fermented vegetables extracts

Sr.No	**Compounds**	**Retention Time**	**Control**	***E. hirae***
1	Amino Acid	18.70	2.052 ± 0.22	69.186 ± 0.063
2	Fatty Acid	20.1	9.447 ± 0.03	3.781 ± 0.298
3	Organic Acid	10.12	6.133 ±0.06	0.124 ±0.008
4	Sugar	20.35	720.563 ± 0.60	763.801 ± 0.851
5	Sugar Alcohol	30.22	13.099 ± 0.60	1.057 ± 0.055

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

Sr.No	**Compounds**	**Retention Time**	**Control**	***S. rubrolavendulae***
1	Amino Acid	18.70	2.052 ± 0.22	14.961±1.777
2	Fatty Acid	20.1	9.447 ± 0.03	3.805 ± 0.021
3	Organic Acid	10.12	6.133 ±0.06	0.337 ±0.120
4	Sugar	20.35	720.563 ± 0.60	870.455±0.693
5	Sugar Alcohol	30.22	13.099 ± 0.60	7.219 ± 0.004

TABLE 4.9: Relative presence of in *S. fradiae*the fermented and non-fermented vegetables extracts

Sr.No	Compounds	Retention Time	Control	***S. fradiae***
1	Amino Acid	18.70	2.052 ± 0.22	97.351± 2.770
2	Fatty Acid	20.1	9.447 ± 0.03	4.885 ± 0.443
3	Organic Acid	10.12	6.133 ±0.06	0.453 ± 0.016
4	Sugar	20.35	720.563 ± 0.60	772.924 ±0.702
5	Sugar Alcohol	30.22	13.099 ± 0.60	9.802 ± 0.377

TABLE 4.10: Relative presence of in *P. acidilactici*the fermented and non-fermented vegetables extracts

Sr.No	Compounds	Retention Time	Control	***P. acidilactici***
1	Amino Acid	18.70	2.052 ± 0.22	81.289 ± 0.131
2	Fatty Acid	20.1	9.447 ± 0.03	4.772 ±1.145
3	Organic Acid	10.12	6.133 ±0.06	0.120 ±0.017
4	Sugar	20.35	720.563 ± 0.60	921.351±0.811
5	Sugar Alcohol	30.22	13.099 ± 0.60	9.847 ± 0.536

4.10 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 [1]. This protects our body from toxins and bacteria keeping us healthy. It is not enough to say that these bacteria "help" our digestion as that would greatly downplay their importance. These bacteria modulate our own immune system, activating the gut defense system, fighting against infectious diseases. These bacteria do not harm our body in any

way and are completely comfortable in our digestive track; they are able to pass through the elementary canal, as they are resistant to gastric acid and bile acid, are able to colonize the intestinal epithelia without damaging it, for they able to adhere to the epithelial cells and are able to survive only on the nutrients present in a healthy human's diet. These bacteria are completely non-pathogenic and non-carcinogenic [2].

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. Through our research we are trying to prove that these people would be able to get the necessary probiotic bacteria from fermented green vegetable like cabbages [10]. To test for the presence of probiotic bacteria which could be eaten by lactose intolerant people or vegans we would have to isolate and identify probiotic strains from fermented vegetables, estimate their properties and identify their surface layer proteins [21].

- We started off by fermenting cabbages to use in our experiments.
- We serially diluted the fermented solution to get a series of solutions and estimate the concentration of probiotic bacteria in fermented cabbage.
- We then readied petri dishes for our diluted solutions containing bacteria.

The agar used for the petri dish was MRS agar which is important, as MRS agar is a selective which only allows the growth of Probiotic bacteria, therefore any colonies seen in the agar would be colonies of probiotic bacteria. The colonies produced are purified via inoculation so that the colonies formed are of isolated bacteria strains. The morphological characteristics of the bacteria in these colonies has shown us that two strains of probiotic bacteria are present. These strains underwent biochemical testing to understand their biochemical properties. The pure

strains underwent the CaCO3 test to check if they produce lactic acid which came put as negative showing that they do not produce lactic acid. These strains also gave a negative result for the catalase test. To get further detail on the bacteria Inoculation of pure strains into MRS broth was done to get biomass of bacterial isolates and its supernatant which helps in identification of metabolites secreted by our strain. Centrifugation of MRS broth inoculated with purified bacterial strain gives supernatant and bacterial culture [20].

Afterward our target was to estimate number and size of proteins in bacterial culture via SDS PAGE, and to estimate the metabolites present in our bacterial strain by testing supernatant via HPLC. SDS PAGE also referred to as sodium dodecyl sulphate-polyacrylamide gel electrophoresis was done to estimate the sizes of different proteins. It separates proteins with molecular weight of about 5 and 250kDa our target was the separation of surface layer proteins as they play a key role in growth, longevity of bacterial strain, maintaining integrity of the bacterial cell, helping bacterial strain to build commensal relationship with host, attaching the bacterial cell to active sites of GIT and to protect the GIT from pathogenic bacteria or have antagonistic activity [22] [27].

Results of SDS PAGE shows excessive dark bands as compared to lighter one. Band size ranges from 254 kda till 20kda dark band indicates excessive amount of specific protein of respective sizes. Whereas light band indicates minimum amount of protein of respective sizes [30].

Chapter 5

Conclusions and Recommendations

Probiotics are vital for the healthy growth and life of living many organisms which include humans. Probiotics are easily accessible in milk products and in an even more vast quantity in fermented milk products. Our goal is to see whether or not non-dairy sources mainly fermented vegetables can serve as a good source of probiotic bacteria for people who are unable to consume milk. For that we need to isolate the strains of probiotic bacteria which we did by taking fermented cabbage and reading cultures of it in MRS media to grow our probiotics bacteria. Then we needed identify the bacteria isolated. We also tested the bacteria to learn more about their physiochemical properties. We conducted CaCO3 test on the bacteria which came out negative. We also had the bacteria undergo urease which was also negative. The indole test was also done which was negative followed by the catalase test which was also negative. These tests gave us a deep insight on the properties of the probiotics. We learn from the CaCO3 test that the probiotics do not produce lactic acid, the urease test showed us that these probiotics are non-lactose-fermenting. We also tried to estimate the surface layer proteins present on the surface of the bacteria as these proteins play a crucial part in the probiotics function of these bacteria in the host's body. These effects range from antagonistic behavior towards pathogens to adhering to the GIT.

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An Appendix

Table 5.1

E. hirae	***P. acidilactici***	***S. fradiae***
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

E. hirae	***P. acidilactici***	***S. fradiae***
0	1	0
0	0	0
1	0	0
0	2	0
0	0	1
0	0	2
2	1	0
0	0	0