EVALUATION OF METABOLIC PROPERTIES OF

"FUNCTIONAL STARTER CULTURE"

BY-

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CERTIFICATE

This is to certify that the work entitled, "EVALUATION OF METABOLIC PROPERTIES OF FUNCTIONAL STARTER CULUTRE" submitted by *Sanjoli Doneria (091707)* and *Ankita Rana (091552)* in partial fulfilment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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ABBREVIATIONS	EXPANDED FORM
AMP	Adenosine Mono Phosphate
BSHs	Bile salt hydrolases
conc.	Concenteration
CFU	Colony Forming Unit
FDA	Food And Drug Administration
GIT	Gastro Intestinal Tract
GALT	Gut Associated Lymphoid Tissue
LAB	Lactic Acid Bacteria
MTCC	Microbial Type Culture Collection
MLN	Mesenteric Lymph Node
MRS	Man Rogosa Sharpe
Ml	Milli litres
Mg	Milli grams
mM	Milli Molar
min.	Minutes
nM	Nano Molar
Nm	Nanometer
Rpm	Rotations per minute
sp.	Species
var.	Variety
WHO	World Health Organisation
w/v	Weight/volume
µg	Micro grams
%	Percentage
°C	Degree Celsius

ABSTRACT

Functional food (FF) is a natural food, to which a component has been added/removed or a food in which the bioavailability of the components has been modified by technological or biotechnological means. People with flourishing intestinal colonies of beneficial bacteria are better equipped to fight the growth of disease causing bacteria. It is imperative therefore that research and technology is now focusing a lot of attention on fermentation technologies and their products with an aim of tapping into the possible associated health benefits where functional foods come into picture and Lactic Acid Bacteria play an important role in this trend. Hence, probiotics are the driving force of the functional food market. The study was aimed at evaluating the metabolic properties of functional starter culture in order to investigate the potential probiotic biomarkers of the three *Lactobacillus* strains, *Lactobacillus delbrueckii, Lactobacillus plantarum* and *Lactobacillus rhamnosus* obtained from Institute of Microbial Technology (IMTECH), Chandigarh. The biomarkers for these strains were checked are Bile salt Hydrolase activity, Exopolysaccharide production activity, Antibiotic susceptibility, Cholesterol reduction ability and Acid tolerance activity. The semi quantitative assay for Bile Salt Hydrolase activity was performed and all the 3 strains were found to be positive for BSH activity. *Lactobacillus delbrueckii* shows the highest EPS production followed by *Lactobacillus rhamnosus* and *Lactobacillus plantarum* which saturates after 72 hours of incubation. After 48 hours of incubation, there is a considerable percentage decrease in the concentration of cholesterol by 62%, 52.79% and 38.6% for *L. rhamnosus, L. plantarum* and *L. delbrueckii*, respectively. Each strain was tested for tolerance to acid at pH 2 for about two hours as the transit time of food from stomach is 2-3 hrs. *L. delbrueckii* showed a significant tolerance to the acidic environment followed by *L. plantarum* and *L. rhamnosus* with 97.08%, 91.22% and 90.75% respectively after 120 minutes. Thus *L. delbrueckii, L. plantarum* and *L. rhamnosus* can be further explored as functional starter cultures with potential probiotic efficiencies.

CHAPTER - 1

INTRODUCTION

The microbiota of the human gastrointestinal (GI) tract plays a pivotal role in human health. Different biologic functions, such as digestion of essential nutrients, maturation of intestinal epithelial cells, and impact on baseline physiologic parameters, including systemic effects on blood lipids, inhibition of harmful bacteria, and stimulation of the immune system, have been attributed to the microbiota through careful scientific evaluations over many [1]. However, the declined birth rates and longer life expectancy in developed countries have led to increased prevalence of chronic disorders like cardiovascular disease and different metabolic disorders (WHO, 2003). Moreover, several respiratory and foodborne infections, and chronic diseases like urinary tract and *Helicobacter pylori* infections are still emerging [2]. Although the prevalence of *H. pylori* infection has been declining in Estonia [3] gastritis, peptic ulcer disease and its general consequences on health such as gastric malignancies still need attention. All this requires population based new preventive approaches, namely infection control and improved nutrition. Functional food is the food that contains some health-promoting components beyond traditional nutrients.

Functional starter cultures are microbes that possess functional properties which aimed at improving the quality of the end product. This functionality is viewed with respect to two things: firstly, from food technology point of view where the taste, aroma, texture and the shelf life of the product is considered and secondly, the potential health benefits from these starter cultures.

Fermentation is one of the oldest technologies used for food preservation. The fermentation process results in the production of acids and probable bacteriocins that prevent growth of microorganisms hence increasing the shelf life of fermented products [4].This is a very valuable attribute especially in rural areas where advanced food preservation technologies such as refrigeration are not affordable, also people have began to appreciate more of naturally preserved than chemically preserved foods.

Fermented foods are associated with 'good bacteria' referred to as probiotics [5]. Probiotics, as defined in a FAO/WHO (2002) report, are live 'microorganisms which when administered in adequate amounts confer a health benefit on the host'.

Probiotics are beneficial bacteria in that they favourably alter the intestinal microflora balance such as reconstruction of normal intestinal microflora after disorders caused by diarrhoea, antibiotic therapy and radiotherapy, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection [5]. Other physiological benefits of probiotics

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include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients [6].

People with flourishing intestinal colonies of beneficial bacteria are better equipped to fight the growth of disease causing bacteria. It is imperative therefore that research and technology is now focusing a lot of attention on fermentation technologies and their products with an aim of tapping into the possible associated health benefits where functional foods come into picture and Lactic Acid Bacteria play an important role in this trend. Hence, probiotics are the driving force of the functional food market [7].

Lactic Acid Bacteria are an important group of starter cultures applied in the production of fermented foods and they display interest in functional properties such as acidification, proteolysis, aroma formation, etc [8].

The observation that host responses are dramatically influenced by bacterial fermentation conditions triggered our research group to develop a functional genomics fermentation platform that allows the identification and optimization of expression of probiotic functionality parameters.

The general goals of our research project are

1. To evaluate the metabolic properties of functional starter cultures from some Indian fermented food.

2. To evaluate the fermentation responses and interaction of these starter cultures with the food matrix. Here we are considering two aspects: Nutritional value and Potential health benefits.

The aims of the present study are:

1. To evaluate the functional properties of *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus delbrueckii*, like, Bile salt Hydrolase Activity, Exopolysaccharide production, Antibiotic susceptibility, Cholesterol Reducing Activity, Acid Tolerance Activity.

2. Screening out the best strain based on these assays.

The selection of potential probiotic strains that would be capable of performing effectively in the gastrointestinal tract is a significant challenge. Strain selection has generally been based on the above mentioned in vitro assays.

CHAPTER - 2

REVIEW OF LITERATURE

2.1 BENEFICIAL INTESTINAL BACTERIA

The intestine harbors an ecosystem composed of the intestinal mucosa, the digestive secretions and the commensal microbiota. The normal gut ecosystem can efficiently block intrusion of many pathogenic bacteria. This has been termed 'microbial interference' or 'colonization resistance' *Lactobacillus* sp. and *Bifidobacterium* sp. are microorganisms that form part of the human microbiota, having an important role in the first line of defence against opportunistic and invasive pathogens [9]. Moreover, the diseases and disorders such as inflammatory bowel disease, irritable bowel syndrome and obesity are associated with human gut microbiota where aberrations could be improved by consuming probiotic lactobacilli and bifidobacteria [10]. The underlying mechanisms depend on particular functional properties of different strains of the mentioned genera and species.

2.1.1 *Lactobacillus* spp.

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria, many of them having received a generally recognized as safe (GRAS) or qualified presumption of safety (QPS) –status. These bacteria are widely found in nature, including the GI and urogenital tracts of humans and animals, and are present in many fermented foods like salted gherkins, marinated olives, capers and salami, and different milk based products such as cheese and yoghurt [11].

LAB are gram-positive, acid-tolerant and non-spore forming cocci and rods. They are a heterogeneous group of bacteria comprising about 20 genera within the phylum *Firmicutes*. From the practical point of view the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* have been considered as the principal LAB [12].
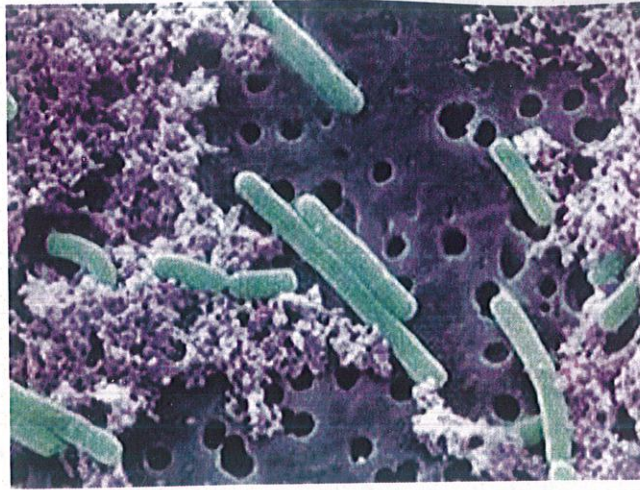
Fig 2.1 *Lactobacillus sp.* in raw milk

The classical way to distinguish between species of lactobacilli is based on the phenotypic properties of lactobacilli. According to carbohydrate fermentation patterns and growth at certain temperatures, the genus *Lactobacillus* is divided into homofermentative lactobacilli (OHOL), facultatively heterofermentative lactobacilli (FHEL), and obligately heterofermentative lactobacilli (OHEL) subgroups [12].

2.2 FUNCTIONAL FOOD

Diet and nutrition are important factors in the promotion and maintenance of good health throughout the entire life-course. However, rapid changes in diets and lifestyles have a significant impact on the health and nutritional status of populations. While the standards of living have improved, food availability has expanded and become more diversified. There have also been significant negative consequences in terms of inappropriate dietary patterns, decreased physical activities and increased tobacco use, and a corresponding increase in non communicable diet-related chronic diseases (*e.g.* obesity, diabetes mellitus type 2, cardiovascular disease, hypertension and stroke, and some types of cancer) (WHO, 2003).

Functional food (FF) is a natural food, to which a component has been added/removed or a food in which the bioavailability of the components has been modified by technological or biotechnological means. FF includes conventional foods, modified foods (fortified, enriched, or enhanced), medical foods, and foods for special dietary use [13]. FF can play an important role in the risk reduction of non-communicable diseases and can prolong remission in IBD (including Crohn's disease and

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ulcerative colitis) and alleviate allergic conditions by providing benefits beyond usual nutrition as well as in optimising health and general well-being [14].

The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) proposed a working definition of functional food: a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern: it is not a pill, a capsule or any form of dietary supplement.

Essential attributes or characteristics of functional foods are the following: form and sensory characteristics (colour, texture, consistency and flavour, including appearance in conventional food). Second, contain nutrients and/or other substances that confer a physiological benefit over and above their basic nutritional properties. Third, possess functional benefits that can be scientifically proven and accepted by the relevant regulatory authority. Fourth, possess functional benefits that can be derived by consuming normal amounts of the foods. Fifth, contain an adequate amount of 'functional' nutrients and/or other substances that produce the claimed effect/in relation to the claimed effect and last, have been proven to be safe during long term usage by the intended target population, based on existing science. FF should not be intended for medical or therapeutic use.

The most promising targets for FF are the GI functions and particularly control of nutrient bioavailability [15]. However, FF may affect different other systems in the body: balanced colonic microbiota, control of transit time and mucosal motility, bowel habits; modulation of epithelial cell proliferation, balance of redox and antioxidant systems, metabolism of macronutrients, especially amino acids, carbohydrates and fatty acids.

2.3 REGULATION AND GUIDELINES FOR THE EVALUATION OF PROBIOTICS

2.3.1 EU regulation on nutrition and health claims

Regulation (EC) No 1924/2006 of the European Parliament and Council of 20 December 2006 on nutrition and health claims made on foods covers all foods, including food supplements and foodstuffs for particular nutritional uses, and concerns nutrition and health claims in advertisements, labelling and presentation of foods to consumers. Regulation 1924/2006 identifies two categories of claims on foods: nutrition claims and health claims. In the context of EU Regulation 1924/2006, health claims are claims that state, suggest or imply a relationship between a food or food category and health. Examples hereof are function claims, reduction of risk of disease claims, or claims referring to the growth and development of children. Nutrition claims are claims that state, suggest or imply that a food has particular beneficial nutritional properties due to the energy it provides or

the nutrients it contains [16]. Health claims on (functional) foods must be scientifically substantiated. The European Food Safety Authority (EFSA) provides scientific advice to the European Commission for health claims. The settled regulations also demand more scientific evidence based research regarding probiotics and prebiotics.

2.3.2 Guidelines for evaluation of probiotics

A joint working group of the FAO and the WHO developed guidelines to assess the efficiency and safety of probiotics in food. The FAO and WHO focused on guidelines and recommendations for the criteria and methodologies required to identify and define probiotics and to establish the minimum requirements needed to accurately substantiate health claims (Figure 2.1) (FAO/WHO, 2002).
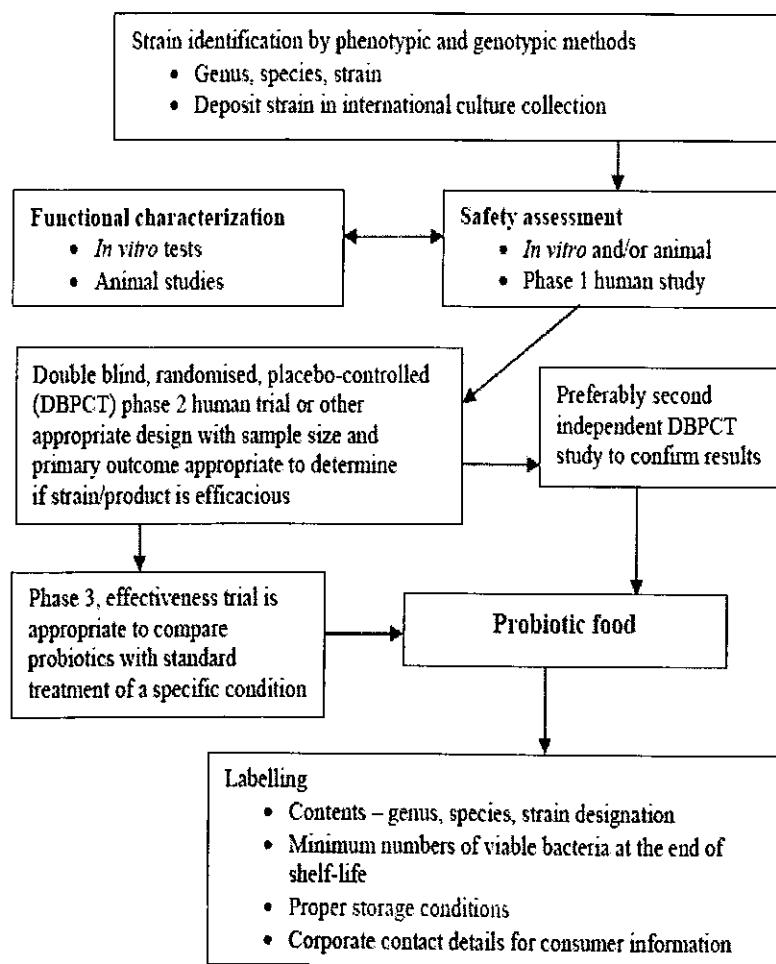
Fig 2.2 Guidelines for evaluation of probiotic strain

2.4 SAFETY OF PROBIOTICS

Lactobacilli belong to the human normal microbiota of the GI and urogenital tracts. Moreover, lactobacilli have been used for many centuries in food fermentation processes and have a long history. Lactobacilli are generally regarded as safe [17]. Therefore, the US Food and Drug Administration (FDA) has classified lactobacilli as GRAS (generally recognized as safe) organisms (Donohue, 2004). In Europe, the European Food Safety Authority (EFSA) has taken responsibility to launch the European initiative toward a "qualified presumption of safety" (QPS) concept which, similar to the GRAS system in the United States, is aimed to allow strains with an established history and safety status to enter the market without extensive testing requirements (EFSA, 2011b).

In order to establish safety guidelines for probiotic organisms, recognizing that many are Generally Recognized as Safe, the FAO and WHO recommended that probiotic strains be characterized at a minimum with a series of tests:

1) determination of antibiotic resistance pattern,

2) assessment of certain metabolic activities (d-lactate production, bile salt deconjugation),

3) assessment of side effects in humans,

4) epidemiological surveillance of adverse incidents in consumers,

5) testing for toxin production,

6) testing for haemolytic activity, and

7) infectivity in immunocompromised animal models (FAO/WHO, 2002).

The safety of probiotics should be confirmed in studies of humans. Although many research tools based on animal models or *in vitro* techniques are available, data from studies of humans are preferred whenever possible.

2.5 BIOMARKERS OF PROBIOSIS

2.5.1 BILE SALT TOLERANCE

The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection, and a number of bile salt hydrolases (BSHs) have been identified and characterized.

Bile is a yellow-green aqueous solution whose major constituents include bile acids, cholesterol, phospholipids, and the pigment biliverdin. It is synthesized in the pericentral hepatocytes of the liver, stored and concentrated in the gallbladder interdigestively, and released into the duodenum after food intake.

Bile functions as a biological detergent that emulsifies and solubilizes lipids, thereby playing an essential role in fat digestion. This detergent property of bile also confers potent antimicrobial activity, primarily through the dissolution of bacterial membranes.

The primary bile acids, **cholic and chenodeoxycholic acid** , are synthesized de novo in the liver from cholesterol. The solubility of the hydrophobic steroid nucleus is increased by conjugation as an *N*-acyl amidate with either glycine (glycoconjugated) or taurine (tauroconjugated) prior to secretion (Fig. 2.3). The resulting molecules are therefore amphipathic and can solubilize lipids to form mixed micelles [18].
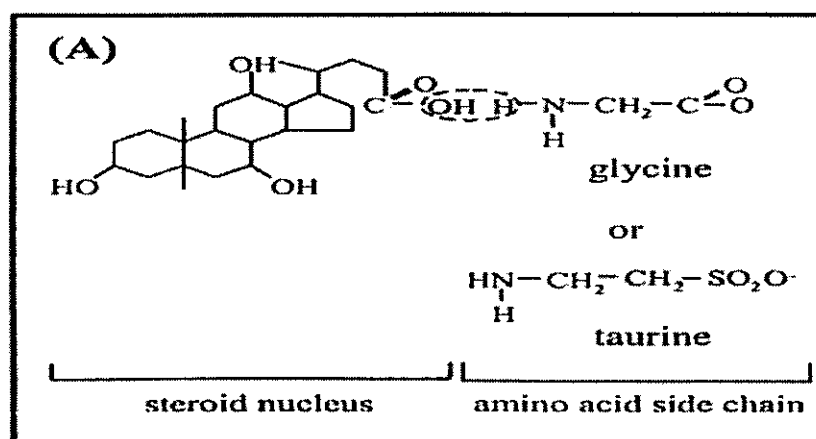
Fig.2.3 - Chemical structure of bile acids.

Primary bile acids are synthesized in the liver from cholesterol and are conjugated with either glycine or taurine prior to secretion. The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond. One important transformation is deconjugation, a reaction

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that must occur before further modifications are possible. Deconjugation is catalyzed by bile salt hydrolase (BSH) enzymes (EC 3.5.1.24), which hydrolyze the amide bond and liberate the glycine/taurine moiety from the steroid core (Fig.2.4).

(B)

conjugated bile acid →BSH→ unconjugated bile acid + glycine/taurine

↓
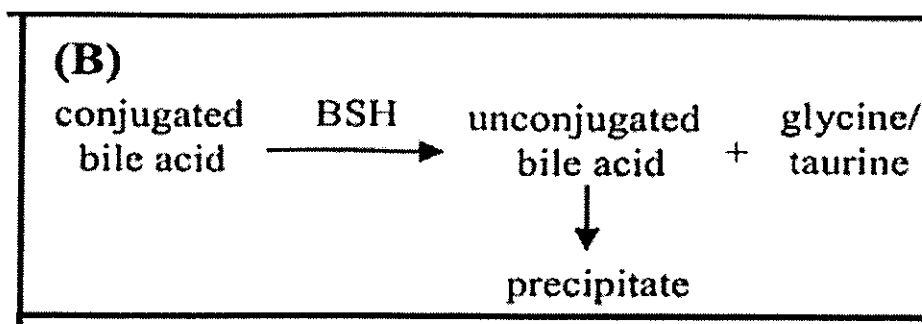
precipitate

Fig 2.4 - Reaction catalyzed by BSH enzymes.

BSHs cleave the peptide linkage of bile acids, which results in removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low pH. BSH activity has been detected in *Lactobacillus*, *Bifidobacterium, Enterococcus* , *Clostridium* , and *Bacteroides* spp. Lactobacilli and bifidobacteria are routinely used as probiotic strains, while *Bacteroides*, *Clostridium*, and *Enterococcus* spp. are also commensal inhabitants of the gastrointestinal tract. It has been documented to play number of role in order to help sustain organism in the gut although still research is going on, to have better understanding of its role. Some of its role is enlisted in Table 2.1 [18].

Table 2.1 - Role of BSH

Role or impact of BSH activity	References
Microbial role	
	Ahn *et al.*.2003 , Begley *et al.*.2005
Bile detoxification	
Gastrointestinal persistence	Bateup e al.1995,Begley *et al.*.2005
Nutritional role	
M Membrane alterations (may increase Resistance to bile, intestinal defensins,	Gilliland *et al.* 1977, Huijghebaert *et al.* 1982
Lysozyme, etc.)	Boggs, J. M. *et al.* 1987., Dambekodi *et al.* , 1998

Impact on the host	
Altered digestive functions (lipid Mal absorption, weight loss)	D De Smet *et al.* 1994 , Feighner *et al.* 1987
Cholesterol lowering	D De Smet *et al.* 1994, De Smet *et al.* 1995
Cancer/activation of carcinogens	B fernstein *et al.* 2005 , Huijghebaert *et al.* 1982
Formation of gallstones	
	Berr, F., *et al.* 1962 , Low-Beer *et al.* 1987

Now it's important to find out whether this particular biomarker is present in different Lactobacillus *sp.* or not.

2.5.2 EXOPOLYSACCHARIDE PRODUCTION

Several lactic acid bacteria (LAB) are able to produce extracellular polysaccharides (EPS) that either encapsulate the bacterial or are excreted into the extracellular environment.

The strains of lactic acid bacteria synthesize two types of exoploysaccharides:

homopolysaccharies

heteropolysaccharides

In case of homopolysaccharides there is a glucide which repeats, and for heteropolysaccharides different monoglucides repeat. EPS characteristics and amount can be influenced by several factors such as composition of the medium, incubation temperature, pH, time.

The use of EPS-producing starter cultures is one means of enhancing the viscosity of yogurt during manufacture [19]. Also, the health benefits of LAB have been attributed to the production of EPS which has anti-tumor, anti-ulcer, immunomodulating and cholesterol-lowering activities. Consequently, EPS-producing probiotic cultures can contribute to human health by positively impacting the gut microflora.

On the other hand, they probably have a protective function in the natural environment, e.g. against desiccation, phagocytosis and predation by phage attack, antibiotics or toxic compounds and

osmotic stress [19]. Another physiological benefit is that EPS is retained longer in the gastrointestinal tract, so that colonization by probiotic bacteria can be enhanced.

The lactic acid bacteria are food-grade organisms, and the Exopolysaccharides that they produce contribute to the specific rheology and texture of fermented products. When added to food products, polysaccharides function as thickeners, stabilizers, emulsifiers, gelling agents, and water binding agents [20].

2.5.3 ANTIBIOTIC SUSCEPTIBILITY

There is concern about the antibiotic resistance of lactic acid bacteria being transferred to possibly pathogenic bacterial species, complicating the treatment of infection and leading to the spread of antibiotic-resistant bacteria [21]. Therefore, all probiotic products intended for use as feed or food additives must be examined to establish the susceptibility of the component strain(s) to a relevant range of antibiotics, using internationally recognised and standardised methods [22]. There is a list of antibiotics, namely ampicillin, gentamicin, streptomycin, kanamycin, erythromycin, clindamycin, tetracycline, chloramphenicol, ciprofloxacin and quinupristin with dalfopristin, that the European Food Safety Authority (EFSA) has suggestsed for detecting the antibiotic susceptibility pattern of lactobacilli (EFSA, 2008). These antibiotics belong to a group of broad-spectrum antibiotics that are intended for treatment of gram-negative pathogens.

The disc diffusion method is most widely used for antibiotic resistance test because of its high degree of reliability towards the standardization of the antibiotic concentration and its relative case of performance. The primary role of the culture medium in this technique is to supply an optimal nutrient environment to support the growth of test organism. In addition it should also provide a suitable gel matrix to allow the reproducible and uniform diffusion of the antibiotic agent, hereby, minimising the possible chemical interaction between undefined medium components and the antibiotic gradient.

2.5.4 CHOLESTEROL REDUCTION ABILITY

Probiotics are live organisms that are primarily used to improve gastrointestinal disorders such as diarrhoea, irritable bowel syndrome, constipation, lactose intolerance, and to inhibit the excessive proliferation of pathogenic intestinal bacteria. However, recent studies have suggested that probiotics could have beneficial effects beyond gastrointestinal health, as they were found to improve certain metabolic disorders such as hypertension .Hypercholesterolemia and obesity are

strongly associated with primary hypertension [23]. Mann and Spoerry were the first researchers to illustrate the hypocholesterolemic effect of wild Lactobacillus-fermented milk. Emerging evidence has indicated that *lactobacilli* are not the only ones that exhibit hypocholesterolemic effects, but bifidobacteria could also cause a significant reduction in serum cholesterol when cholesterol is elevated. This is because cholesterol synthesis and absorption mainly occurs in the intestines, therefore intestinal microflora have profound effects on lipid metabolism.

Cholesterol-lowering effects can be partially ascribed to BSH activity other possible mechanisms include assimilation of cholesterol by the bacteria, binding of cholesterol to the bacterial cell walls, or physiological actions of the end products of short chain fatty acid fermentation [18]. The assimilation of cholesterol by probiotics in the small intestine could reduce serum cholesterol by reducing cholesterol absorption in the intestines. One beneficial effect that has been suggested from human consumption of probiotics is a reduction in serum cholesterol levels (reviewed by Pereira and Gibson). This effect can partially be ascribed to an enzymatic deconjugation of bile acids and other possible mechanisms include assimilation of cholesterol by the bacteria, binding of cholesterol to the bacterial cell walls, or physiological actions of the end products of short chain fatty acid fermentation. De conjugated bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterparts, which results in excretion of larger amounts of free bile acids in faeces. Also, free bile salts are less efficient in the solubilization and absorption of lipids in the gut. Therefore, the deconjugation of bile acids by probiotic bacteria could lead towards a reduction in serum cholesterol either by increasing the demand of cholesterol for de novo synthesis of bile acids to replace that lost in faeces or by reducing cholesterol solubility and, thereby, absorption of cholesterol throughout the intestinal lumen.

Probiotics must be viable and growing, in order to be able to remove or assimilate cholesterol [24]. Liong and Shah reported that cholesterol could be removed from media by *L. acidophilus* not only through assimilation during growth, but also through binding of cholesterol to the cellular surface. So we tested our Lactobacillus strains for cholesterol reducing activity to check that other than sustaining in the gut whether they can be of any beneficence to the host or not.

2.5.5 ACID TOLERANCE

Probiotic organisms encounter various environmental conditions upon ingestion by the host and during transit in the GIT, firstly, they need to survive the harsh conditions of the stomach. Humans secrete approximately 2.5 litres of gastric juice each day, generating a fasting pH of 1.5, increasing to pH 3 to 5 during food intake [25].

The effect of acid stress on the physiology of the organism can be varied like lowering of the intracellular pH reduces the transmembrane pH difference, which determines the proton motive force used as an energy source in numerous transmembrane transport processes. Also Internal acidification reduces the activity of acid-sensitive enzymes and results in damage to proteins and DNA of the organism thus a probiotic organism in order to perform its function has to sustain high acidic conditions of the gut and for testing the tolerance of an organism *in vitro* experiments are performed giving similar stress conditions in order to see their response.

CHAPTER - 3

MATERIALS AND METHODS

An overview of the material and methods used in this study are described as follows:

3.1 MATERIALS

3.1.1 **BACTERIAL STRAINS**

Three strains *Lactobacillus debrueckii* MTCC 911, *Lactobacillus plantarum* MTCC 2941 and *Lactobacillus rhamnosus* MTCC 1408 were obtained from Institute of Microbial Technology, Chandigarh in lyophilized form. Lyophilized forms were stored as glycerol stocks for long term preservation.

For this, 50% glycerol was prepared and autoclaved. For each glycerol stock, 0.85 ml glycerol and 0.15 ml water were put in an eppendorf tube and the lyophilized culture was inoculated and stored at -80 $^\circ$ C.

Table 3.1 – Strains along with their MTCC number

STRAINS	MTCC No.
Lactobacillus delbrueckii	911
Lactobacillus plantarum	2941
Lactobacillus rhamnosus	1408

3.1.2. CHEMICALS

MRS Broth (HiMedia)

INGREDIENTS	GRAMS
Peptone	10.0
Yeast Extract	05.0
D – Glucose	20.0
Polysorbate-80	01.0
K_2HPO_4	02.0
Sodium Acetate	05.0
Tri – ammonium citrate	02.0
$MgSO_4.7H_2O$	0.1
$MnSO_4.4H_2O$	0.05
Distilled Water	1000 mL

MRS Agar (HiMedia)

INGREDIENTS	GRAMS
Peptone	10.0
Beef Extract	8.0
Yeast Extract	5.0
Ammonium citrate	2.0
Sodium Acetate	5.0
$MgSO_4.7H_2O$	0.20
$MnSO_4.4H_2O$	0.05
K_2HPO_4	2.0
D- Glucose	20.0
Polysorbate 80	1.0
Distilled Water	1000 mL

Crystal violet (primary stain)

Iodine solution/Gram's Iodine (mordant that fixes crystal violet to cell wall)

Decolorizer (e.g. ethanol)

Safranin (secondary stain)

Distilled Water (preferably in a squirt bottle)

Saline

Pepsin

Pepsin was used at a concentration of 0.3% w/v in saline.

Glacial Acetic Acid (Analar Grade)

Conc.H2SO4

Tri chloro acetic acid

Absolute Ethanol

Phenol

Antibiotics

>Streptomycin (10 μg/ml)

>Ampicillin (10 μg/ml)

>Chloramphenicol (30 μg/ml)

>Tetracycline (30 μg/ml)

Ferric Chloride Solution

0.84% solution of ferric chloride ($FeCl_3.6H_2O$) was prepared in glacial acetic acid, 10 ml of this stock solution was diluted to 100 ml with glacial acetic acid.

Standard Cholestrol Solution

200 μg/ml of Cholestrol stock solution was prepared by dissolving 10 mg of cholesterol in 50 ml of glacial acetic acid and was filter sterilized.

Bile Salt

Sodium deoxychocholate (1mM concenteration).

Glycine

Glycine stock was made at a concenteration of 10 μmol/ml

Diethylthriotol (10 mM)

10 mM working solution was prepared from 100 mM stock of DTT.

Sodium Phosphate Buffer (0.1 M sodium-phosphate buffer pH 7.0)

100ml stock solution of Sodium Phosphate Buffer was made by adding 1.56 grams of Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) (monobasic) , 1.4 grams of Di Sodium hydrogen phosphate(Na_2HPO_4) (dibasic) in 50ml of distilled water. The pH of the solution was adjusted to 7 by addition of sodium hydroxide solution (1 N) and volume was made up to 100 ml with distilled water.

Sodium Phosphate Buffer (0.1 M sodium-phosphate buffer pH 6.0)

100ml stock solution of Sodium Phosphate Buffer was made by adding 1.56 grams of Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) (monobasic) , 1.4 grams of Di Sodium hydrogen phosphate(Na_2HPO_4) (dibasic) in 50ml of distilled water. The pH of the solution was adjusted to 6 by addition of Hydro chloride solution (1 N) and volume was made up to 100 ml with distilled water

Sodium Citrate Buffer (0.5 M sodium-citrate buffer pH 5.5)

200 ml stock solution of Sodium Citrate Buffer was made by adding 21.01 grams of citric acid (monohydrate), 29.41 grams of sodium citrate (dihydrate) in 150 ml of distilled water.The pH of the solution was adjusted to 5.5 by addition of sodium hydroxide solution (1 N) and volume was made up to 200 ml with distilled water

Ninhydrin Reagent

200 ml stock of ninhydrin reagent was prepared by adding 52.63 ml of 1% (wt/vol) ninhydrin in 0.5M sodium-citrate buffer,pH 5.5, 126.28 ml of glycerol, and 21.052 ml of 0.5 M sodium-citrate buffer pH 5.5.

TCA solution

TCA solution was made up of 15% (wt/vol) in distilled water

3.2. METHODS

3.2.1 GRAM STAINING

The bacterial smear was made on the slide and was allowed to dry for five minutes. Five drops of crystal violet stain was added over the fixed culture and was made to stand for 60 seconds. The stain was poured off and the excess stain was washed off with water gently. Five drops of the iodine solution was added on the smear, enough to cover the fixed culture. It was made to stand for 30 seconds. The iodine solution was poured off and the slide was washed with running water. A few drops of decolorizer were added so the solution trickles down the slide, and was rinsed off with water after 5 seconds and counterstained with five drops of the safranin solution for 20 seconds. And red safranin was washed off with water. Slide was blotted with blotting paper to remove the excess water.

3.2.2 GROWTH KINETICS

L.delbrueckii, L.plantarum and *L.rhamnosus* were inoculated in MRS Broth. The inoculated cultures were kept in a rotary shaker at 37°C. Aliquots were taken for the determination of absorbance and cell counts at regular intervals. Cell count of all the three strains was performed in MRS Agar. Plates were incubated at 37°C for 48 hours.
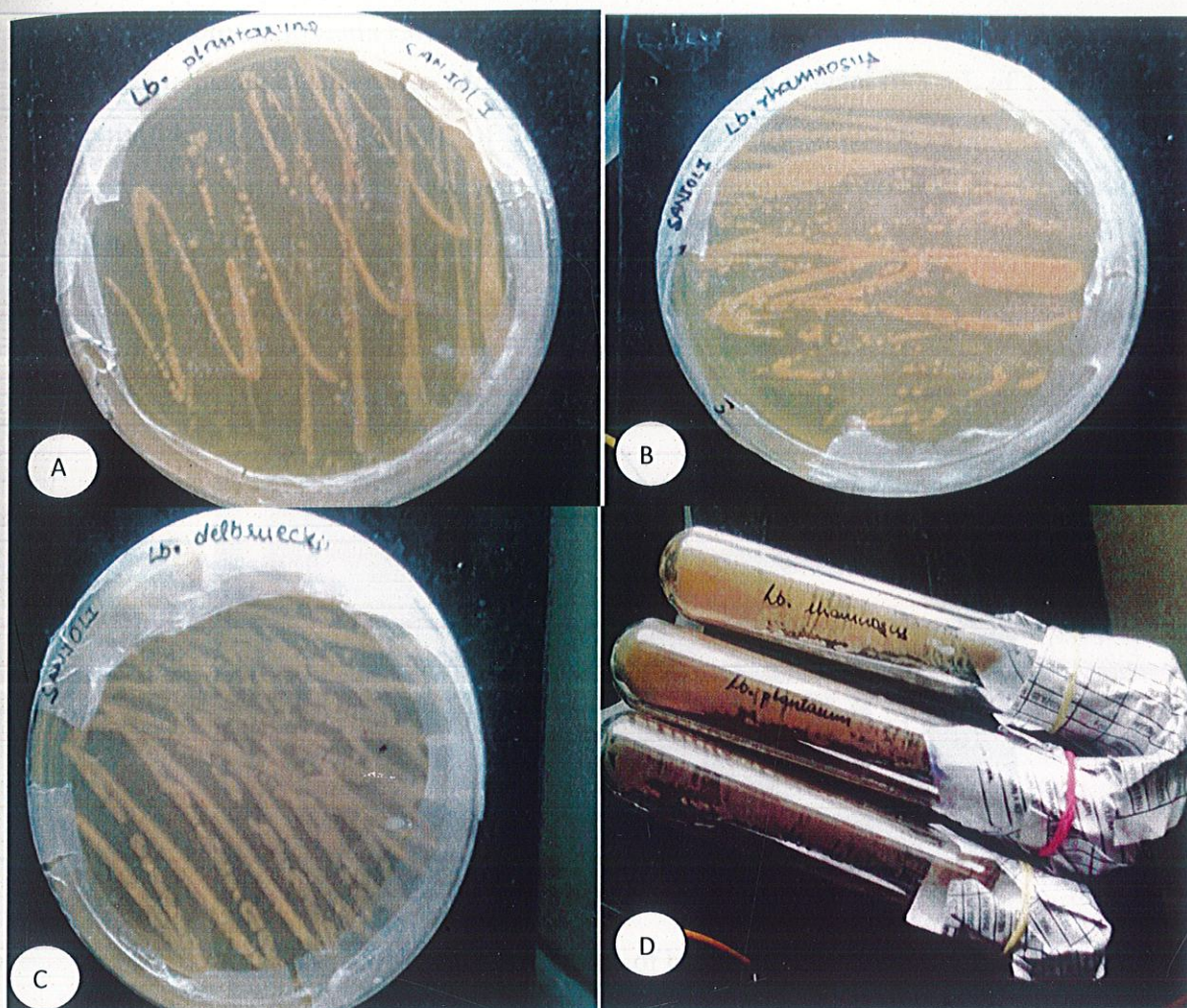
Fig 3.1 - Plates and slants showing the growth of bacterial cells. A- *L. rhamnosus*, B- *L. plantarum*, C- *L.delbrueckii* and D- slants of all 3 strains

3.2.3 BILE SALT HYDROLASE ASSAY

BSH activity was measured by determining the amount of amino acids liberated from conjugated bile salts by lactobacilli strains as described by Tanaka et al. [26] with some modifications. Briefly, cells grown in MRS broth for 20 h were centrifuged at 10000g at $4^{\circ}C$ for 10 min. The cell pellet washed twice before suspension into 10mL of 0.1 M phosphate buffer (pH 7.0). The cell concentration was adjusted to an OD value of 1 unit at 600 nm. Five millilitres of cell suspension was sonicated for 3 min with constant cooling in ice, followed by centrifugation at 10000f at $4^{\circ}C$ for 10 min. To 0.1mL of appropriately diluted supernatant obtained, 1.8 mL of 0.1 M sodium phosphate buffer (pH 6.0) and 0.1mL of conjugated bile salt, i.e., Sodium deoxycholate was added. The mixture was incubated at $37^{\circ}C$ for 30 min. Enzymatic reaction was terminated by adding

0.5mL of trichloroacetic acid (15% w/v) to 0.5mL of the sample. The mixture was centrifuged and 0.2mL of supernatant obtained was added to 1mL of ninhydrin reagent (0.5 mL of 1% ninhydrin in 0.5M citrate buffer pH 5.5, 1.2mL of 30% glycerol, 0.2 mL of 0.5M citrate buffer 5.5). The preparation was vortexed and boiled for 14 min. After subsequent cooling, the absorbance was determined at 570 nm against glycine as standard.
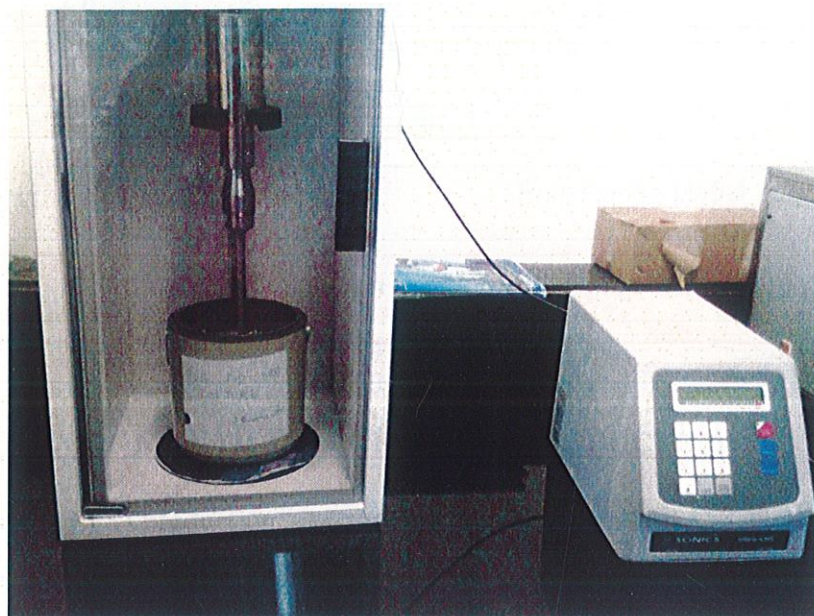

Fig 3.2 - Sonication of bacterial cells.

One unit of BSH activity is defined as the amount of enzyme that liberates 1nano mol of the amino acid from substrate per min per ml and is given as –

$$ \text{Enzyme Activity} = \frac{\text{amount of Amino acids liberated (nano moles)}}{\text{Time (min) x ml}} $$

(R. Suresh Kumar *et al.* 2006;H. TANAKA *et al.* 1999).

3.2.3.1 GLYCINE STANDARD CURVE

Estimation of Amino Acid (Glycine) was done by ninhydrin method. Ninhydrin, also known as triketohydrindene hydrate reacts with amino acids to give a purple coloured complex (Ruhemann's purple) with an absorption maximum at 570 nm. However, amino acids such as proline and hydroxyl-proline yield a yellow colour with an absorption maximum at 440 nm.

Ninhydrin oxidizes the amino acid (glycine) to aldehyde, releasing carbon dioxide and ammonia. During the course of reaction, ninhydrin gets reduced to hydridantin. The hydridantin formed

condenses with ninhydrin in the presence of ammonia to yield a purple coloured complex –
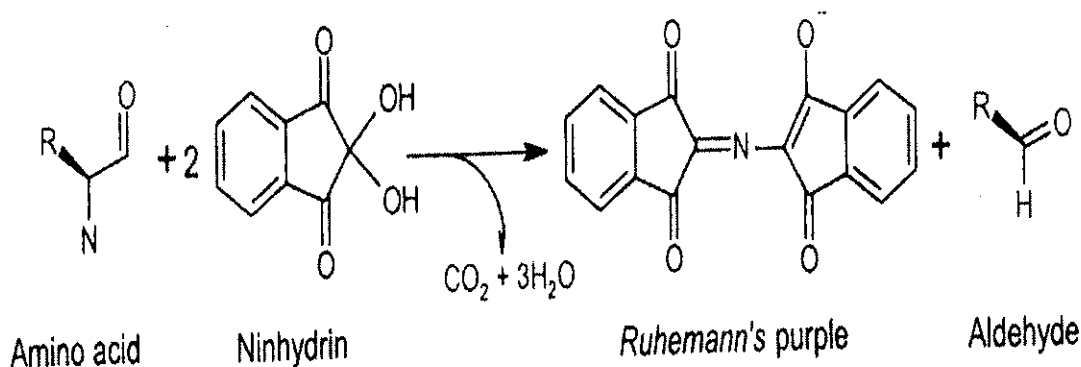Ruhemann's complex.

Fig 3.3 - Ninhydrin reaction with amino acid.

3.2.4 ESTIMATION OF EXOPOLYSACCHARIDES PRODUCTION.

Exopolysaccharide (EPS) production was determined according to the method of Savadogo et al.
[27]. Isolates showing a slimy growth in MRS agar were selected for this test. Isolates were cultured
in MRS broth and incubated aerobically at 37°C for 24 h. The samples were boiled at 100° C for 10
min. Then they were maintained for 10 min at room temperature (25°C), treated with 17 % (v/v) of
85 % trichloracetic acid solution and centrifuged at 13,000 rpm. After removal of the cells and
protein by centrifugation, the EPS was precipitated with ethanol (90 %). The EPS was recovered by
centrifugation at 4 °C at 14,000 rpm for 20 min. Total EPS (expressed as mg/l) was estimated in
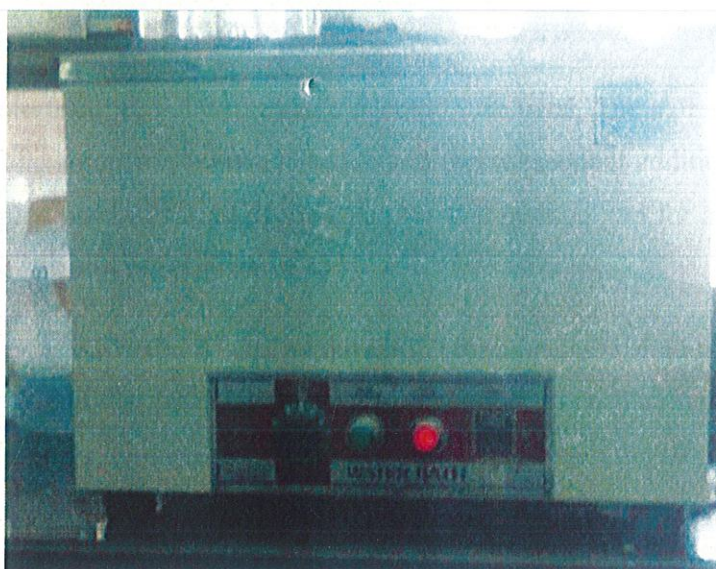each sample by phenol-sulphuric method using glucose as standard [28].

Fig 3.4 - Boiling the samples at 100°C.

3.2.5 ANTIBIOTIC SUSCEPTIBILTY TEST

In this test, each strain was spread uniformly on the surface of MRS agar plate. Stocks of all the antibiotics were made and were filter sterilized by 0.2 μ filter. A filter disc impregnated with the standard amount of an antibiotic was applied to the surface of the plate and each antibiotic was allowed to diffuse into the medium. We checked the resistance of four antibiotics, namely, Ampicillin (10 μg/ml), Streptomycin (10 μg/ml), Tetracycline (30 μg/ml) and Chloramphenicol (30 μg/ml). Following this, the plates were incubated for 24 hours at 37° C, and were observed for the Zone of Inhibition. The size of zone of inhibition is dependent on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganisms and the growth rate of the bacterium [29]. The test was performed under standardized conditions and the standard zones of inhibiton have been established for each antibiotic. If the zone of inhibition is greater than or equal to the standard the organism is considered to be sensitive to the antibiotic. If the zone of inhibition is less than the standard, the organism is considered to be resistant.

3.2.6 <u>CHOLESTEROL REDUCTION ASSAY</u>

For checking of Cholesterol Reduction, Zack's Method was used. The extraction and oxidation of cholesterol by an acidic solution of ferric chloride and the subsequent addition of sulphuric acid to form a colored complex has been used as the basis for this method and for a number of laboratory analysis for the determination of cholesterol.

25ml of Cholesterol (stock-200µg/ml) was added into 25ml of each broth to have final concentrations of 100µg/ml was inoculated with 2% net weight of each culture and were incubated for 24 hours. After 24 hours, cultures were centrifuged at 10,000 rpm for 10 minutes, and supernatant was stored.

500 µl of supernatant was taken, added to 500 µl of Glacial Acetic Acid in a test tube. To this mixture, 4 ml $FeCl_3$ was added. The tubes were cooled in an ice bath for 2 minutes. 4 ml of conc. $H_2 SO_4$ was then added to the tubes and after a lapse of 30 minutes absorbance was read at 560 nm against a suitable blank [30].

3.2.6.1 CHOLESTEROL STANDARD CURVE

To obtain the standard curve for Cholesterol, aliquots in the range of 10 µg/ml to 100 µg/ml were pipetted into different tubes from a stock solution of 200 µg/ml. The volume of each tube was made to a final volume to 1 ml with glacial acetic acid. 4 ml $FeCl_3$ solution was added to each tube. The tubes were cooled in an ice bath for 2 minutes. 4 ml of conc. H_2SO_4 was then added to the tubes and after a lapse of 30 minutes and then absorbance was read at 560 nm against a suitable blank.

3.2.7 <u>ACID TOLERANCE TEST</u>

All three cultures were grown at their desired conditions. Then, vials with preweight were taken and each strain was put in respective vials, centrifuged at 10,000 rpm for 10 minutes, supernatant was discarded, so as to get 2% net weight of each culture. Gastric juice was prepared by adding Saline (0.5% w/v NaCl) and Pepsin (0.3% w/v). Following this pH of gastric juice was adjusted to 2.0 and the gastric juice was then filter sterilized by 0.2µ filters.

After retaining the pellet, it was washed with Sodium phosphate buffer (pH 7). Gastric juice was added to each vial and then strains were kept at 30, 60, 90, 120 minutes of incubation. After completion of the incubation, all the vials were centrifuged at 10, 000 rpm for 10 minutes. The

supernatant was discarded and to each vial autoclaved distilled water was added and the solution was made homogenous by vortexing [31].
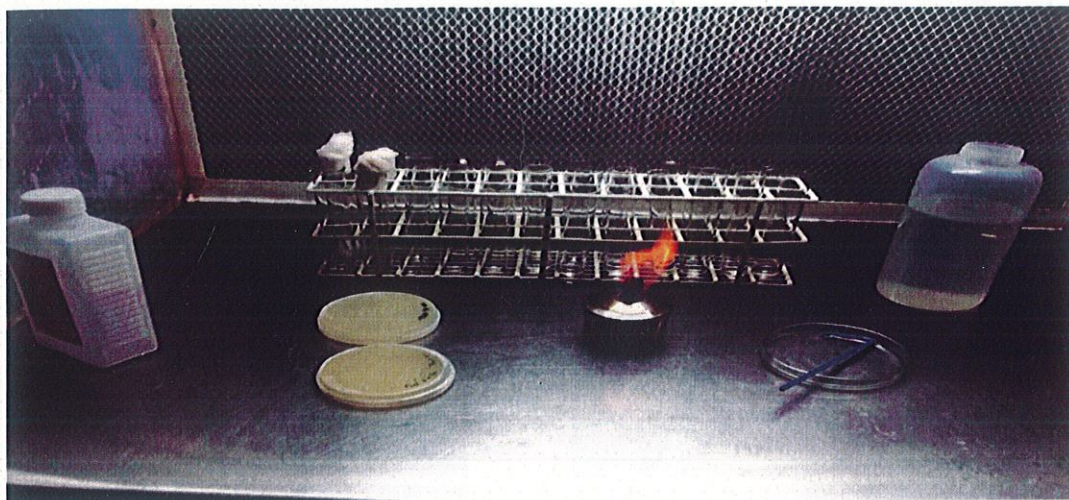

Fig 3.5 - Serial dilution done for each strain after each time interval.

Then, we took 1 ml of this homogenous solution and was serially diluted (for *Lactobacillus* strains dilution was done till 10^{-8} and as shown in Figure 4. After this, CFU was done from 10^{-4}, 10^{-6}, 10^{-8} dilution for *Lactobacillus* strains.

CHAPTER - 4

RESULTS

There are several biomarkers for probiosis, among all we have selected few, in order to test the probiotic potential of our test organisms i.e., *Lactobacillus delbrueckii*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus*.

4.1 <u>GRAM STAINING</u>

It was done for the morphology characterization of the strains.
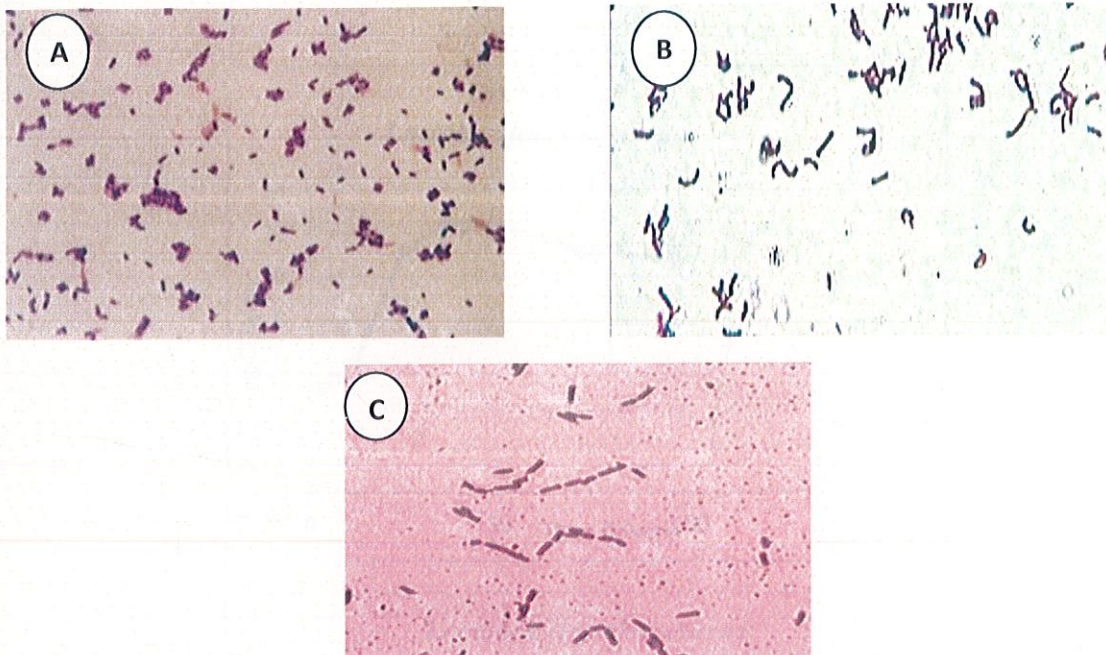
Fig 4.1 - Gram Staining of A- Lactobacillus rhamnosus, B – Lactobacillus delbrueckii and C-Lactobacillus plantarum

4.2 <u>GROWTH KINETICS</u>

Lactobacillus delbrueckii, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* showed exponential phase at 22 hrs with a log (cfu/ml) of 10.75, 10.96 and 10.10 respectively. Further the stationary phase for Lactobacillus species was observed after 36hrs.
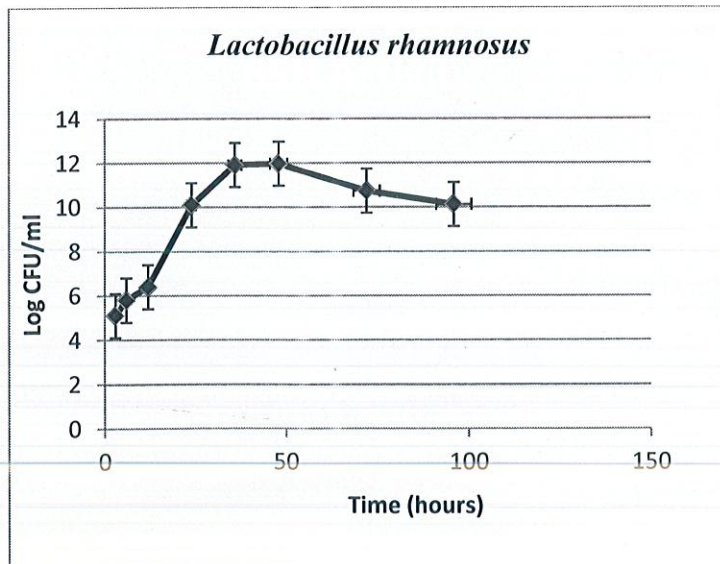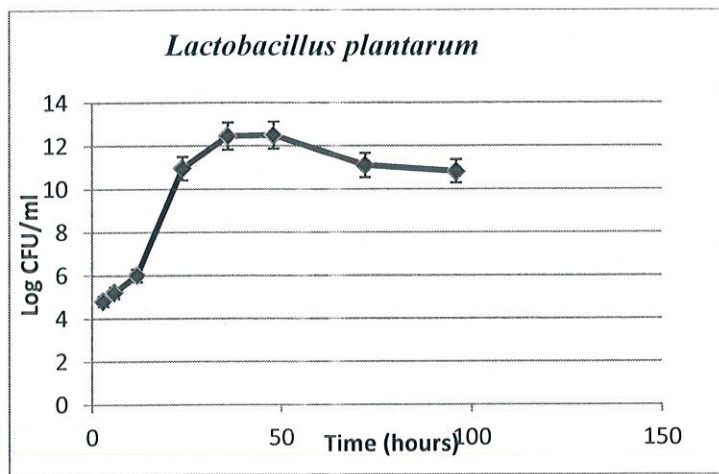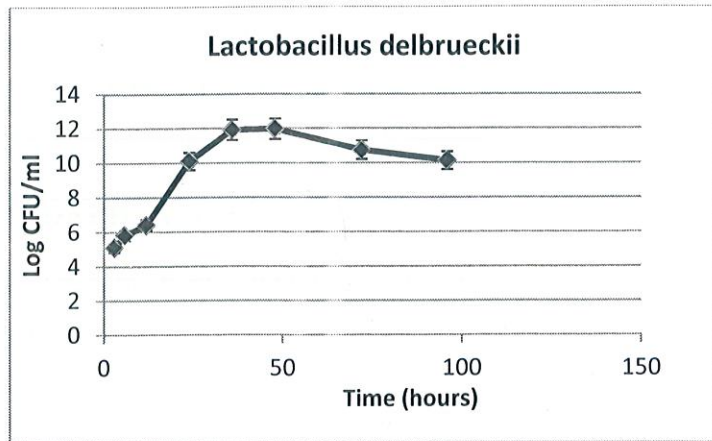
Fig 4.2 Growth curve showing Log CFU/ml versus Time (in hours)

4.3 BILE SALT HYDROLASE ASSAY

BSH is an intracellular enzyme which leads to de conjugation of conjugated Bile salts in the gut thus providing tolerance to organism against bile acids not only this it have number of other benefits as well, therefore it is a important marker for probiosis and its presence is critical for the survival of organism in the gut.

4.3.1 GLYCINE STANDARD CURVE

Amino acid standard plot was made by Ninhydrin Test using glycine as standard.
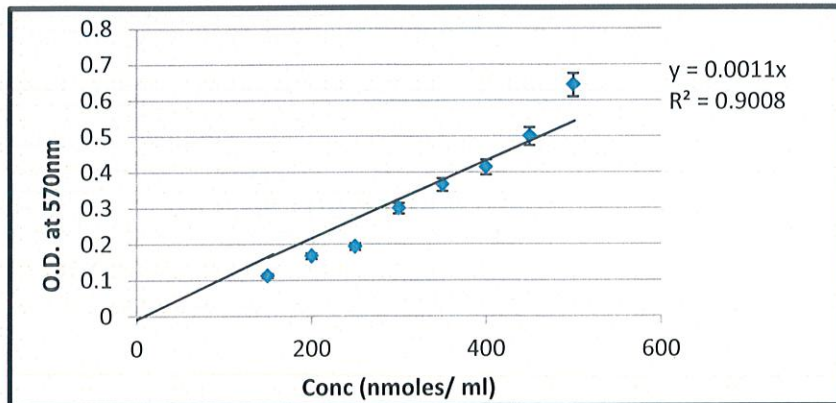
Fig 4.3 Glycine standard curve

4.3.2 BSH ACTIVITY
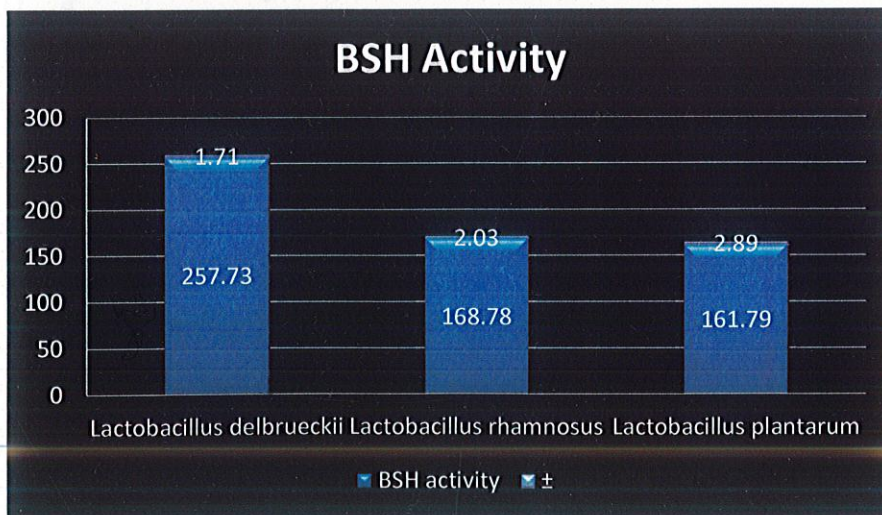
Fig 4.4 Comparison of BSH activity of the three strains

4.4 EXOPOLYSACCHARIDE PRODUCTION

Microbial exopolysaccharides are extracellular polysaccharides which are either associated with the cell surface in the form of capsules or secreted into the extracellular environment in the form of slime. EPS in their natural environment are thought to play a role in the protection of the microbial cell against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (e.g. toxic metal ions, sulfur dioxide, ethanol), predation by protozoans, osmotic stress, adhesion to solid surfaces and biofilm formation, and also in cellular recognition (e.g. via binding to a lectin). This polysaccharides may contribute to human health, either as non-digestible food fraction or because of their antitumoral , antiulcer, immunomodulating or cholesterol-lowering activity . Therefore EPS from LAB have potential for development and exploitation as functional food ingredients with both health and economic benefits.

4.4.1 GLUCOSE STANDARD CURVE
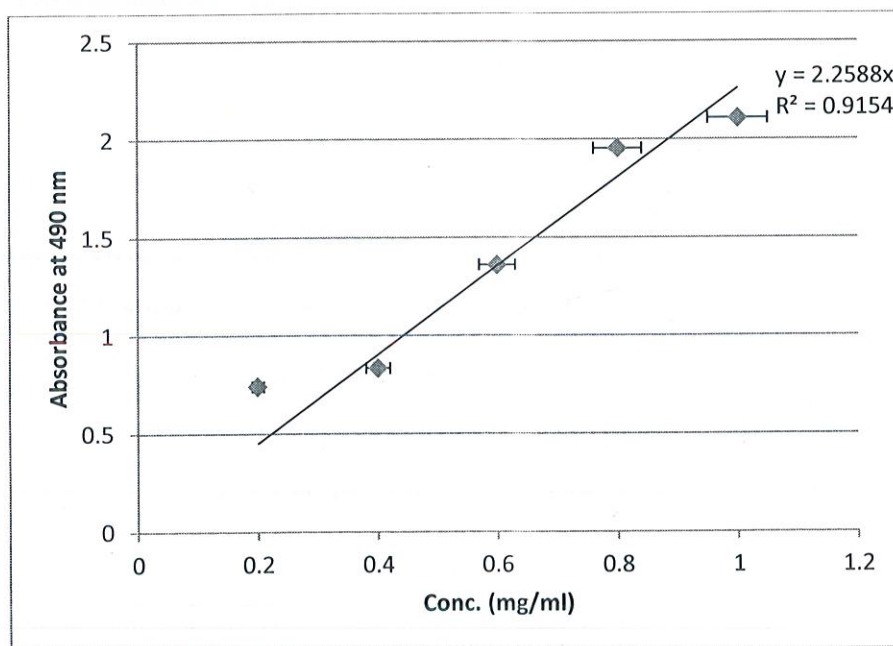
Fig 4.5 Glucose standard curve

4.4.2 EPS produced

Exoploysaccharide production (mg/ml)
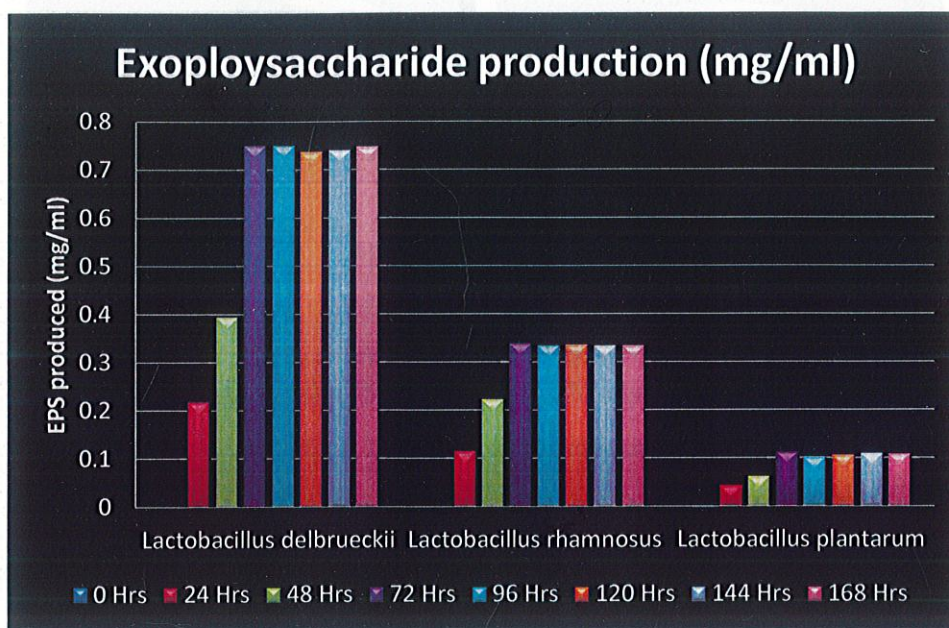
Fig 4.6 Comparison of EPS produced by 3 strains.

4.5 ANTIBIOTIC RESISTANCE

Antibiotic resistance character of all the strains was determined using Ampicillin, Streptomycin, Tetracycline and Chloramphenicol by Disc Diffusion Method.

Table 4.1 - Antibiotic resistance character of the three strains

Antibiotic Conc.	Lb. delbrueckii	Lb. rhamnosus	Lb. plantarum
Ampicillin (10 µg/ml)	20	15	10
Streptomycin(10 µg/ml)	------	-------	--------
Tetracycline (30 µg/ml)	18	10	8
Chloramphenicol (30 µg/ml)	14	11	9
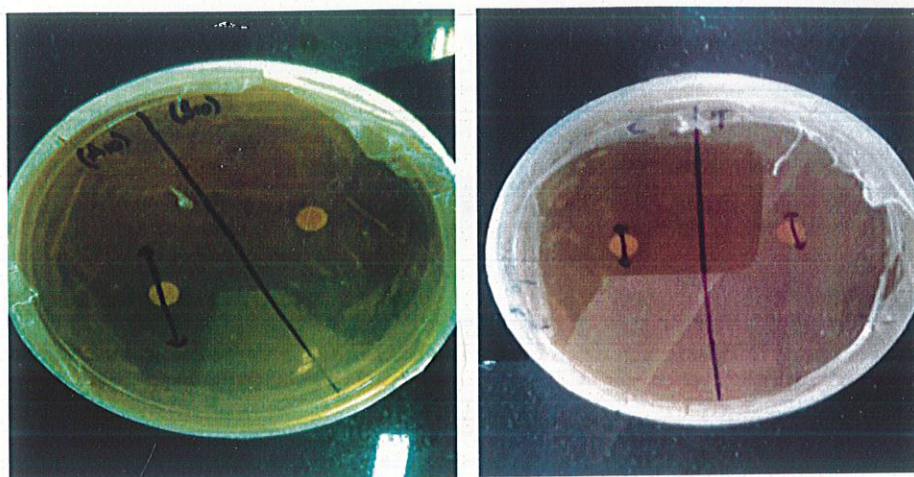
Fig 4.7 - Results of disc diffusion

It was inferred by looking at the diameter of zone of inhibition that L.plantarum and L.rhamnosus were resistant to Ampicillin, Tetracycline and Chloramphenicol, whereas L.delbrueckii is moderately resistant to Ampicillin, Tetracycline and Chloramphinicol. All the three strains were highly resistant to streptomycin since there was no formation of zone of inhibition.

4.6 CHOLESTEROL REDUCTION ASSAY

It was done by Zack's Method by taking cholesterol as standard.

4.6.1 Cholesterol standard curve

Different working concentrations of cholesterol (µg/ml) were taken as 10, 20, 30, 40,..... 100 µg/ml and Zack's reaction was carried out and O.D was taken at 560nm.
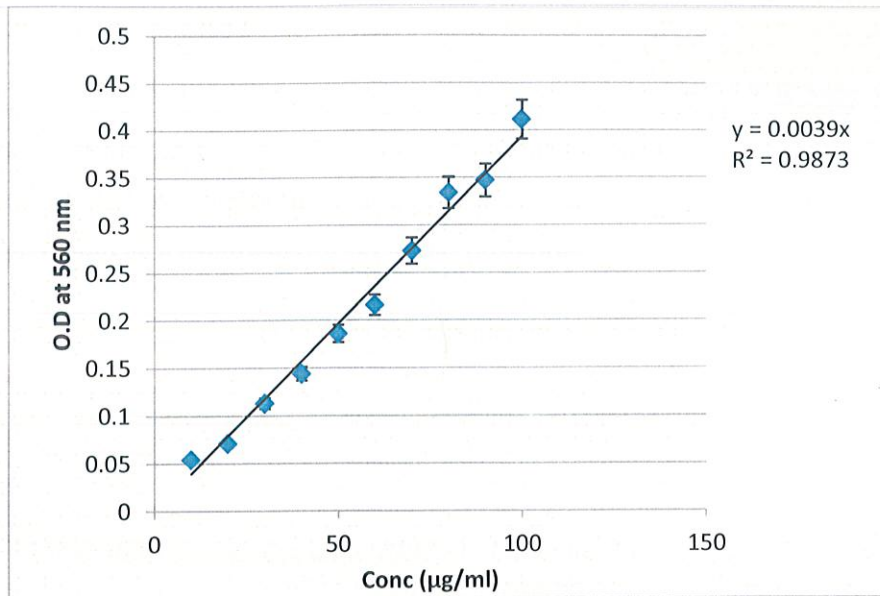
Fig. 4.8 – Cholesterol standard curve

4.6.2 Cholesterol Reduction
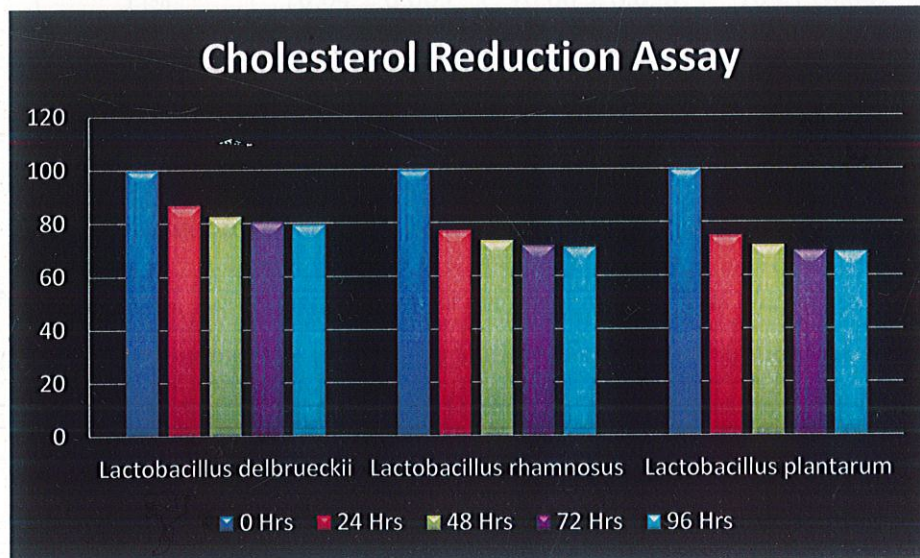
Fig 4.9 – Comparison of the cholesterol reduced by 3 strains

After 48 hours of incubation, there is a considerable percentage decrease in the concentration of cholesterol by 62%, 52.79% and 38.6% for L. rhamnosus, L. plantarum and L. delbrueckii, respectively.

4.7 ACID TOLERANCE TEST

For the organism to sustain in the gut and to be a potential probiotic it is very necessary that it tolerates various stresses one of which is the Gastric juice, which is secreted in stomach having a pH as low as 2, so it's very important that an organism should be checked for gastric tolerance in order to be considered as a candidate for potential probiotic.

Table 4.2 - Acid Tolerance Activity

| Acid Tolerance (Log_{10} cfu of viable bacteria ml^{-1})			
Incubation Time (min)	*Lactobacillus delbrueckii*	*Lactobacillus rhamnosus*	*Lactobacillus plantarum*
T_0	10.96 ± 0.084	11.62 ± 0.049	11.47 ± 0.035
T_{30}	10.87 ± 0.042	11.47 ± 0.035	10.79 ± 0.028
T_{60}	10.85 ± 0.021	10.74 ± 0.056	10.76 ± 0.042
T_{90}	10.80 ± 0.063	10.67 ± 0.028	10.65 ± 0.049
T_{120}	10.64 ± 0.063	10.60 ± 0.028	10.41 ± 0.042
% Survival after 120 min	97.08	91.22	90.75

Acid tolerance done for three strains, CFU/ml is calculated by plating 100µl of 10^{-8} dilution on MRS Agar Plates; plates incubated at 37^{0}C for 24hrs; colonies were counted (done in duplicates)

Lactobacillus delbrueckii, Lactobacillus plantarum and Lactobacillus *rhamnosus* were subjected to simulated gastric juice treatment at pH 2.0. Evolution of cell concentrations of all the three strains under gastric juice treatment is presented in Table 4.1. The percentage survival after 120min of exposure for *Lactobacillus delbrueckii, Lactobacillus plantarum* and Lactobacillus *rhamnosus were* 97.08%, 91.22% and 90.75% respectively.

DISCUSSION

The three strains obtained from IMTECH Chandigarh are well characterized *Lactobacillus sp.* which were isolated from fermented food product was morphologically characterized again using Gram staining technique and were found to be Gram positive rod shaped organism, retaining the crystal violet stain and thus showing that their cell wall is rich in peptidoglycan. Lactic acid bacteria are potential probiotic and are used as health adjuncts in food to provide a wide variety of health benefits.

The study was aimed at evaluating the metabolic properties of functional starter culture in order to investigate the potential probiotic biomarkers of the three *Lactobacillus* strains, *Lactobacillus delbrueckii*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus.*

Bile tolerance is considered to be an important characteristic for a probiotic that enables it to survive and then grow and exert its action in the small intestine (Pereira et al.,2003). Probiotics such as Lactobacillus sp. excrete bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), the enzyme that catalyzes the hydrolysis of glycine- and taurine-conjugated bile salts into amino acid residues and free bile salts (bile acids). The semi quantitative assay was performed and all the 3 strains were found to be positive for BSH activity.

Several lactic acid bacteria are able to produce extracellular polysaccharides (EPS) that either encapsulate the bacterial or are excreted into the extracellular environment(Hatice Bokee et al.,2010).The health benefits of LAB have been attributed to the production of EPS which has anti-tumor, anti-ulcer, immunomodulating and cholesterol-lowering activities. With reference to EPS, a protective function has been indicated especially in the natural environment, e.g. against desiccation, phagocytosis and predation by phage attack, antibiotics or toxic compounds and osmotic stress (Ruas-Madiedo et al.,2002). Another physiological benefit is that EPS is retained longer in the gastrointestinal tract, so that colonization by probiotic bacteria can be enhanced (Looijesteijn et al., 2001). Exocellular polysaccharides (EPS) produced by lactic acid bacteria (LAB) have been the subject of much research in recent years because of their immunogenic properties, their role in the texture of fermented dairy products and their potential use as thickening and gelling agents in place of exopolysaccharide produced by non food-grade organisms. Our results showed increased production of EPS with time and ultimately saturated after 72 hours of incubation. Out of the three strains tested by us, we found that *Lactobacillus delbrueckii* shows the highest EPS production followed by *Lactobacillus rhamnosus* and *Lactobacillus plantarum.*

The significance of antibiotic resistance of probiotic bacteria is currently unclear. Antibiotic resistance of bacteria used for food, feed and probiotic applications are being proposed as risky, due to the potential probability of resistance gene transfer especially to other pathogenic bacteria. The

antibiotic resistance potential was evaluated using disk diffusion tests for *L. delbrueckii, L. plantarum* and *L. rhamnosus.* It was inferred that the organisms were resistant to many of them ((Hemalatha *et al.* 2010). Since the usefulness of antibiotic resistance for the strains to be probiotic is debatable and may not be important criteria for the selection of potential probiotic strains. Therefore, with regard to general concerns on biosafety, further research is required to ascertain the location and potential transferability of their antibiotic resistance determinants.

Bile salt Hydrolase (BSH) has been found to have significant contribution in cholesterol reduction. In recent years, interest has risen in the possibility of using bile salt deconjugation by lactic acid bacteria to lower serum cholesterol level in hypercholesterolemic patients and prevent hypercholesterolemia in normal people (Liong et al., 2004). Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract. Conjugated bile salts are secreted into the small intestine for absorption of dietary fat, hydrophobic vitamins and other fat-soluble compounds. A small fraction of bile salts that are not absorbed is lost as free bile salts in feces. Free bile salts were less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen. Thus, in a steady-state situation, deconjugation of bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation. After 48 hours of incubation, there is a considerable percentage decrease in the concentration of cholesterol by 62%, 52.79% and 38.6% for L. rhamnosus, L. plantarum and L. delbrueckii, respectively.

Strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile acids, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits. Therefore, each strain was tested for tolerance to gastric juice at ph 2 for about two hours as the transit time of food from stomach is 2-3 hrs. *L. delbrueckii* showed a significant tolerance to the gastric juice followed by *L. plantarum* and *L. rhamnosus* with 97.08%, 91.22% and 90.75% respectively after 120 minutes.

All the three *Lactobacillus* strains showed a potential probiotic potential confirming the presence of biomarkers like Bile Salt Hydrolase, production of Exopolysaccharide, Antibiotic resistance, Cholesterol reduction and Acid tolerance. Thus *L. delbrueckii, L. plantarum* and *L. rhamnosus* can be further explored as functional starter cultures with potential probiotic efficiencies.

CONCLUSION

A heightened awareness among consumers of the link between diet and health and the fact that probiotic-containing foods are generally perceived as "safe" and "natural," the global market for such foods is on the increase. Lactic acid bacteria isolated from fermented foods have shown combinations of potential probiotic attributes. Interest in the field of probiotics has boomed in recent years, paralleling the renewed interest in studies focusing on microbial ecology of the gut and evaluation of these probiotic strains by *in vitro* studies

We tested *Lactobacillus delbrueckii, Lactobacillus plantarum* and *Lactobacillus rhamnosus* for various probiotic markers.

> Morphological characterization showed that these strains belong to gram positive family of bacteria.

> Bile tolerance is considered to be an important characteristic for a probiotic, also BSH has been found to be an important player in cholesterol reduction. All our three strains showed a positive BSH activity with a consequent reduction in serum cholesterol level.

> Upon testing for exopolysaccharide production ability, the three strains showed increased production of exopolysaccharides with time.

> Studies on antibiotic resistance suggested that the organisms were resistant to many of the antibiotics.

> The strains showed significant tolerance to the gastric juice with maximum tolerance shown by Lactobacillus *delbrueckii.*

Therefore, from our results we can infer that *Lactobacillus delbrueckii, Lactobacillus plantarum* and *Lactobacillus rhamnosus* have the potential probiotic features. Thus, an appropriate selection of probiotic strains is the basic for the further development of new probiotic products as well as for planning human clinical trials. *In vitro* studies are important to assess the safety and efficiency of probiotics and also, they are useful to expand the knowledge of specific properties of tested strains.

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