A STUDY OF CURD MICROFLORA AND ITS PROBIOTIC PROPERTIES

A Project Report

Submitted by

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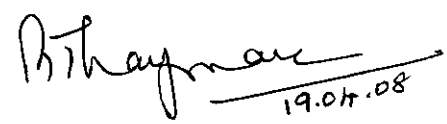

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The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was evaluated and confirmed to be their original work. It was submitted for evaluation and viva-voce held on 24-4-2008

(INTERNAL EXAMINER) **(EXTERNAL EXAMINER)**

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ABSTRACT

Curd, a fermented milk product is a traditional food made in every household in India and is perceived as being healthful. However the microbial diversity and the probiotic potential of curd have not been clearly understood. Our aim was to study the microbial diversity of curd at different stages of maturation, the effect of starter culture and milk source on microbial composition of curd and to assess the probiotic potential of the curd isolates *in vitro*. Nine different strains of *Lactobacillus* and 2 different strains of *Leuconostoc* were isolated. All the stains were sensitive to pH 1 but remained unaffected by pH 3, pancreatin and bile salts. All the stains were sensitive to pepsin individually but they showed resistance when grouped. None of the strains were haemolytic. All the strains were resistant to Vancomycin, Tetracycline, Gentamicin, Rifampicin, Chloramphenicol and Norfloxacin. Most of the strains were sensitive to Imipenem, Ampicillin and Augentin. Several strains formed zones of inhibition against *S.typhimurium* and *V.cholerae*. All the strains were able to adhere to Caco-2 cell lines. Most of the strains were able to suppress IL-8 secretion induced by V. cholerae in Thp-1 cell line. Three *Lactobacillus* strains namely *L.fermentum 1*, *L.acidophilus 1*, and *L.delbrueckii ssp delbrueckii* were therefore found, *in vitro* to possess desirable probiotic properties.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

GI	Gastro Intestinal tract
SCFA	Short Chain Fatty Acids
MRS	de Mann Rogosa Sharpe medium
BHI	Brain Heart Infusion medium
SS	Salmonella- Shigella agar
SDS	Sodium Dodecyl Sulphate
PBS	Phosphate-Buffered Saline
RPM	Revolutions Per Minute
PCR	Polymerase Chain Reaction
DNA	Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
UV-Vis	Ultra Violet- Visible
RBC	Red Blood Corpuscles
DMEM	Dulbecco's Modified Eagle Medium
TCBS	Thiosulphate Citrate Bile salt Sucrose medium
IL-8	Interleukin-8
IL-10	Interleukin-10
ELISA	Enzyme Linked Immuno Sorbent Assay
FBS	Foetal Bovine Serum
cDNA	Complementary Deoxyribo Nucleic Acid
dNTP	deoxyribo Nucleoside Tri Phosphate
dATP	deoxyribo Adenosine Tri Phosphate
dGTP	Deoxyribo Guanosine Tri Phosphate
dCTP	Deoxyribo Cytosine Tri Phosphate
dTTP	Deoxyribo Thymidine Tri Phosphate
RT	Reverse Transcriptase
EHEC	Entero Haemorrhagic *E.coli*

INTRODUCTION

1. INTRODUCTION

The human gut comprises billions of bacteria that belong to as many as 400 diverse species. These microorganisms are referred to as the gut flora. Bacteria have been estimated to constitute 35-50% of the total volume of the human colon. The dominant genera are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus*. The subdominant genera include *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus*, etc (Salminen *et al.*, 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 suggests 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. These bacteria exert 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 invasion by alien microbes. Viruses, fungi and protozoa can also be a part of the gut microflora, but these normally form only a minor component of the total resident population of microorganisms in healthy individuals.

In recent years, the balance between the harmful bacteria and the beneficial ones has been disturbed. Illness, dietary changes, stress, aging, 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 diarrhoea, inflammatory bowel disease(Shanahan, 2000) colon cancer and other gastrointestinal disorders.

One way of maintaining a balance of the gut microflora is to consume a diet supplemented with beneficial bacteria. For thousands of years microbial cultures have been used to ferment foods and 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 environments and help support normal intestinal balance. 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 and Kim, 1984), 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. Other possible health benefits include balance of pH, prevention and treatment of diseases like acute diarrhoea, inflammatory bowel diseases(Linskens *et al.*, 2001; Noverr and Huffnagle, 2005) and other GI disorders.

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. 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 (Maragkoudakis *et al.*, 2006). 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(Parvez *et al.*, 2006) and remain viable in the final food product. Lactic acid bacteria are capable of modulating specific immune responses in gut- associated lymphoid tissue(Meydani and Ha, 2000). This effect depends on the degree of contact with lymphoid tissues and their ability to survive in the GI tract.

Curd is a staple constituent of Indian diet that harbors large numbers of lactic acid bacteria such as *Lactobacillus* sp., *Leuconostoc* sp. and *Streptococcus* sp(Adolfsson *et al.*, 2004). It is prepared primarily by inoculating the previous day's curd into fresh milk and fermented overnight. Curd is easily available and is known for its beneficial properties through years of consumption by all classes of people.

2. OBJECTIVES

The present investigation was initiated with the following objectives.

1. To determine the diversity of microorganisms in curd
2. To quantitate the bacteria in curd
3. To evaluate the probiotic potential of the isolates

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Overview of the gut flora

The gut flora are the microorganisms that normally live in the digestive tract and can perform a number of useful functions for their hosts. 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). Bacteria make up most of the flora in the colon and 60% of the mass of faeces (Guarner *et al.*, 2003). Fungi and protozoa also make up a part of the gut flora, but little is known about their activities.

Research suggests that the relationship between gut flora and humans is not merely commensal (a non-harmful coexistence), but rather is a mutualistic, symbiotic relationship (Sears, 2005). 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 vitamin 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 *et al.*, 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). Most of the bacteria in the 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 *et al.*, 2004), while the

latter part mostly breaks down proteins and amino acids (Gibson, 2004). 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 (Guarner *et al.*, 2003). 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, 2003), but in the cecum aerobic bacteria reach high densities (Guarner *et al.*, 2003).

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. 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 *et al.*, 2003). Species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut, suggesting that this 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' faeces (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} per gram of faeces. 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 microflora 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.

2.1.4 Functions of the gut flora

2.1.4.1 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). Carbohydrates that humans cannot digest without bacterial help include certain starches, fiber, oligosaccharides, lactose, 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 vitamin like vitamin K.

2.1.4.2 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, an activity termed the "barrier effect". 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, 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 *et al.*, 2003).

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. Indigenous gut flora also produce bacteriocins, 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.

2.1.4.3 Immunity

Gut flora have a continuous and dynamic effect on the host's gut and systemic immune systems. The bacteria are the key 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 tissue 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).

2.2 Alterations in balance of gut flora

Many factors, such as diet or climate, aging, medication, illness, stress, pH, infection, geographic location, race, socioeconomic circumstances, lifestyle can upset the balance of the gut flora. A state of balance within the microbial population within the GI tract can be called "eubiosis" while an imbalance is termed "dysbiosis".

2.2.1 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 *et al.*, 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.

2.2.2 Changes in lifestyle

It is increasingly evident that human diseases are most often related to lifestyle. The stress of modern life, our reduced physical activity, our consumption of manipulated and processed foods, and of chemicals including pharmaceuticals all contribute to our decreasing resistance to disease. Our modern lifestyle has dramatically reduced the availability of foods produced by natural fermentation. After the early identification of microbes, they were mainly regarded as a source of disease, and unwanted in commercially manufactured food. Furthermore, the desire of the food industry to prolong the shelf life promoted alternative methods of production such as the use of enzymes instead of live bacteria. Combined with extensive hygiene measures practised during delivery and in child care, children in urban societies may have difficulty developing a satisfactory protective indigenous gut flora. It is suspected that this could be connected to the rising incidence of allergy and infections seen among such children.

2.3 Modulation of gut flora

Burkitt and Trowell (1978) published their revolutionary epidemiological study on non-contagious diseases in traditional African population groups whose diet was characterized by a high percentage of plant fiber. From the low incidence of these diseases in the African population groups studied, Trowell formulated the hypothesis that the low-fiber diet in the industrial developed countries is the cause of numerous lifestyle-related diseases (Trowell, 1978). This picture yielded many new

insights on the bacterial colonization of the human large intestine and its role in the mechanism of action of dietary fiber. In the early 1990s, the term "prebiotics" was established to designate types of dietary fiber. The inclusion of prebiotic foods like nondigestible oligosaccharides and fructooligosaccharides in the diet can make a contribution to stabilizing or exerting a beneficial effect on the intestinal flora. They have been shown to stimulate the growth of endogenous bifidobacteria, which, after a short feeding period, become predominant in human faeces. Moreover, these prebiotics modulate lipid metabolism, most likely via fermentation products.

Because the human gut microbiota can play a major role in host health, there is currently some interest in the manipulation of the composition of the gut flora towards a potentially more remedial community. Attempts have been made to increase bacterial groups such as *Bifidobacterium* and *Lactobacillus* that are perceived as exerting health-promoting properties.

Probiotics, defined as microbial food supplements that beneficially affect the host by improving its intestinal microbial balance, have been used to change the composition of colonic microbiota. By combining the rationale of pro- and prebiotics, the concept of synbiotics is proposed to characterize some colonic foods with interesting nutritional properties that make these compounds candidates for classification as health-enhancing functional food ingredients.

2.4 Probiotics

Probiotics, which means "for life", have been used for centuries as natural components in health-promoting foods. The original observation of the positive role played by certain bacteria was first introduced by Russian

scientist and Nobel laureate Eli Metchnikoff, who in the beginning of the 20th century suggested that it would be possible to modify the gut flora and to replace harmful microbes by useful microbes. Metchnikoff had observed that certain rural populations in Europe who lived largely on milk fermented by lactic-acid bacteria were exceptionally long lived. Based on these facts, Metchnikoff proposed that consumption of fermented milk would "seed" the intestine with harmless lactic-acid bacteria and decrease the intestinal pH which in turn would suppress the growth of harmful bacteria.

Henry Tissier, also from the Pasteur Institute, was the first to isolate a *Bifidobacterium*. He isolated the bacterium from a breast-fed infant and named it *Bacillus bifidus communis*. This bacterium was later renamed *Bifidobacterium bifidum*. Tissier showed that bifidobacteria are predominant in the gut flora of breast-fed babies, and he recommended administration of bifidobacteria to infants suffering from diarrhea.

Kollath first introduced the term "probiotics" in 1953 (Hamilton *et al.*, 2003). Probiotics were defined as 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 beneficially affects the host animal by improving its intestinal microbial balance". In the 1960s the dairy industry began to promote fermented milk products containing *Lactobacillus acidophilus*. In subsequent decades other species have been introduced including *Lactobacillus rhamnosus, Lactobacillus casei,* and *Lactobacillus johnsonii*, because they are intestinal species with beneficial properties.

The mechanisms by which probiotics beneficially affect the host are multiple. Probiotic functional foods can improve specific physiologic functions in the human gastrointestinal tract, e.g., the host immune defense,

thereby reducing the risk of contracting illnesses (Marteau, 2000). Specific probiotic bacteria were shown to promote non-specific host resistance to microbial pathogens. Several probiotic strains were shown to induce in vitro the release of proinflammatory cytokines, tumor necrosis factor α, and interleukin 6, which reflects the stimulation of non-specific immunity (Miettinen, Vuopio-Varkila *et al.*, 1996).

In balancing the gut microecology, the incidence of slower gastric emptying and partial hydrolysis of lactose during fermentation may be associated with the documented alleviation associated with symptoms of secondary lactose intolerance in adults. The best-documented clinical application of probiotics is in the treatment of acute diarrhea and as adjunct therapy in gut related inflammatory conditions.

There is an increasing appreciation of the role of cytokines in regulating inflammatory responses at a local and systemic level. The ingestion of probiotic bacteria can potentially stabilize the immunologic barrier in the gut mucosa by reducing the generation of local proinflammatory cytokines (Shanahan, 2000). Preliminary evidence suggests that a combination of strains rather than a single organism (Prantera, Scribano *et al.*, 2002) may alleviate symptoms of inflammatory bowel diseases(IBD), displaying the potential role of probiotics.

2.4.1 Lactobacilli as probiotics

Lactobacillus species are commonly used as probiotics. It has been shown that the strains of *L. paracasei* administered with a symbiotic dietary supplement are able to survive through the gastrointestinal tract and to persist for at least a few days. In general terms, a group of requirements have been identified as important properties for lactobacilli to be effective

probiotic organisms. These include the ability to (i) adhere to cells; (ii) exclude or reduce pathogenic adherence; (iii) persist and multiply; (iv) produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth; (v) be safe and therefore noninvasive, noncarcinogenic, and nonpathogenic; and (vi) coaggregate and form a normal, balanced flora.

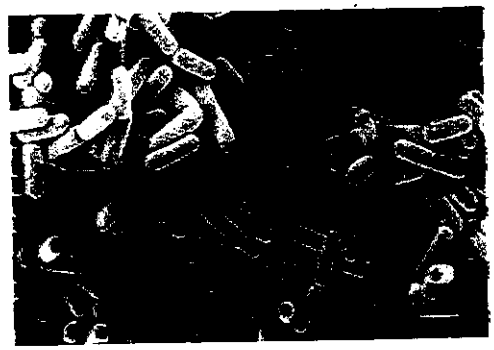

Figure 1: *Lactobacillus acidophilus*

L. rhamnosus GG, *L. acidophilus* NCFM, *L. casei* Shirota, *L. reuteri* MM53, *L. casei* CRL431, *L. rhamnosus* GR-1, *L. fermentum* RC-14 are reported to possess the above mentioned properties (Reid 1999). It has been indicated that the two strains of *Lactobacillus* from human origin namely *Lactobacillus acidophilus* UO 001 and *Lactobacillus gasseri* UO 002 present important properties for survival in, and colonization of, the gastrointestinal tract, that make them potential probiotics(Fernandez, Boris *et al.*, 2003).

Probiotic bacteria are thought to occupy binding sites on the gut mucosa, preventing pathogenic bacteria from adhering to the mucosa. Lactobacilli also produce proteinaceous compounds-bacteriocins that act as local antibiotics against more pathogenic organisms. Studies with *L. casei* Shirota injected into mice showed a significant increase in natural killer cell activity from mesenteric node cells but not of Peyer's patch cells or spleen

cells(Matsuzaki and Chin, 2000), supporting the concept that some probiotic strains can enhance the innate immune response.

2.5 Curd

Curd is a commonly consumed dairy product that is known to harbor large numbers of lactic acid bacteria. Curd, also known as yoghurt is defined by Codex Alimentarius in 1992 as a coagulated milk product that results from fermentation of lactose in milk by *Lactobacillus bulgaricus, Streptococcus thermophilus* and other lactic acid bacteria (Adolfsson *et al.*, 2004). Curd is a good source of vitamin, minerals and folic acid. Some minerals like calcium are more bioavailable from curd than milk (Cano, Aguero *et al.*, 2002; Elli *et al.*, 2006). Curd has less lactose, more lactic acid, galactose, and peptides, free amino acids and free fatty acids. The nutrient composition of curd is affected by many factors such as genetic and mammalian differences, feed, stage of lactation, age, and environmental factors (Meydani and Ha, 2000). Curd consumption improves lactose digestion and eliminates symptoms of lactose intolerance. It may enhance the immune response in immuno-compromised populations such as the elderly. Curd intake has been shown to induce measurable health benefits linked to the presence of live bacteria (Bourlioux and Pochart, 1988).

MATERIALS & METHODS

3. MATERIALS & METHODS

3.1 Microbial growth conditions

Microbial strains isolated from curd were routinely cultured on Lactobacillus MRS broth (Himedia). They were incubated for 16 h at 37°C with 10% CO_2 under anaerobic conditions.

For the antimicrobial production screening, inhibition of pathogen adhesion to Caco-2 cells and modulation of IL-8 response in HT-29 cells, pathogenic strains of clinical origin were included in this study. These included an *Escherichia coli* strain, a *Vibrio cholerae* strain (O139), a *Salmonella typhimurium* strain and a *Shigella flexneri* strain. The *Vibrio* strain was routinely grown in Alkaline Peptone Water while the remaining pathogenic strains were grown in BHI broth (Himedia) at 37°C.

Total viable counts of the curd isolates were determined using MRS agar (Himedia) at 37°C. *Shigella* and *Salmonella* were enumerated on SS agar (Himedia), *E.coli* on MacConkey agar (Himedia) and *V.cholerae* on TCBS agar (Himedia) after 18 h at 37°C.

3.2 Effect of starter culture and milk source on microbial diversity of curd

The objective of this experiment was to establish the cause for microbial diversity in curd. There are two factors that may influence the composition of the curd, one being the starter culture and the other being the type of milk used. The test was performed by varying these two factors and observing the extent of microbial diversity.

Materials:

1. Starter cultures (Curd samples)

2. Milk source- Pasteurized and Unpasteurized milk
3. Lactobacillus MRS agar
4. Columbia agar containing 5% (w/v) human blood
5. MacConkey agar
6. Petri plates
7. Conical Flasks
8. Inoculation loop
9. Anaerobic jar

Procedure:

1. Two different starter cultures were inoculated separately into 150 ml of pasteurized milk at a concentration of 1 % (w/v). Also, one starter culture was inoculated into 150 ml of unpasteurized milk.
2. The milk was allowed to curdle for 48 hr at room temperature.
3. Every 4 h, 1.5 ml of the curdling milk was aliquoted and stored at -20°C for DNA isolation.
4. The samples were also streaked on MRS agar, MacConkey agar & Blood agar plates.
5. MRS agar plates were incubated for 24 h at 37°C in anaerobic conditions with 10% CO_2.
6. MacConkey agar & Blood agar plates were incubated for 48 h at 37°C.
7. After incubation, colonies were enumerated and variations in colony morphologies at different durations were noted.

3.3 DNA extraction from curd

DNA was isolated from curd samples collected over a period of 48 hr. DNA extraction was accomplished with a combination of physical, chemical and enzymatic steps (Apajalahti *et al.*, 1998).

Materials:

1. Centrifuge
2. Lysozyme
3. Proteinase K
4. SDS 10% (w/v)
5. Equilibrated Phenol (pH 8.0)
6. Chloroform
7. Isoamyl alcohol
8. Ammonium acetate (10.0 M)
9. Absolute alcohol
10. Microfuge tubes (sterile)
11. Ethanol 70% (v/v)
12. Sterile water

Reagents:

1. Tris-Cl (1.0 M): 121.1g of Tris base was dissolved in 800 ml of water and the pH was adjusted to 8.0 by adding 42 ml of 1N HCl. The volume of the solution was adjusted to 1 litre with water. The solution was dispensed into aliquots and sterilized by autoclaving.
2. Phosphate-buffered saline (PBS): 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2HPO_4 were dissolved in 800 ml of distilled water and the pH was adjusted to 7.4 with HCl. The volume

was made up to 1 litre with water. The solution was dispensed into aliquots and sterilized by autoclaving. The buffer was stored at room temperature.

3. SDS 10% (w/v): 10 g of SDS was dissolved in 100 ml of water and heated at 65°C to assist dissolution. Sterilization was not required.
4. Lysozyme: The lyophilized lysozyme (Cat no. B3M, Genei, Bangalore) was dissolved at a concentration of 10 mg/ml in 10 mM Tris-Cl (pH 8.0) immediately before use. 500 µl volumes of the enzymes were aliquoted into microfuge tubes and stored at -20°C for long periods.
5. Proteinase K: The lyophilized proteinase K (Cat no. PK1, Genei, Bangalore) was dissolved in 10 ml of sterile water to obtain a concentration of 10mg/ml. 500 µl volume was aliquoted into microfuge tubes and was stored at -20°C for long periods.
6. Ammonium acetate: 77 g of ammonium acetate was dissolved in 80 ml of water and the volume was adjusted to 100 ml with water and sterilized by filtration. The solution was stored in tightly sealed bottles at 4°C.
7. Phenol:Chloroform:Isoamyl alcohol (25:24:1): A mixture consisting of equal parts of equilibrated phenol and chloroform : isoamyl alcohol (24:1) was frequently used to remove proteins from preparations of nucleic acids.

Procedure:

1. Curd sample of 1.5 ml volume was centrifuged for 5 min at 1500 rpm and the supernatant was drained.

2. The pellet was resuspended in 500 µl of PBS; 20 µl of lysozyme (10 mg/ml) was added to the supernatant and incubated at 37°C for 1 hour.
3. SDS 0.5% (w/v) and 30 µl of proteinase K (14 mg/ml) were added followed by incubation for 2-3 h at 55°C.
4. Extraction was done twice with 150 µl of equilibrated phenol and 150 µl of chloroform-isoamyl alcohol (24:1) and once with 150 µl of chloroform. About 90% of the upper aqueous layer was drained into a clean tube taking care not to disturb the proteins at the aqueous-phenol interface. The aqueous phase was extracted for a second time with an equal volume of 1:1 equilibrated phenol: chloroform, centrifuged and drained into a new tube.
5. Two volumes of absolute alcohol and 2M ammonium acetate were added to the extract in a 1.5 ml microfuge tube, inverted to mix and placed at -20°C for 30 min at this stage.
6. After centrifuging at 10000 rpm for 15 min at 4°C, the supernatant was decanted and blotted dry on a paper towel. 70% ethanol (corresponding to about 2 volumes of the original sample) was added and the mixture was placed at room temperature for 5-10 min followed by centrifugation for 5 min at 8100 rpm. The tube was decanted and blotted dry.
7. DNA was dissolved in 200 µl of sterile water and maintained at 50°C for an hour before use.

3.4 Quantification of bacteria by Real-time PCR

An attempt was made to estimate the number of lactobacilli present relative to the total bacteria in curd. The DNA samples of curd collected at

various time intervals were subjected to real time PCR using three sets of primers-Universal, *Lactobacillus* genus specific and *Lactobacillus acidophilus* specific primers.

Materials:

1. Forward and Reverse primers
2. PCR Master mix (dNTPs, $MgCl_2$, Taq polymerase, SYBR® green and buffer)
3. DNA samples
4. Sterile water
5. 96-well plate
6. Real time PCR machine

Procedure:

1. A 20 µl reaction volume mix consisting of 1X Master mix, 250 nM of forward and reverse primers, 2 µl of template and sterile water was prepared and loaded on a 96-well plate.
2. The following program was run on the Real time PCR (Bio Rad):

   Step 1: Incubated at 50°C for 2 min.

   Step 2: Incubated at 95°C for 10 min.

   Step 3: Incubated at 95°C for 15 sec.

   Step 4: Incubated at 58.9°C for 30 sec.

   Step 5: Incubated at 72°C for 30 sec.

   Step 6: Plate read.

   Step 7: Repeated steps 3-6 for 44 more times.

   Step 8: Incubated at 72°C for 10 min.

Step 9: Melting curve from 40°C to 95°C, read every 1°C, held 10 sec.

Step 10: Incubated at 72°C for 5 min.

Step 11: Incubated at 10°C for 20 min.

Step 12: End.

3.5 Characterization of curd isolates

Curd samples were collected from 20 different sources based on three criteria, namely socio-economic status, location and commercial establishments. The samples were plated on MRS agar and morphologically different colonies were isolated. These colonies underwent preliminary screening by gram staining and the catalase test followed by biochemical characterization.

3.5.1 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 the crystal 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 counterstain of safranin was applied to the smear to give decolorized gram-negative bacteria a pink color.

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 one 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 oil immersion microscopy.

3.5.2 Catalase test

The catalase test identifies organisms which produce the catalase enzyme; 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 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 strains
3. Glass slide
4. Inoculation loop

Procedure:

1. A small drop of water was placed on the 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 evolution.

3.5.3 Biochemical characterization

API 50 CH is a standardized system, associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. API 50 CH

is used in conjunction with API 50 CHL Medium for the identification of *Lactobacillus* and related genera.

The API 50 CH strip consists of 50 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids). The fermentation tests are inoculated with API 50 CHL Medium or which rehydrates the substrates. During incubation, fermentation is revealed by a color change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the chosen medium. The first tube, which does not contain any active ingredient, is used as a negative control.

Materials:

1. API 50CH strips
2. API 50CH 2ml suspension medium
3. API 50CH 5ml suspension medium
4. API 50CH 10ml suspension medium
5. Micropipette & tips
6. Fresh microbial cultures
7. Anaerobic jar
8. Inoculation loop

Procedure:

1. Using a sterile inoculation loop, bacterial colonies were picked from the culture plate and mixed with API 50CH 2ml suspension medium till the suspension medium turned turbid.

2. A certain volume of API 50CH 2ml suspension medium was transferred to API 50CH 5ml suspension medium until the turbidity in this medium matched that of McFarland Standard 2.
3. Twice the volume of the 2ml suspension medium added to the 5ml medium was transferred to the 10ml API 50CH medium.
4. The 10ml medium was transferred into the cupules on the API 50CH strips.
5. The tubules were filled with paraffin oil to provide an anaerobic environment.
6. About 10 ml of distilled water was distributed into the honeycombed wells of the incubation tray to create a humid atmosphere.
7. The strips were placed in the tray and incubated for 24-48 h at 37°C under anaerobic conditions with 10% CO_2.
8. The strips were observed for colour changes after 24 and 48 h respectively.

3.6 Growth curve analysis

The strains identified by the API 50CH system were 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 flasks
3. Fresh microbial cultures
4. Cuvettes

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 48 h at 37°C in anaerobic conditions.
3. The absorbance was measured at 600 nm using UV-Vis spectrophotometer (Shimadzu) every 2 h.
4. A graph was plotted between the time and absorbance values for each of the cultures and by observing the curve obtained, the log phase of the bacterial cultures was ascertained.

3.7 Survival under conditions simulating the human GI 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 little loss to 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 exhibit antimicrobial activity against pathogens. It must be capable of modulating intestinal mucosal immune responses. The following experiments were designed to test the probiotic potential of the isolated strains.

3.7.1 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 1 and pH 3 was assessed.

Materials:

1. 16 h microbial cultures
2. Sterile Phosphate-buffered Saline (pH 1, pH 3 & pH 7.2)
3. MRS agar plates
4. McFarland standard 2
5. Micropipette & tips
6. Anaerobic jar
7. Microfuge tubes
8. L-rods

Procedure:

1. Broth cultures of 2ml volume were centrifuged at 10000 rpm for 2 min.
2. The supernatant was drained off and the pellet was re-suspended in 2ml of sterile PBS (pH 7.2) and centrifuged at 10000 rpm for 1 min.
3. Step (2) was repeated.
4. To the pellet obtained, PBS of specific pH (pH 1 & pH 3) was added until the turbidity matched that of McFarland standard 2.
5. The suspension was incubated at 37°C for 3 h.
6. The suspension was spread plated at 0, 1 & 3 h on MRS agar plates. The plates were incubated at 37°C for 24 h in anaerobic conditions with 10% CO_2.
7. Colonies were enumerated after incubation.

3.7.2 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 simulate the gut conditions, the test strains were exposed to pepsin at pH 2 to evaluate their resistance to the enzyme.

Materials:

1. 16 hour broth cultures
2. Sterile Phosphate-buffered Saline (pH 2 [supplemented with 3mg/ml Pepsin] & pH 7.2)
3. MRS agar plates
4. McFarland standard 2
5. Micropipette & tips
6. Anaerobic jar
7. Microfuge tubes
8. L-rods

Procedure:

1. Broth cultures of 2ml volume were centrifuged at 10000 rpm for 2 min.
2. The supernatant was drained; the pellet was re-suspended in 2ml of sterile PBS (pH 7.2) and centrifuged at 10000 rpm for 1 min.
3. Step (2) was repeated.
4. To the pellet obtained, PBS of pH 2[supplemented with 3mg/ml pepsin] was added until the turbidity matched that of McFarland Standard 2.
5. The suspension was incubated at 37°C for 3 h.

6. Suspension was spread plated at 0, 1 & 3 h on MRS agar plates. The plates were incubated at 37°C for 24 h in anaerobic conditions with 10% CO_2.
7. Colonies were enumerated after incubation.

3.7.3 Pancreatin resistance test

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

Materials:

1. 16 hour broth cultures
2. Sterile Phosphate-buffered Saline (pH 8 [supplemented with 1mg/ml Pancreatin] & pH 7.2)
3. MRS agar plates
4. McFarland standard 2
5. Micropipette & tips
6. Anaerobic jar
7. Microfuge tubes
8. L-rods

Procedure:

1. Broth cultures of 2ml volume were centrifuged at 10000 rpm for 2 min.

2. The supernatant was drained; pellet was re-suspended in 2ml of sterile PBS (pH 7.2) and centrifuged at 10000 rpm for 1 min.
3. Step (2) was repeated.
4. To the pellet obtained, PBS of pH 8 [supplemented with 1mg/ml pancreatin] was added until the turbidity matched that of McFarland Standard 2.
5. The suspension was incubated at 37°C for 3 h.
6. Suspension was spread plated at 0 and 4 h on MRS agar plates. The plates were incubated at 37°C for 24 h in anaerobic conditions with 10% CO_2.
7. Colonies were enumerated after incubation.

3.7.4 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 serve to reduce cholesterol levels, emulsify lipids and fat soluble vitamin 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. 16-hour broth cultures.
2. MRS agar plates
3. MRS broth supplemented with 0.3% (w/v) Ox Gall.
4. Phosphate-buffered Saline (pH 7.2)
5. Microfuge tubes.
6. L-rods.

Procedure:

1. 2ml volumes of broth cultures were centrifuged at 10000 rpm for 2 min.
2. The supernatant was drained; pellet was re-suspended in 2ml of sterile PBS (pH 7.2) and centrifuged at 10000rpm for 1 min.
3. Step (2) was repeated.
4. The pellet was re-suspended in 2 ml of supplemented MRS broth for 3 h at 37°C.
5. The suspension was spread plated on MRS agar every 0, 1 & 3 h. The plates were incubated at 37°C for 24 h in anaerobic conditions with 10% CO_2.
6. Colonies were enumerated after incubation.

3.7.5 Haemolytic activity test

Haemolysis refers to the breaking open of red blood cells and the release of haemoglobin into the surrounding fluid. Microorganisms can cause different patterns of haemolysis on blood agar plates. Alpha haemolysis is shown by a greenish halo around the colony and is the result of haemoglobin reduction to methaemoglobin in red blood cells. Beta haemolysis is shown by a clear halo around the colony and is produced by complete haemolysis of the RBCs. Gamma haemolysis is shown as no haemolysis or discoloration of the blood. A crucial safety requirement for probiotic strains is that they do not cause the haemolysis of RBCs.

Materials:

1. Columbia blood agar plates (supplemented with 5% human blood)
2. 16-hour broth cultures.

3. Anaerobic jar
4. Inoculation loop

Procedure:

1. The broth cultures were quadrant streaked on Columbia Blood Agar plates.
2. The plates were incubated at 37°C for 48 h in anaerobic conditions with 10% CO_2. After incubation, plates were observed for signs of hydrolysis around the colonies formed

3.7.6 Antibiotic sensitivity test

To determine the sensitivity or resistance of the strains to the antibiotics, the Kirby-Bauer method was followed (Maragkoudakis *et al.*, 2006). Nine commonly used antibiotics, namely Ampicillin, Augentin, Gentamicin, Chloramphenicol, Rifampicin, Norfloxacin, Vancomycin and Imipenem were used in the study.

Materials:

1. Antibiotic discs (fixed antibiotic concentrations)
2. 16 hour broth cultures
3. MRS agar plates
4. Anaerobic jar

Procedure:

1. Bacterial cultures were swabbed onto the MRS agar plates using a sterile swab.
2. Antibiotic discs were placed on the inoculated media plates.

3. The plates were incubated for 24 h in anaerobic conditions with 10% CO_2 at 37°C.
4. After incubation, plates were observed for zones of inhibition around the antibiotic discs.

3.7.7 Antimicrobial 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 the organic acids and other metabolic end products they generate. The test strains were examined for the extent of their antimicrobial activity against the pathogens *Escherichia coli, Salmonella typhimurium, Vibrio cholerae* and *Shigella flexneri* by the well-diffusion method.

Materials:

1. 16 hour broth cultures
2. Pathogen broth cultures (*E.coli, S.typhimurium, V.cholerae* V0139 & *Shigella flexneri*)
3. BHI soft agar plates
4. Gel puncher

Procedure:

1. BHI soft agar plates were prepared containing 1% (v/v) of pathogenic cultures.
2. Broth cultures of the test strains were centrifuged at 10000 rpm for 5 min and the supernatants were retained.
3. Wells were punched in the BHI soft agar plates using a sterile gel puncher.

4. 50µl of supernatant was added to each well.
5. Plates were incubated at 37°C for 24 h. After incubation, plates were observed for zones of inhibition around the wells.

3.7.8 Adhesion to Caco-2 cells

Caco-2 refers to a human colorectal adenocarcinoma cell line. This cell line is used to assess the extent of microbial adhesion *in vitro*. From this adhesion study, the degree of microbial adherence to the intestinal epithelial cells *in vivo* can be understood. A probiotic strain must be able to adhere to the intestinal cells in order to exert its beneficial effects on the host.

Materials:

1. 16 hour broth cultures
2. Caco-2 cells
3. *Escherichia coli* culture
4. DMEM medium
5. Tissue culture plates
6. Phosphate-buffered saline (pH 7.2)
7. Triton X – 100
8. MRS agar plates
9. MacConkey agar plates

Procedure:

1. Caco-2 cells were routinely grown in DMEM(Siga), supplemented with 10% (v/v) foetal calf serum inactivated at 56°C for 30 min, 1 %(v/v) non-essential amino acids, 1 %(v/v) glutamine and 20 µg/ml of streptomycin and penicillin.

2. Cells were incubated at 37°C in a 10% CO_2/90% air atmosphere.
3. For the adhesion assay, monolayers of Caco-2 cells were prepared in a 24-well tissue culture plate(BD Falcon), seeded(1 ml) at a concentration of 1 million cells per ml inside the wells, and incubated for 7 days, with the culture medium changed once in two days.
4. 2 ml volumes of 16 hour broth cultures were harvested at 10000 rpm for 5 min; pellets were washed twice with PBS (pH 7.2).
5. Cells were resuspended in 1 ml of PBS and then properly diluted in non-supplemented DMEM to achieve a concentration corresponding to McFarland standard 3.
6. The growth medium in the 24-well tissue culture plates of Caco-2 monolayers (7 days old) was aspirated and the cells were washed twice with DMEM.
7. 1 ml of microbial-DMEM suspension was transferred onto the Caco-2 monolayers.
8. The plates were incubated at 37°C in a 10% CO_2/90% air atmosphere for 90 min.
9. The microbial suspension was aspirated and the Caco-2 monolayers were washed twice with DMEM.
10. 1 ml of Triton-X 100 was added to detach the adhered microbial cells.
11. The cells were plated on MRS agar at 10^2 and 10^4 dilutions and incubated at 37°C in a 10% CO_2/90% air atmosphere for 24 h.
12. The colonies were enumerated after incubation. *E.coli* was used as a positive control and the adhesion of the test strains was expressed relative to the *E.coli* adhesion value.

3.7.9 Inhibition of Pathogen adhesion to Caco-2 cells

A probiotic strain must be able to inhibit the adhesion of pathogenic microorganisms to the intestinal cells. This may be accomplished by a competition for nutrients and space as well as bacteriocin and organic acid production by the probiotic strain.

Materials:

1. 16 hour broth cultures
2. Caco-2 cells
3. *Vibrio cholerae* O139 culture
4. DMEM medium
5. Tissue culture plates
6. Phosphate-buffered saline (pH 7.2)
7. Triton X – 100
8. MRS agar plates
9. TCBS agar plates

Procedure:

1. Caco-2 cells were routinely grown in DMEM(Siga), supplemented with 10% (v/v) foetal calf serum inactivated at 56°C for 30 min, 1 %(w/v) non-essential amino acids, and 20 μg/ml of streptomycin and penicillin.
2. Cells were incubated at 37°C in a 10% CO_2/90% air atmosphere.
3. For the adhesion assay, monolayers of Caco-2 cells were prepared in a 24-well tissue culture plate(BD Falcon), seeded(1 ml) at a concentration of 1 million cells per ml inside the wells, and incubated for 7 days, with the culture medium changed once in two days.

4. 2 ml volumes of 16 hour broth cultures were harvested at 10000 rpm for 5 min; pellets were washed twice with PBS (pH 7.2).
5. Cells were resuspended in 1 ml of PBS and then properly diluted in non-supplemented DMEM to achieve a concentration of 109 cells.
6. The growth medium in the 24-well tissue culture plates of Caco-2 monolayers (7 days old) was aspirated and the cells were washed twice with DMEM.
7. 1 ml of microbial-DMEM suspension was transferred onto the Caco-2 monolayers.
8. The plates were incubated at 37°C in a 10% CO_2/90% air atmosphere for 90 min.
9. The microbial suspension was aspirated and the Caco-2 monolayers were washed thrice with DMEM.
10. *Vibrio cholerae* cells were harvested and washed twice with PBS (pH 7.2). The pellet was resuspended in PBS and then properly diluted in non-supplemented DMEM to achieve a concentration of 10^8 cells.
11. 1 ml of *Vibrio*-DMEM suspension was transferred onto the Caco-2 monolayers.
12. The plates were incubated at 37°C in a 10% CO_2/90% air atmosphere for 90 min.
13. The microbial suspension was aspirated and the Caco-2 monolayers were washed thrice with DMEM.
14. 1 ml of Triton-X 100 was added to detach the adhered microbial cells.
15. The cells were plated on MRS agar and TCBS agar at 10^2 dilutions and incubated at 37°C in a 10% CO_2/90% air atmosphere for 24 h.

w/v) non-essential amino acids, and 20 μg/ml of streptomycin and penicillin.

2. Cells were incubated at 37°C in a 10% CO_2/90% air atmosphere.
3. Monolayers of HT-29 cells were prepared in a 24-well tissue culture plate(BD Falcon), seeded(1 ml) at a concentration of 1 million cells per ml inside the wells, and incubated for 3 days, with the culture medium changed once in two days.
4. 2 ml volumes of 16 hour broth cultures were harvested at 10000 rpm for 5 min; pellets were washed twice with PBS (pH 7.2).
5. Cells were resuspended in 1 ml of PBS and then properly diluted in non-supplemented DMEM to achieve a concentration of 10^9 cells.
6. The growth medium in the 24-well tissue culture plates of HT-29 monolayers (3 days old) was aspirated and the cells were washed twice with DMEM. The monolayers were incubated for 2 h with non-supplemented DMEM.
7. The medium was aspirated from the wells; 1 ml of microbial-DMEM suspension was transferred onto the HT-29 monolayers.
8. The plates were incubated at 37°C in a 10% CO_2/90% air atmosphere for 2 h.
9. The microbial suspension was aspirated and the HT-29 monolayers were washed thrice with DMEM.
10. *Vibrio cholerae* cells were harvested and washed twice with PBS (pH 7.2). The pellet was resuspended in PBS and then properly diluted in non-supplemented DMEM to achieve a concentration of 10^8 cells.
11. 1 ml of *Vibrio*-DMEM suspension was transferred onto the HT-29 monolayers.

16. The colonies were enumerated after incubation. *V.cholerae* was used as a positive control and the inhibition of *Vibrio* adhesion by the test strains was expressed relative to the *V.cholerae* adhesion value.

3.7.10 Modulation of IL-8 response in HT-29 cells

HT-29 refers to a human colonic adenocarcinoma cell line. The intestinal epithelium is an important factor of gut mucosal barrier and participates in innate immunity. Intestinal epithelia are capable of releasing some pro-inflammatory cytokines such as IL-8 when stimulated by enteric pathogens which in turn direct the movement of inflammatory cells of the lamina propria. Probiotic strains may modulate intestinal mucosal immune response and play a protective role by inhibiting the adhesion of pathogenic bacteria to intestinal epithelia. This experiment was aimed at investigating the effect of the test strains on IL-8 secretion of HT-29 cells induced by V.cholerae.

Materials:

1. 16 hour broth cultures
2. HT-29 cells
3. *Vibrio cholerae* O139 culture
4. DMEM medium
5. Tissue culture plates
6. Phosphate-buffered saline (pH 7.2)

Procedure:

1. HT-29 cells were routinely grown in DMEM(Siga), supplemented with 10% (v/v) foetal calf serum inactivated at 56°C for 30 min, 1 %(

12. The plates were incubated at 37°C in a 10% CO_2/90% air atmosphere for 4 h.
13. The suspensions were aspirated from the wells and stored at -20°C till ELISA was carried out to determine IL-8 levels.

3.7.10.1 Estimation of IL-8 production by ELISA

Enzyme-Linked ImmunoSorbent Assay, or ELISA, is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. In sandwich ELISA, a specific primary antibody is affixed to a surface, and then an unknown amount of antigen is washed over the surface so that it can bind to the antibody. A secondary antibody linked to an enzyme is washed over the primary Ab-Ag complex and in the final step a substrate is added that the enzyme can convert to a coloured product. The absorbance measured denotes the amount of protein present in the sample.

Materials:

1. 96-well BD FalconTM ELISA plates (Cat No. 353279)
2. Microplate reader (Perkin Elmer; Wallac Victor3 V- 1420 Multilabel counter)
3. Precision pipettes
4. Distilled water
5. Wash bottle and automated washer (Bio Rad)

Reagents:

1. Coating buffer: 0.1 M sodium carbonate, pH 9.5; 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3; made up to 1 litre; pH to 9.5.
2. Assay diluent: PBS with 10% FBS, pH 7.0.

3. Wash buffer: PBS with 0.05% Tween-20
4. Substrate solution: Tetramethyl benzidine (TMB) and hydrogen peroxide
5. Stop solution: 2 N H_2SO_4

Procedure:

1. Wells were coated with 100 µl per well of capture antibody diluted in coating buffer. The plates were sealed and incubated overnight at 4°C.
2. Wells were aspirated and washed three times with 300 µl per well wash buffer. After the last wash, the plates were inverted and blotted on absorbent paper to remove any residual buffer.
3. Plates were blocked with 200 µl per well assay diluent and incubated at room temperature for 1 hour.
4. Wells were aspirated and washed as in Step 2.
5. Samples of 100 µl volume were diluted in 900 µl in assay diluent. The assay diluent was used as control. The control and samples were loaded into appropriate wells.
6. Plates were sealed and incubated for 2 h at room temperature.
7. Wells were aspirated and washed as in Step 2, but with a total of 5 washes.
8. Prepared working detector of 100 µl volume (dectection antibody + SAv-HRP reagent) was added to each well. The plates were sealed and incubated for 1 hour at room temperature.
9. Wells were aspirated and washed as in Step 2, but with a total of 7 washes. The wells were soaked in wash buffer for 30 sec for each wash.

10. Substrate solution of 100 µl volume was added to each well. The plates were incubated (without plate sealer) for 30 min at room temperature in the dark.
11. Stop solution of 50 µl volume was added to each well. The absorbance was read at 450 nm within 30 min of stopping the reaction.

3.7.11 Modulation of cytokine response in THP-1 cells

THP-1 is a human mononuclear leukemia cell line. An important negative regulator of proinflammatory cytokines is the anti-inflammatory cytokine IL-10. IL-10 is secreted under different conditions of immune activation by a variety of cell types, including T cells, B cells, and monocytes/ macrophages. Our study was aimed at estimating IL-10 levels in THP-1 cells secreted in response to the test strains.

Materials:

1. 16 hour broth cultures
2. THP-1 cells
3. RPM1 medium
4. Tissue culture plates
5. Phosphate-buffered saline (pH 7.2)
6. TRI REAGENT™

Procedure:

1. THP-1 cells were seeded (1 ml) in a 24-well tissue culture plate (BD Falcon) at a concentration of 1 million cells per ml inside the wells, and incubated for one hour.

2. 2 ml volumes of 16 hour broth cultures were harvested at 10000 rpm for 5 min; pellets were washed twice with PBS (pH 7.2).
3. Cells were resuspended in 1 ml of PBS and then properly diluted in non-supplemented RPM1 to achieve a concentration of 10^8 cells.
4. 1 ml of microbial-RPM1 suspension was transferred into the wells.
5. The plates were incubated at 37°C in a 10% CO^2/90% air atmosphere for 2 h.
6. The microbial suspension was aspirated from the wells. 20 ng/ml of gentamicin in RPM1 medium was added to each well.
7. The plates were incubated at 37°C in a 10% CO_2/90% air atmosphere for 4 h.
8. The wells were aspirated and the suspensions were centrifuged at 8100 rpm for 10 min at 4°C.
9. To the pellets, 300 µl of TRI reagent was added and stored at -80°C for RNA isolation.

3.7.11.1 RNA extraction using TRI REAGENT™

TRI REAGENT™ is a convenient reagent for use in the simultaneous isolation of RNA, DNA and protein. It involves a single-step liquid phase separation and is an improvement of the method reported by Chomczynski and Sacchi (1993) for total RNA isolation. TRI REAGENT™, a mixture of guanidine thiocyanate and phenol in a mono-phase solution, effectively dissolves DNA, RNA and protein from lysed cells. After adding chloroform and centrifuging, the mixture separates into three phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins. RNA is then isolated after separating the phases.

Materials:

1. Sterile microfuge tubes
2. Pipette and tips
3. Gloves

Reagents:

1. Chloroform
2. Isopropanol
3. Ethanol, 75% (v/v)
4. SDS solution, 0.5% (w/v)

Procedure:

1. Cells were isolated by centrifugation and lysed in 1 ml of TRI REAGENT™ by repeated pipetting.
2. The samples were allowed to stand for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes.
3. 0.2 ml of chloroform was added to the samples, shaken vigorously for 15 sec and allowed to stand for 2-15 min at room temperature.
4. The resulting mixture was centrifuged at 8100 rpm for 10 min at 4°C.
5. The mixture separated into three phases on centrifugation: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).
6. The aqueous phase was transferred to a fresh tube; 0.5 ml of isopropanol was added and allowed to stand for 5-10 min at room temperature.

7. The samples were centrifuged at 8100 rpm for 5 min at 4°C. The RNA precipitate formed a pellet on the side and bottom of the microfuge tube.
8. The supernatant was removed and the RNA pellet was washed by adding 1 ml of 75% ethanol.
9. The samples were vortexed and centrifuged at 8100 rpm for 5 min at 4°C.
10. The pellets were briefly air-dried for 5-10 min. 20 µl of water was added to the pellets and placed in a water bath for 10-15 min at 60°C to facilitate dissolution.
11. The RNA samples were stored at -80°C for future use.

3.7.11.2 cDNA conversion and evaluation of cytokine gene expression

The RNA samples were subjected to cDNA conversion using a Reverse Transcriptase Core kit (Eurogentec). Expression of the housekeeping gene actin was then monitored to confirm cDNA conversion. A real-time PCR was carried out for the cDNA products to check for IL-10, IL-12 and TNF-α gene expression.

Materials:

1. 96-well PCR plate
2. Pipette and tips
3. Microfuge tubes
4. Gloves
5. End-point and Real time PCR machine

Reagents:

1. 10 X reaction buffer containing KCl and Tris-HCl
2. Euroscript reverse transcriptase- Moloney Murine Leukemia virus reverse transcriptase, 6250 U at 50U/µl
3. RNase inhibitor- 4000 U at 20U/µl
4. 2.5 mM dNTP mix containing dATP, dGTP, dCTP and dTTP in autoclaved, deionized water titrated with NaOH at pH 7.0
5. 25 mM $MgCl_2$
6. 50 µM oligodeoxynucleotides of sequence d (T) 15 VN in 10 nM Tris-HCl, pH 8.3
7. 50 µM short oligonucleotides of random sequence (d (N) 9) in 10 mM Tris-HCl, pH 8.3
8. RNase free water
9. PCR Master mix (dNTPs, $MgCl_2$, Taq polymerase, SYBR® green and buffer)
10. Actin-Hu forward and reverse primers
11. IL-10, IL-12 and TNF-α gene primers

Procedure:

1. All reagents were thawed and put on ice, except for the EuroScript, which was kept in the freezer until required. All the reagents were mixed by inversion and spun down prior to pipetting.
2. A 10 µl RT reaction mix was prepared as follows:

Component	Volume (µl)	Final concentration
10 X reaction buffer	1.0	1 X
25 mM MgCl2	2.0	5 mM
2.5 mM dNTP	2.0	500 µM each dNTP

Random nonamer	0.5	2.5 µM
RNase inhibitor	0.2	0.4 U/µl
Euroscript RT	0.25	1.25 U/µl
RNase free water	3.75	-
Template	1.0	10 – 20 ng Total RNA

3. All the components were added together except for the template, mixed thoroughly by inversion and spun down.
4. The reaction mix was added to each well; the template was added to individual reactions and spun down.
5. The following program was used in the PCR thermocycler:

Initial step	10 min 25°C
Reverse transcriptase step	30 min 48°C
Inactivation of the RT enzyme	5 min 95°C

6. The PCR products were then run with the Actin-Hu housekeeping gene primers to confirm cDNA conversion. A 20 µl reaction volume consisting of 1X Master mix, 250 nM of Actin-Hu forward and reverse primers, 2 µl of template and sterile water was prepared and loaded on a 96-well plate.
7. The following program was run on the Real time PCR (Bio Rad):

   Step 1: Incubated at 50°C for 2 min.

   Step 2: Incubated at 95°C for 10 min.

   Step 3: Incubated at 95°C for 15 sec.

   Step 4: Incubated at 58.9°C for 30 sec.

   Step 5: Incubated at 72°C for 30 sec.

   Step 6: Plate read

   Step 7: Steps 3-6 were repeated for 44 more times.

   Step 8: Incubated at 72°C for 10 min.

Step 9: Melting curve from 40°C to 95°C, read every 1°C, held 10 sec.

Step 10: Incubated at 72°C for 5 min.

Step 11: Incubated at 10°C for 20 min.

Step 12: End.

8. Following confirmation of conversion, cDNA products were run with cytokine gene specific primers. A 20 µl reaction volume consisting of 1X Master mix, 250 nM of forward and reverse primers, 2 µl of template and sterile water was prepared and loaded on a 96-well plate. The above program was followed.

9. The c (t) values of all the cytokine gene amplification curves were expressed relative to those of Actin-Hu.

RESULTS

4. RESULTS

4.1 Effect of starter culture and milk source on microbial diversity of curd

The MRS agar plates showed a higher colony count when compared to MacConkey and Blood agar plates (Fig.2). All the colonies that grew on MacConkey agar were lactose fermenters, i.e, they appeared pink in colour. All three types of haemolysis were observed on the blood agar plates with a majority of the colonies being alpha haemolytic (partial haemolysis- greenish yellow zones), some gamma haemolytic (no haemolysis- no zones) and a few being beta haemolytic (complete haemolysis- clear yellowish zones).

When a single curd source was inoculated into pasteurized and unpasteurized milk, there was not much variation in the types of colonies observed. On the other hand, when two different curd samples were inoculated into pasteurized milk alone, a great diversity in colonies was noted.

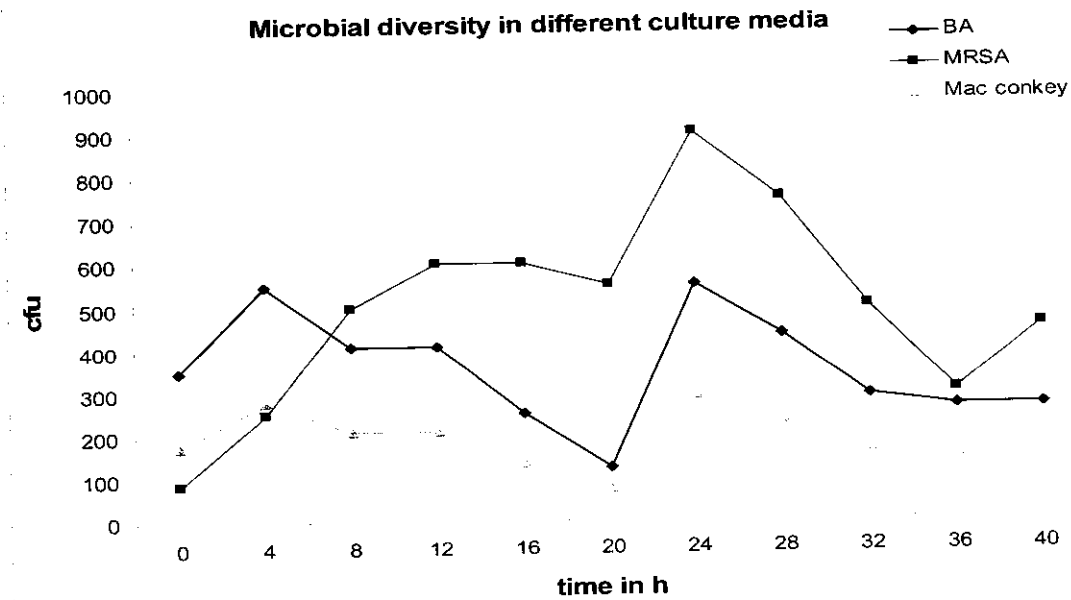

Fig.2: Microbial diversity on different culture media

4.2 Characterization of curd isolates

4.2.1 Gram staining

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. Several yeasts were also isolated. All gram-positive rod shaped bacteria were chosen for further characterization. A gram-positive coccoid bacterium and a yeast colony were also included in the study.

4.2.2 Catalase test

The catalase test was performed on the gram postive bacilli. Most strains were catalase negative while a few were catalase positive; the catalase positive 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 the catalase negative bacilli were selected for biochemical characterization.

4.2.3 Biochemical Characterization

Gram-positive, catalase negative bacilli were subjected to the API 50CH biochemical test. Following 48 h of incubation, changes in medium colour in each of the 50 cupules were noted (Fig.3). The biochemical profiles for the strains were then identified using the **apiweb™** identification software. The organisms identified after biochemical characterization are presented in Table 1.

Strain Number	Strain Name
Strain 1	*Lactobacillus fermentum 1*
Strain 10	*Leuconostoc lactis*
Strain 14	*Lactobacillus acidophilus 3*
Strain 17	*Lactobacillus acidophilus 1*
Strain 18	*Lactobacillus delbrueckii ssp delbrueckii*
Strain 19	*Lactobacillus confusus*
Strain 24	*Lactobacillus lindneri*
Strain 27	*Lactobacillus fermentum 1*
Strain 28	*Lactobacillus fermentum 1*
Strain 30	*Leuconostoc mesenteroides ssp cremoris*
Strain 32	*Lactobacillus helveticus*
Strain 33	*Lactobacillus delbrueckii ssp lactis*
Strain 39	*Lactobacillus delbrueckii ssp bulgaricus*
Strain 40	*Lactobacillus acidophilus 3*

Table 1: Microorganisms identified by API 50CH biochemical test

4.3 Growth curve analysis

A growth curve analysis was performed for the identified strains. *L.fermentum 1, Leuconostoc lactis, L.acidophilus 1, L.confusus* and *L.delbrueckii ssp bulgaricus* were among the fast growing strains having exponential growth between 4 and 10 h. *L.acidophilus 3, L.delbrueckii ssp delbrueckii, L.lindneri, Leuconostoc mesenteroides ssp cremoris* and *L.helveticus* were in the log phase between 6 and 24 h. On an average, 16-hour cultures were chosen to perform the remaining experiments (Fig.4).

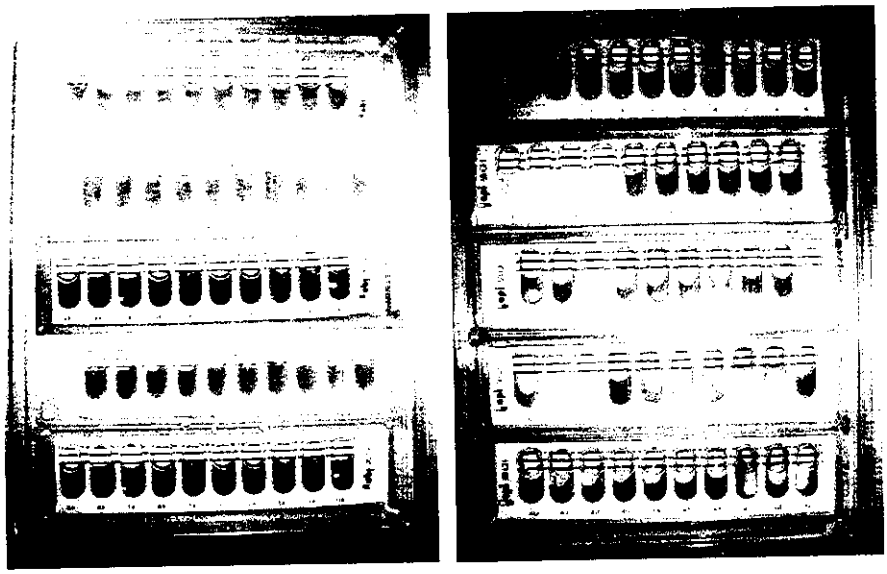

Fig.3: API 50CH biochemical kit

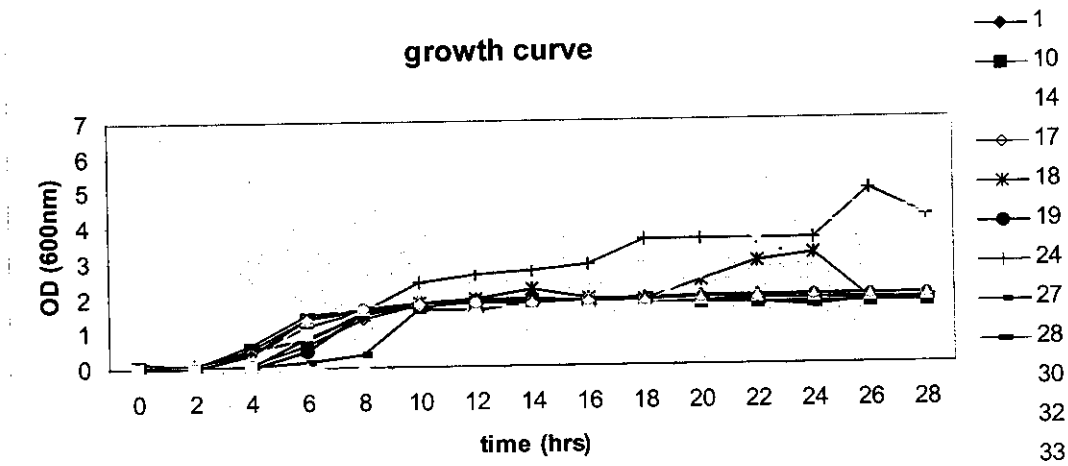

Fig.4: Growth curves of the identified strains

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 must be resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestines.

4.4.1 Resistance to gastric acidity

The strains were exposed to pH 1 and pH 3 for a period of three h. Several strains retained viability even after three h of exposure to pH 3 (Figs.5 and 6). On the contrary, no strains were capable of surviving at pH 1.

4.4.2 Resistance to pepsin

The strains were tested for their resistance to pepsin at pH 2. All strains (Fig.7) except *Lactobacillus delbrueckii ssp delbrueckii* (Figs.8 and 9) lost viability within an hour of exposure as determined by a decline in colony count on MRS agar plates.

4.4.3 Resistance to pancreatin

The strains were tested for their tolerance to pancreatin at pH 8. All the strains were resistant to pancreatin, as even after 4 h of exposure they retained their viability (Figs.10 and 11).

4.4.4 Test for haemolytic activity

The cultures were streaked on human blood agar. After 24 h of incubation in anaerobic conditions, the plates were observed for any sign of haemolysis. All strains were found to be gamma-haemolytic, i.e., no zones of haemolysis were formed (Fig.12).

4.4.5 Tolerance to bile salts

The strains were exposed to bile salts over a period of three h, and plated on MRS agar at regular intervals. On performing a colony count, it was found that there was no reduction in viability. The strains were thus tolerant to bile salts (Fig.13).

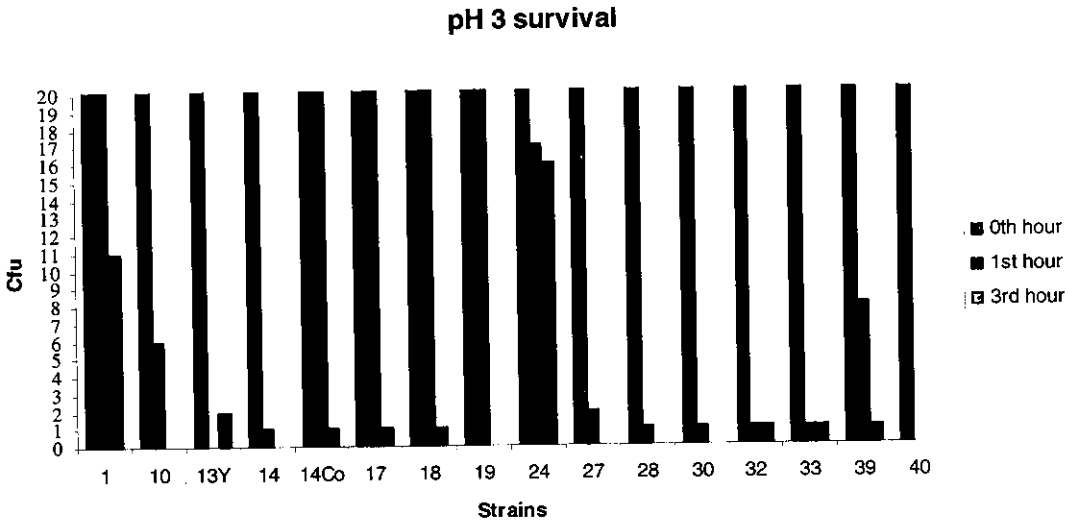

Fig.5: Resistance to pH 3

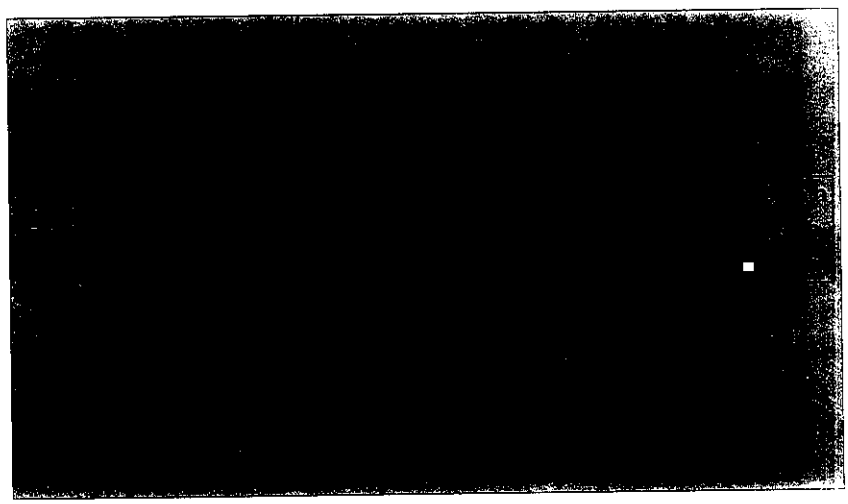

Fig.6: Resistance to pH 1

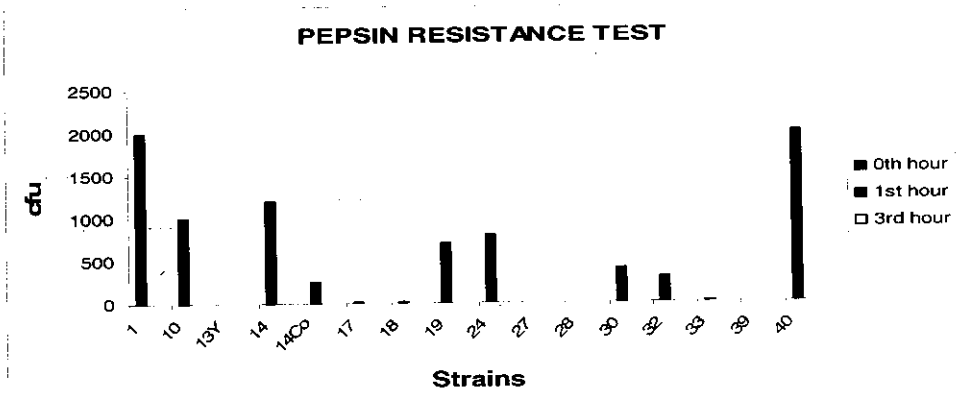

Fig.7: Resistance to pepsin

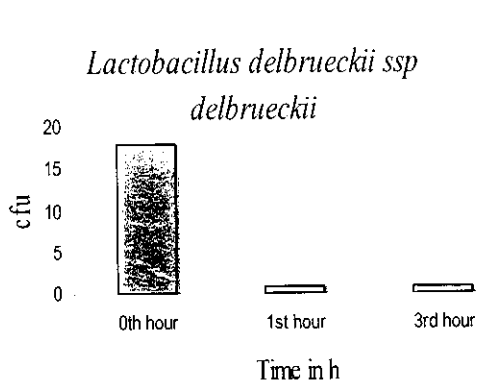

Fig.8 **Fig.9**

***L.delbrueckii ssp* showing resistance to pepsin**

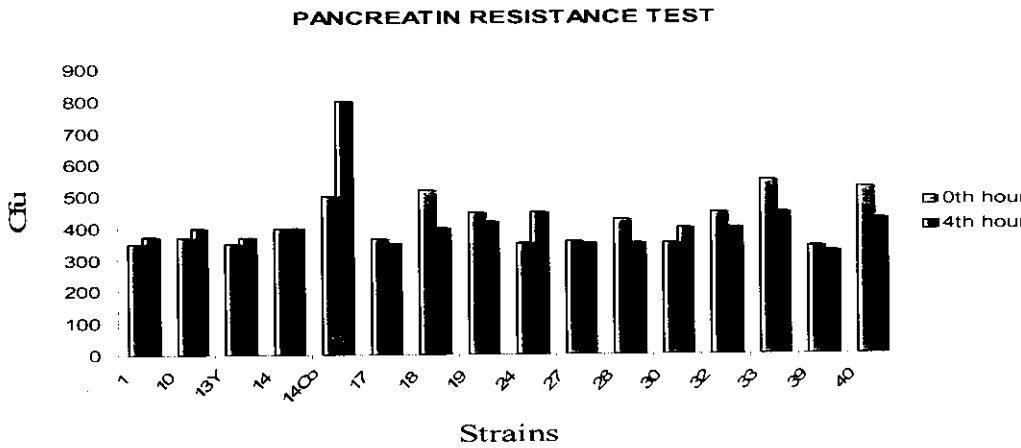

Fig.10: Resistance to pancreatin

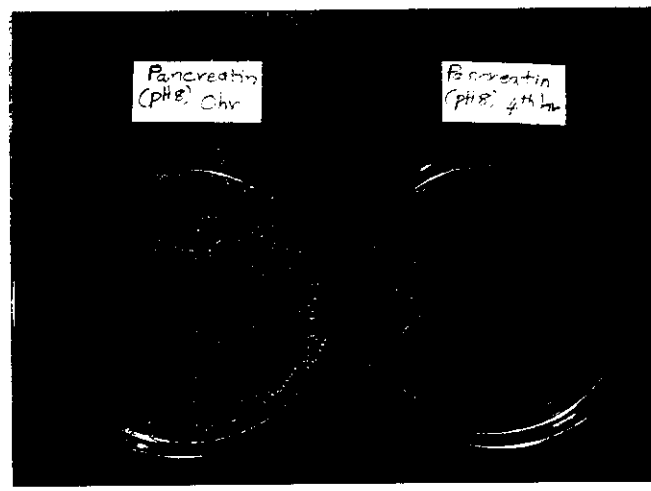

Fig.11: MRS agar plates showing growth at 0^{th} and 4^{th} hours of exposure to pancreatin

4.4.6 Antibiotic sensitivity test

The strains were subjected to the Kirby Bauer method to determine their sensitivity to 9 different antibiotics. Following incubation for 24 h, the zones of inhibition formed with the various antibiotics were compared with standard charts. All the strains were resistant to Vancomycin and Norfloxacin whereas most of them were sensitive to Imipenem. A majority of the strains were resistant to Ampicillin, Chloramphenicol, Gentamicin, Rifampicin, Tetracycline and Augentin (Table 2, Fig.14).

Table 2: Resistance of strains to antibiotics

Strain	Amp	Chl	Gen	Imi	Nor	Rif	Van	Tet	Aug
1	R	R	I	S	R	R	R	R	R
10	I	S	R	I	R	R	R	I	S
14	I	R	R	S	R	R	R	R	S
17	R	R	R	S	R	R	R	R	S
18	R	R	S	S	R	R	R	R	R
19	R	R	R	S	R	R	R	R	R
24	R	R	R	S	R	I	R	R	R
27	R	R	R	S	R	S	R	R	R
28	R	R	R	S	R	R	R	R	S
30	R	R	R	S	R	R	R	R	R
32	R	R	R	S	R	R	R	R	S
33	R	R	R	I	R	R	R	R	R
39	R	R	R	S	R	R	R	R	R
40	R	R	R	S	R	R	R	R	R
13 Y	I	R	R	S	R	R	R	R	S
14Co	R	R	R	S	R	R	R	R	R

Amp-Ampicillin, Chl-Chloramphenicol, Gen-Gentamicin, Imi-Imipenem, Nor-Norfloxacin, Rif-Rifampicin, Van-Vancomycin, Tet-Tetracycline, Aug-Augmentin R-Resistant, I-Intermediate, S-Sensitive

4.4.7 Antimicrobial Activity

The well diffusion method was carried out for all strains to determine their inhibitory effect on four pathogens namely, *E.coli* strain EHEC, *Vibrio cholerae* O139, *Salmonella typhimurium* and *Shigella* sp. Zones of inhibition were formed against *S.typhimurium* by most of the strains. A few strains showed an inhibitory effect towards *V.cholerae* 0139. Only *Lactobacillus fermentum 1*(Strain 1) and *Lactobacillus lindneri* were able to inhibit *E.coli* and *Shigella* sp (Fig.15, Fig.16).

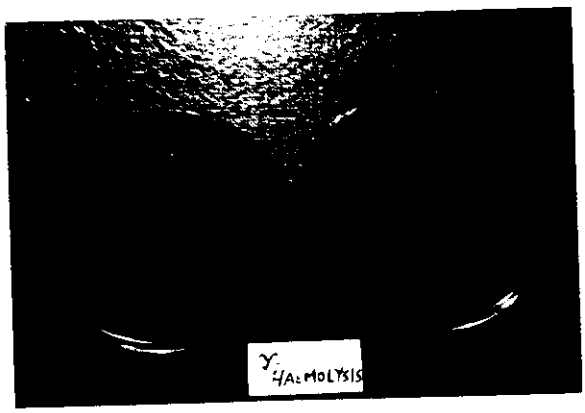

Fig.12: Gamma-haemolytic colonies on Blood agar plates

Fig.13: MRS agar plates showing growth at 1st and 3rd hours after exposure to bile salts

Fig.14: *L.acidophilus* 1 showing zones of inhibition with different antibiotics

4.4.8 Adhesion to Caco-2 cells

All strains except *Lactobacillus delbrueckii ssp lactis* were capable of adhering to Caco-2 cells. The strains were capable of increased adhesion when taken in groups (Figs.17-19).

4.4.9 Inhibition of pathogen adhesion to Caco-2 cells

Most of the strains were able to inhibit the adhesion of *Vibrio cholerae* O139 to Caco-2 cells. *L.acidophilus 1* and *L.delbrueckii ssp delbrueckii* showed the greatest extent of inhibition to VO139 adhesion (Fig.20).

4.4.10 Modulation of IL-8 response in HT-29 cell line

The strains were co-cultured with *V.cholerae* O139 on HT-29 cells. Following a 4-hour incubation, the supernatants were examined for IL-8 secretion using an ELISA kit. Basal cytokine levels were determined in HT-29 cells in order to serve as controls. The level of IL-8 secreted by cells infected with *Vibrio cholerae* was 443.595 ng/ml and was taken as the positive control. There was a considerable decrease in IL-8 production when the test strains and *V.cholerae* were co-cultured on HT-29 cells. *Lactobacillus acidophilus 3* reduced IL-8 levels to almost one-fourth (108.614 ng/ml) of the positive control value (Table 3, Fig.21).

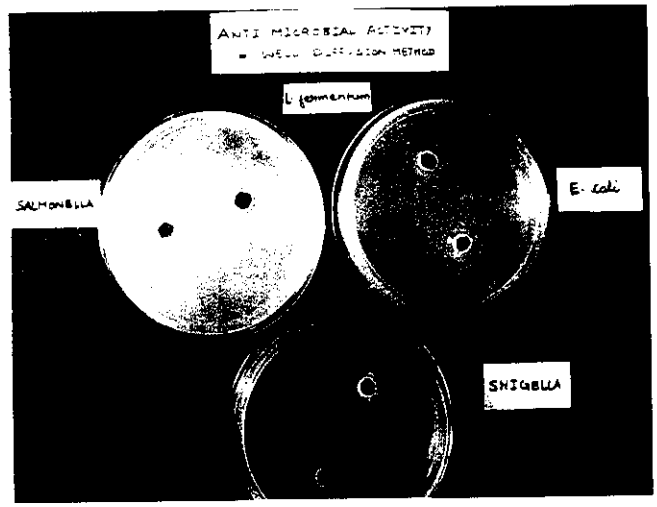

Fig.15: *L.fermentum* 1 forming zones of inhibition against pathogens

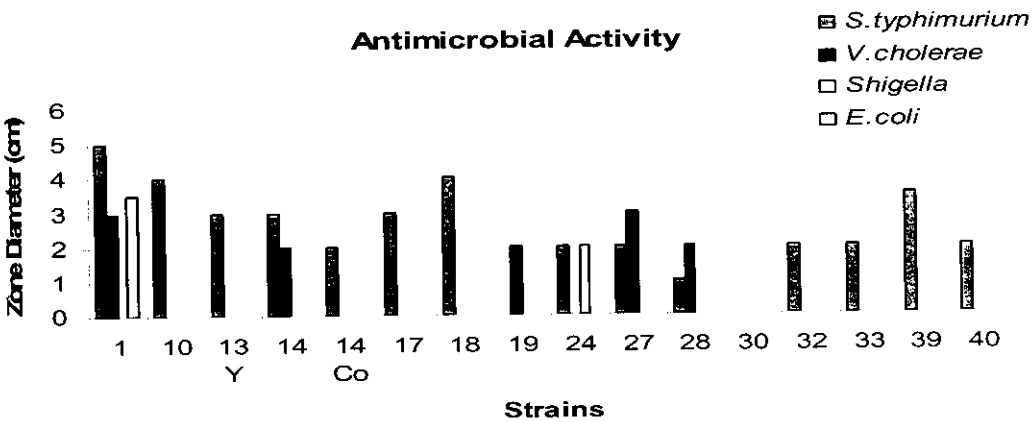

Fig. 16: Antimicrobial activity of test strains

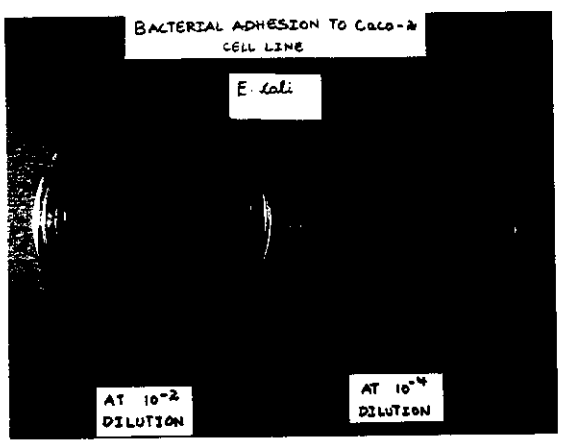

Fig.17: Adhesion of *E.coli* to Caco-2 cells-postive control

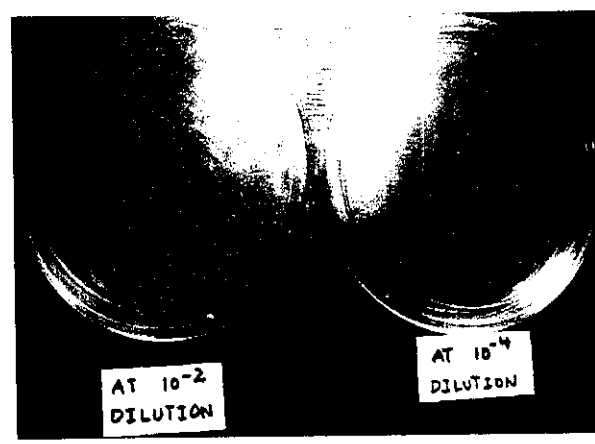

Fig.18: Adhesion of *L.fermentum* 1 to Caco-2 cells

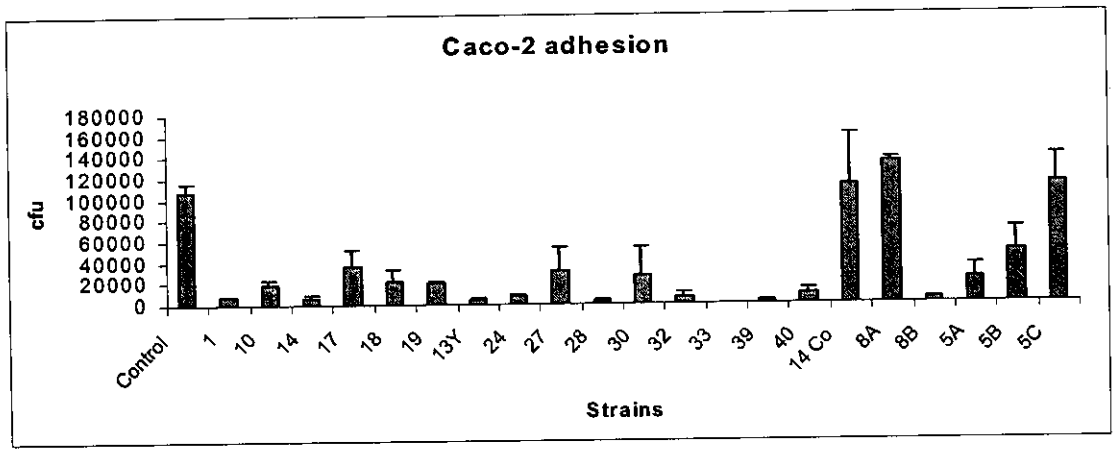

Fig. 19: Adhesion of test strains to Caco-2 cells

Samples	IL-8 (ng/ml)
Control	69.202
VO139	443.595
L. fermentum 1	64.737
L. fermentum 1 + VO139	195.896
Leuconostoc lactis	28.717
Leuconostoc lactis + VO139	167.668
L.acidophilus 3	56.525
L.acidophilus 3 + VO139	108.614
L.acidophilus 1	61.911
L.acidophilus 1 + VO139	380.947
L.delbrueckii ssp delbrueckii	82.443
L.delbrueckii ssp delbrueckii + VO139	257.587
L.lindneri	384.686
L.lindneri + VO139	242.095
Leuconostoc mesenteroides	159.243
Leuconostoc mesenteroides + VO139	187.497
L.helveticus	41.130
L.helveticus + VO139	205.606
L.delbrueckii ssp lactis	26.411
L.delbrueckii ssp lactis + VO139	141.120
L.delbrueckii ssp bulgaricus	35.409
L.delbrueckii ssp bulgaricus + VO139	128.557
L.acidophilus 3	35.607
L.acidophilus 3 + VO139	134.669

Table 3: IL-8 secretion of HT-29 cells induced by *V.cholerae* 0139

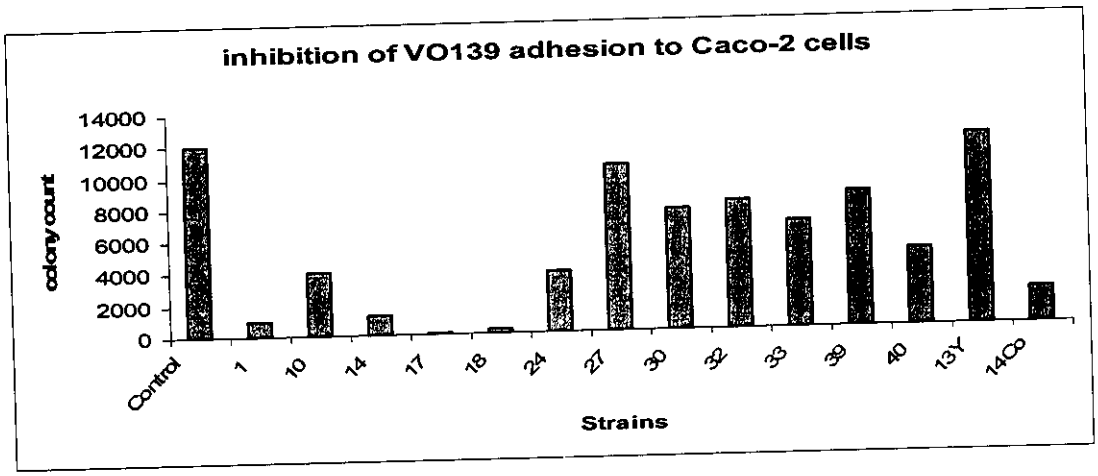

Fig.20: Inhibition of *V.cholerae* O139 adhesion to Caco-2 cells

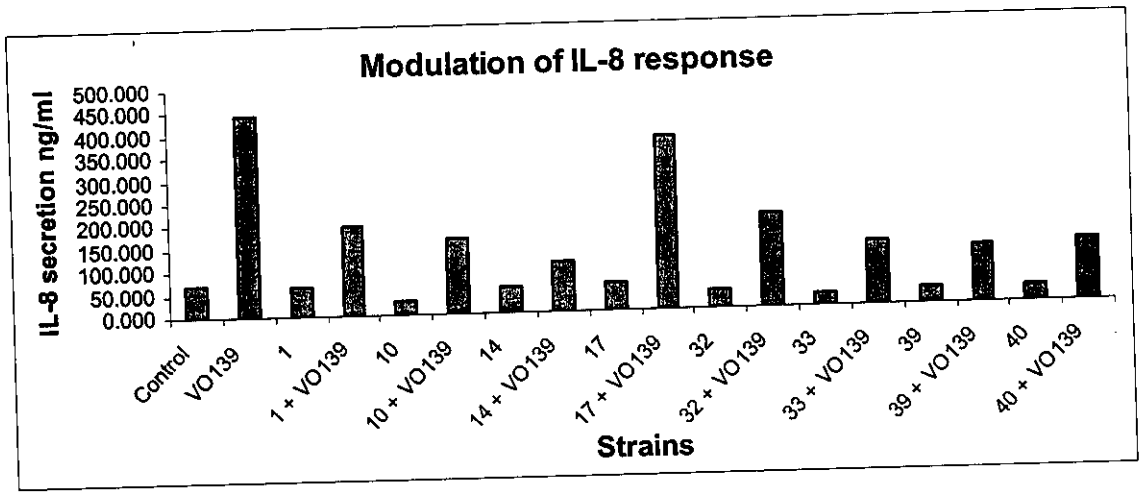

Fig.21: Modulation of IL-8 response in HT-29 cells by *V.cholerae* O139 and test strains

DISCUSSION

5. DISCUSSION

The human gut flora constitutes a dynamic and ecologically diverse environment with nearly 500 species of microorganisms. Microbial interactions between the members of the gut microflora and the host maintain homeostasis. In recent years, the balance has been lost due to various changes in dietary patterns, life style changes etc. The balance of gut flora can be regained by the use of probiotics. The term probiotic is a relatively new word meaning "for life" and is defined as "A live microbial feed supplement, which beneficially affects the host animal by improving its intestinal balance", (Fuller, 1989). 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 and substantiation of health claims relating to their consumption. Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly used, but not exclusively, as probiotic microorganisms and a growing number of probiotic foods are available to the consumer.

Curd was chosen for our study to evaluate the probiotic potential of its microbiota. Curd is well suited to promoting the positive health image of probiotics since it is easily affordable, readily available and consumed by a majority of the people in South India. Besides, consumers are already aware of the fact that curd contains beneficial microorganisms and can thus be encouraged to consume fermented foods containing probiotics.

In Indian households, curd is made by adding a small quantity of the previous day's curd to fresh milk. This is a practice that is carried out everyday so that each household may have a unique curd microflora, i.e., a distinct starter culture. One of the objectives of the study was to determine the microbial diversity in curd and establish the cause for such diversity, if

present. We chose two variables that could possibly influence the microbial composition of the curd, namely the kind of milk and the starter culture. A single curd source was inoculated into pasteurized and unpasteurized milk, and two curd samples were inoculated into pasteurized milk. The curdling milk was observed for microbial composition at regular intervals, and it was established that only a change in starter culture influenced the diversity of the microbial population. A change in the milk source, on the other hand, resulted in little variation in microbial diversity.

Twenty different curd samples were therefore collected from various locations, various classes of people and commercial establishments. Following the isolation of microorganisms from the different curd samples, characterization of the morphologically different colonies was carried out. Eleven distinct species of bacteria were identified, namely *Lactobacillus fermentum 1, L.acidophilus 1, L.acidophilus 3, L.helveticus, L.lindneri, L.confusus, L.delbrueckii ssp delbrueckii, L. delbrueckii ssp lactis, L. delbrueckii ssp bulgaricus, Leuconostoc lactis* and *Leuconostoc mesenteroides ssp cremoris*. A yeast colony and a gram-positive coccoid bacterium were also identified in the study. The curd samples at different hours of maturation were quantitated for their bacterial population using PCR, the results of which are yet to be interpreted.

The second objective of the study was to evaluate the probiotic potential of the strains isolated from the curd samples. The *in vitro* criteria for the selection of candidate probiotics have been described in 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 probiotics, the committee suggested that the following guidelines be used. 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 they must be resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestines, or be consumed in a food vehicle that allows them to survive passage through the stomach and exposure to bile. For the selection of highly potent probiotic strains, safety and functionality properties such as antibiotic resistance, adhesion to intestinal cell lines, antimicrobial activity and inhibition of pathogenic adhesion, as well as immunomodulation potential, are highly important and should be studied using reliable *in vitro* screening methods. Performance of the strains, selected by the above protocols, should be confirmed through in vivo studies and/or clinical studies. The type of the in vivo study as well as the experimental parameters (e.g., mode of administration) can be determined most effectively by the outcome of the above in vitro test results.

The pH in human stomach ranges from 1 during fasting to 4.5 after a meal, and food ingestion can take up to 3 h. Although most of the examined strains were completely resistant to pH 3 even after 3 h of exposure, all strains displayed loss of viability when exposed to pH 1 for 1 h. 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 2.5–4.0, but displayed loss of viability at lower pH values (Conway *et al.*, 1987; Du Toit *et al.*, 1998; Jacobsen *et al.*, 1999; Dunne & Mahony, 2001). Only one strain, namely Lactobacillus delbrueckii ssp delbrueckii was resistant to pepsin over a period of three h. 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 effect of a 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 enzyme alone, or in synergy with low acidity. 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 (Conway *et al.*, 1987; Charteris *et al.*, 1998; Fernandez *et al.*, 2003). Also, 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 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. In contrast to pepsin, all the strains examined in this study could survive well in a pancreatin solution at pH 8.0 or in the presence of bile salts (0.3%, w/v), simulating the near neutral small intestine environment. Most studies so far have shown that the majority of the strains survived well under such conditions, suggesting a potential recuperation of the initial levels during the passage of the small intestine (Charteris *et al.*, 1998; Du Toit *et al.*, 1998; Jacobsen *et al.*, 1999; Fernandez *et al.*, 2003).

One of the safety requirements for a probiotic strain is that it should not have β-haemolytic activity. All the strains were gamma-hemolytic when grown on blood agar plates, thereby suggesting that they have no undesirable interaction with our red blood cells. Many a time, medications have included a course of antibiotics and probiotic supplements. It is thus essential that the probiotic strains be resistant to the antibiotics prescribed. We determined that our strains were resistant to most of the commonly used antibiotics, thus validating their potential as probiotics.

Probiotic bacteria prevent the growth of pathogenic species by competing for nutrition and attachment sites to the epithelium of the colon. Symbiotic bacteria are better adapted to survival in this ecological niche and are thus more successful in the competition. Some of these probiotics also produce bacteriocins, substances which kill harmful microbes. In our study, the supernatants of several strains inhibited the growth of the pathogenic organisms *S. typhimurium* and *Vibrio cholerae* O139, using the well diffusion assay. However, few strains were capable of inhibiting the growth of *E.coli* and *Shigella* sp. Inhibition effects cannot be explained by bacteriocin action alone and are probably due to the production of organic acids as well.

Adhesion of lactobacilli has been claimed to be essential for the exertion of a beneficial (probiotic) effect in the large intestine. Previous studies have reported adhesive strains such as *L. johnsonii* La1, *L. rhamnosus* GG, as well as *L. casei* Shirota and *L. casei* Imunitass (Tuomola & Salminen, 1998; Juntunen *et al.,* 2001; Ouwenhand *et al.*, 2001). In our study, where viable adherent bacteria were measured by enumeration, most of the strains displayed adhesion to Caco-2 cells.

The *in vitro* inhibition of Gram-negative pathogens by probiotic strains, and also the inhibition of pathogen adhesion to eukaryotic cell lines, has already been reported for strains such as *L. johnsonii* La1, *Bifidobacterium* CA1 and F9, and *L. acidophilus* LB (Bernet- Camard *et al.*, 1997).

Direct inhibitory activity has been linked to possible bacteriocin production by the probiotic strains and/or to their competitive adherence to epithelial cells, resulting in the inability of the pathogen to bind to its normal attachment site (Tuomola & Salminen, 1998; Ouwenhand *et al.*, 2001). The

adhesion of *V.cholerae* O139 to Caco-2 cells was reduced to more than 50%. Strains with higher adhesive ability might well occupy common adhesion sites and thus prevent the further adhesion of the pathogenic bacterium. On the other hand, even the weak binding of a *Lactobacillus* strain on a Caco-2 cell could stimulate cellular mechanisms, which may lead to increased colonisation resistance. Further studies are required to elucidate the adhesive mechanism and the inhibition of pathogen adhesion observed in this study.

Intestinal epithelia constitute the mucosal barrier of the bowel, and participate in inflammatory or immune responses in the gut (Gordon *et al.*, 1997; Campbell *et al.*, 1999). In some gastrointestinal infections and inflammatory conditions, inflammatory cells including monocytes and lymphocytes are activated and accumulated in lamina propria. The cells secrete excessive inflammatory products such as Th1 type cytokines, chemokines and several active oxides. Overproduction of cytokines could affect the biological action of epithelial cells.

In order to imitate the inflammatory condition of the gut *in vitro*, we used *V.cholerae* O139 to stimulate human colonic adenocarcinoma HT-29 cells which have basically the same biological properties as normal colonic epithelia. Most of the probiotic strains were capable of reducing IL-8 secretion indicating that they trigger anti-inflammatory pathways within the gut epithelium. To test this hypothesis further, we are currently investigating induction of IL-10 secretion by our strains in THP-1 cells.

CONCLUSION

6. CONCLUSION

Curd from different sources was collected to study its microflora. Two different milk sources- pasteurized and unpasteurized milk were compared with the starter culture to ascertain which of these influenced the microbial diversity and composition. Microorganisms from curd were isolated in the MRS enrichment medium. We observed that changing the starter culture showed diverse microbial groups while alteration of milk source had little influence on the diversity of the microbial population. We identified 11 *Lactobacillus* species and 2 *Leuconostoc* species using API 50 CH biochemical kit. Since yeasts and cocci are also perceived to have desirable probiotic properties, they were also included in our study.

The isolated strains were exposed to an acidic environment, gastric enzymes and bile salts to simulate the conditions prevailing in the human GI tract. The adhesion capability of the strains to Caco-2 cells and their antimicrobial properties were evaluated. Their ability to modulate immune responses in HT-29 and THP-1 cells was also investigated.

From our study, we have inferred that the strains *L.fermentum* 1, *L.acidophilus 1, L.delbrueckii ssp delbrueckii* and the coccoid bacterium are found to possess desirable probiotic properties *in vitro*. These strains are good candidates for further investigation in *in vivo* studies to elucidate their potential health benefits and their application as novel probiotic strains in the food industry.

APPENDIX

APPENDIX

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
Manganese sulphate	-	0.05g
Dipotassium phosphate	-	2g
pH	-	6.5±0.2

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

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

pH	-	6.5±0.2

Columbia Blood Agar Base (g/1000ml)

Pancreatic digest of casein	-	10g
Meat peptic digest	-	5g
Heart pancreatic digest	-	3g
Yeast extract	-	5g
Corn starch	-	1g
Sodium chloride	-	5g

Agar	-	15g
pH	-	7.3±0.2

MacConkey agar (g/1000ml)

Animal tissue peptic digest-		20g
Lactose	-	10g
Bile salts	-	5g
Sodium chloride	-	5g
Neutral red	-	0.07g
Agar	-	15g
pH	-	7.5±0.2

Brain Heart Infusion Broth (g/1000ml)

Calf brain infusion	-	200g
Beef heart infusion	-	250g
Protease peptone	-	10g
Dextrose	-	2g
Sodium chloride	-	5g
Disodium phosphate	-	250g
pH	-	7.4±0.2

Brain Heart Infusion agar (g/1000ml)

To Brain Heart Infusion broth, add 15g agar powder.

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

Dissolve the following in 800ml MilliQ water.

Disodium hydrogen phosphate - 1.44g

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