ISOLATION OF LACTIC ACID BACTERIA FROM VARIOUS SOURCES AND INCORPORATING INTO PLANTAIN PITH JUICE

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

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ABSTRACT

Lactic acid bacteria have been reported to be useful as a health adjunct and are commonly added to food as the mode of delivery. In this study, LAB strains were collected from various locations in and around Coimbatore and their biochemical properties were observed. Probiotics are live microorganisms which when administrated in adequate amounts confer a health benefit on the host. Fruit juices have been suggested as ideal media for probiotic growth because they inherently contain essential nutrients, good-looking and have good taste. Our objective of this study is to develop a probiotic juice of plantain pith by incorporating LAB in the juice. Powder form of the probiotic juice was prepared using freeze drying. Physiochemical properties of the juice powder were analyzed and the shelf-life of the probiotic juice powder was observed. By the result of various packaging study Aluminium foil packets were selected as a suitable package. Result of our study indicates that the probiotic juice with the combination of plantain pith juice and LAB strain could serve as a good health beneficial food product.

Key words: probiotic juice powder, plantain pith, biochemical properties.

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MRS	De Mann Ragosa Sharpe media
LAB	Lactic Acid Bacteria
RPM	Revolutions Per Minute
WHO	World Health Organisation
FAO	Food and Agricultural Organisation
gm	Grams
ml	milli litre
l	Litre
µl	Micro litre
Std	Standard
NTU	Nephalometric Turbidity units

CHAPTER 1

INTRODUCTION

Human intestinal flora contains as many as 10^{14} bacteria classified into 400-500 species, which are ten times higher than all the cells in the human body. Some bacteria of the intestinal flora such as *Clostridia, Proteus* and *Pseudomonas* can be harmful, while others like *Bifidobacteria* and *Lactobacilli* belonging to the so-called probiotic strains are favorable for the life of the host organism. The micro flora in the large intestine plays an important part in the life of the host organism (Saxelin, 1996 and Lidbeck, 1993). Its composition may change several times during the life of an individual. However, it can still be regarded as nearly constant.

Bacteria have been estimated to constitute 35-50% of the total of the human colon. The dominant genera are *Bacteroides, Bifidobacteria, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus* and *Ruminococcus*. The subdominant genera include Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus, Proteus, etc (Salminen and Tuomla, 1998). It has been found that every individual has hundreds of species of these genera with the combinations of the predominant genera being uniquely different in each individual. Research suggests that there is a symbiotic relationship between the gut flora and the host. The microbial inhabitants of the gut profoundly influence nutritional, physiologic and protective processes. The bacteria exerts several benefits to the host such as breaking down of the food remains that have not been digested earlier in the digestive system, fermentation of sugars, production of vitamin like biotin, vitamin K , mediation of immune responses and production of the host against invasions by alien microbes. Viruses and protozoa can also be a part of the gut micro flora, but these normally form only minor components of the total resident population of microorganism in healthy individuals.

Non-pathogenic and potentially pathogenic microorganism living in a state of equilibrium determined by their own ecosystem within the large intestine take part in the local immunological and metabolic processes as well as in those affecting the organism as a whole (Benno *et al.,* 1996).

In recent years the balance between harmful bacteria and beneficial ones has been distributed. Illness, dietary changes, stress, ageing, food poisoning and the use of medications can contribute to this imbalance. The indiscriminate use of antibiotics is particularly detrimental to the gut flora since they are unable to distinguish between beneficial and harmful bacteria. They can wipe out the beneficial bacteria which leaves the bowel vulnerable for invasion by potentially dangerous bacteria. This can lead to side effects such as diarrhea, inflammatory bowel disease, colon cancer and other gastrointestinal disorder (Shananhan, 2002).

One way of maintaining a balance of the gut micro flora is to consume a diet supplemented with beneficial bacteria. For thousands of years microbial cultures have been used in the 19^{th} century with the aim of preventing and curing diseases. However, it was only at beginning of this century that this practice was first put onto a scientific basis by the work of Metchnikoff at the Pasteur institute in Paris. He observed longevity in Bulgarian peasants and associated this with their elevated intake of soured milks what is now known as probiotics.

Probiotics are bacteria which when consumed in certain amounts confer health benefits beyond inherent nutrition. These probiotics works to repopulate internal environment and help support normal intestinal balance. The best known probiotics are the Lactic Acid Bacteria (LAB) especially the *Lactobacillus* sp. and *Bifidobacterium* sp. and some fungal species has also been proven to be used as probiotics. A number of potential benefits arising through the consumption of probiotics include prevention of colon cancer (Guarner and Malagelada, 2003), alleviation of lactose intolerance (Gilliland, 1990), reduction of cholesterol levels, lowering of blood pressure, improvement of immune function, increased resistance to infectious disease, particularly of the intestine and reduction in allergy. Possible health benefits include balance of pH, prevention and treatment of disease like acute diarrhea, inflammatory bowel diseases and other GI disorders.

Oral consumption of health-promoting lactic acid bacteria or probiotics has been associated with the prevention, alleviation, or cure of diverse intestinal disorders such as viral and bacterial diarrhea, adverse effects of abdominal radiotherapy, constipation, inflammatory bowel disease and food allergy (Gilliland, 1990; Hammes and Tichaczek, 1994; Salminen *et al.,*1996). Much of the early evidence on the actual health effects of probiotics was anecdotal, but during the last few years, data based on rigorous clinical studies indicating real health-

promoting properties of certain well-characterized strains has started to accumulate (Lee and Salminen, 1995).

Commercial products contain probiotic strains like *Lactobacillus, Bifidobacterium* and certain species of *Streptococcus.* Lactic acid bacteria are widely used as a probiotics to confer health benefits to the consumer. Lactic acid bacteria are known to be of practical importance. One of the most important applications of Lactic acid bacteria (LAB) is its use as probiotics. From all LAB types, *Lactobacillus* has some useful characteristics which make it useful for industrial applications (Stiles, 1996). In order to influence human health, the lactic acid bacteria must survive passage through the upper region of Gastrointestinal (GI) tract and persist in the colon. There must neither be any adverse immune response to the bacteria nor must they be affected by metabolic end product. The probiotics not should be antagonistic to mutagenic or pathogenic organism in the gut. They must be genetically stable and remain viable in the final food product.

Some limitations of the use of dairy products to deliver probiotics are the presence of the allergens and requirement of cold environments. This fact has led to the launch of new products based on non-dairy matrices. Some claims related to probiotic products are lactose intolerance and fat content. Some matrices have been used in the non-dairy probiotics products such as fruits, vegetables, legumes and cereals. Fruits and Vegetables can be considered good matrices since they contain nutrients such as minerals, vitamins, dietary fibers and antioxidants. The development of different probiotic fruit juices has been studied. (Pandey, 2000) described a revision about a variety of non-dairy probiotic beverages.

It has been suggested that fruit juices could serve as suitable media for cultivating probiotic bacteria (Mattila-Sandholm *et al.*, 2002). Fruit juices have an established market sector as functional drink through sale of calcium and vitamin-fortified juices and they are consumed regularly, which is essential if the full benefits attributed to probiotics are to be experienced (Sheehan *et al.*, 2007).

Different studies have been carried out to explore the suitability of fruit juices such as tomato, beet and cabbage juices as raw materials for the production of probiotic drinks. *L.plantarum, L. acidophilus* and *L. casei* have been employed as probiotic bacterial cultures. Results have indicated that all the strains are capable of growth in fruit juices mentioned and as a result, the microbial population increases significantly after 48 hours of fermentation. Moreover, *L. plantarum, L. acidophilus* and *L. delbruekii* have shown to be resistant to the

high acidic and low pH conditions during storage periods at 4^{o}C. However, results on *L.* casei have indicated that this strain loses its viability during cold storage (Yoon *et al*., 2004, 2005, 2006). Enrichment of the fruit juice-based medium with nutritive substances has also been studied.

Beverages of new generations are those obtained by means of a controlled fermentation process performed by selected bacterial strains derived from the human digestive tract and manifesting probiotic characteristics, primarily from the genera *Lactobacillus, Bifidobacterium* and *Enterococcus.* The probiotic species representatives include *L. acidophilus, L. johnsonii, L. gasseri, L. plantarum, L. rhamnosus, Bifidobacterium longum, B. breve, B. bifidum, B. infantis and Enterococcus faecium* (Kaur IP *et al.*, 2002). The consumption of probiotic beverages increases the lactic acid bacteria in the large intestine. Lactic acid bacteria are capable of inhibiting many pathogens, such as *Escherichia coli, Salmonella* sp. (Zamfir M *et al.,* 1999), *Staphylococcus aureus* and *enterococci* frequently present in the fermented food (Bengmark S., 2000) that cause acute diarrhea in children. In recent years, a positive effect of probiotic bacteria on the human immunological system, when used in children with innate HIV, has been indicated (Rundles-Cunningham S *et al.,* 2000).

Musa paradisiaca (Family: Musaceae), commonly known as "plantain" is a perennial herb widely distributed in the tropics. Due to the enriched food value and versatile medicinal value, it is one of the most important vegetable crops of several countries. Fruits, leaves, peels, root and stalks from plantain plants have been used orally or topically as a medicine for treating diarrhoea and dysentery in the healing of intestinal lesions in colitis antilithic inflammation, pains and snakebite, anti-ulcerogenic activity, hypoglemic effect, hypolipidemic and antioxidant actions (Santosh Kumar Singh *et al.,* 2007). A constituent hydroxyanigorufone obtained from *M. paradisiaca* showed to be a potential cancer chemo-preventive agent. The stem juice of *M. paradisiaca* is also used in traditional medicine for immediate arrest of bleeding and in wound management in Ghana, but this pharmacological activity has not been tested in controlled experiments (Weremfo *et al.,* 2011).

The most frequently manufactured beverages and preparations containing probiotic lactic acid bacteria are based on milk and its derivatives. However, such products might prove unsuitable for people with an intolerance of lactose or an allergy to milk proteins.

Fermented vegetable products, e.g. vegetables and fruit juices, are an alternative for this group of consumers.

The utilization of the white central portion of the banana stem, called plantain pith has been limited to the generation of biogas, ethanol production and paper making. Plantain stem juice has been reported to have excellent therapeutic efficacy such as dissolving preformed stones and preventing stones in urinary bladder (Prasobh and Revikumar, 2011). It has also been found to contain condensed tannins and pectin which have anti-helminthic and hypoglycemic properties, respectively. Current study was carried out to expand the utility of plantain stem, which is a low cost underutilized agricultural waste.

Lactic acid bacteria (LAB) are commonly used as starter cultures for food fermentation. Freeze drying is usually used in the preservation of LAB starters. However, this technique brings about undesirable side effects such as changes in the physical state of membrane lipids and structure of sensitive proteins and decreasing of cell viability. Consequently, some compounds such as polyols, polysaccharides, disaccharides, amino acids, proteins, vitamins and various salts have been examined for their potential role to improve the survival of LAB throughout freeze drying process. Glucose, lactose, sucrose, sorbitol, trehalose, skim milk and egg yolk have been used for cryopreservation to improve survival of certain bacterial cultures. However, soy milk which contains several nutrients including protein, fat, carbohydrate, calcium, phosphorus, riboflavin, iron, thiamine and niacin has never been studied for its protective effect on microorganisms during freeze drying (Gomathy *et al.,* 1989). Therefore, understanding the effect of these lyoprotectants and their formulations is important to develop viable freeze-dried *Lactococcus lactis* and *Lactobacillus sakei* as starter cultures. Stress adaptation of microbial cells enables the cells to survive better when they are subsequently exposed to the same stress or other types of stresses. Lactic acid bacteria have been shown to induce adaptive response after exposing to some stresses. (Suree nanasombat and Niracha Sriwong, 2007) Suree nanasombat demonstrated that following cold adaptation at 10°C, *L. lactis* showed increased resistance to freezing stress. (Suree nanasombat and Niracha Sriwong, 2007) Niracha Sriwong reported that preincubation of *Lactobacillus acidophilus* at low temperature (22°C) for 6 hours led to development of cryotolerance during freezing treatment at -80°C for 24 h. This bacterial species was reported to be protected from osmotic stress by glycine, betaine and intracellular osmolyte. This work aimed at quantifying the effect of lyoprotectants and lyoprotectants formulations on survival

of *L. lactis* and *L. sakei* after freeze drying and storage at -80°C and determining the effect of osmotic and cold adaptation on survival of these bacterial strains (Amenan *et al.,* 2009).

Freeze-drying is used to preserve products containing water or solvents. Good preservation of fragrances, flavourings and ingredients with nutritional value is crucial in the food industry. With proper packaging and storage, freeze-dried products can be stored for a very long time without any appreciable loss of quality even at room temperature. Weight reduction will be up to 92%. Owing to their extremely large internal surface area, freeze-dried products have an optimal capacity to take up water.

The principle of freeze/sublimation-drying is based on this physical fact. The ice in the product is directly converted into water vapour (without passing through the "fluid state") if the ambient partial water vapour pressure is lower than the partial pressure of the ice at its relevant temperature. The freeze-drying process is divided into two different physical process steps. Step one is the freezing of the material below it solidification temperature and step two removes the ice or solvent crystals at very low temperatures. The freezing process has a great influence on the quality of the finished product and the time that will be required to dry the material. For continuously operating freeze-drying installations with high throughputs the most familiar freezing systems will be continuous blast air freezing belts. In addition, fluidised bed systems, freezing channels or chambers will be used (Myrbo and Shane, 2004).

CHAPTER 2

LITERATURE REVIEW

In recent years, there has been an increase in awareness among consumers about alternative therapies to antibiotics. In the present era of antibiotic-resistance, pathogens and other looming microbial threats, the value of prevention of the infection and disease are recognized (Isolauri *et al*., 2001). Hence, there has been more emphasis on preventive methods for disease occurrence rather than curative measures. Currently, antibiotic therapy can result in an altered intestinal microbial balance causing several unpleasant side effects that can persist even after the cessation of the treatment. The condition of the gastrointestinal tract is essential for our well being. It boosts the body's ability to resist infection, prevents morbidity and decrease antibiotic use. Exposure of the immune cells of the intestinal tract to the right types of microbes in infancy may be important for the prevention of allergy development later in life. In addition to being a serious threat to human health, resistance to antimicrobial agents is a significant economic threat as well. These facts therefore suggest that intervention at the level of intestinal micro flora may be important to enhance and sustain human health. Therefore, people all over the world are now opting to prevent or reduce the risk of diseases as against treating diseases. With this view in mind, the World Health Organization (WHO) recommends global programs to reduce the use of antibiotics in animals, plants and fishes, for promoting livestock growth. In human medicine, WHO recommends a reduction in the intake of antibiotics and an increase in efforts to prevent the disease through immunization with existing vaccines and through the development of newer and more effective and safer approaches.

One such newer approach is the use of probiotics. Probiotics may play a very important role in helping the body protect itself from infection, especially along the colonized mucosal surfaces of the gastrointestinal tract. The evidence in the role of probiotics in maintenance of health or prevention of the disease is mounting and is supported in some cases by the blinded, placebo controlled human trials (Isolauri *et al*., 2001).

The relationship between certain foods health benefits has been investigated for many years. Development of foods that promote health and well-being is one of the key research

priorities of food industry (Klaenhammer and Kullen, 1999). This trend has favored consumption of foods enriched with physiologically active components such as prebiotics, probiotics, vitamins, minerals, dietary fibers, fish oil and plant sterol (Betoret *et al*., 2003). Probiotics are defined as live microbial feed supplement that beneficially affects the host by improving its intestinal balance (Fuller, 1989). The majority of probiotics recommended are the species of *Lactobacillus* including *L. acidophilus, L. plantarum, L. casei* and *Streptococcus lactis, etc.* (Sindhu and Khetarpaul, 2001). Probiotication is one the methods used to produce fermented functional foods. Addition of probiotics to food provides several health benefits including reduction in the level of serum cholesterol, improvement of gastrointestinal function, enhancement of immune system and reduction in risk of colon cancer (Berner and O Donnell, 1998; Saarela *et al*., 2002; McNaught and MacFie, 2001; Rafter, 2003). For health benefits, probiotic bacteria must be viable and available at a high concentration, typically 10^6 CFU/g of the product (Shah, 2001).

Currently, probiotic products are usually marketed in the form of fermented milk and yogurt. However, lactose intolerance and cholesterol content are two drawbacks related to their consumption. It has been suggested that fruit juice could serve as a good medium for cultivating probiotics (Mattila-Sandholm, *et al.,* 2001). Fruits and vegetables are healthy foods, because they are rich in anti-oxidants, vitamins, dietary fibers and minerals. Furthermore, fruits and vegetables do not contain any dairy allergens that might prevent usage by certain segments of the population (Luckow & Delahunty, 2004).

2.1. History

The history of probiotic bacteria and their beneficial properties of live microbial supplements such as fermented milks go back many centuries. The use of live bacteria for health benefits is not a new concept. They were used in the treatments of body ailments, which have been mentioned even in biblical scriptures. Similarly in Ayurveda, one of the pioneering medical sciences that back to around 2500 BC, the consumption of yogurt has scientists in the early ages as Hippocrates considered fermented milk not only as food products in terms of nutrition but also medicine.

Elie Metchnikoff is regarded as the pioneer in the development of modern day probiotics. As early as 1907 Metchnikoff stated in his book "The prolongation of life" that

through the consumption of large quantities of cultured foods such as sour milk, butter milk and yogurt containing the microorganisms termed as *Bulgarican bacillus* helped to maintain good health and long life of peasants. Metchnikoff suggested that the Lactic acid bacteria in fermented milks eliminate putrefactive bacteria from the gastrointestinal tract, thereby laying the foundation of modern day probiotics. Later, the *Bulgarican bacillus* was renamed *Lactobacillus bulgaricus* and is now called as *L. delbrueckii* sub *sp. bulgaricus*. Metchnikoff proposed that consumption of fermented milk would "seed" the intestine with harmless lactic-acid bacteria and decrease the intestinal pH and that this would suppress the growth of proteolytic bacteria (Saxelin, 1996 and Lidbeck, 1993).

Kollath first introduced the term "Probiotics" in 1953 (Hamilton *et al.,* 2003). Probiotics are microbial derived factors that stimulate the growth of other microorganisms. Later a definition of probiotics that has been widely used: "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance".

2.2. Definition

The World Health Organization defines a probiotic as "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). The bacteria that exert a beneficial effect in intestinal function and promote a good health are therefore known as probiotic bacteria.

The three main aspects of the definitions are:

- The microorganisms(bacteria) are alive,
- The bacteria are administered orally,
- The bacteria should be capable of reaching the intestine alive, in order to have an influence on the microbial balance.

There are also situations where the cell viability is not required for probiotic activities such as improved digestion of lactose, immune system modulation and anti-hypertensive effects. In these situations, health beneficial effects have been linked to non-viable cells or to cell components, enzyme activities or fermentation products. There is also debate as to whether the concept of probiotic bacteria should include dead microorganisms or bacterial fragments. In this regard, the concepts of LAB that have a capacity to interact with the host mucosa and may beneficially modulate the immune system independent of its viability.

2.3. Description of LAB

In general, *Lactobacilli* are the largest genus of the lactic acid bacteria group and over 50 species. *Lactobacilli* commonly inhabit the gastrointestinal (GI) tract, oral cavity of animals and humans.

2.3.1. Family: Lactic Acid Bacteria

The genera *Lactobacillus, Carnobacterium, Enterococcus* and *Sporolactobacillus* can then be divided into species, variants and strains. Early genus and species have different characteristics but they are generally chained cocci or rod shaped gram positive, non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of metabolism.

Lactic acid bacteria use lactose as their main source of carbon to produce energy. Lactic acid bacteria use energy to transfer lactose (main sugar of milk) through their cell membrane. The lactose is metabolized to lactic acid in some species also acetic acid, ethanol and carbon dioxide. Lactic acid bacteria that only produce lactic acid as an end product are called homo fermentative; those that also produce acetic acid, ethanol and carbon dioxide are termed hetero fermentative. The by-product of this reaction is energy that the bacteria use for growth. The end products of fermentation ultimately change taste and texture of food.

2.3.2. Genus: Lactobacillus

There are many species of *Lactobacillus* bacteria that are found in a variety of environments, from dairy products to the GI tract. They are either micro-aerophilic or anaerobic and strictly fermentative. The optimum growth occurs within 35-40 $^{\circ}$C. The optimum pH for growth is between 5.0 and 5.6 and its acid tolerance is from 0.3-1.9% titrable acidity.

Species of *Lactobacillus* that have been isolated from the GI tract are *L. acidophilus, L. fermentum, L. plantarum, L. brevis, L. leichmanii, and L. casei.* Some of the most common

Lactobacilli used for dietary intake are *L. acidophilus, L. casei, L. paracasei, L. plantarum and L. rhamnosus.*

Lactobacilli are able to live in highly acidic environment of pH 4-5 or lower. This pH is well below the pH of other lactic acid bacteria can live in and because of this; *Lactobacillus* is responsible for the final stages of fermentative in products.

2.3.3. Nutrition Supplementation

Nowadays, several strain of probiotic are known, but their utilization is restricted by the fact that an effective probiotic is supposed to proceed along the acidic pH of the stomach and is able to resist the digestion of bile and pancreatic juice and finally sticks to the surface of some cells in the intestinal wall. The lifespan of the stuck probiotic is short, ranging from a few weeks and it usually lasts for a short period of time following its regular intake. Colonizing probiotics compete with other microorganism for nutrients and appropriate binding site. Only probiotics capable of colonizing temporarily in the intestine can exert the required immunological effect (Saxelin, 1996). The *in-vitro* efficacy of the well-known probiotic strains has already been proven in clinical practice (O'Mahony *et al., 2001)*. The most widely used strains are those taken from sour dairy products of the intestinal system. The most frequently studied species include various species of *Lactobacilli, Streptococcus, Bifidobacteria* and *Saccharomyces boulardii,* but under certain conditions other strains in the intestinal microflora, such as *Escherichia coli.,* can also be used as probiotics (Floch, 2003 and Kruis *et al., 2004)*.

In order to become suitable for producing health foods, namely functional foods, individual probiotic strains need to meet certain requirements (Ouwehand and Salminen, 1999 and Saxelin *et al.,* 1996 and Salminen *et al.,* 1999).

- Maintenance of the biotic potential.
- Good taste and flavor following fermentation.
- Mild acidity in the course of storage.
- Preservation of the capability of colonizing in the course of food processing technology and storage.
- High degree of stability during processing.

- Stability in the course of freeze-drying or other drying procedures.
- Accurate and reliable determination of the strain.
- The dose-dependent effects.

Lactic bacteria supplements are available in different forms, including tablets, capsules and freeze-dried preparations. It is estimated that a dose of at least 108 (0ne billion) of live bacteria delivered to the appropriate site (Floch, 2003).

2.3.4. Significance

Lactobacilli have many important roles in industry. They contribute to the production of some cheese, yogurt and other products. The lactic acid produced by *Lactobacilli* inhibits the growth of other organisms and lowers the pH of the products. The starter cultures for such products are carefully cultivated and maintained because their metabolic end products contribute to the flavor of the final food product. Additionally, some of the *Lactobacilli*'s metabolic reactions are intentionally manipulated to breakdown milk proteins during cheese production. *Lactobacillus* have been examined for their effectiveness in the prevention and treatment of a diverse spectrum of GI disorders such as antibiotic-associated diarrhea (including *Clostridium difficile* associated intestinal diseases), infectious bacterial and viral diarrhea (including diarrhea caused by rotavirus, *Shigella, Salmonella,* enterotoxigenic *E. coli*, *Vibrio cholerae* and human immunodeficiency virus (HIV), acquired immunodeficiency disorder, enteral feeding diarrhea, *Helicobacter pylori* gastroenteritis, sucrose maltase deficiency, inflammatory bowel disease, irritable bowel syndrome, small bowel bacterial overgrowth and lactose intolerance (Lidbeck, 1993).

A very modern issue of LAB is there use as beneficial live components in so-called "healthy eating". The central aim behind this idea is to modulate the human gut micro flora by administering health-relevant bacteria or by stimulating their growth by providing specific substrates of oligo and polysaccharide nature which cause an increase of their population (Shananhan, 2002).

Interaction of host micro-organisms occurs by enzyme production which helps digestion (lactose), diminish production of ammonia, amines or toxic enzymes and favor a good functioning of the intestinal mucosa (Gregor *et al.,* 2003).

Lactobacillus has been found to inhibit intestinal enzymes involved in the synthesis of colonic carcinogens. It can help the body by protecting against colon cancer and the adverse effects of chemotherapy and radiotherapy. There are many mechanisms by which probiotics enhance intestinal health, including stimulation of immunity, competition for limited nutrients, and inhibition of antimicrobial substances. Furthermore, they can be taken as a preventive against food poisoning when traveling (Gilliland, 1990).

Lactic acid bacteria are an important barrier against pathogenic microorganisms passing through the digestive tract, preventing their colonization by blocking the specific sites and by consuming the nourishing substrate. The favorable microorganisms stabilize pH in the digestive tract, obstructing the development of pathogenic organism and favour the growth of useful micro-organisms (Ducluzeanu and Raibaud, 1989).

There are 2 ways to reduce the formation of pathogenic germs is by:

1. The production of substances with selective anti-bacterial effects (organic acid or hydrogen peroxide which is favorable).
2. Competition for the active sites on the digestive lumen walls (usually won by the organisms which are predominant).

2.3.5. Mechanisms of Action

The mechanisms by which probiotics exert biological effects are still poorly understood but the nonspecific terms such as colonization resistance or competitive exclusions are often used to explain their mode of action (Elo *et al., 1991).* Colonization resistance or competitive exclusion describes a phenomenon whereby the indigenous anaerobic flora limits the concentration of potentially pathogenic (mostly aerobic) flora in the digestive tract (Vollaard and Clasener, 1994). The concept of competitive exclusion was first developed during the early 1970's, when it was discovered that the administration of mixed adult intestinal microorganisms conferred adult-type resistance against *Salmonella* infection to newly hatched chicks (Nurmi *et al.,* 1992). Oelschlaeger (2010) reported that the effects of probiotics may be classified in three modes of action:

(i) Probiotics might be able to modulate the host's defenses including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention and therapy of infectious disease but also for the treatment of (chronic) inflammation of the digestive tract or parts thereof. In addition, this probiotic action could be important for the eradication of neoplastic host cells.

(ii) Probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases of importance for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut.

(iii) Finally, probiotic efforts may be based on actions affecting microbial products like toxins and host products, e.g. bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of host and food components in the gut. The same author also stated that the kind of effect(s) a certain probiotic executes depends on its metabolic properties, the molecules presented at its surface or on the components secreted. Even integral parts of the bacterial cell such as DNA or peptidoglycan might be of importance for its probiotic effectiveness. The individual combination of such properties in a certain probiotic strain determines a specific probiotic action and as a consequence its effective application for the prevention and/ or treatment of a certain disease.

2.5.4. Probiotics and Gut Disorder

The gastrointestinal tract is a complex ecosystem having diverse and highly evolved microbial community composed of hundreds of different microbial species. The interactions that occur between this complex microbial community and the human host have become the focus of scientific research due to increase in the incidence of illnesses associated with deficient or compromised microflora (e.g. GI tract infections, inflammatory bowel disease (Crohn's disease and ulcerative colitis), irritable bowel syndrome, antibiotic-induced diarrhea, constipation, food allergies, cardiovascular disease and certain cancers).

Effective multidisciplinary research programs now complement conventional microbiology with molecular ecology techniques to provide culture-independent analysis of the gastrointestinal ecosystem.

Furthermore, as we acquire an understanding of gut microflora composition and processes such as intestinal adherence, colonization, translocation and immune-modulation, we are elucidating mechanisms by which these can be influenced. This knowledge not only allows scientists to define the activities and interactions of "functional food" – borne beneficial bacteria in the gut, but will also provide the scientific basics for the development of innovative biotechnology-based products tailored to prevent specific diseases and promote overall human gastrointestinal health (Hammes and Tichaczek, 1994).

Under normal conditions, the resident flora of the gut comprises up to more than 10^{12} cells per g dry weight faces. It has been recognized that LAB and *bifidobacteria* play a major role in this complex environment since they may contribute considerably to the microbiological balance (Stiles, 1996).

2.5.5. Probiotics enhance anti-infective defenses in the Gastro Intestinal tract

Several clinical studies have demonstrated the therapeutic and/or prophylactic efficacy or specific probiotics against acute viral gastroenteritis and antibiotic-associated diarrhea (including *Clostridium difficile* infection). Emerging evidence also suggests beneficial effects against *Helicobacter pylori* infection (Mattila-Sandholm *et al*., 2002).

The evidence of efficacy against traveler's diarrhea remains, however, inconclusive. The precise mechanisms by which probiotics potentiate host GI defenses and mediate protection are not fully known. There is evidence to suggest, however, that probiotics might contribute to host defense by reinforcing non-immunological defenses and stimulating both specific and non-specific host immune responses.

2.5.6. Antagonistic property of LAB

The antagonistic property is attributed to the lower pH, undissociated acids and production of other primary and secondary antimicrobial metabolites produced by LAB. The metabolites produced by the fermentation process, except the volatile ones, are kept in the foods and result in growth inhibition of plant spoilage or poisoning bacteria and

detoxification of noxious compounds of plant origin. The primary antimicrobial effect exerted by LAB is the production of Lactic acid and reduction of pH. In addition LAB produce various anti-microbial compounds, which can be classified as Low-Molecular-Mass (LMM) compounds such as hydrogen peroxide, carbon dioxide, diacetyl(2,3-utanedione), uncharacterized compounds and High-Molecular-Mass (HMM) compounds like bacteriocin (Yoon *et al*., 2004).

2.5.7. Lactose Intolerance

There is convincing evidence from several studies that lactose intolerance symptoms reduce with the consumption of probiotic dairy products. The mechanism of action of probiotic bacteria and fermented product include lower concentration in the product resulting in high lactose activity increasing the active lactose enzyme entering the small intestine. The bacterial enzyme, beta-galactosidase found in the ileum after consumption of fermented milk products with viable probiotic bacteria is the major factor that improves digestibility by lactose hydrolysis. Therefore, there is good scientific evidence on the alleviation of lactose intolerance symptoms by specific probiotic bacteria (Yoon *et al*., 2006).

2.5.8. Rotavirus Diarrhea

Several studies have been selected probiotics such as *L. reuteri, L. casei, B. lactis* and *L. delbrueckii* sub sp. *Bulgaricus* can shorten the duration of rotavirus diarrhea using the probiotic strain *L. rhamnosus* GG is perhaps the best documented probiotic effect. The mechanism behind this favorable outcome is associated with enhancement of Immunoglobulin A (IgA) to rotavirus and serum IgA antibody level at convalescence. It is therefore suggested that certain strains of probiotic bacteria promote systematic and local immune response to rotavirus, which may be of importance for protective immunity against re-infection (Guarner and Malagelada, 2003).

2.5.9. Allergy Prevention and Alleviation

The prevalence of allergic disease has been on the rise in the past decades and is likely to continue to do so. It has been found that differences in intestinal microbial composition precede the development of some allergic diseases (Bjorksten *et al*., 2001).

Administration of *L. rhamnosus* and *B. lactis* has been found to be more effective than un-supplemented formula on eczema alleviation in infants with atopic eczema (Isolauri *et al.,* 2001).

2.5.10. The function of Food Supplements in our body

1. Improve efficiency of our digestive system.
2. Manufacture vitamins such as biotin and vitamin K.
3. Produce antibacterial substance which kill or deactivate harmful bacteria.
4. Help maintain normal bacteria balance in lower intestines.
5. Kill yeast and fungus on contact.
6. May lower cholesterol.
7. May clear up skin problems.
8. May extend life span.
9. May enhance immunity.
10. May reduce symptoms from spastic colon.
11. May reduce diarrhea to long-term antibiotic use.

Research shows that stomach acid kills many *Lactobacillus* strain on contact with prevents those strains from reaching the intestinal tract. Also, many of the other strains that do survive the stomach acid do not adhere to the intestinal lining.

2.5.11. Functional Foods

In the recent years, the stressful lifestyles, deterioration in personal health and lack of exercise has led to increased self medication and personal responsibility for health care. Research in nutrition has led to a number of discoveries on food ingredients that can be incorporated for health benefits. This education has made it challenging for the food industry globally to introduce food products with ingredients that are not only just nutritional but which combine taste and appearance with positive health benefit, such foods are termed as

functional foods. A functional food is a food or a food ingredient, not necessarily a nutrient, with a demonstrated health benefit, or with the capacity to protect against disease, beyond the fundamental nutritional attributes (Benno *et al.,* 2002). Among the most promising targets for functional foods are the gastrointestinal functions including those that control of nutrient bio-availability. This can in turn modify the gastrointestinal immune activity. Some other functions are lipoid homeostasis, which indirectly influence nutrient digestion or fermentation. The functional food market has also been successful in UK, Japan and Australia.

2.5.12. Plantain pith

Plantain stems are taken from the inner parts of the plantain tree after removing the outer layers which are greenish in colour.

Figure 2.1: Plantain Pith

The inner stem which is used for cooking purposes is off-white or creamish. Just like the fruits and leaves of the plantain/banana tree stem is also very useful. The stem is full of edible fiber roughages helps to eliminate the waste from our body smoothly and efficiently. This stem is a natural diuretic which helps in removal of excess fluids from the body and in treating of sciatica, lymphatic swelling, pre-menstrual syndrome, high blood pressure, diluting and breaking down of kidney stones, gonorrhea and liver disorders. It helps to increase the urination and through that detoxifies our body. This stimulates the kidneys into better removal of uric acid also. By helping the kidneys do their job cleanly the

plantain stems help reduce high cholesterol levels and the astringent quality helps in blood coagulation. Stem juice is also used in nervous affectations like epilepsy, hysteria and in dysentery and diarrhea. It has also been found to contain condensed tannins and pectin which have anti-helminthic and hypoglycemic properties (Geetawatal, 2007).

2.5.13. Health benefits of Plantain Pith

1. Control of obesity,
2. Removal of urinary stones,
3. Reduction of ulcers,
4. Prevention of burning sensation,
5. Stabilizing acidity,
6. Control of diabetes and
7. Prevention of bleeding disorders.

2.5.14. Properties of Plantain Pith

1. Is a natural diuretic
2. Reduces cholesterol
3. Its astringent quality helps in blood coagulation
4. Is an alkalizer

2.5.15. Prebiotics

The application of probiotics has been supplemented with the concept of prebiotics. A prebotic is defined as a non-digestible food ingredient that beneficially affects the host selectively stimulating the growth, activity or both of one or a limited number of bacterial species already resident in the colon (Dimer and Gibson, 1998). The most commonly used

prebiotics are carbohydrate substrates with the ability to promote the components of the normal intestinal micro flora which may evince a health benefit to the host. However, prebiotics can also be non-absorbable substrates which stimulate the growth of probiotics. When these two are applied together the concept is defined as synbiotic. At present, most of the prebiotics are directed towards the growth of lactic acid bacteria due to their purposed health promoting properties.

The prebiotics identified as non-digestible carbohydrates include lacunose, insulin resistant starch and a range of oligosaccharides that supply a source of fermentable carbohydrate for probiotic bacteria in the colon.

2.5.16. Synbiotics

The benefits of prebiotics on probiotics have rise to the concept of synbiotics, in which probiotics and prebiotics are used in the combination (Gibson and Roberfroid, 1995). The live microbial additions (probiotics) may be used in conjunction with specific substrates (prebiotics) for the growth (e.g. a fructoligosaccharide in conjunction with a *Bifidobacterial* strain).

It has been claimed that this combination could substantially improve the survival of probiotic bacteria as well as offer the advantages of microecological balance of the gut microflora.

2.3.17. Freeze-drying

Freeze-drying (also known as lyophilization, lyophilization or cryodesiccation) is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase.

2.3.18. The origins of freeze drying

Freeze-drying was first actively developed during World War II. Serum being sent to Europe for medical treatment of the wounded required refrigeration, but because of the lack of simultaneous refrigeration and transport, many serum supplies were spoiled before reaching their intended recipients. The freeze-drying process was developed as a commercial technique that enabled serum to be rendered chemically stable and viable without being refrigerated. Shortly thereafter, the freeze-dry process was applied to penicillin and lyophilization became recognized as an important technique for preservation of biological materials. Since that time, freeze-drying has been used as a preservation or processing technique for a wide variety of products. The applications include the following the processing of food, pharmaceuticals, and diagnostic kits; the restoration of water damaged documents; the preparation of river-bottom sludge for hydrocarbon analysis; the manufacturing of ceramics used in the semiconductor industry; the production of synthetic skin; the restoration of historic/reclaimed boat hulls (Myrbo, 2004).

2.3.16. The freeze-drying process

There are four stages in the complete drying process:

1. Pretreatment
2. Freezing
3. Primary drying and
4. Secondary drying

2.3.17. Properties of freeze-dried products

If a freeze-dried substance is sealed to prevent the re-absorption of moisture, the substance may be stored at room temperature without refrigeration, and be protected against spoilage for many years. Preservation is possible because the greatly reduced water content inhibits the action of microorganisms and enzymes.

Freeze-drying also causes less damage to the substance than other dehydration methods using higher temperatures. Freeze-drying does not usually cause shrinkage or toughening of the material being dried. In addition, the flavors, smells and nutritional content generally remain unchanged, making the process popular for preserving food. However,

water is not the only chemical capable of sublimation, and the loss of other volatile compounds such as acetic acid (vinegar) and alcohols can yield undesirable results.

Freeze-dried products can be rehydrated (reconstituted) much more quickly and easily because the process leaves microscopic pores. The pores are created by the ice crystals that sublimate, leaving gaps or pores in their place. This is especially important when it comes to pharmaceutical uses. Freeze-drying can also be used to increase the shelf life of some pharmaceuticals for many years (Myrbo, 2004).

2.3.18. Applications of freeze-drying

1. Pharmaceutical companies often use freeze-drying to increase the shelf life of products, such as vaccines and other injectables.
2. Freeze-drying is used to preserve food, the resulting product being very lightweight.
3. In chemical synthesis, products are often freeze-dried to make them more stable, or easier to dissolve in water for subsequent use.
4. In bacteriology freeze-drying is used to conserve special strains.
5. In high-altitude environments, the low temperatures and pressures can sometimes produce natural mummies by a process of freeze-drying.

OBJECTIVES

1. To Isolate and Identify Probiotic bacteria from various milk sources and dairy effluents.
2. To dry the plantain pith juice and LAB separately using freeze drier.
3. To optimize the composition of the fruit juices with probiotics in *in-vitro* conditions.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sterilization of glass ware

Glass wares like sampling bottles, flasks, Petridishes and test tubes after washing with detergent and autoclaved at 121□C for 15 minutes.

3.2. Isolation of Lactic acid bacteria

Milk samples from various locations in and around Coimbatore were taken and microbial strains were isolated from MRS agar (Mann, de Ragosa, Sharpe, 1990). Single pure colonies were isolated for further study.

Materials:

1. Milk
2. Lactobacillus MRS agar
3. Petri plates
4. Conical flasks
5. Inoculation loop

Procedure:

1. 1 ml of milk sample was taken and serially diluted.
2. The 4^{th} and 5^{th} dilutions were spread plated on MRS agar.
3. The plates were incubated overnight at 37□C and observed for colonies.
4. From the MRS agar plates, the colonies were taken and Quadrant streaking was done to isolate individual colonies.

3.2.1. Isolation of pure culture

1. From the plates, single colonies with good zone of inhibition were selected.
2. The colonies were streaked in MRS slants.
3. The test tubes were incubated over night at 37□C.
4. The slants were observed for growth.
5. These colonies were sub-cultured and the original slants were preserved for future use.

3.3. Characterization of *Lactobacillus*

The samples were placed on MRS agar and the colonies were isolated. These colonies underwent preliminary screening by gram staining and the catalase test.

3.3.1. Gram staining

Gram staining is an empirical method of differentiating bacteria into two large groups based on difference in their cell walls. The cell walls for Gram-positive microorganisms have a higher peptidoglycan and lower lipid content than gram-negative bacteria. Bacteria cell walls were stained by the crystals violet. Iodine was subsequently added as a mordant to form the crystal violet iodine complex, so that the dye was not removed easily. However, subsequent treatment with ethanol dissolved the lipid layer from the gram-negative cells and enhanced the leaching of the primary stain from the cells. In contrast, the solvent dehydrated the thicker Gram-positive cell walls, closing the pores as the cell wall shrunk during dehydration. As a result, the diffusion of the violet-iodine complex was blocked, and the bacteria remained stained. Finally, a counter stain of safranin was applied to the smear to give decolorized gram-negative bacteria, a pink colour.

Materials:

1. Microbial strains
2. Crystal violet

3. Gram's iodine
4. Safranin
5. Ethanol (95%)
6. Glass slides
7. Inoculation loop
8. Bunsen burner

Procedure:

1. A small drop of distilled water was placed on the surface of a clean glass slide.
2. Using a sterile loop, a thin smear of the culture was made on the slide.
3. The smear was flooded with crystal violet for 1 min after which the stain was washed-off under running water.
4. The dye was fixed with Gram's Iodine and washed with water after 1 min.
5. The smear was decolorized using ethanol (95%) and washed under running water.
6. Finally, the smear was counter-stained with safranin for 30 sec and then washed with water.
7. The smear was air dried and visualized under 100X magnification.

3.3.2. Acid-Fast Staining:

The lipoid capsule of the acid-fast organism takes up carbolfuschin and resists decolorization with a dilute acid rinse. The lipoid capsule of the mycobacteria is of such high molecular weight that it is waxy at room temperature and successful penetration by the aqueous based staining solution (such as Gram's) is prevented.

Materials:

1. Ziehl-Neelsen carbol-Fuchsin Solution
2. 1% Acid Alcohol
3. Distilled water
4. Methylene Blue Solution

Methods:

1. Deparaffinize and hydrate to distilled water.
2. Place a few drops of Carbol-fuchsin solution and microwave for 45 seconds, allow slides to stand in hot solution for 5 minutes.
3. Wash in running tap water.
4. 1% Acid alcohol until light pink and colour stops running.
5. Wash in running tap water for 5 minutes.
6. Rinse in distilled water.
7. Dehydrate, clear, and coverslip.

3.3.3. Spore formation test:

Materials:

1. Agar
2. Dipotassium Hydrogen phosphate
3. Potassium Dihydrogen phosphate
4. Ammonium sulphate
5. Glucose
6. Sodium citrate
7. Magnesium sulphate
8. Distilled water

Methods:

1. Prepare the minimal agar media by dissolving the Davis Minimal Agar in distilled water.
2. Sterilize the media by keeping at 121^0C for 15 minutes.
3. Allow it to cool for 10 minutes.
4. Pour the media into the petriplates and allow it to solidify.
5. Inoculate the LAB culture into the media by using an inoculum loop.
6. Incubate at 35^0C for 48 hrs and observe the results.

3.3.4. Acid tolerance test

Acid tolerance ability of LAB was determined by exposing to different pH levels. Acid tolerance was evaluated by determining the viable counts on selective media at different time interval of 0, 30, 60 and 120 min.

Materials:

1. HCl

2. 1N NaOH
3. pH meter and electrode
4. Standard buffers- pH 4.0 and 7.0
5. Sample
6. Control
7. MRS agar
8. MRS broth

Procedure:

1. Harvest the cells by centrifugation, at 10,000 rpm for 5 min.
2. Prepare MRS broth at different pH levels 2.0, 3.0, 4.0 and 6.8(normal MRS broth pH) using diluted HCL and NaOH.
3. Inoculate the cell pellets (1%) into MRS broth acidified with HCL.
4. Collect 1ml of the sample after inoculating into MRS broth at different time interval of 0, 30, 60 and 120 min.
5. Inoculate the sample in MRS agar and incubate at 37□C.
6. Use cell count method for determine viability of the cells.

3.3.5. Bile tolerance

Materials:

1. Ox bile salt
2. MRS broth
3.

Procedure:

1. Obtain fresh culture of LAB by growing in broth overnight at optimum growth temperature (37◦C).

2. The actively grown cells were harvested by centrifugation (10,000 rpm for 5 min).
3. Re-suspend the cells in equal volume of broth supplemented with 0.5, 1.0, 1.5 and 2.0% (w/v) ox bile.
4. Collect 1ml of the sample after inoculating into MRS broth at different time interval of 0, 30, 60 and 120 min.
5. Culture the sample in MRS agar plate and incubate at 37◦C.
6. Determine viability of cells by plate count method.

3.3.6. Catalase test

The catalase test identifies the organisms which produce the enzyme catalase; this enzyme converts hydrogen peroxide to oxygen gas and water. Catalase helps to protect bacterial cells against hydrogen peroxide. Hydrogen peroxide is a highly reactive compound which damages cell components. It is sometimes formed when the electron transport chain is used to produce energy. When a catalase positive organism is exposed to hydrogen peroxide, the hydrogen peroxide will bubble.

Materials:

1. Hydrogen peroxide
2. Microbial strain
3. Glass slide
4. Inoculation loop

Procedure:

1. LAB colonies were air dried, heat fixed on a glass slide.
2. Crystal violet stain was added and washed with water after 1 min.
3. Iodine solution (mordant) was added and washed with water.
4. Decoloriser (95% Ethanol) was added until no violet color remains.

5. Safranin was added and washed with water after 1 min.

3.3.7. Glucose fermentation test

Materials:

1. Peptone
2. Sodium chloride
3. Glucose
4. Bromthymol blue
5. Agar
6. Di-potassium phosphate

Procedure:

I. Oxidative-fermentative test using the media with glucose

A. Inoculation of media

Two tubes of oxidative-fermentative medium are inoculated by stabbing "half way to the bottom or ¼-inch from the bottom with the test organism. Overlay one of the two tubes with 1 cm of mineral oil. This overlay prevents the diffusion of oxygen into the medium and creates an anaerobic condition in the tube.

B. Incubation conditions

Incubation of the samples at 35°C for 48 hours is recommended for most gram-negative rods. Slow growing bacteria may take 3 to 4 days for results.

1. Fermentative results

Bacteria that can ferment glucose give a fermentative result as indicated by acid production in both the open (aerobic) and oil covered (anaerobic) tube. The acid produced (pH 6.0) changes the pH indicator, bromothymol blue, from green to yellow. The semisolid consistency of the medium also allows for detection of motility. Note: Hazy growth away from the stab line.

2. Oxidative results

Non-fermenting bacteria that metabolize glucose through oxidative metabolism give an oxidative result. This result is indicated by a small amount of acid production in the open tube. The acid produced (at pH 6.0) changes the pH indicator, bromthymolblue, from green to yellow. After 24-hour incubation the change in pH is observed at the surface of the open tube where the growth in the presence of oxygen is observed. With prolonged incubation (more than 48 hours), the concentration of agar in the medium decreases and allows eventual diffusion of the weak acid throughout the whole tube.

3. Negative results

Nonsacchrolytic bacteria give a negativeresult. The negative result is indicated by no colour change in the oil-covered tube and in some cases an increase in pH of about 7.6 changing the bromothymol blue from green to blue in the top of the open tube. The increase in pH is due to amine production by bacteria that break down the peptone (protein) in the medium. Other bacteria give a negative result which is indicated by no growth or colour change in the medium.

II. The oxidative-fermentative test using carbohydrates other than glucose

Non-fermenting gram-negative rods that have been shown to give an oxidative result in the glucose test can be further tested for its ability to metabolize other carbohydrates oxidatively. The glucose is replaced by maltose, lactose, mannitol, or sucrose in the medium and only one tube per carbohydrate is inoculated. A heavy inoculum should be used, as many of these non-fermenters are slow growing. As the result being detected is based on aerobic respiration no mineral oil or agar layer is used.

A positive result is indicated by an acid production and a change in the pH in the top of the tube after 24 hours. Some slow growing non-fermenters may take several days to produce enough acid to be detected by the bromothymol blue.

3.3.8. Antimicrobial Test

Materials:

1. LAB sample
2. TGE soft agar
3. Indicator organism

Procedure:

1. Active pure culture of LAB is grown in MRS broth for 16-18 h at 37◦C.
2. Cell free culture supernatant (CFCS) are prepared by centrifugation at 10,000 rpm for 10 min at 4◦C.
3. Heat culture supernatant at 90◦C for 5-7 min to kill any live bacterial cell.
4. Prepare fresh culture of indicator bacteria by growing for 16-18 h and further inoculate it for its active growth at optimum temperature for 3-4 h.
5. 50 µl of this culture is mixed with 7ml of melted and tempered (45◦C) TGE soft agar and poured onto the previously dried TGE agar plates.
6. The soft agar is allowed to solidify and 5µl of CFCS is directly spotted on the lawns of indicator organism.
7. The plates are kept undisturbed for 2h and subsequently incubated at 37^0C.
8. After 24 h of incubation, a 5mm or more diameter of the growth inhibition zones are considered as positive inhibition.

3.3.9. Selection of Lactobacilli for inoculation in the juice

Materials:

1. Isolated Lactobacillus slant
2. Lactobacillus MRS broth
3. Plantain pith juice
4. pH meter
5. Nephelometer
6. Centrifuge

Procedure:

1. Loop-full of organism was taken from the sub cultured slant of LAB and inoculated in the broth.
2. The tubes were incubated overnight at 37□C.
3. 1 ml of the broth was taken and centrifuged at 10000 rpm for 10 mins.
4. The supernatant was discarded and the pellets were washed.
5. The organism was inoculated in 10 ml of juice and incubated at 37□C for 48 h.
6. The pH and turbidity were noted before and after fermentation.

3.4. Preparation and Pasteurization of Plantain Pith Juice

Materials:

1. Fresh Plantain pith
2. Mixer with juicer jar
3. Sterilized glass bottles
4. Distilled water
5. Sterilized knife
6. Glass funnel
7. Filter paper
8. Hot water bath
9. Thermometer

Procedure:

1. Plantain pith was peeled to remove the sap.
2. Small slices of pith were made with a sterilized knife.
3. The pith was juiced to pulp in a mixer.
4. Clear juice was extracted from the pulp with a filter paper and a glass funnel.
5. The glass bottles with juice were sealed tight.
6. The bottles were placed in a water bath and the juice was pasteurized at 63^oC for 30 minutes in a hot water bath.
7. The fresh juice was stored at 4^oC in a refrigerator.

3.4.1. Lyophilization of LAB culture and Plantain pith juice

Materials:

1. Lyophilizer (or) Freeze drier (Model: Shimadzu FD – 1206, Capacity: 10 liters)
2. Cooling centrifuge
3. Round bottom flasks
4. 500ml LAB culture
5. 500ml Plantain pith juice sample
6. Deep freezer
7. Petri plates

Procedure:

1. 500ml of LAB culture grown in MRS broth was centrifuged in batches at 10,000 RPM for 5 minutes to pellet the organism.
2. The pellets were collected and spread on a Petri plate and froze at -20^{o}C in a deep freezer.
3. 100ml of juice sample was frozen in a deep freezer in a round bottom flask.
4. The Lyophilizer is switched on and set to pre-cooling to -40^{o}C.
5. A negative pressure of 0.005 Torr is maintained prior to sample exposure.
6. The frozen juice sample is connected to the Vacuum chamber and the valve was set to open, the Petri plate with LAB culture was placed on the tray of the freeze drier.
7. Freeze drying was carried out for 12 hours.
8. The residual moisture was removed by the product heater.

3.4.2. Packaging Composition

Materials:

1. Weighing balance
2. Aluminum foil packets
3. Grinded Palm Sugar
4. Lyophilized LAB culture
5. Lyophilized Plantain Pith juice

Procedure:

For 100 grams of lyophilized juice sample, 2% concentration of lyophilized LAB culture was added. Based on trial and error basis the palm sugar concentration of 25g/100g of juice sample gave a palatable taste without losing the products characteristics. 1g of the finished probiotic juice was sufficient to make-up 100ml of active probiotic plantain pith juice.

3.4.3. Packaging of Final Product

Materials:

1. Aluminum foil packets
2. Heat sealer
3. Label

Procedure:

1. 5 grams of finished probiotic juice powder was added to aluminum foil packets.
2. The packets were sealed by heating with a sealer.
3. Products were labeled for content and packaging information.
4. The foil packets were stored inside zip-lock pouches and stored in a dry are away from sunlight.

3.5.Determination of pH

pH is the measurement of H^{+} ion activity. It measures active acidity. pH may be determined by measuring the electrode potential between glass and reference electrodes; pH meter is standardized using standard pH buffers. Use homogenized sample for the determination of pH.

Materials:

1. pH meter and electrode
2. standard buffers- pH 4.0 and 7.0
3. sample
4. control

Procedure:

Standardization of pH meter:

1. press **mode** to select pH
2. Press **setup** twice and then press **enter to** clear the existing standardization buffers 4.0.
3. Press **std** again to initiate standardization. After the reading is stable, the meter will return to the measure screen. Remove electrode from buffer. Rinse off with distilled water and blot dry with filter paper.
4. Repeat steps 2 and 3 with buffer 7.0 and sample.

3.6. Determination of turbidity

Turbidity is measured by nephelometry. This method is based on a comparison of light scattered by a standard reference suspension under the same conditions. The higher the intensity of scattered light, the higher is the turbidity.

Materials:

1. Laboratory or process nephelometer consisting of a light source for illuminating the sample and one or more photoelectric detectors with a readout device to indicate intensity of light scattered at 90E to the path of the incident light. An instrument designed to minimize stray light reaching the detector in the absence of turbidity and to be free from significant drift after a short warm up period is used. The sensitivity of the instrument should permit detecting turbidity differences of 0.02 NTU or less in the lowest range in waters having a turbidity of less than 1 NTU. Several ranges may be necessary to obtain both adequate coverage and sufficient sensitivity for low turbidities. Differences in instrument design will

cause differences in measured values for turbidity even though the same suspension is used for calibration. To minimize such differences, the following design criteria are observed:

i. Light source- Tungsten-filament lamp operated at a color temperature between 2200 and 3000EK.
ii. Distance traversed by incident light and scattered light within the sample tube should not be exceed 10 cm.
iii. Angle of light acceptance by detector – Centered at 90E to the light path and not exceed 30E from 90E. The detector and filter system, if used, shall have a spectral peak response between 400 and 600 nm.

2. Sample cells:

Sample cells or tubes of clear, colorless glass or plastic are used. The cells are kept scrupulously clean, both inside and outside and discard if scratched or etched. Tubes with sufficient extra length or with a protective case are used, so that they may be handled properly. The cells that are filled with samples and standards have been agitated thoroughly and sufficient time is allowed for bubbles to escape.

3. Control and sample.
4. Distilled water.

Procedure:

1. General measurement techniques

Turbidity is measured immediately to prevent temperature changes and particle flocculation and sedimentation from changing sample characteristics. If flocculation is apparent, the aggregates are broken by agitation. Dilution should be avoided whenever possible. Particles suspended in the original sample may dissolve or otherwise change characteristics when the temperature changes or when the sample is diluted. Air or other entrained gases in the sample are removed before measurement. Air bubbles should not be removed by letting the sample stand for a period of time because during standing, turbidity-causing particulates may settle and sample temperature may change. Both of these conditions alter sample turbidity, resulting in a non representative measurement.

Condensation may occur on the outside of surface of a sample cell when a cold sample is being measured in a warm, humid environment. This interferes with turbidity measurement. All moisture from the outside of the sample cell should be removed before placing the cell in the instrument. If fogging recurs, sample should let to warm slightly by letting it stand at room temperature or by partially immersing it in a warm water bath for a short time. Sample should be well mixed again.

2. Measurement of turbidity:

1. Set 'o' using distilled water as blank.
2. The control is gently agitated.
3. The control is poured into the cell after the air bubbles disappear.
4. The turbidity is read directly from the instrument display.
5. The steps 1to 3 are repeated with the samples.
6. The readings are tabulated.

3.7. Determination of titratable acidity

OH^- from the base reacts with the H^+ in solution to form water, more H^+ will break loose from the undissociated portion of the acid to take its place. To measure the total acidity, also called base neutralizing capacity (BNC) of a sample, it has to be titrated with the base. That is, a solution of a base whose concentration is known must be added to the sample slowly until the neutralization is completed by measuring the volume of base added. It can be figure out the original concentration of acid.

Materials:

1. 0.1 N Sodium Hydroxide
2. 1% Phenolphthalein

Procedure:

1. 1ml of sample was taken in a conical flask.
2. 5ml of water was added and few drops of phenolphthalein.
3. The burette was rinsed and filled with 0.1N NaOH and titrated with the contents of conical flask.
4. The end point was noted by the colour change (pale pink).
5. The titration was repeated until the concordant value was obtained.

3.8. Determination of total carbohydrates

Carbohydrates are the important and structural material in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharide's which cannot be split by hydrolysis into simpler sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharide. Carbohydrates were first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms a green color with anthrone and is measured at 630nm.

Materials:

1. 2.5 N-HCl.
2. Anthrone reagent: dissolve 200 mg anthrone in 100ml of ice cold 95% H_2SO_4. Prepare fresh before use.
3. Standard Glucose: Stock – Dissolve 100mg in 100ml water. Working standard- 10ml of stock diluted to 100ml distilled water. Store this in refrigerated condition after adding few drops of toluene.

Procedure:

1. Weigh 100mg of the sample into a boiling tube.
2. Hydrolyze it by keeping in boiling water bath for 3 hours with 5ml of 2.5 N-HCL and cool to room temperature.
3. Neutralize it with solid sodium carbonate until the effervescence ceases.
4. Make up the volume to 100ml and centrifuge.
5. Collect the supernatant and take 0.5ml and 1ml aliquots for analysis.
6. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard '0' serves as blank.
7. Make up the volume to 1ml in all the tubes including the sample tubes by adding distilled water.
8. Then add 4ml of anthrone reagent.
9. Heat for eight minutes in a boiling water bath.
10. Cool rapidly and measure thedark green color at 630nm.
11. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
12. From the graph calculate the amount of carbohydrate present in the sample.

Calculation:

$$\text{Amount of carbohydrate present in 100mg of the sample} = \frac{\text{"x" mg of glucose} \times 100}{\text{Volume of test sample}}$$

3.9. Microbial viability

Materials:

1. Lactobacillus MRS Agar.
2. Petri plates.
3. Juice sample.
4. Inoculation loop.

Procedure:

1. The Petri plates were sterilized by autoclaving at 121□C for 15 minutes.
2. MRS agar is prepared by adding 2% agar to MRS broth and autoclaving at 121□C for 15 minutes.
3. 15-20ml of the agar is poured in each plate were cooled.
4. 100µl of the Probiotic juice sample was taken and pour plated in the Petriplate.
5. Incubate at 37^0C for 24 hrs.

3.10. Microbial analysis

Viable cell count (CFU/ml) was determined by the standard count plate method with lactobacillus MRS medium.

Materials:

1. Sample
2. MRS agar
3. Petriplates
4. Test tubes

Procedure:

1. The test tubes and the Petriplates were sterilized by autoclaving at 121□C for 15 minutes.
2. MRS agar is prepared by adding 2% agar to MRS broth and autoclaving at 121□C for 15 minutes.
3. 15-20ml of the agar is poured in each plate were cooled.
4. The sample was serially diluted to 10^{-4} concentration.
5. 1ml of the serially diluted sample was taken and pour plated in the Petriplates.
6. The plates were counted for every three days for a period of 15 days.

3.11. Sensory evaluation

The method used for sensory evaluation is the 9 point hedonic scale rating. Hedonic rating relates to pleasurable or unpleasurable experiences. The hedonic scale rating is used to measure the consumer acceptability of food products. The hedonic scale rating samples 1-4 are served to the panelist at one session. They are asked to rate the acceptability of the product on a scale, usually of 9 points, ranging from 'like extremely' to 'dislike extremely'. Scales with different ranges and other experience phrase could also be used. The results are analyzed for preference with data from large untrained panels. Semi-trained panels in smaller number are used to screen a number of products for selecting a few for consumer preference studies.

Procedure:

The sample and the control were distributed among panel members and they were asked to rate them for various parameters such as color, flavor, taste, mouth feel, overall acceptability. The following hedonic rating sheet was distributed among the panel members to rate the sample and control.

Specimen evaluation card

HEDONIC RATING SCALE

Name: …….. Date: ……

Product: ……..

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Isolation of Lactic acid bacteria

Eleven different milk samples was collected in and around Coimbatore and were serially diluted to 10^{-4} and 10^{-5} dilutions and streaked on Lactobacillus MRS agar. Growth of milky white colonies were observed on all samples after incubating at 37^{o}C for 24 hours.

Figure 4.1: Isolated LAB (S6)

4.2. Isolation of pure culture

Single colonies were isolated from the quadrant streaked culture plates and slant cultures were prepared on Lactobacillus MRS Agar. The slants were observed for growth and were sealed then preserved at -20^{o}C in a deep freezer. They were collected later for further analysis.

Figure 4.2: Slant preparation of LAB (S5)

4.3. Characterization of Lactobacillus

4.3.1. Gram staining

Morphologically different colonies on MRS agar plates were subjected to Gram staining. Crystal violet is used as the primary stain and Safranin is used as the counter stain. Most of the stained colonies were observed to be gram positive in nature and differing in morphology as rods or cocci. All gram positive bacteria's were chosen for further characterization.

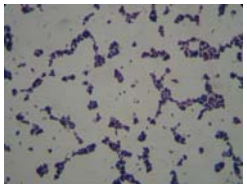

Figure 4.3: Gram positive cocci (S2)

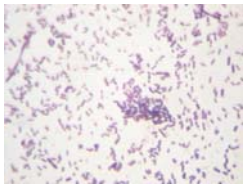

Figure 4.4: Gram positive rods (S6)

The Gram positive bacteria's retain the primary stain as they have a thick peptidoglycan layer which is more resistant ethanol, thus retaining the colour which helps in differentiating bacteria based on the cell wall characteristics.

4.3.2. Spore formation test

Minimal agar, Davis composition (appendix 6.1) was prepared and all the cultures were plated and incubated at 37^{o}C for 24 hours. No spores were observed in any samples. The minimal agar consists of all the essential nutrition in a low quantity, thus making the bacteria suffocate without enough nutrition to survive and push them to form spores (if capable of) or die. From the result, it is clear that the bacteria were all dead and no identifiable spores were formed and the sample is not capable of forming spores.

Figure 4.5: Spore formation test (S4)

4.3.3. Acid fast staining

Ziehl-Neelsen staining was performed on all the collected samples. Sample No.1 was found to be acid fast and the remaining bacterial samples were non - acid fast cultures. The lipoid capsule of the acid-fast organism takes up carbol-fuchsin and resists decolorization with a dilute acid rinse. The lipoid capsule of the acid-fast positive bacteria are of high molecular weight and waxy in nature and prevents penetration by the aqueous based staining solutions. Thus from result we could confirm that the sample does not belong to *Mycobacterium* family.

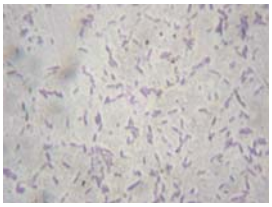

Figure 4.6: Non – Acid fast organism (S6)

4.3.4. Catalase test

The catalase test was performed on the bacterial samples. Strains of sample No. 3, 5, 7, 10 and 11 were catalase positive bacteria and sample No. 1, 2, 4, 6, 8 and 9 were catalase negative. The catalase positive bacteria converted hydrogen peroxide to water and oxygen gas resulting in the formation of bubbles. Since LAB is known to be catalase negative bacteria, only the catalase negative bacteria were selected.

$$H_2O_2 \xrightarrow{\text{Catalase}} O_2 + H_2O$$

4.3.5. Glucose fermentation activity

Glucose Fermentation media (appendix 6.2) was prepared and samples were inoculated separately in normal and oil covered tubes. The tubes were incubated at 35^{o}C for 48 hours and analyzed to find the following results

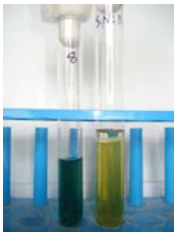

Figure 4.7: Oxidative result (S1)

Figure 4.8: Fermentative result (S9)

Tabulation 4.1: Glucose fermentation test

Sample No.	Oxidative	Fermentative
1	+	-
2	-	+
3	-	+
4	+	-
5	-	+
6	-	+
7	+	-
8	+	-
9	-	+
10	-	+
11	+	-

Legend: "+" - Positive result, **"-"–** Negative result.

Bacteria that can ferment glucose give a fermentative result as indicated by the acid production in both the open (aerobic) and oil covered (anaerobic) tube. The acid produced (pH 6.0) changes the pH indicator, bromothymol blue, from green to yellow. Acid productions in both the tubes indicate a fermentative result. The fermentative bacteria were preferred and selected for further analysis.

Note: Based on the tests conducted, **Sample No. 6** was more suitable for the study and is chosen for further analysis.

4.4. Testing for Probiotic attributes

4.4.1. Acid Tolerance test

The acid tolerance test was conducted to screen organisms which are more tolerant to highly acidic condition that naturally prevails in stomach. The organism from sample No.6 was exposed to varying HCl concentrations for various time and then cultured on MRS broth tubes which are then incubated for 24 hours at 37^{o}C. The nephalometric readings observed per each tube is as follows

Tabulation 4.2: Acid tolerance results

pH			
Time (in minutes)	**7.0** (NTU)	**3.0** (NTU)	**2.0** (NTU)
0	26	12	01
30	20	01	00
60	22	02	00
120	26	23	00

Control reading - 01 NTU.

It is observed from the tabulation that the number of viable organisms decrease with the increase of exposure time and decreasing pH levels. The organism from sample No.6 is more viable in the time exposure of 120 minutes and at a pH of 3.0, which is suitable for probiotic application of organism through oral path.

4.4.2. Bile tolerance test

Bile tolerance is very important for a probiotic organism to survive in the harsh intestinal condition with exposure to bile. The sample No.6 was tested for tolerance to Ox bile of various concentrations dissolved in MRS media and exposed for different time and

then cultured on MRS broth tubes which are then incubated for 24 hours at 37°C. The nephalometric readings observed per each tube is as follows

Tabulation 4.3: Bile Tolerance results

Ox bile (In %) **Time** (In minutes)	**0.5** (NTU)	**1.0** (NTU)	**1.5** (NTU)	**2.0** (NTU)
0	23	18	11	09
60	20	15	09	07
120	18	15	08	09

Control reading - 01 NTU.

It is observed from the tabulation that the number of viable organisms decrease with the increase of exposure time and increasing Ox bile concentration levels. The organism from sample No.6 could tolerate Ox bile concentration of 2.0% for 120 minutes and still survive in a considerable amount, which is suitable for probiotic application of organism through oral path.

4.5. Post Lyophilization analysis

4.5.1. Composition and Preparation

The final composition of Probiotic juice which is prepared and packed and stored for 5 days in dark and away from sunlight consist of,

Tabulation 4.4: Probiotic Juice Composition

Components	**Dissolved in g / 100ml of water**
Lyophilized Plantain pith juice powder	78 g
Lyophilized LAB culture	2 g

Figure 4.10: Probiotic Juice

4.5.3. Determination of pH

The pH of the diluted juice (1g/100ml) was analyzed and found to be 4.92 using a pH meter (ELICO Li-120).

Figure 4.11: pH determination

4.5.4. Determination of Titratable Acidity

Titration was carried out with 0.1N NaOH against the probiotic juice sample with phenolphthalein as indicator. The juice was neutralized with 0.5 ml of 0.1N NaOH solution. The acidity of the juice is found to be by the formula

Grounded Palm sugar (sweetener)	20g

The preparation of probiotic juice was optimized to be by diluting 1 gram of probiotic juice powder to 100ml with water. This was achieved by trial and error method,

Trial 1: Dissolving 5g of probiotic juice in 100ml of water.

Inference: Juice solution too thick, difficult to consume.

Trial 2: Dissolving 5g of probiotic juice in 300ml of water.

Inference: Juice solution thick, difficult to consume.

Trial 3: Dissolving 5g of probiotic juice in 500ml of water.

Inference: Juice in good colour, flavor and is consumable.

Figure 4.9: Packed Probiotic juice powder

4.5.2. Physical appeal

The prepared probiotic juice was light brown in colour and a palatable sweet taste.

$$V_1 * N_1 = V_2 * N_2$$

V_1 (sample) = 1.0 ml V_2 (NaOH) = 0.05 ml (by titration)

N_1 (sample) = **?** N_2 (NaOH) = 0.1 N

$$N_1 = (V_2 * N_2) / V_1$$

The acidity of the solution was found to be 0.05N or 0.05 mol/L.

Figure 4.12: Titratable acidity

4.5.5. Determination of total carbohydrate

Total carbohydrate content of the probiotic juice was determined by using anthrone. The green colour formed was colorimetrically measured at 630nm. A standard graph was prepared by using standard glucose solution. 1 ml of the sample from the probiotic juice solution was analyzed to find the total sugar concentration.

Tabulation 4.5: Determination of Total Carbohydrate

S.No	**REAGENTS**	**BLANK**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**TEST**
1.	Vol. of Standard (ml)	-	0.2	0.4	0.6	0.8	1.0	-
2.	Conc. of Standard (µg/ml)	-	20	40	60	80	100	-

3.	Vol. of Sample (ml)	-	-	-	-	-	-	0.1
4.	Vol. of distilled water (ml)	-	0.8	0.6	0.4	0.2	-	0.9
5.	Vol. of Anthrone reagent (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Keep in boiling water bath for 8 minutes								
6.	Observe OD at 630nm							

Tabulation 4.6: Determination of Total Carbohydrate

S. No.	Label	Concentration of Standard glucose (µg/ml)
1.	Blank	0
2.	0.2	2
3.	0.4	4
4.	0.6	6
5.	0.8	8
6.	1.0	10

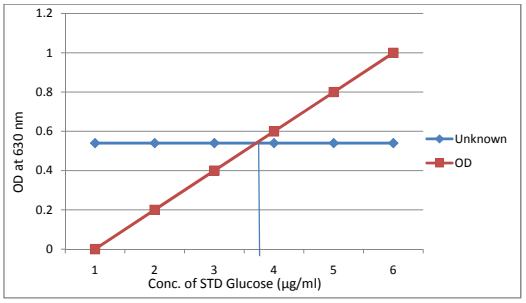

Figure 4.13: Total Sugar Concentration

From the graph, it is observed that the total sugar concentration present in the probiotic juice (1g in 100ml dilution) is found to be 3.3 µg/ml.

4.5.6. Lyophilization Product Yield

The product yield in the Lyophilizer is stated as follows;

Plantain pith juice (in grams):

Before Lyophilization = 50 g

After Lyophilization = 7.6 g Yield (in %) = 15.2 %

LAB culture (in grams):

Before Lyophilization = 20 g

After Lyophilization = 2.2 g Yield (in %) = 11.0 %

4.5.7. Sensory Evaluation

Sensory parameters such as colour, flavor, taste, mouth feel and overall acceptability of the control and the sample inoculated with LAB were determined. The tests were performed using 9 point hedontic scale. It was found that the control had a high rating of 8when compared with the sample which had a rating of 7. The sample had higher rating for all other parameters such as flavor, taste, mouth feel and overall acceptability when compared to the control. The rating of flavor for sample was 8 and that of control was 7. The rating for taste of the sample was 7 and that of control was 6. The rating for mouth feel of sample was 8 and that of control was 7. The overall acceptability rating of the sample was 8 and for the control were 7.

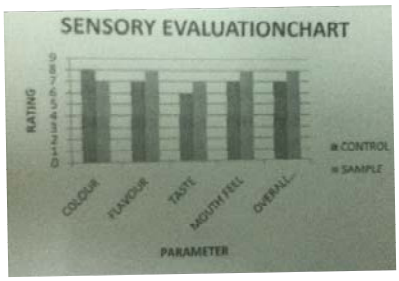

Figure 4.14: Sensory evaluation results

CHAPTER 5

CONCLUSION

The lactic acid bacterial strain for inoculation in the plantain pith juice was isolated from milk samples and dairy effluents. Characterization of Lactobacillus was done by Gram and Acid-fast staining. Spore formation, Acid tolerance, Bile tolerance, Catalase, Glucose fermentation and anti-microbial tests were carried out to screen the suitable probiotic organism for adding to juice mixture. The probiotic *Lactobacillus* was then lyophilized to obtain LAB powder which was sealed, stored and analyzed. Juice was extracted from fresh plantain pith and Pasteurization was carried out at 63^{o}C for 30 minutes. Lyophilization was carried out with the fresh juice to obtain freeze-dried plantain pith juice powder, which was then sealed, stored and analyzed. The optimal composition for mixing juice powder with LAB powder was studied by trial and error method and finalized. Palm sugar was added as a sweetener to the probiotic mixture. The final product of probiotic juice was then studied for pH, titratable acidity, total carbohydrates, microbial viability and sensory evaluation was performed based on 9 point hedonic scale which showed that the sample was more acceptable than control in terms of colour, flavor, mouth feel, taste and overall acceptability. Thus the isolation of LAB from various sources and incorporating into plantain pith juice was performed and studied.

CHAPTER 6

APPENDIX

6.1. *Lactobacillus* de Mann, Rogosa & Sharpe broth (g/1000ml)

Protease peptone	10g
Beef extract	10g
Yeast extract	5g
Dextrose	20g
Polysorbate 80	1g
Ammonium citrate	5g
Sodium acetate	5g
Magnesium sulphate	0.1g
Dipotassium phosphate	2g
Manganese sulphate	0.05g
pH	6.5±0.2

6.2. *Lactobacillus* de Mann, Rogosa & Sharpe agar (g/1000ml)

To the components of *Lactobacillus* de Mann, Rogosa & Sharpe broth, add 12g of agar powder.

pH - 6.5±0.2

6.3. Gram's staining reagents:

Crystal violet stain (g/100ml):

Solution A:	
Crystal violet	2g
Ethanol (95%)	20ml
Solution B:	
Ammonium oxalate	0.8g
Distilled water	80ml
Mix solutions A and B; store for 24 hours before use.	
Gram's Iodine solution (g/300ml)	
Iodine crystals	1g
Potassium iodide	2g
Distilled iodide	300ml
Grind dry iodide crystals and potassium iodide thoroughly in a mortat, adding water (few ml at a time) and rinse into a new bottle with the rest of the water.	
Safranin solution (g/100ml) Stock Safranin solution:	
Safranin	0.25g
Ethanol (95%)	100ml

6.4. 2%Bile Media (g/1000ml):

Protease peptone	10g
Beef extract	10g
Yeast extract	5g
Dextrose	20g
Polysorbate 80	1g
Ammonium citrate	5g
Sodium acetate	5g
Magnesium sulphate	0.1g
Dipotassium phosphate	2g
Manganese sulphate	0.05g
pH	6.5±0.2
Ox Bile	20g

6.5. Minimal Agar, Davis Composition (g/1000ml)

Agar	15.0g
K_2HPO_4	7.0g
KH_2PO_4	2.0g
$(NH4)_2SO_4$	1.0g
Glucose	1.0g
Sodium citrate	0.5g
$MgSO_4.7H_2O$	0.1g

6.6. Glucose Fermentation Media (g/100ml)

Peptone	0.2g
NaCl	0.5g
Glucose	1.0g
Bromothymol blue	0.003g
Agar	0.3g
Dipotassium Hydrogen phosphate	0.03g

6.7. Samples and their collection point

S. No.	Abbreviation	Collected Location
1.	S1	Saravanampatti, Coimbatore
2.	S2	Perinayackampalayam, Coimbatore
3.	S3	Erode
4.	S4	Saibaba Colony, Coimbatore
5.	S5	Saravanampatti, Coimbatore
6.	S6	Vadhambachery, Erode
7.	S7	Vadhambachery, Erode
8.	S8	Vadhambachery, Erode
9.	S9	Vadhambachery, Erode
10.	S10	Vadhambachery, Erode
11.	S11	Perur

CHAPTER 7

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