PROBIOTIC CHARACTERISTICS OF *Lactobacilli* ISOLATE FROM TRADITIONAL FERMENTED CURD RICE

A PROJECT REPORT

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ABSTRACT

Curd rice, a fermented milk product is a traditional food commonly made in every household in India. The microbial diversity and probiotic potential of curd rice have not been clearly elucidated. Hence a study was carried out to know in detail about the probiotic characteristics of Lactobacilli from curd rice. Hetero and homo fermentative Lactic acid bacteria are found widely distributed in milk and other diary products. In recent years attention is focused o the probiotic characteristics of these bacterial isolates because of their beneficial effects. In this project, *Lactobacillus* has been evaluated for its acid and bile salt tolerance. The isolated *Lactobacillus* was exposed to an acidic environment, bile salts and gastric enzymes like pepsin and pancreatin. The organism is able to survive in mild acidic conditions (pH 4.0), can tolerate the effect of bile salt (0.3% oxgall for 3h), pepsin (3mg/ml for 1h) and pancreatin (1mg/ml for 4h) before reaching the intestinal tract. The organism is found to resist and grow in presence of most common antibiotics and some potential intestinal pathogens like *Escherichia coli* and *Bacillus sp.* From our study, we inferred that the isolated Lactobacilli is found to possess desirable probiotic properties in vitro.

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GI	Gastro Intestinal Tract
HMM	High Molecular Mass
LAB	Lactic Acid Bacteria
LMM	Low Molecular Mass
MRS	De Mann Rogosa Sharpe Medium
PBS	Phosphate Buffer Saline
RPM	Revolutions Per Minute
SCFA	Short Chain Fatty Acids
UV-Vis	Ultra Violet – Visible
WHO	World Health Organisation

INTRODUCTION

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 aeruginosa* can be harmful, while others like Bifidobacteria and Lactobacilli belonging to the so-called probiotic strains are favorable for the organism. The microflora 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 our life. 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 Bacterioides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus and Ruminococcus. The subdominant genera include Escherichia, Enterobacter, Entoercoccus, 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 species being uniquely different in each individual. Research suggest that there is a symbiotic relationship between the host and gut flora. 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 protection 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 microorganisms in healthy individuals.

Non-pathogenic, pathogenic and potentially pathogenic microorganisms 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 and Collin *et al.,* 2002). Some strains produce metabolites, such as short chain fatty acids and bacteriocins, which are of antibacterial effects. As a consequence of the relationship between cells of the mucous membrane and the microflora, the expression of certain mucosal genes may change, the cytokine release may increase, the proliferation of mucous membrane may change and produce a significant effect on the intestine-associated lymphoid t which is the largest immune organ of the organism containing 80% of cells producing antibodies (Duffy *et al.*, 1999).

In recent years the balance between the harmful bacteria and beneficial ones has been disturbed. 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 diarrhoear, inflammatory bowel disease (Shanahan, 2002) colon cancer and other gastrointestinal disorder.

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 to ferment foods and to prepare alcoholic beverages. Microorganisms were used in the 19th century with the aim of preventing and curing diseases. However it was only at the 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 work 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.* Other bacterial species like *Escherichia coli, Streptococcus sp, Enterococcus sp* and Bacteroides and some fungal species are 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 diseases, particularly of the intestine and reduction in allergy. Possible health benefits include balance of pH, prevention and treatment of diseases like acute diarrhea, inflammatory bowel diseases and other GI disorders.

Oral consumption of health-promoting lactic acid bacteria or probiotic 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 have 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 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 regions of the 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 products. The Probiotics should be antagonistic to mutagenic or pathogenic organisms in the gut. They must be genetically stable and remain viable in the final food product. Lactic acid bacteria are capable of modulating specific immune responses in gut associated and lymphoid t .This effect depends on the degree of contact with lymphoid ts and their ability to survive in the GI tract.

Curd rice is a staple constituent of Indian diet that harbours large numbers of lactic bacteria such as *Lactobacillus sp, Leuconostoc sp* and *Streptococcus sp*. It is prepared primarily by inoculating the previous day's curd into fresh milk and fermented overnight. Curd rice is easily available

and is known for its beneficial properties through years of consumptions by all classes of people.

LITERATURE REVIEW

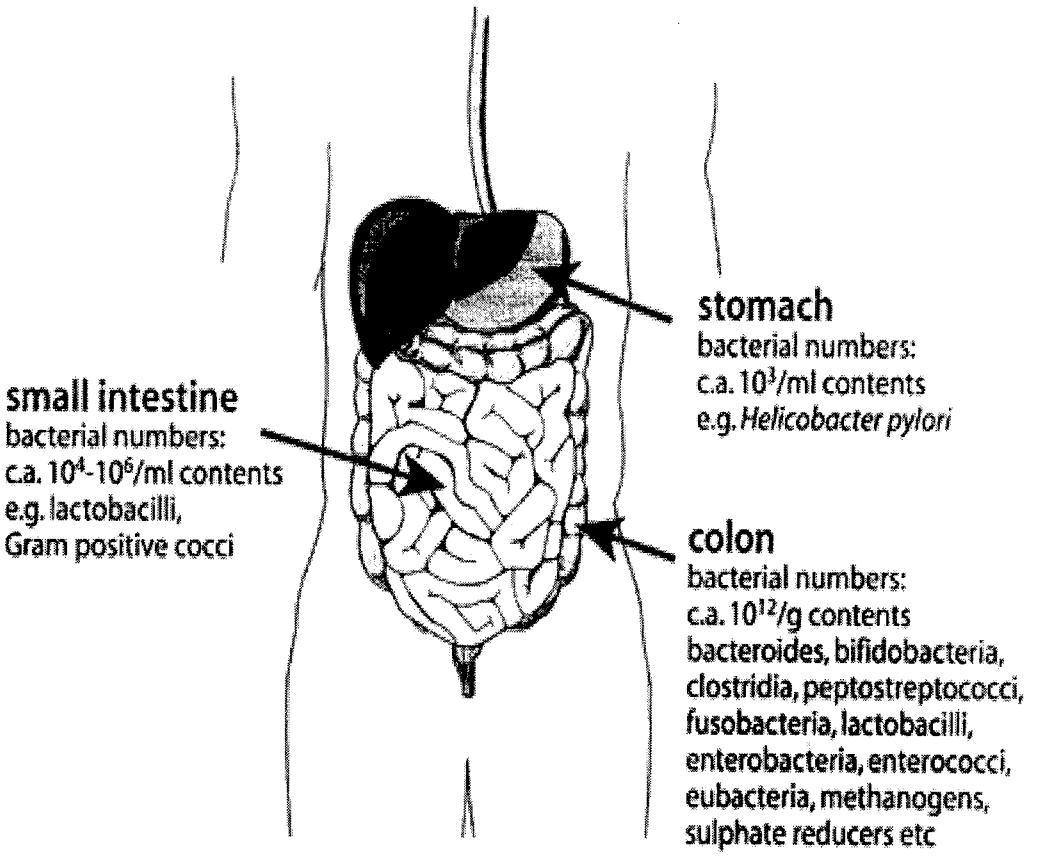
2. LITERATURE REVIEW

2.1 Microbes of the Gut flora

The intestinal microflora is a metabolically active but as yet unexplored organ of host defence. The average human body, consisting of about 10^{13} cells, has about ten times that number of microorganisms in the gut (Bjorksten *et al.*, 2001). Although bacteria are distributed throughout the intestine, the major concentration of microbes and metabolic activity is found in the large intestine. From culture based data it has been shown that the mouth harbours a complex microflora consisting of facultative and strict anaerobes, including Streptococci, Bacteroides, Lactobacilli, and yeasts. The upper bowel (stomach, duodenum, and jejunum) has a sparse microflora with content of up to 10^5 colony forming units/ml. From the ileum on, bacterial concentrations gradually increase, reaching 10^{11} to 10^{12} colony forming units/g in the colon.

The human small intestine contains approximately 10^5 bacterial cells per gram while the colon contains 10^{11} bacterial cells per gram of contents. The metabolic activity performed by these bacteria is equal to that of a virtual. Bacteria make up most of the flora in the colon and 60% of the dry mass of feces (Guarner and Malagelada, 2003). Somewhere between 300 and 1000 different species live in the gut, with most estimates at about 500. It is probable that 99% of the bacteria come from about 30 or 40 species. Predominate human microflora include *Bacteroides*, *Lactobacillus*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia*, and *Veillonella*. Fungi and protozoa also make up a part of the gut flora, but little is known about their activities.

Figure 1: Human Gastrointestinal ecology



Microbial colonisation commences immediately after birth. The maternal vaginal and intestinal flora constitutes a source of bacteria colonising intestine of the newborn. Colonisation is also determined by contact with the surrounding environment. At this stage, the dominating strains are facultative anaerobes such as the Enterobacteria, Coliforms, and Lactobacilli. The diet may exert a major effect on the composition and activity of the gut microflora. In infants, it is thought that those who are breast fed have a natural predominance of Bifidobacteria, while formula fed infants have a profile more complex and similar to the adult microflora, with Enterobacteria, Lactobacilli, Bacteroides, Clostridia, Bifidobacteria, and Streptococci (Harmsen *et al.*, 2000).

After weaning, the composition of the microflora gradually alters to resemble that of the adult. The indigenous bacteria have sometimes been classified as potentially harmful or health promoting; most of them, however, are part of the commensal flora. The strains with beneficial properties include principally Bifidobacteria and Lactobacilli. The most common probiotics are Bifidobacteria and Lactobacilli, and some of these exhibit powerful anti-inflammatory capabilities (Isolauri *et al.,* 2001). Moreover, the same genera have been attributed with other beneficial aspects such as stimulation of the immune response and competitive exclusion of pathogens, whereby non-specific host resistance to microbial pathogens is promoted.

The relationship between gut flora and humans is not merely commensal, but rather is a mutualistic, symbiotic relationship (Sears, 2005). Though people can survive with no gut flora, the microorganisms perform a host of useful functions, such as fermenting unused energy substrates, training the immune system, preventing growth of harmful species (Guarner *et al.,* 2003), regulating the development of the gut, producing vitamins for the host (such as biotin and vitamin K), and producing hormones to direct the host to store fats. However, in certain conditions, some species are thought to be capable of causing disease by causing infection or increasing cancer risk for the host (Guarner and Malagelada, 2003).

2.1.1 Localization

The colon has the greatest numbers of bacteria and the most different species, and the activity of these bacteria make the colon the most metabolically active organ in the body (Gibson, 2004). The acid in the

stomach, as well as bile and pancreatic secretions, hinder colonization of most bacteria in the stomach and proximal small intestine. Most of the bacteria in the distal small intestine are Gram-positive, while those in the colon are mostly Gram-negative (Riordan *et al.*, 2001). The first part of the colon is mostly responsible for fermenting carbohydrates (Beaugerie and petit, 2004), while the latter part mostly breaks down proteins and amino acids. Bacterial growth is rapid in the cecum and ascending colon, which has a low pH, and slow in the descending colon, which has an almost neutral pH. The body maintains the proper balance and locations of species by altering pH, the activity of the immune system, and peristalsis. Over 99% of the bacteria in the gut are anaerobes, (Vedantam and Hecht, 2003) but in the cecum, aerobic bacteria reach high densities.

2.1.2 Microbial species in the gut

Populations of species vary widely among different individuals but stay fairly constant within an individual over time, even though some alterations may occur with changes in lifestyle, diet and age. An effort to better describe the microflora of the gut and other body locations has been initiated; see Human microbiome project. Most bacteria come from the genera *Bacteroides*, *Clostridium*, *Fusobacterium*. *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium*. Other genera such as *Escherichia* and *Lactobacillus* are present to a lesser extent (Guarner and Malagelada, 2003). Species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut, suggesting that that genus is especially important in the functioning of the host (Sears, 2005). The currently known genera of fungi of the gut flora include *Candida*, *Saccharomyces*, *Aspergillus* and *Penicillium*.

2.1.3 Acquisition of gut flora in human infants

The gastrointestinal tract of a normal fetus is sterile. During birth and rapidly thereafter, bacteria from the mother and the surrounding environment colonize the infant's gut. Immediately after vaginal delivery, babies have bacterial strains in the upper gastrointestinal tract derived from the mothers' feces (Bettelheim *et al.,* 1974). Infants born by caesarean section may also be exposed to their mothers' microflora, but the main exposure is from the surroundings (Schwiertz *et al.,* 2003). All infants are initially colonized by large numbers of *E. coli* and streptococci. Within a few days, bacterial numbers reach 10^8 to 10^{10} cfu per gram of feces. During the first week of life, these bacteria create a reducing environment favorable for the subsequent bacterial succession of strict anaerobic species mainly belonging to the genera *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Ruminococcus* (Favier *et al.* 2002). Breast-fed babies become dominated by bifidobacteria, possibly due to the contents of bifidobacterial growth factors in breast milk (Coppa *et al.*, 2004). In contrast, the microbiota of formula-fed infants is more diverse with high numbers of *Enterobacteriaceae*, enterococci, bifidobacteria, *Bacteroides*, and clostridia (Harmsen *et al.*, 2000). After the introduction of solid food and weaning, the microflora of breast-fed infants becomes similar to that of formula-fed infants. By the second year of life the fecal microflora resembles that of adults.

2.2 The Role of Microorganisms in the Intestinal Tract

Introduction numerous factors influence the interactions among intestinal microorganisms as well as those between microorganisms and their hosts. The cumulative effects of these interactions control the

composition and metabolic activity of the intestinal microflora. An optimum 'balance' in microbial population has been associated with good nutrition and health. There is increasing evidence indicating that certain microorganisms can help maintain such a favorable microbial profile. The microorganisms most associated with this 'balance' are Lactobacilli and bifidobacteria.

Intestinal Flora Competition among microorganisms in the large intestine is a major consideration since the highest numbers of bacteria occur here.

Bifidobacteria are the predominant organisms in the large intestine of breast-fed infants, accounting for about 99% of the cultivatable flora. Lactobacilli, Enteterocci, and Coliforms comprise about 1% of the flora.

Bifidobacteria are a major component in the large intestine of adolescents and adults, while Lactobacilli, Enterococci, and Coliforms are a smaller component of the flora. Bifidobacteria are reduced significantly in the stools of old people, but Clostridia, Streptococci, and Coliforms are increased.

Lactobacilli are the predominant organisms in the small intestine. Lactobacilli have important metabolic activities, although they may occur in smaller numbers than Bifidobacteria in the upper and lower intestines combined. The different Lactobacilli species are aerotolerant, non pathogenic, and do not produce toxic substances or toxins. They can resist weak acids (Milton *et al* 2003), pH 3.5 to 6.5 and the yield of lactic acid is 90%. They are highly used in controlled fermentations (Nicole Roos and Martijn Katan, 2000).

2.3 The Role of Lactobaccilli in the Intestinal Tract

2.3.1 Antibiotic Production

Inhibits the pathogenic flora by production of the following antibiotics

Lactolin (*L. plantarum*)
Lactobrevin (*L. brevis*)
Bulgarican (*L. bulgaricus*)
Acidophilin (*L. acidophilus*)
Lactocidin (*L. acidophilus*)
Acidolin (*L. acidophilus*)
Lactolin (*L. acidophilus*)

The strains vary in their ability to produce these substances and cultural conditions will influence the amount produced.

In vitro inhibitory activity has been reported against the following intestinal pathogens:

Salmonellae
Shigellae
Staphylococci
Proteus
Kelbsiella
Pseudomonads
Enteropathogenic *Escherichia coli*
Bacilli

Clostridium perfringens

Vibrio

2.3.2 Organic Acid Production

Lactobacilli produce lactic acid. Organic acetic and lactic acids which are produced by lactic acid bacteria will inhibit the growth of many bacteria, especially pathogenic gram-negative types. Lactobacillus acidophilus produces DL-lactic acid which is metabolized to a limited extent.

2.3.3 Lower pH and Oxidation Reduction

Inhibition of pathogens by Lactobacilli is attributed to the lowering of the pH values by the liberation of acids, resulting in antimicrobial action (altering oxidation-reduction potential).

2.3.4 Competitive Antagonists

Lactobacilli may compete with other bacteria for nutrients and occupy the sites, making them unavailable to other microorganisms.

In particular, Lactobacilli consume certain B-vitamins and biotin, decreasing their availability for other organisms.

2.3.5 Bile Deconjugation

The role of Lactobacilli in the deconjugating of bile acids was studied. The results indicated that Lactobacilli can liberate (Deconjugate) free bile acids in the intestinal tract and can exert an influence on the balance of bacteria present It shows how these friendly organisms can be helpful in

Osteoporosis, cholesterol problems, with antibiotic action, with lactose intolerance, nutrient deficiencies, anxiety, cancer, skin problems, liver detoxification, diarrhea, children's needs and other uses.

2.4 Functions of the gut flora

2.4.1 Carbohydrate fermentation and absorption

Without gut flora, the human body would be unable to utilize some of the undigested carbohydrates it consumes, because some types of gut flora have enzymes that human cells lack for breaking down certain polysaccharides (Sears, 2005). Rodents raised in a sterile environment and lacking in gut flora need to eat 30% more calories just to remain the same weight as their normal counterparts. Carbohydrates that humans cannot digest without bacterial help include certain starches; fiber; oligosaccharides and sugars that the body failed to digest and absorb like lactose in the case of lactose intolerance and sugar alcohols, mucus produced by the gut, and proteins (Gibson, 2004).

Bacteria turn carbohydrates they ferment into Short Chain Fatty Acids, or SCFAs (Gibson, 2004). These materials can be used by host cells, providing a major source of useful energy and nutrients for humans. They increase the gut's absorption of water, reduce counts of damaging bacteria, increase growth of human gut cells, and are also used for the growth of indigenous bacteria. Evidence also suggests that bacteria enhance the absorption and storage of lipids (Sears, 2005). Bacteria also produce and help the body absorb needed vitamins like vitamin K

2.4.2 Repression of pathogenic microbial growth

Another important role of helpful gut flora is that they prevent species that would harm the host from colonizing the gut. Yeasts and harmful bacterial species such as *Clostridium difficile* (the overgrowth of which can cause pseudomembranous colitis) are unable to grow too much due to competition from helpful gut flora species to adhere to the mucosal lining of the intestine, thus animals without gut flora are infected very easily. The barrier effect protects humans from both invading species and species normally present in the gut at low numbers, whose growth is usually inhibited by the gut flora (Guarner and malagelada, 2003).

The most important role of LAB is its protective role against infections and against the colonization of pathogen microorganism in the digestive tract. In most cases the inoculum passively transits the gastro-intestinal tract. The microbial interactions are mainly responsible for the maintenance or the alteration of the intestinal microflora of the host; they form the basis of the mechanisms by which the stabilized microflora opposes the stability of microorganisms that the host organism ingests daily (Fuller 1993). The first line of defense of the host against an intestinal infection consists of colonization resistance, competitive exclusion or barrier effect. The barrier effect of the intestinal microflora can be preventive or curative depending on its manifestation of pathogen organism (Vamanu *et al.,* 2006). If this effect is drastic, the undesirable bacteria are totally eliminated. If it is permissive, then the pathogen organisms are maintained in the gastro-intestinal tract at a population level below one which would be attained in the absence of the barrier effect (Vamanu *et al.,* 2006). In this case, the

development of the pathogenic microorganisms is repressed but not eliminated by the intestinal flora (Fuller, 1989).

Helpful bacteria prevent the growth of pathogenic species by competing for nutrition and attachment sites to the epithelium of the colon. Symbiotic bacteria are more at home in this ecological niche and are thus more successful in the competition. The indigenous bacteria send chemical signals to the host about the amount of nutrients they need, and the host provides only that much, so harmful bacteria are starved out. Indigenous gut floras also produce bacteriocins which are proteinacious toxins that inhibit growth of similar bacterial strains, substances which kill harmful microbes and the levels of which can be regulated by enzymes produced by the host. The process of fermentation, since it produces fatty acids, also serves to lower the pH in the colon, preventing the proliferation of harmful species of bacteria and facilitating that of helpful species.

2.4.3 Immunity

Gut flora have a continuous and dynamic effect on the host's gut and systemic immune systems. The bacteria play a key role in promoting the early development of the gut's mucosal immune system both in terms of its physical components and function and continue to play a role later in life in its operation. The bacteria stimulate the lymphoid t associated with the gut mucosa to produce antibodies to pathogens. The immune system recognizes and fights harmful bacteria, but leaves the helpful species alone, a tolerance developed in infancy (Shanahan, 2002).

Certain strains of bacteria are capable of improving the barrier function of the mucous membrane and increase the differentiation of B cells as well as IgA secretion (Collin *et al.*, 2002). The probiotics can influence the unspecific immunity, which consists of 2 systems, one which acts through the antibodies secreted by lymphocytes B (humoral immunity) and another which acts directly through lymphocytes T (cell mediated immunity). The 2 systems communicate through chemical substances named interleukines. The increase in the specific immune response corresponds with the activity of lymphocytes B and T, which leads to an increase of interleukine and the levels of circulating antibodies (immunoglobulins M and immunoglobulins G). The probiotics also have an effect on the production of antibodies (mainly immunoglobulins A) in the intestinal lumen. Immunoglobulins A are very important in the digestive tract, being the first defense against infection, in contact with the antigens present in the digestive tract (Vamanu *et al.,* 2004). Immunoglobulins A can inhibit the adhesion of pathogenic bacteria on the surface of the mucus membrane of the digestive tract by the agglutination of bacteria, fixation on adhesive proteins which are present on the surface of bacteria, and the interference in the interaction of adhesive substance/cellular receptors (Pedersen and Roos, 2004).

Lactic bacteria have antitumor properties inactivating or inhibiting the carcinogenic compounds in the gastrointestinal tract, the stimulation of the immune response, reduction of the enzymatic activity: b-glucuronidase, azoreductase and nitroreductase which are known to convert precarcinogens into carcinogens.

2.5 Effects of antibiotic use

Altering the numbers of gut bacteria, for example by taking broad-spectrum antibiotics, may affect the host's health and ability to digest food (Carman *et al.,* 2004). People may take the drugs to cure bacterial illnesses or may unintentionally consume significant amounts of antibiotics by eating the meat of animals to which they were fed. Antibiotics can cause antibiotic-Associated Diarrhea (AAD) by irritating the bowel directly, changing the levels of gut flora, or allowing pathogenic bacteria to grow (Beaugerie and petit, 2004). Another harmful effect of antibiotics is the increase in numbers of antibiotic-resistant bacteria found after their use, which, when they invade the host, cause illnesses that are difficult to treat with antibiotics.

2.6 Probiotics

2.6.1 Introduction

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 preventative 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 SStreatment. The condition of the gastrointestinal tract is essential for our well being. The microflora limits the ability of potential pathogens to infect. It boosts the body's ability to resist infection, prevents morbidity and

decrease antibiotic use (the spread of antibiotic resistant pathogens). 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 microflora 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 new 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, placebocontrolled human trials.

2.6.2 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 treatment of body ailments, which has been mentioned even in Biblical scriptures. Similarly in Ayurveda, one of the pioneering medical sciences that date back to around 2500 BC, the consumption of yogurt has been recommended for the maintenance of overall well being. Well-known scientists in the early ages such as Hippocrates considered fermented milk not only as a food product in terms of nutrition but also as a 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 consumption of large quantities of cultured foods such as sour milk, butter milk and yogurt containing the microorganism termed as *Bulgarican bacillus* helped to maintain good health and long life of peasants. Metchnikoff suggested that the lactic acid bacteria in fermented milks eliminated 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 *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.

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

2.6.3 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). Bacteria that exert a beneficial effect in intestinal function and promote good health are therefore known as probiotic bacteria.

The three main aspects of the definition are:

- The micro-organisms (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 activity such as improved digestion of lactose, immune system modulation and antihypertensive 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 concept of Probiotic-Active Substance was introduced as a cellular complex of lactic acid bacteria that have a capacity to interact with the host mucosa and may beneficially modulate the immune system independently of the viability of Lactic acid bacteria.

2.6.4 Description

In general, Lactobacilli is the largest genus of the lactic acid bacteria group and includes over 50 species. Lactobacilli commonly inhabit the gastrointestinal (GI) tract, oral, and vaginal regions of humans and animals.

Family: Lactic Acid Bacteria

L. acidophilus is a member of one of the eight main genera of lactic acid bacteria. The genera, Lactobacillus, Streptococcus, Lactococcus, Leuconostot, Bifidobacterium, Carnobacterium, Enterococcus and Sporolactobacillus can then be divided into species, subspecies, variants and strains. Each genus and species have different characteristics but they are generally chained cocci or rod shaped gram positive, nonmotile, nonsporulating bacteria that produce lactic acid as a major or sole product of fermentative 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 and in some species also acetic acid, ethanol and carbon dioxide. Lactic acid bacteria that only produce lactic acid as an end product are called homofermentative; those that also produce acetic acid, ethanol and carbon dioxide are termed heterofermentive. 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.

Genus: Lactobacillus

There are many species of Lactobacillus bacteria that are found in a variety of environments, from dairy products to the human gastrointestinal tract (GI). They are either micro-aerophillic or anaerobic and strictly fermentative. The G + C content of their DNA is between 32 and 51 mol %. The optimum growth occurs within 35-40°C but it can tolerate temperatures as high as 45°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. plantarunm, L. brevis, L. caesi, L. leichmanii*, and *L. mintus*. 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 environments of pH 4-5 or lower. This pH is well below the pH other lactic acid bacteria can live in and because of this; Lactobacillus is responsible for the final stages of fermentation in products.

Species: *L. acidophilus*

L. acidophilus is probably the best well-known species of Lactobacillus. *L. acidophilus* is naturally found in the human and animal GI tract, mouth and vagina. It can also be found in certain dairy products and freeze dried in capsule and tablet form in grocery and health food stores. *L. acidophilus* is characterized as being a rod shaped motile bacteria grows in

or without the presence of oxygen. *L. acidophilus* is also characterized as a homo fermentative that only produces lactic acid as its sole product.

2.6.5 Dairy Industry

There are many types of fermented dairy products that use *L. acidophilus*. Sweet acidophilus milk is consumed by individuals who suffer from lactose maldigestion and intolerance, a condition that effect approximately 75% of the world's population Maldigestion and intolerance occurs when enzymes (Lactase) cannot break down lactose or milk sugar in the intestine. Failure to digest lactose results in discomfort, cramps and diarrhea.

Sweet acidophilus milk is made by inoculating milk with the *L. acidophilus* bacteria. After inoculation, the milk sets for 24 hrs and yields a type of buttermilk that has a low content of lactose. *L. acidophilus* is also used in the preparation of yogurt. *L. acidophilus* along with other lactic acid bacteria is added to milk to decrease the pH. When milk becomes acidic, proteins in the milk break down and coagulate to form a gel.

Lactobacillus acidophilus and other Lactic acid bacteria are important in the fermentation of many foods from dairy products to fruits and vegetables. Fermentation occurs when bacteria break down sugars and carbohydrates to produce alcohol, carbon dioxide and lactic acid. These by-products are responsible for the unique taste of fermented foods and help preserve and increase palatability.

In recent years the market potential of probiotic dairy products has increased rapidly throughout Europe, North America, Japan, Australia and other developed countries. The probiotic food market is worth $ 1.3 billion Europe and growing in most countries. The enhanced awareness among consumers on the role of probiotic bacteria in disease prevention and health maintenance, increase in aging population, the rising costs of health care and stressful lifestyles has created a huge potential for the rapid increase in probiotic food market in the coming years.

2.6.6 Nutritional Supplementation

The natural health industry claims that *L. acidophilus* is a probiotic or 'friendly' organisms that helps the body fight disease and restore health. There are an enormous amount of health claims associated with taking L. acidophilus, these claims vary from relieving constipation to killing deadly strains of *E.coli* and Salmonella.

L. acidophilus can help maintain a healthy balance of intestinal flora by increasing acidity of the intestine, killing off harmful bacteria. It was demonstrated to have anti-microbial effects against pathogens and fungal microorganisms (Buttris, 1997). Also since *L. acidophilus* is able to survive in environments of pH 4-5 or below, it is able to survive the harsh conditions of the stomach and pass through to the small intestine. Eating foods high in *L. acidophilus*, or taking supplements, boost our immune system.

Nowadays several strains of probiotic bacteria are known, but their utilization is restricted by the fact that an effective probiotic is supposed to proceed along the acidy 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 days to a few weeks and it usually lasts for a short period of time following its regular intake. Colonizing probiotics compete with other microrganisms for nutrients and appropriate binding sites. Only probiotics capable of colonizing even if only temporarily, can exert the required immunological effect (Saxelin, 1996). The *in vitro* efficacy of the well-known probiotic strains has already been proved in clinical practice (O'Mahony *et al.,* 2001). The most widely used strains are those taken from sour dairy products and the intestinal system. The most frequently studied species include various species of Lactobacilli, Streptococcus, Bifidobacteria, *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 improving foods, namely functional foods, individual probiotic strains need to meet certain requirements (Ouwehand and Salminen, 1999 and Saxelin *et al.,* 1999). The following criteria are listed for effective probiotic bacteria (Salminen *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 technology and storage
- High degree of stability during storage
- 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 (one billion) of live bacteria delivered to the appropriate site - the large intestine for *Bifidobacterium bifidium*, the small intestine for *Lactobacillus acidophilus* - is required for efficacy.

2.6.7 Significance

Lactobacilli have many important roles in industry. They contribute to the production of some cheeses, yogurt, and other products. The lactic acid produced by Lactobacilli inhibits the growth of other organisms and lowers the pH of the product in these 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 Lactobacilli's metabolic reactions are intentionally manipulated to breakdown milk proteins during cheese production.

Lactobacillus acidophilus, is one of the most important bacteria found in our body, and generally resides in our digestive tract. It has been suggested that for bacteria to act as probiotics they must arrive in the intestines alive and in sufficient number to have an effect or they should adhere or implant and multiply.

Lactobacillus have been examined for their effectiveness in the prevention and treatment of a diverse spectrum of gastrointestinal disorders such as antibiotic-associated diarrhea (including *Clostridium difficile*-

associated intestinal disease), infectious bacterial and viral diarrhea (including diarrhea caused by rotavirus, *Shigella, Salmonella*, enterotoxigenic *E.coli*, *Vibrio cholerae* and human immunodeficiency virus/acquired immunodeficiency disorder, enteral feeding diarrhea, *Helicobacter pylori* gastroenteritis, sucrase maltase deficiency, inflammatory bowel disease, irritable bowel syndrome, small bowel bacterial overgrowth and lactose intolerance.

Interaction of host / microorganisms occurs by enzyme production which helps digestion (lactase), 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 bacterial 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, inhibition of epithelial and mucosal adherence, inhibition of epithelial invasion and production of antimicrobial substances. Other Furthermore, they can be taken as a preventive against food poisoning when traveling.

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 favoring the growth of useful microorganisms (Ducluzeanu and Raibaud 1989).

There are 2 ways to reduce the formation pathogenic germs:

1. Production of substances with selective antibacterial effect (organic acids or hydrogen peroxide which are favorable)

2. Competition for the active sites on the digestive lumen walls (usually won by the organisms which are predominant)

2.6.7.1 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 increases in the incidence of illnesses associated with deficient or compromised microflora (e.g Gsastrointestinal 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 immunomodulation, we are also 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 basis for the development of innovative biotechnology-based products tailored to prevent specific diseases and promote overall human gastrointestinal health.

2.6.7.2 Probiotics Enhance Anti-infective Defences in the GI Tract.

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

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

2.6.7.3 Antagonistic property of lactobacilli

The antagonistic property is attributed to the lowered pH, the 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 food 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 antimicrobial compounds, which can be classified as Low-Molecular-Mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-utanedione), uncharacterized compounds, and High-Molecular-mass (HMM) compounds like bacteriocin.

2.6.7.4 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 lactose concentration in the product resulting in high lactase activity increasing the active lactase 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 hyrolysis. Therefore, there is good scientific evidence on the alleviation of lactose intolerance symptoms by specific probiotic bacteria.

2.6.7.5 Rotavirus diarrhea

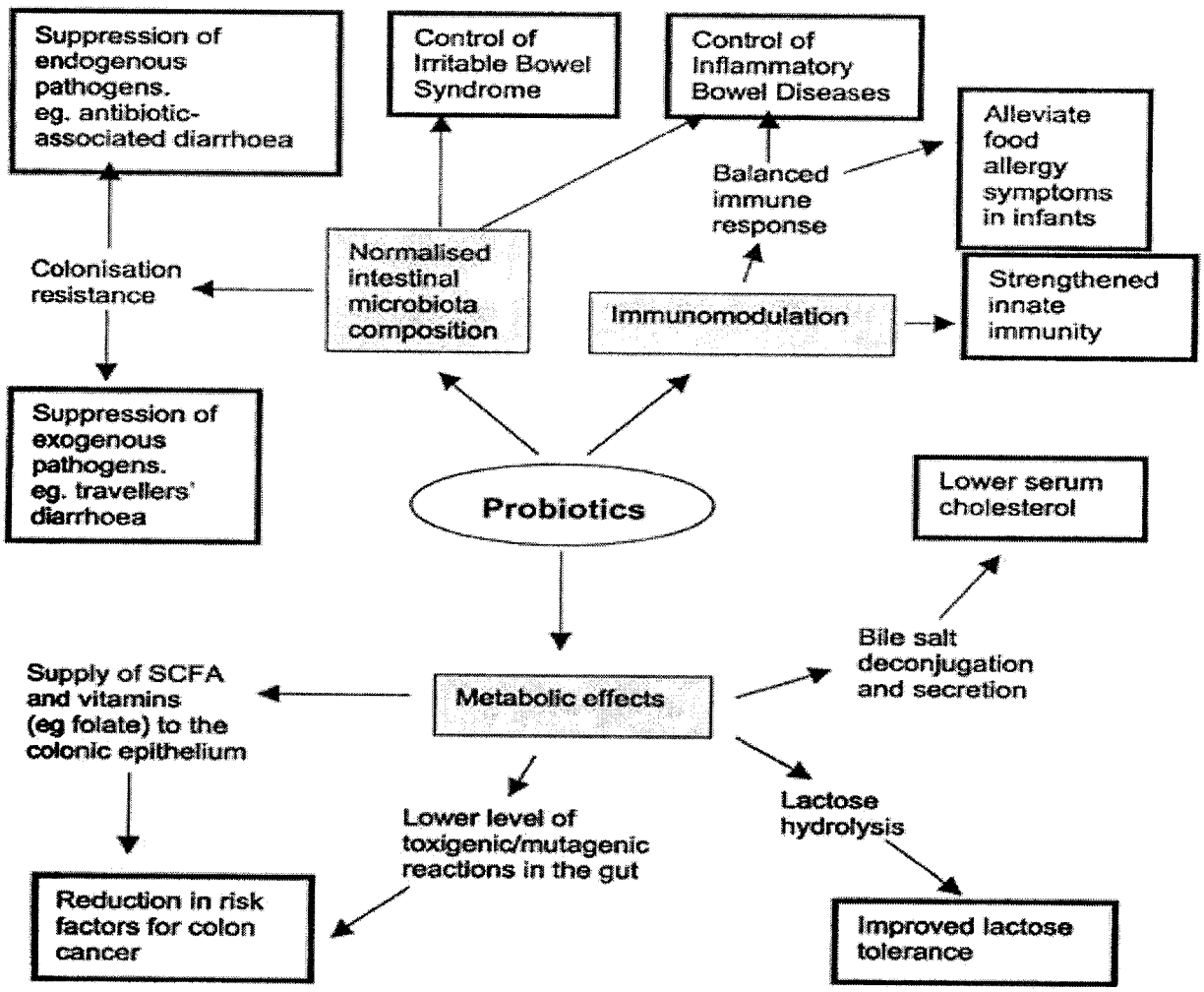
Several studies have shown selected probiotics such as *L. reuteri, L. casei Shirota, B. lactis* Bb12 and *L. delbrueckii* sub sp. *bulgaricus* can shorten the duration of rotavirus diarrhea by approximately one day. Shortening of the duration of rotavirus diarrhea using the probiotic strain *L. rhamnosus* GG is perhaps the best documented probiotic effect. The mechanism behind this favourable outcome is associated with enhancement of 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-infections.

2.6.7.6 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 microbiota composition precede the development of some allergic diseases (Bjorksten *et al.,* 2001). This therefore indicates potential application of probiotic bacteria in this area. Administration of *L. rhamnosus* GG and *B. lactis* Bb12 prenatally to mother and during the first few months to infants with high risk of atopic disease, reduced the prevalence of atopic eczema (Isolauri *et al.,* 2001). Additionally, supplementation of extensively hydrolyzed whey formula with *L. rhamnosus* GG or *B. lactis* Bb12, has been found to be more effective than unsupplemented formula on eczema alleviation in infants with atopic eczema (Isolauri *et al.,* 2001).

Figure 2: Therapeutic benefits of probiotic bacteria



Proposed health benefits stemming from probiotic consumption.

2.7 How This Food Supplement Works in our Body

1. Improve efficiency of our digestive system.
2. Manufacture vitamins, such as, biotin and vitamin K.
3. Produce antibacterial substances 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 related to Long-term antibiotic use.

Research shows that stomach acid kills many Lactobacillus strains on contact which 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.

Even if there is scientific proof that a certain strain has probiotic characteristics, it does not mean that the strain is effective in every person, as each person has his/her own intestinal flora, which may limit the effectiveness of a probiotic product.

Figure 3: *Lactobacillus acidophilus*

2.8 Safety of probiotics for human administration

In spite of the fact that *L. acidophilus* and *Bifidobacterium* spp. are the most common probiotic dietary cultures, *Saccharomyces boulardii*, *Escherichia coli* and *Enterococcus* strains have also been reported to have probiotic qualities. These strains have also been used to treat traveller's diarrhea and inflammatory bowel disease. The benefits of probiotic bacteria should however usually outweigh any potential risks. Probiotic strains for human consumption should be proven to be of low risk in inducing or being associated with any disease (FAO / WHO, 2002). As compared to the other probiotic species, Lactic acid bacteria have a good safety record and are rarely involved in disease. The most commonly used probiotics, which include *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp. have been accorded the GRAS (Generally Recognized As Safe) status (Salminen *et al.,* 1998). It is normally difficult for one strain to satisfy all the desirable criteria, but most *L. acidophilus* and *Bifidobacterium* spp exhibit all these characters (Sears, 2005).

2.9 Introduction to Functional foods

In the recent years, the stressful lifestyles, deterioration in personal health and lack of exercise has led to an increase in the consumer awareness about foods that are nutritional and have dietary benefits. The reduction on health care expenditures by government has also 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 benefits. 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. Among the most promising targets for functional foods are the gastrointestinal functions including those that control transit time, bowel habits, balanced colonic microflora with control of nutrient bioavailability. This can in turn modify the gastrointestinal immune activity. Some other functions are lipid homeostasis, which indirectly influence nutrient digestion or fermentation. The functional food market has also been successful in UK, Japan and Australia.

2.10 Prebiotics

The application of probiotics has been supplemented with the concept of prebiotics. A prebiotic is defined as a nondigestible 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 microflora which may evince a health benefit to the host. However, prebiotics can also be non-absorbable substrates which stimulate the growth of probiotics. When the two are applied together the concept is defined as synbiotic. At present, most prebiotics are directed towards the growth of lactic acid bacteria due to their purported health promoting properties.

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

2.11 Synbiotics

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

OBJECTIVES

OBJECTIVE

1. To quantitate the bacteria in curd rice.
   - -Total bacterial count.
   - -Lactobacilli count.
2. To isolate pure lactobacilli.
3. To characterize organism through gram staining and catalase test.
4. To evaluate the probiotic potential of the isolates by testing the viability of organism against
   - -Pathogens
   - -pH, Bile salt and
   - -Other condition in GI tract.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Preparation of Curd Rice

Milk was inoculated with a small amount of curd sample and allowed to form curd. Then it was mixed with equal amount of boiled rice and allowed to ferment overnight. This fermented curd rice is used as the source of Lactobacilli.

3.2 Isolation of Lactic acid bacteria from curd rice

Microbial strain was isolated from curd rice and cultured on MRS broth (de Mann, Rogosa and Sharpe, 1990). They were incubated for 16 h at 37°C under anaerobic conditions. A single pure culture was isolated for further study.

Materials

1. Starter cultures (curd rice samples)
2. Lactobacillus MRS agar
3. Nutrient agar
4. Petri plates
5. Conical flask
6. Inoculation loop

Procedure

1. One gm of fermented curd rice sample is taken and serially diluted.
2. 5^{th} and 6^{th} dilutions were spread plated on Nutrient agar and MRS agar.

3. Plates were incubated overnight in anaerobic condition at 37°C and observed for colonies.
4. Total bacteria and Lactobacilli count was determined.

3.3 Selection of Lactobacilli

From the MRS agar plates colonies were taken and Quadrant streaking was done to isolate individual colonies.

3.4 Characterization of Curd Isolates

The curd samples were plated on MRS agar and morphologically different colonies were isolated. These colonies underwent screening by gram staining and the catalase test.

3.4.1 Catalase test

Catalase test was done as per the general procedure and is as follows, The Catalase test identifies organisms which produce the enzyme Catalase; this enzyme converts hydrogen peroxide to water and oxygen gas. Catalase helps protect bacterial cells against hydrogen peroxide. Hydrogen peroxide is a highly reactive compound which damage 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. A small drop of water was placed on glass slide.
2. Using a sterile inoculation loop a bacterial colony was smeared on the slide.
3. Using a dropper a few drops of hydrogen peroxide were added to the smear and observed for any signs of bubble evaluation.

3.4.2 Gram staining

Gram staining is an empirical method of differentiating bacteria into two large groups based on differences 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 crystal violet. Iodine was subsequently added as a mordent 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 decolorised gram negative bacteria a pink color.

Materials

1. Microbial stain
2. Crystal violet

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

Procedure

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

3.5 Growth curve analysis

The strain was subjected to growth curve analysis in order to determine the time period during which they experienced exponential growth. The following experiments were then carried out when the strains were in their log phase.

Materials

1. MRS broth
2. Conical flask
3. Fresh microbial culture
4. Cuvette
5. UV-Vis spectrophotometer
6. Micropipette & tips

Procedure

1. Broth cultures of 1 % (w/v) of were inoculated into 100ml sterile MRS broth.
2. The broth was incubated for 48h at 37°C.
3. The absorbance was measured at 600 nm using UV-Vis spectrophotometer every 2h.
4. A graph was plotted between the time and absorbance values for each of the cultures and by observing the curves obtained, the log phase of the bacterial cultures was ascertained.

3.6 Survival under conditions of human intestinal tract

In order for a probiotic strain to exert its beneficial effects on the host, it must be able to traverse the GI tract with minimum loss of viability. In other words, it must be capable of survival in an acidic environment, be resistant to bile salts and gastric enzymes. It must adhere to and colonize the intestinal epithelium and exert antimicrobial activity against pathogens. The following tests were designed to test the probiotic potential of the isolated strain.

3.6.1 Acid resistance test

The stomach has a pH typically ranging from 1.0 during fasting to 4.5 after a meal. Lactobacilli and related species are capable of growth at a pH of 4.5. Therefore, the resistance of the microorganisms to pH ranging from 1.0 to 5.0 was assessed.

Materials

1. 16h microbial culture

2. MRS broth at pH ranging from 1.0 to 5.0
3. MRS agar plates
4. Micropipette & tips
5. L-rod

Procedure

1. Sterile MRS broth at different acidic condition (pH 1.0 to pH 5.0) was prepared.
2. 16h Lactobacilli culture was inoculated and grown for 24h at 37°C
3. Optical density reading at 600nm was determined.
4. Plating was done to find the effect of acid on Lactobacilli growth.

3.6.2 Bile salt tolerance test

The bile salts sodium glycocholate and sodium taurocholate are produced by the liver from cholesterol and are then stored in the gall bladder. Bile salts serves to reduce cholesterol levels, emulsify lipids and fat soluble vitamins and aid in the reduction of bacterial flora found in the small intestine and biliary tract. Thus, the test strains are required to be tolerant to bile salts in order to adhere to and inhabit the small intestine.

Materials

1. 16h microbial culture
2. MRS broth supplemented with 0.3% (w/v) Ox Gall
3. MRS agar plates
4. Sterile Phosphate buffered saline (pH 7.2)
5. Micropipette and tips
6. L-rod

Procedure

1. 2ml of 16h growth culture was centrifuged at 10000 rpm for 2min.
2. The supernatant was drained. To the pellet, sterile Phosphate buffer saline (pH 7.2) was added and centrifuged at 10000 rpm for 1min.
3. Step 2 was repeated.
4. The pellet was re-suspended in 2ml of MRS broth (with 0.3% oxgall) for 3h at 37°C.
5. The suspension was spread plated on MRS agar at 0h and 3h. The plates were incubated at 37°C for 24h.
6. Colonies were enumerated after incubation.

3.6.3 Antibiotic sensitivity test

To determine the sensitivity or resistance of the strain to the antibiotics, the Kirby-Bauer method was followed (Maragkoudakis et al., 2006). Six commonly used antibiotics, namely Amikacin, Bacitracin, Chloramphenicol, Gentamicin, Penicillin, Tetracycline were in the study.

Materials

1. Antibiotic discs (fixed antibiotic concentrations)
2. 16h broth culture
3. MRS agar plates

Procedure

1. Bacterial culture was streaked onto the MRS agar plates using a inoculation needle.
2. Antibiotics discs were placed on the inoculated media plates.
3. The plates were incubated for 24h at 37°C.

4. After incubation, plates were observed for zones of inhibition around the antibiotics discs.

3.6.4 Antibacterial activity test

Some strains of lactic acid bacteria produce bacteriocins which are antimicrobial compounds. They are also capable of limiting pathogen growth by means of organic acids and other metabolic end products they generate. The test strain was examined for the extent of their antibacterial activity against *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Bacillus sp* plates by the agar well diffusion method.

Materials

1. 24h broth culture
2. Pathogen broth cultures (*Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Bacillus sp*)
3. Gel puncher
4. Micropipette and tips

Procedure

1. *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Bacillus sp* plates were prepared.
2. 2ml of 24h broth culture was centrifuged at 10000 rpm for 2min and supernatant was retained.
3. Wells were punctured in plates using a sterile gel puncher.
4. Cell free supernatant was added to the wells formed on the plates, in varying amounts like 10 µl, 20 µl and 30 µl.
5. The plates were incubated for 24h at 37°C.

6. After incubation, plates were observed for zones of inhibition around the wells.

3.6.5 Pancreatin resistance test

Pancreatin is a mixture of several digestive enzymes produced by exocrine cells of the pancreas. It is composed of amylase lipase and protease. Pancreatin digests food only at alkaline pH. The test strain was exposed to pancreatin at pH 8.0 to find out if they are tolerant to the enzyme mixture.

Materials

1. 16h microbial culture
2. MRS agar plates
3. Sterile Phosphate buffered saline (pH 8 [supplemented with 1mg/ml pancreatin] and pH 7.2)
4. Microfuge tubes
5. Micropipette and tips
6. L-rod

Procedure

1. 2ml of 16h growth culture was centrifuged at 10000 rpm for 2min.
2. To the pellet sterile phosphate buffer (pH 7.2) was added and centrifuged at 10000 rpm for 1min.
3. Step 2 was repeated.
4. To the pellet obtained, 2ml of PBS of pH 8.0 (supplemented with 1mg/ml of pancreatin) was added.
5. The suspension was incubated at 37°C for 4h.

6. Spread plating was carried out at 0h and 4h on MRS agar plates. The plates were incubated at 37°C for 24h.
7. Colonies were enumerated after incubation.

3.6.6 Pepsin resistance test

Pepsin is a digestive protease that functions to degrade proteins into peptides. The stomach cells release pepsinogen which is activated in the presence of hydrochloric acid. To stimulate the gut conditions, the test strain was exposed to pepsin at pH 2.0 to evaluate their resistance to the enzyme

Materials

1. 16h microbial culture
2. MRS agar plates
3. Sterile Phosphate buffered saline (pH 2 [supplemented with 3mg/ml pepsin] and pH 7.2)
4. Microfuge tubes
5. Micropipette and tips
6. L-rod

Procedure

1. 2ml of 16hr growth culture was centrifuged at 10000 rpm for 2min.
2. To the pellet sterile phosphate buffer (pH 7.2) was added and centrifuged at 10000 rpm for 1min.
3. Step 2 was repeated.
4. To the pellet obtained, 2ml of PBS (Phosphate Buffer Salin) of pH 2.0 (supplemented with 3mg/ml of pepsin) was added.

5. The suspension was incubated at 37°C for 1h.
6. Spread plating was carried out at 0h and 1h on MRS agar plates.
7. Colonies were enumerated after incubation at 37°C for 24h.

RESULTS

4. RESULTS

4.1 Enumeration of bacterial population in curd rice/gm of dry weight

Table 1: Enumeration of bacteria in curd rice

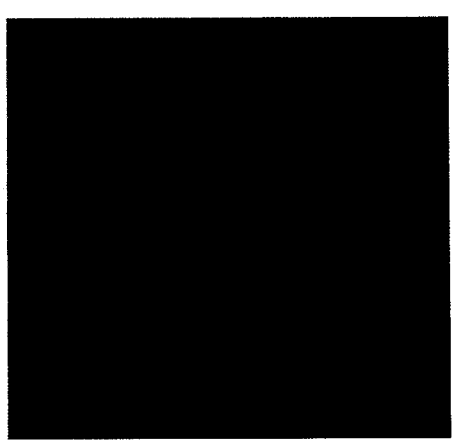
Dilution	**Total bacteria**		***Lactobacilli***	
	10^{-5}	10^{-6}	10^{-5}	10^{-6}
Cfu/g of dry wt	8955	895	16119	2313

Figure 4: *Lactobacilli* colony

4.2 Characterization of Curd Isolate

4.2.1 Gram staining

Figure 5: Isolated *Lactobacillus* cells



Morphologically different colonies on MRS agar plates were subjected to Gram staining. Most of the colonies were gram positive in nature, differing in morphology as rods or cocci. A single gram positive bacterium was chosen for further study (Fig 5).

4.2.2 Catalase test

Bacteria converted hydrogen peroxide to water and oxygen gas resulting in the formation of bubbles. Since Lactobacilli are known to be catalase negative bacteria, only catalase negative bacteria are chosen for further study.

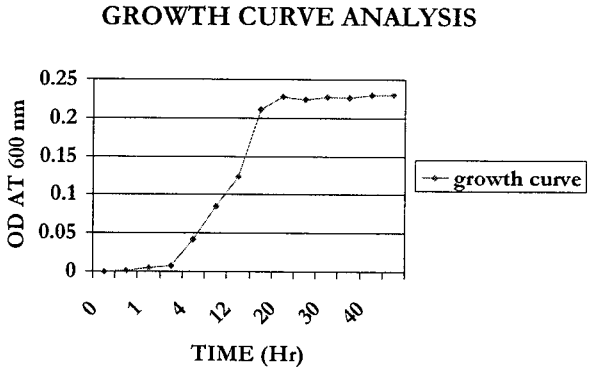
4.3 Growth curve analysis

The growth curve analysis was done for the isolated strain. The log phase was found after 16h of growth at 37°C (Fig 6).

Table 2: Growth curve analysis

Time(hr)	OD at 600nm
0	0.000
0.25	0.001
01	0.005
02	0.007
04	0.042
08	0.086
12	0.123
16	0.211
20	0.227
24	0.224
30	0.227
34	0.226
40	0.230
48	0.230

Figure 6: Growth curve analysis



4.4 Evaluation of probiotic potential

For use in foods, probiotic microorganisms should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut. This means that they should be resistantto gastric juices and be able to grow in the presence of bile under conditions in the intestines.

4.4.1 Resistance to gastric juice

The strain was exposed to pH ranging from 1.0 to 5.0. The strain is unable to survive under mild acidic conditions. In this test, maximum survival was found at pH 5.0 (Fig 7).

Table 3: Survival of *Lactobacillus* under different acidic conditions

pH	OD at 600nm
1.0	0.002
2.0	0.008
3.0	0.014
4.0	0.096
5.0	0.121

4.4.2 Resistance to bile salt

The strain was exposed to bile salt over a period of 3h and plated on MRS agar at regular intervals. On performing a colony count, it was found that there was no reduction in viability. The strain was thus tolerant to bile salt.

Table 4: Survival of *Lactobacillus* after bile treatment

S.No	Time (hr)	cfu/ml ($x10^2$)
1	0	155
2	1	142
3	3	124

4.4.3Antibiotic sensitivity test

The strain was subjected to the Kirby bauer method to determine their sensitivity to 6 antibiotics. Following incubation for 24h, the zones of incubation formed with the various antibiotics were compared with standard charts (Fig 8).

Table 5: Resistance of *Lactobacillus* to antibiotics

S.No	Name of the Antibiotic	Zone of inhibition (cm)
1	Tetracycline	1.8
2	Gentamycin	1.7
3	Penicillin	1.7
4	Amikacin	1.4
5	Chloramphenicol	1.3
6	Bacitracin	-

4.4.4 Antibacterial activity test

The well diffusion method was carried out for the strain to determine their inhibitory effect on four pathogens namely *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Bacillus sp.* Zone of inhibition was formed against *Escherichia coli* and *Bacillus sp* but not against *Staphylococcus aureus* and *Salmonella typhi.* (Fig 9).

Table 6: Inhibition zone of *Lactobacillus* against *Bacillus sp*

S.No	Supernatant (µl)	Zone radius (mm)
1	10	1
2	20	2
3	30	2

Table 7: Inhibition zone of *Lactobacillus* against *Escherichia coli*

S.No	Supernatant (µl)	Zone radius (mm)
1	10	2
2	20	3
3	30	5

4.4.5 Pancreatin tolerance test

The strain was tested for their tolerance to pancreatin at pH 8.0. The strain was resistant to pancreatin even after 4 hr of exposure since they retained their viability (Fig 10).

Table 8: Survival of *Lactobacillus* after Pancreatin treatment

S.No	Time (hr)	Cfu/ml ($x10^2$)
1	0	135
2	4	120

4.4.6 Pepsin resistance test

The strain was tested for its resistance to pepsin at pH 2.0. The strain retained its viability even after 1h of exposure as determined by colony count on MRS agar plates (Fig 11).

Table 9: Survival of *Lactobacillus* after Pepsin treatment

S.No	Time (hr)	Cfu/ml ($x\ 10^2$)
1	0	146
2	1	115

Figure 7: Growth of *Lactobacillus* at pH 5.0

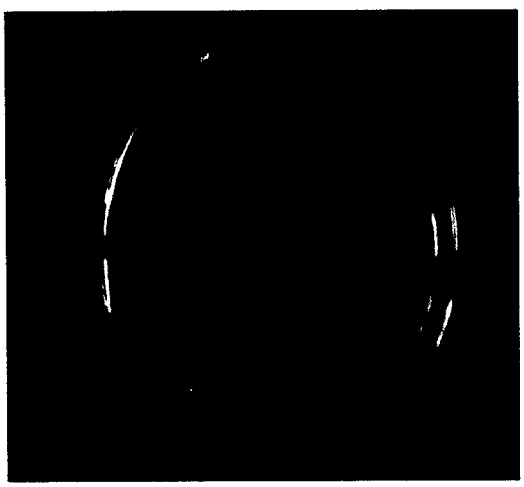
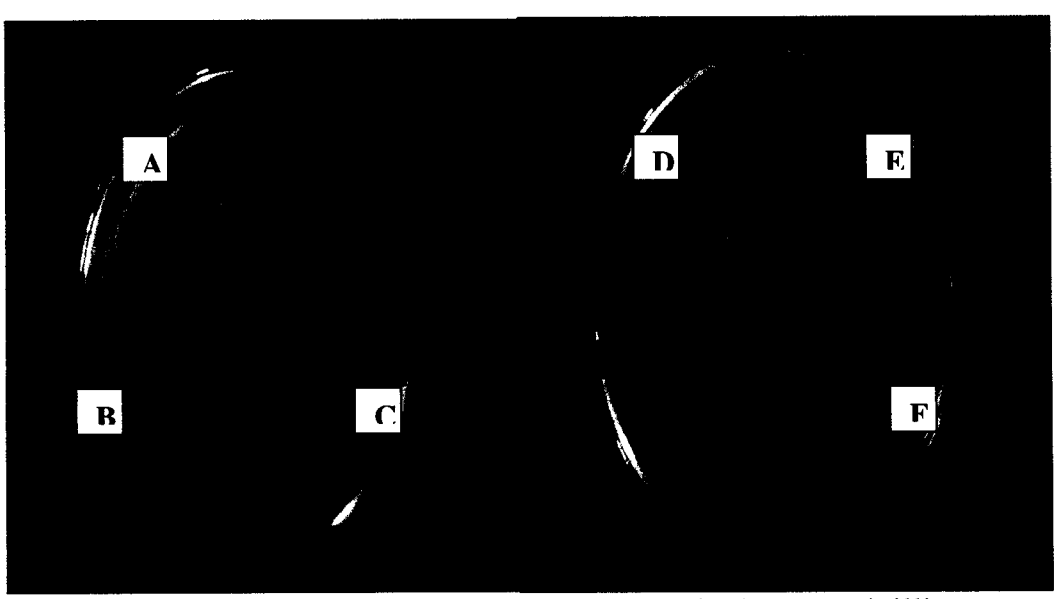
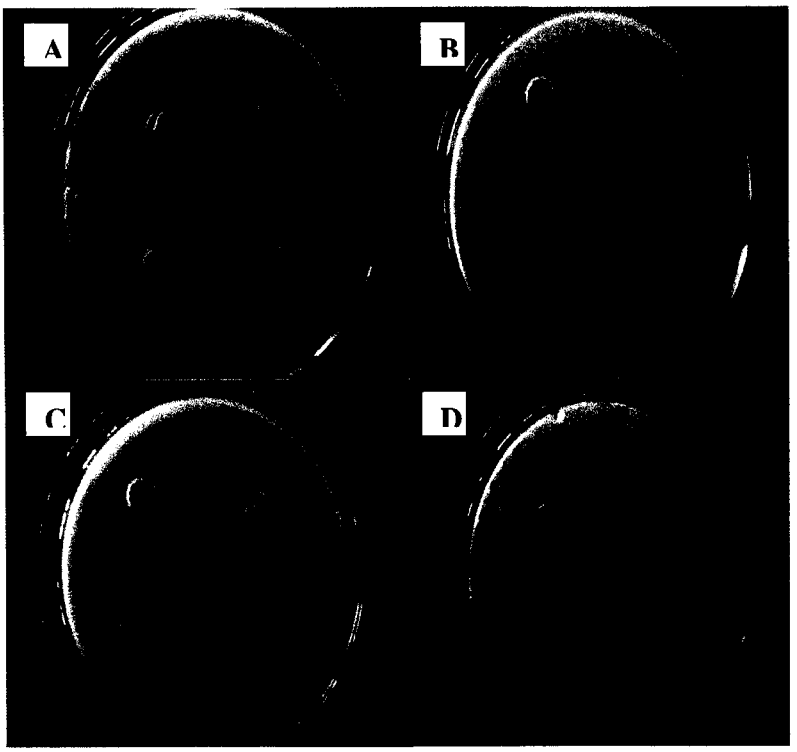


Figure 8: Antibiotic disc assay showing inhibition zone



A: Amikacin, B: Tetracycline, C: Chloramphenical, D: Penicillin, E: Gentamycin and F: Bacitracin.

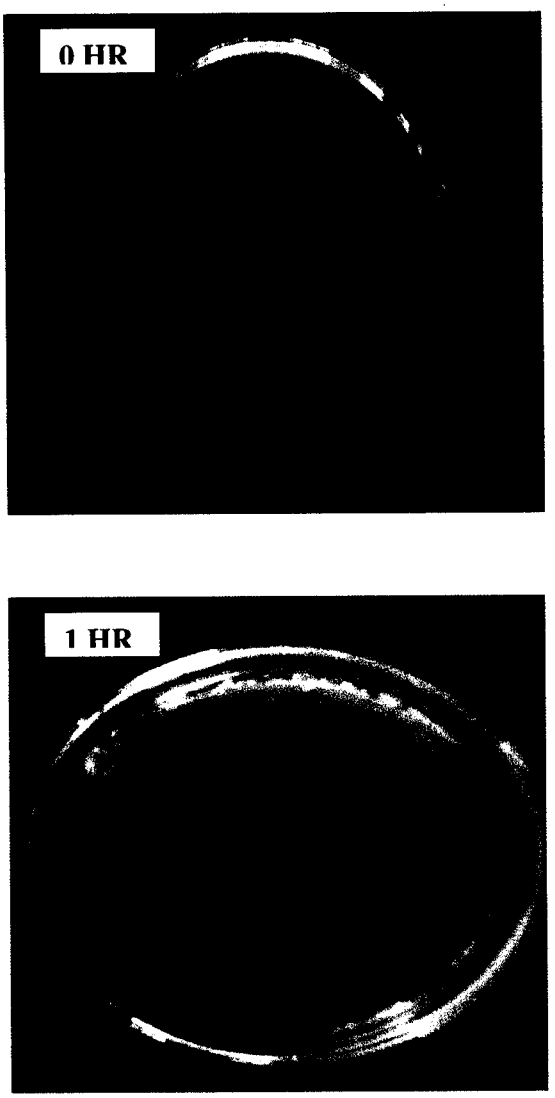
Figure 9: Antibacterial activity against pathogens



A: *Escherichia coli,* B: *Salmonella typhi,* C: *Bacillus sp* and D: *Staphylococcus aureus*

Figure 10: Pancreatin treated plates at 0 hr and 4 hr

Figure 11: Pepsin treated plates at 0 hr and 1 hr



DISCUSSION

5. DISCUSSION

Probiotic bacteria should be resistant against acid (stomach), bile, capable of growing under anaerobic conditions and be non-toxic. These criteria limit the number of bacterial species and strains to the following groups of bacteria: Lactobacillus, Streptococcus and Bifidobacterium species, although some other species can be used in some cases. All three genera are part of the lactic acid bacteria and are naturally present in the intestine as well as in many fermented (mainly acid) dairy products. In recent years research in the probiotic area has progressed considerably and significant advances have been made in the selection and characterization of specific probiotic cultures.

The objective of this study is to evaluate the probiotic potential of Lactobacillus isolated from curd rice. This source is readily available easily affordable and also has major health benefits it contains beneficial microorganisms and thus can be an effective probiotic provided it is able to survive and adhere to the GI tract

Curd is made by adding a small quantity of the previous days curd to fresh milk. This is a practice that is carried out every day so that each household may have a unique curd microflora. One of the objectives of the study was to determine the effect of starch on the growth and number of microflora in curd rice. A single curd source was inoculated into warm milk and allowed to form curd. Then the curd was mixed well with boiled rice and was allowed to ferment overnight. Using this as the source, total bacterial count and Lactobacilli count was determined. Lactobacillus was isolated and was evaluated for its probiotic characteristics.

Following the isolation of Lactobacilli from the curd rice sample, characterization of the morphologically different colonies was carried out. A single Lactobacillus species was isolated and was used for further experiments.

The *in vitro* criteria for the selection of candidate probiotic have been described in the previous studies and are referred to as selection guidelines by the FAO / WHO committee (Joint FAO / WHO Working Report, 2002). In order to assess the properties of probiotic, the committee suggested certain guidelines. Probiotic micro-organisms should not only be capable of surviving while passing through the digestive tract but also have the capability to proliferate in the gut. This means that they must be resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestine or be consumed in a food vehicle hat allows them to survive passage through the stomach an exposure to bile. For the selection of high potent probiotic strain safety and functionality properties such as antibiotic resistance anti-microbial activity and inhibition of pathogenic adhesion are highly important and should be studied using reliable *in-vitro* screening methods.

The pH in human stomach ranges from 1 during fasting to 4.5 after a meal and food ingestion can take up to three hours. The strain displayed loss of viability when pH decreases drastically. These results are in agreement with those obtained from previous similar studies where Lactobacilli were able to retain their viability when exposed to pH values of 3 to 5, but displayed loss of viability at lower pH values (Conway *et al.,* 1987; Du Toit and franz, 1998; Jacobsen *et al.,* 1999; Dunne *et al.,* 2001). The gastric juice itself may offer some degree of protection when compared with low pH

buffers (Conway *et al.,* 1987). In this context, even strains that are not able to survive at pH 1 *in vitro* may exhibit substantial viability when consumed as starters or adjuncts in a matrix of fermented milk. It should be mentioned, however that probiotic bacteria are mainly consumed in the presence of milk proteins. Milk proteins have a protective effect on the starters and thus support bacterial survival in the acidic environment of the stomach.

Our findings on the viability of the Lactobacilli in the presence of Pepsin at pH 2 are also in agreement with the existing literature data (Charteris *et al.*, 1998; Fernandez and Barbes, 2003). The combined effects of pepsin-pH solution aims at simulating the gastric juice, although it is not clear whether the decrease of viability conferred by the pepsin solution at pH 2 was due to the activity of the enzyme alone, or in synergy with low acidity.

In contrast with pepsin, the strain examined in the study could survive well in a pancreatin solution (1% w/v) at pH 8 and also in the presence of bile salts (0.3% w/v), simulating the near neutral small intestine environment.

Many a times, medications have included a course of antibiotics and probiotic supplements in our day to day life. It is thus essential that the probiotic strain be resistant to the antibiotics prescribed. We determined that our strain was resistant to most of the commonly used antibiotics, thus validating their potential as effective probiotics.

Probiotic bacteria prevent the growth of pathogenic species by competing for nutrition and attachment sites to the epithelium of the colon. Some of the probiotics produce bacteriocins, substances which kill harmful

microbes. In our study, the supernatant of the strains inhibited the growth of pathogenic organisms like *Escherichia coli and Bacillus sp.* Inhibition effects cannot be explained by action of the bacteriocins action alone and may probably be due to the production of organic acids as well.

CONCLUSION

6. CONCLUSION

Microorganism from fermented curd rice was isolated in the MRS enriched medium. The characteristics of the bacteria were studied through catalase test and gram staining. Then a single Lactobacilli isolate was taken for the further study of its probiotic characteristics.

The isolated Lactobacilli was exposed to an acidic environment, bile salts and gastric enzymes like pepsin and pancreatin. The organism is able to survive in mild acidic conditions, can tolerate the effect of bile salt, pepsin and pancreatin before reaching the intestinal tract. The organism is found to resist and grow in the presence of most common antibiotics and some potential intestinal pathogens like *Echerichia coli* and *Bacillus sp.*

From our study, we inferred that the isolated Lactobacilli is found to possess desirable probiotic properties *in vitro.* This bactrium is a good candidate for further investigation in *in vivo* to elucidate their potential health benefits and their application as a novel probiotic stain in food industry.

APPENDIX

7. APPENDIX

Lactobacillus de mann, rogosa and 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
Manganese sulphate	-	0.05g
Dipotassium phosphate	-	2g
pH	-	6.5±0.2

Lactobacillus de mann, rogosa and sharpe agar (g/1000ml)

To components of Lactobacillus de mann, rogosa and sharpe broth, add 12g of agar powder.

pH	-	6.5±0.2

Nutrient agar (g/1000l)

Meat extract	-	3g
Peptic digest of animal Tissue	-	5g
Agar	-	15g
pH	-	6.8±0.2

Phosphate Buffered Saline [PBS] (g/1000ml)

Dissolve the following in 800ml of distilled water.

Disodium hydrogen phosphate - 1.44g

Potassium dihydrogen phosphate - 0.24g

Sodium chloride - 8g

Potassium chloride - 0.2g

Adjust pH, make up to1000ml with distilled water, sterilise in autoclave and store at room temperature.

Gram stain 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 h before use.

Gram's iodine solution (g/300ml)

Iodine crystals - 1g

Potassium iodide - 2g

Distilled water - 300ml

Grind dry iodine crystals and potassium iodide thoroughly in a mortar, adding water (few ml at a time) and rinse into a new bottle with the rest of the water.

Safranin solution (g/1000ml)

Stock safranin solution:

Safranin - 0.25g

Ethanol (95%) - 100ml

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8. REFERENCE

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