

**STUDIES ON THE HYPOCHOLESTEROLEMIC  
EFFECT OF *LACTOBACILLUS* ISOLATES FOR  
POTENTIAL APPLICATION AS PROBIOTICS**



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IN  
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# **Studies on the hypocholesterolemic effect of *Lactobacillus* isolates for potential application as probiotics**

By

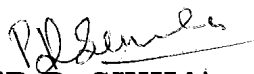
**Ab. Gani Lone**

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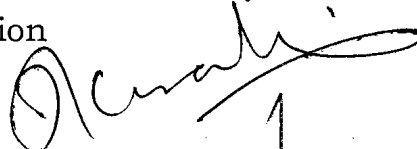

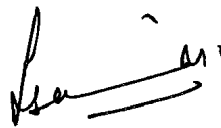
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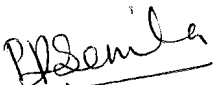
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This is to certify that the thesis entitled, "**STUDIES ON THE HYPOCHOLESTEROLEMIC EFFECT OF LACTOBACILLUS ISOLATES FOR POTENTIAL APPLICATION AS PROBIOTICS**" submitted by **SHRI AB. GANI LONE** in partial fulfilment of the requirements of the award of the degree of **DOCTOR OF PHILOSOPHY (DAIRYING)** in **ANIMAL BIOCHEMISTRY** of **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance, and no part of the thesis has been submitted for any other degree or diploma.

  
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## ABSTRACT

Efforts are being devoted worldwide to foods, which, beyond organoleptic and nutritional properties, can impart health-promoting benefits. Central to the development of such foods has been the growing application of probiotics, which in addition to promotion of gut-health, have recently been shown to aid beneficially in affecting the cardiovascular disease risk factors. Keeping this in mind, 50 strains of lactobacilli were screened for their probiotic properties which included evaluating the strains for: tolerance to pH levels of 1 to 4 and bile concentrations of 0.1 to 0.5%, cell surface hydrophobicity, antimicrobial activity against common enteric pathogens and food spoilage microorganisms, and bile salt (sodium taurocholate) deconjugating and plasma cholesterol assimilating capabilities. The strains with maximum probiotic potential were subjected to protoplast fusion, which resulted in no significant improvement in fusants over parents. Therefore, among the 50 strains, three, *Lactobacillus casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20, showing the best gut colonizing and cholesterol lowering potential, were selected for feeding to diet-induced hypercholesterolemic rats to check their hypocholesterolemic effect. *L. plantarum* 20 followed by *L. acidophilus* 14 exhibited a decreasing trend in the concentrations of total plasma cholesterol, LDL- and VLDL-cholesterol, and in the values of atherogenic index in both cholesterol-enriched diet as well as cream-enriched diet fed groups of rats. *L. casei* ssp. *casei* 19 had no effect on plasma cholesterol fractions of rats fed cholesterol-enriched diet. *L. plantarum* 20 amounted to a significant ( $p < 0.05$ ) fall of 35 and 30% in total plasma cholesterol of cholesterol-enriched and cream-enriched diet induced hypercholesterolemic rats, respectively. Atherogenic index was also significantly suppressed by 46 and 32% in cholesterol-enriched diet fed and cream-enriched diet fed groups, respectively. *L. plantarum* 20 can, therefore, be used as a dietary adjunct to help reduce the development of cardiovascular disease on population basis.

## सारांश

सम्पूर्ण विश्व में ऐसे आहारों पर अध्ययन हो रहा है जो रुचिकर एवं पोषक होने के साथ-साथ स्वास्थ्य की दृष्टि से भी लाभप्रद हैं। इन प्रयासों के केन्द्र में ऐसे प्रजैविक हैं जो आहार नाल के स्वास्थ्य में योगदान के साथ-साथ हृद-संवाही रोगों के खतरे के कारकों को भी प्रभावित करते हैं। उपरोक्त को ध्यान में रखकर लैक्टोबैसिलस के पचास तन्तुओं की प्रजैविक गुणों के लिये जांच की गयी जिसमें तन्तुओं की पी.एच. एक से चार तक तथा पित्त सान्द्रता 0.1-0.5% तक सध्यता, कोशा झिल्ली की सतह की सामान्य आंत्र रोगजन एवं भोज दूषी रोगाणुओं के विपरीत प्रतिजैविक सक्रियता, पित्त लवण (सोडियम टॉरोकॉलेट) वियुग्मन एवं कॉलेस्टेरॉल के स्वांगीकरण क्षमतायें शामिल थी। सर्वाधिक प्रजैविक सम्भाव्यता वाले तन्तुओं का जीवद्रव्य-युग्मन कराया गया जिसके परिणाम स्वरूप युग्मों का जनकों की अपेक्षा संज्ञेय सुधार नहीं पाया गया। तदनन्तर पचास तन्तुओं में से तीन (लैक्टोवैसिलस कैसाई स्पे-कैसाई 19, लै0 एसिडोफिलस, 14, एवं लै0 प्लैण्टेरम 20) का श्रेष्ठ प्रजैविक एवं आंत्रवासी तथा कॉलेस्ट्राल न्यूनकारी प्रवृत्तियों के आधार पर अति-कॉलेस्ट्राली चूहों को खिलाने के लिये चयन किया गया। सम्पूर्ण द्रव्यी कॉलेस्ट्राल, एल.डी.एल एवं वी.एल.डी.एल. कॉलेस्ट्राल तथा एथेरोजनक सूचकांक की लै0 प्लैण्टेरम 20 एवं लै0 एसिडोफिलस 14 में क्रमशः अवरोही प्रवृत्तियां पायी गयीं। कॉलेस्ट्राल प्रचुर एवं क्रीम मिश्रित आहार ग्रहण करने वाले चूहों के समूहों में लै0 कैसाई स्पे0 कैसाई 19 का द्रव्यी कॉलेस्ट्राल पर कोई प्रभाव नहीं देखा गया। लै0 प्लैण्टेरम 20 से कॉलेस्ट्राल प्रचुर एवं क्रीम-प्रचुर आहारों से उत्पन्न अतिकॉलेस्ट्रालता पर क्रमशः 35 से 30% न्यूनकारी प्रभाव पड़ा। एथेरो जनक सूचकांक पर भी कॉलेस्ट्राल प्रचुर एवं क्रीम-प्रचुर आहारों पर क्रमशः 46% एवं 32% का उल्लेखनीय प्रभाव पड़ा। अतः लै0 प्लैण्टेरम 20 जनता में हृद एवं संवाही रोगों को कम करने के लिये आहार के साथ दिये जा सकते हैं।

## 1. INTRODUCTION

One key priority in dairying is to increase the development of dairy foods that promote health and well being. The exploding area of functional foods (nutraceuticals) shows considerable promise to expand dairy industry into new arenas. Dairy foods fit naturally with probiotics, which offer a broad range of potential health benefits. Currently much interest has been generated worldwide to assess hypocholesterolemic effect of various lactic acid bacteria, especially lactobacilli, to be used as probiotic dietary adjuncts for the reduction of the risk of atherosclerotic cardiovascular disease (Taranto *et al*, 2000), which has a high mortality rate and is one of the major causes of death in the world (Agerholm-Larsen *et al*, 2000). Hypercholesterolemia is a major risk factor for the disease (Havenaare and Marteau, 1994). Also, drug intervention trials unequivocally demonstrate that drug induced reduction in serum cholesterol concentrations results in significant and marked reduction in mortality from the heart disease (Levin *et al*, 1995).

A high percentage of population has moderately elevated cholesterol levels and is thus at the risk of developing cardiovascular disease (Taylor and Williams, 1998; Agerholm-Larsen *et al*, 2000). It is clear that effective dietary strategies rather than drug approach (which entails many drug induced deleterious side effects) in lowering plasma cholesterol levels, would have considerable benefits in terms of economy in reducing morbidity and mortality from atherosclerotic cardiovascular disease. Current dietary strategies for prevention of atherosclerotic heart disease

advocate adherence to low fat or low saturated fat diets. Although under experimental conditions, low fat diets offer an effective means of reducing blood cholesterol concentrations (Taylor and Williams, 1998), on population basis they appear to be much less effective, largely due to poor long-term compliance attributed to low palatability and acceptability of these diets to the consumer.

The above discussion then leads to identifying dietary components, like probiotics, which can be used, if found hypocholesterolemic, in preventing atherosclerotic cardiovascular disease on population basis. The 83<sup>rd</sup> Indian Science Congress (January, 1996) also recommended for the development of effective solutions for cardiovascular disease and the probiotic dairy foods for their innate therapeutic values. Therefore, the following objectives were undertaken in the Ph. D. research project:

1. Screening of different *Lactobacillus* isolates for their effect on cholesterol reduction and bile salt deconjugation.
2. *In vitro* evaluation of gut colonizing efficiency of selected strains of probiotics.
3. *In vivo* evaluation of hypocholesterolemic effect of different probiotic cultures affecting directly cholesterol and thus the development of cardiovascular disease.

## 2. REVIEW OF LITREATURE

The word 'probiotic' is derived from the Greek meaning 'for life' and was first used by Lilley and Stillwell in 1965 to describe substances secreted by one microorganism, which stimulated the growth of another. Since then, it has had several different meanings over the years. Parker (1974) defined probiotics as organisms and substances, which contribute to intestinal microbial balance. Fuller (1989) redefined probiotics as a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. Salminen *et al* (1999) further elaborated probiotics as "Microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host."

Although the word "probiotics" relating to feed supplements dates from 1974, the history of live microbial feed supplements goes back thousands of years. The beneficial effects of microbial feed supplements were put on scientific basis at the beginning of 20<sup>th</sup> century and the work of Russian Noble Laureate, Elie Metchnikoff (Metchnikoff, 1908), who authored the book, *The Prolongation of Life* (which contained material related to probiotics), can be regarded as the birth of probiotics, i.e. microbes ingested with the aim of promoting good health.

Many health promoting benefits, including disease prophylactic ones, have already been attributed to probiotics; for example, their beneficial effect in urogenital infections, lactose intolerance, diarrhoea, hypertension and cancer (Salminen *et al*, 1998). Currently much interest has been

generated worldwide to assess hypocholesterolemic effect of various lactic acid bacteria, especially lactobacilli, to be used as probiotics to reduce the risk of atherosclerotic cardiovascular disease (Agerholm-Larsen, 2000; Taranto *et al*, 2000).

Hypercholesterolemia is a well known risk factor for cardiovascular heart disease (Havenaare and Marteau, 1994) and because it was proved that atherosclerosis may be reduced by controlling serum cholesterol concentrations (Shiomi *et al*, 1990), numerous active substances with serum cholesterol lowering functions have been investigated. Mann (1977) found that large dietary intakes of yoghurt lowered cholesterol in man. Since then many reports have appeared suggesting strain specific hypocholesterolemic effect of lactic acid bacteria, including lactobacilli (Grunewald, 1982; Chawla and Kansal, 1984; Zommara *et al*, 1996; Fukushima and Nakano, 1996; Taranto *et al*, 1998; Agerholm-Larsen *et al*, 2000).

Although it is not exactly known how some strains of lactic acid bacteria that lower plasma cholesterol do so, but it is suggested by several workers (Gilliland and Walker, 1990; Taranto *et al*, 1998; Usman and Hosono, 1999) that the same mechanism by which they remove cholesterol and deconjugate bile salts in growth medium may be operated *in vivo* by these strains to lower plasma cholesterol by affecting intestinal absorption of dietary cholesterol and enterohepatic circulation of bile acids. Therefore, if a bacterial culture is able to remove cholesterol from or deconjugate bile acids in an artificial growth medium, it may be able to exert hypocholesterolemic effect if fed to animals (Taranto *et al*,

1998), provided it successfully survives harsh environment of the gut and is able to colonize it without any harm to host.

It is difficult to find all the potential gut colonizing and plasma cholesterol lowering properties in a single strain of lactic acid bacteria. Thus, in an attempt to enhance and merge different probiotic properties of two strains into single one, various workers have resorted to protoplast fusion (Iwata *et al*, 1986; Cocconcelli *et al*, 1986), a non-specific gene transfer technique.

In the light of above discussion, various aspects pertaining to plasma cholesterol lowering effect of lactic acid bacteria will be reviewed under following major headings:

1. Gut colonizing and plasma cholesterol lowering potential.
2. Protoplast fusion
3. *In vivo* plasma cholesterol lowering effect.

## **2.1 Gut colonizing and plasma cholesterol lowering potential**

A microorganism exhibiting plasma cholesterol lowering potential, i.e. cholesterol removing and bile salt deconjugating ability in growth medium, may not be able to do so *in vivo*. It may be because, for many microorganisms, the stomach and intestinal tract present a hostile environment that can easily discourage their growth and survival (Brown, 1977; Sandine, 1979). Thus, the following factors ought to be considered for selection of a *Lactobacillus* culture as dietary adjunct to lower plasma cholesterol levels:

### 2.1.1 Acid tolerance

Lactobacilli, which are to be used as dietary adjuncts, must survive the extremely low pH of human stomach (Gilliland, 1979; Klaenhammer, 1982; Kim, 1988).

Several studies have shown that various lactic acid bacteria were acid tolerant (Conway *et al*, 1987; Hood and Zottota, 1988; Clark and Martin, 1993; Lankaputhra and Shah, 1995). The acid tolerance differed among lactic acid bacteria; and at various pH levels, it was strain specific within a species. *L. acidophilus* was shown to have higher tolerance than *L. delbreuckii*, which in turn was more resistant than *Streptococcus salivarius* subsp. *thermophilus* in gastric juice of low pH (Conway *et al*, 1987). Lankaputhra and Shah (1995) reported a strain dependent acid tolerance in lactobacilli and bifidobacteria at pH levels of 1.5 to 3.0, with viability ranging between  $10^7$  cfu/g to  $10^8$  cfu/g of culture for different strains.

Hood and Zottota (1988) examined the effect of various growth media (MRS broth, potassium chloride buffer and potassium phthalate buffer) on acid tolerance of *L. acidophilus* at pH levels of 2.0, 3.0 and 4.0. At pH 2.0 in both buffer and broth, bacterial numbers rapidly declined to zero after 45 minutes of incubation from an initial cell number of  $10^7$  to  $10^8$  cfu/ml, but at pH levels of 3.0 and 4.0 of MRS broth, the cell numbers remained relatively constant over 2 hours of incubation. In a similar study Plockova *et al* (1996) reported better survival of lactobacilli at low pH in MRS broth than in potassium chloride or phthalate buffer. *In vitro* tolerance to acid was also affected by the presence of foods like milk (Conway *et al*, 1987), which may act both as

buffering agent and as an inhibitor of digestive protease activity (Charteris *et al*, 1998). These studies are in conformation to *in vivo* studies of Robins-Browne *et al* (1981) who observed that the survival of *L. bulgaricus* and *L. acidophilus* in gastric fluid after passage through stomach was much lower in fasting subjects than in non-fasting ones.

Acid tolerant strains obtained through natural selection techniques by sequential exposure to hydrochloric acid (pH 3.5 to 7.0) exhibited growth advantages in stability, freezing, lactose utilization, protease activity, aminopeptidase activity, plasmid profile and cell wall fatty acid profile over those of acid sensitive parents (Chou and Weimer, 1999). Lorca and Valdez (2001) observed that some proteins were over expressed and some exclusively expressed in stationary phase of acid resistant strains of food fermentation lactobacilli. Metabolic adaptation of acid tolerant food fermentation lactobacilli was also observed by De Angelis *et al* (2001) and Haller *et al* (2001).

### **2.1.2 Bile tolerance**

Bile tolerance is an important characteristic of probiotic lactic acid bacteria used as dietary adjunct, since this enables them to survive, to grow and to perform their beneficial action in the small intestine (Gilliland and Walker, 1990).

Bile concentration varies in the intestines and its concentration could reach upto 2% during the first hour of digestion of food in the small intestine and then could decrease to 0.5% by the second hour of digestion (Davenport, 1977).

Several studies have shown that various lactic acid bacteria were bile tolerant (Gilliland, 1979; Chateau *et al*,

1994; Clark and Martin, 1994). This tolerance was different among different lactic acid bacteria; and at various bile concentrations, ranging from 0.1 to 1%, of different growth media, it was reported to be strain dependent (Gilliland, 1979; Gilliland *et al*, 1984; Gilliland and Walker, 1990; Jin *et al*, 1998; Jacobson *et al*, 1999; Usman and Hosono, 1999).

The addition of milk proteins, in general, was seen to improve bile tolerance in simulated small intestinal juice and this could be because of milk functioning as buffering as well as digestive protease inhibiting agent (Charteris *et al*, 1998).

Bile tolerant strains have been reported to possess some growth advantages over non-tolerant ones. Noh and Gilliland (1997) concluded that, in the presence of bile, the permeability of cells of *L. acidophilus* increased, permitting more substrate to enter the cells, thus increasing beta-galactosidase activity of the cells. Gupta *et al* (1996) tested seven strains of *L. acidophilus* for various beneficial traits, like bile and pH tolerance, and found that the strains significantly differed in their biochemical and beneficial traits. Chou and Weimer (1999) obtained bile tolerant isolates, using natural selection techniques after sequential exposure to mixed bile salts. They observed that the bile tolerant strains exhibited growth advantages in stability, freezing, lactose utilization, protease activity, aminopeptidase activity, plasmid profile and cell wall fatty acid profile over those of non-tolerant ones. Bile tolerance in some food fermentation lactobacilli was also observed by Haller *et al* (2001).

### **2.1.3 Cell surface hydrophobicity**

Adherence and colonization are important properties of probiotic strains, comparable with viability and metabolic

activity. An important aspect, which is being widely considered in the bacterial adhesion, is the cell surface hydrophobicity (Rosenberg *et al*, 1981; Rijnaarts *et al*, 1993; Prakash *et al*, 1997). This phenomenon may be due to the interaction involving free energy change associated with the process of bringing two infinitely, separated entities to distance of the order of molecular dimensions (Ben-Naim, 1977).

Cell surface hydrophobicity appears to be an important factor in adhesion of bacterial cells to the gastro-intestinal tract. Adhesion of rough enterobacterial strains to intestinal mucosal cells was greater than that of less hydrophobic, smooth wild type cells (Peres *et al*, 1977). Partitioning methods with hydrocarbons, have been used in many investigations as a measure of adherence (Rosenberg *et al*, 1981; Busscher *et al*, 1984). Rosenberg *et al* (1981) observed a positive correlation between the ability of bacterial cells to adhere to intestinal epithelial cells and to hydrocarbons. Several workers have shown that lactobacilli having higher cell surface hydrophobicity exhibited higher adhesion indicating involvement of hydrophobic interactions in the adhesion of these bacteria to epithelial cells (Van Oss, 1998; Wadstrom *et al*, 1987; Rijnaarts *et al*, 1993; Prakash *et al*, 1997).

Using hexadecane, percent cell surface hydrophobicity has been reported from 0.8% to 95% for different lactic acid bacteria (Rosenberg *et al*, 1981; Rijnaarts *et al*, 1993; Prakash *et al*, 1997; Aftabuddin, 1997; Anuradha, 2000).

A decrease in bacterial cell surface hydrophobicity was observed after subculturing lactobacilli in liquid medium

(Reid *et al*, 1992). Spencer and Chesson (1994) reported that lactobacilli showed better attachment ability to porcine enterocytes when grown on agar than when grown in broth culture.

Bhowmik *et al* (1985) reported that high degree of cell surface hydrophobicity in *L. acidophilus* was protein dependent. Christenson *et al* (1985) explored the positive association of lipoteichoic acid-protein complexes with cell surface hydrophobicity in lactobacilli.

#### **2.1.4 Antimicrobial activity**

Among lactobacilli, *L. acidophilus*, *L. gasseri*, *L. reuteri*, *L. casei*, are said to have beneficial effect in the gastro intestinal tract and other parts of the body. These effects include the suppression of undesirable and pathogenic microorganism by competitive exclusion and antagonism (Korasapati, 1998). This inhibition may be due to the cumulative effect of acids, hydrogen peroxide, antimicrobial metabolites and bacteriocins (Klaenhammer, 1993). The *in vivo* effects of lactobacilli and their antimicrobial products, however, remain to be established.

A number of investigators have reported the inhibitory effects of fermented milk products against different pathogenic and spoilage bacteria (Lin *et al*, 1986; Garriga *et al*, 1993; Jack *et al*, 1995). Various organisms like *Staphylococcus aureus*, *Pseudomonas putrefaciens*, *Clostridium sporogenes*, *Salmonella typhi*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Bacillus subtilis*, *Shigella dysenteriae*, *Klebsiella spp.*, *Proteus spp.*, *Micrococcus flavus* and *Listeria monocytogenes* have been reported to be inhibited by strains of *L. bifidus*, *L.*

*acidophilus*, *L. bulgaricus*, *L. helveticus*, *L. casei* and *L. plantarum* (Gandhi and Nambudripad, 1981; Ryser and Marth, 1987). Different workers have associated different bacterial metabolites like lactic acid (Kim, 1988), hydrogen peroxide (Gilliland and Speek, 1977), organic acids (Fuller, 1989) or bacteriocins with the overall antibacterial activity of these organisms.

Bacteriocins are protein or peptide antibiotics of relative high molecular weight mainly working against the same, or closely related species by adsorption to the receptors on the target cell (Ouweland, 1998). Bacteriocins of the colicin type (prototype bacteriocin produced by *E. coli*) possessed a bactericidal mode of action with a narrow inhibitory spectrum (Koninsky, 1982) whereas the bacteriocins of Gram-positive organisms usually had a broader inhibitory spectrum and were also active against other Gram-positive species (Tagg *et al*, 1977). Although some bacteriocin-like substances produced by Gram-positive bacteria (especially some of those produced by lactococci and lactobacilli) appeared to have relatively narrow inhibitory spectra, most were much more broadly active than the colicins. In general, they are active against a wide range of Gram-positive bacteria, and plantaricin F (Fricourt *et al*, 1994) and acidophilin 801 (Zamfor *et al*, 1999) have also been reported to inhibit Gram-negative species as well.

The non-bacteriocin antimicrobials such as acidolin (Hamdan and Mikolajcik, 1974), acidophilin (Shahani *et al*, 1976, 1977) and bulgaricin (Shahani *et al*, 1976; Reddy *et al*, 1983) have been reported to possess a much broader inhibitory spectrum; many gram positive and Gram-negative bacteria (including pathogens such as staphylococci,

salmonella, shigella, pseudomonads) were said to be inhibited by the purified products.

#### **2.1.5 Cholesterol removal and bile salt deconjugation**

Amongst the probiotic effects attributed to lactic acid bacteria, assimilation (removal) of cholesterol and deconjugation of bile acids in the small intestine may be important in lowering the blood cholesterol concentration. Assimilation of cholesterol in the small intestine by the bacteria may be playing an important role in reducing the absorption of dietary cholesterol from the digestive system into the blood, and deconjugation of bile acids in the small intestine may be important in controlling serum cholesterol concentration as deconjugated bile acids do not function as well as conjugated bile acids in solubilization and absorption of lipids (Walker and Gilliland, 1993). Also, several studies have indicated that the cholesterol removal would be related to the ability of cultures to deconjugate bile salts (Gilliland and Speck, 1977; Gilliland *et al*, 1985; Taranto *et al*, 1996).

Results of several studies conducted *in vitro* have shown that many strains of lactic acid bacteria were able to remove cholesterol from and deconjugate bile salts in growth medium (Gilliland *et al*, 1985; Walker and Gilliland, 1993; Taranto *et al*, 1996; Usman and Hosono, 1999). Considerable variation existed among different *Lactobacillus acidophilus* cultures for assimilation of cholesterol and in general, most strains that exhibited cholesterol assimilation expressed bile salt deconjugation and bile resistance as well, but the correlation among the three traits was insignificant statistically (Walker and Gilliland, 1993). Similar results

were obtained with *L. gasseri* (Usman and Hosono, 1999) and with different strains of bifidobacteria (Gopal *et al*, 1996). However, deconjugation of bile salts seems to be a good predictor to select cholesterol-removing strains, since it was reported by Taranto *et al* (1996) that non-deconjugating bacterial strains of *Enterococcus faecium* produced only upto 12% of reduction in cholesterol concentration, whereas the deconjugating ones showed a higher cholesterol removal of upto 56%. Some workers (Walker and Gilliland, 1993; Gopal *et al*, 1996) reported maximum deconjugation of bile salt by different strains of *L. acidophilus* in late exponential phase of their growth, which coincided with the maximum assimilation of cholesterol.

Cholesterol uptake by *L. acidophilus* was also reported to be influenced by aerobicity and bile concentration; cholesterol was removed from broth medium only under anaerobic conditions when bile concentration was more than 0.1% (Gilliland *et al*, 1985). However, Marshal and Taylor (1995) observed that some strains of *L. acidophilus* were able to remove 8 to 15% of cholesterol from the medium in the absence of bile. With some strains of lactic acid bacteria, various workers (Klaver and Meer, 1993; Brashears *et al*, 1998; Grill *et al*, 2000) observed that there was a partial or complete coprecipitation of cholesterol with deconjugated bile acids at low pH. Brashears *et al* (1998) reported that among the four strains tested, two *L. acidophilus* ones were predominantly cholesterol assimilators, whereas *L. casei* ones were cholesterol precipitators. Grill *et al* (2000) reported that in the presence of taurocholic acid, cholesterol uptake and precipitation were observed in two strains, one each from *Lactobacillus* and *Bifidobacterium* species, they tested.

However, in the presence of Ox gall, bacterial uptake and precipitation were observed for *Lactobacillus* but only precipitation occurred for *Bifidobacterium*.

Noh *et al* (1997) found that cholesterol assimilated by a strain of *L. acidophilus* was not metabolically degraded; most of it was recovered with the cells. Cells that were grown in the presence of cholesterol micelles and bile salts were more resistant to lysis by sonication than those grown in their absence, suggesting a possible alteration of the cell walls or membrane. They also observed that increased amounts of Tween 80 decreased cholesterol uptake by the cells and opined that this may be because of adverse effect of Tween 80 on the permeability of cells.

## **2.2 Protoplast fusion in lactobacilli**

Strain improvement by means other than classical mutagenesis and selection is at present impossible because no usable system of transformation, transduction or conjugal transfer has been described for the genus *Lactobacillus*. Under such circumstances, a relatively non-specific gene transfer system such as protoplast fusion is promising, where there is a possibility of transfer of both plasmid as well as chromosomal markers (Hopwood, 1981).

Basic techniques of genetic manipulation involving protoplast fusion and transformation have been developed in group N streptococci (Gassoon, 1980; Kondo and McKay, 1982, 1984; Okamoto *et al*, 1983, 1985) but these are less advanced in lactobacilli (Iwata *et al*, 1986). Protoplast fusion technique involves:

1. Protoplast formation
2. Protoplast regeneration
3. Protoplast fusion and regeneration of fusants

### **2.2.1 Protoplast formation**

Production of protoplasts in lactobacilli was reported by several workers (Tomochicka *et al*, 1982; Shimizu-Kadota and Kado, 1984; Lee-Wickner and Chassy, 1984; Aftabuddin, 1997; Anuradha, 2000).

Using different cell wall hydrolytic enzymes and osmotic stabilizers, several workers have reported protoplast formation in different lactic acid bacteria. Chassy and Guiffrida (1980) reported that lactobacilli could be lysed with lysozyme in the presence of polyethylene glycol which as osmotic stabilizer caused fusion and aggregation of *Lactobacillus* protoplasts. Both lysozyme and mutanolysin were used by Lee-Wickner and Chassy (1984) to produce protoplasts of *L. casei* with raffinose or lactose as an osmotic stabilizer. With lysozyme, protoplasts were produced from *L. reuteri* by Vescovo *et al* (1984). Using both lysozyme and mutanolysin together, Iwata *et al* (1986) reported protoplast formation in *L. plantarum* with raffinose as an osmotic stabilizer. With lysozyme and lactose or sucrose as an osmotic stabilizer, Reed (1987) produced protoplasts of *L. acidophilus* and *Streptococcus lactis*. Some other workers (Kanatani *et al*, 1989; Prasad *et al*, 1990; Aftabuddin, 1997; Anuradha, 2000) also reported protoplast formation with lysozyme and mutanolysin and raffinose as an osmotic stabilizer in different species of lactobacilli.

Depending upon the time of incubation with cell wall hydrolytic enzymes, different workers have reported different percentage of protoplast formation in lactobacilli with highest being 99% (Prasad *et al*, 1990).

### **2.2.2 Protoplast regeneration**

Following protoplast formation, regeneration of the protoplasts into cells is essential for successful fusion to be carried out.

Different workers have reported different percentages of protoplast regeneration. Lee-Wickner and Chassy (1984) were successful in regenerating about 30 to 40% of *L. casei* protoplasts into rod-shaped cells. Using 0.45M lactose as an osmotic stabilizer, Reed (1987) reported 0.45% regeneration in *L. acidophilus*. Depending upon the period of exposure to lysozyme at 37°C, Prasad *et al* (1990) observed a regeneration of 0.4 to 30% in different strains of lactobacilli. In different strains of lactobacilli, Aftabuddin (1997) and Anuradha (2000) found a maximum regeneration percentage of 28 and 26, respectively.

### **2.2.3 Protoplast fusion and regeneration of fusants**

For protoplast fusion in lactobacilli, different workers have used different molecular weight polyethylene glycols with different percentages. Iwata *et al* (1986) were the first to carry out protoplast fusion in lactobacilli. They fused *L. plantarum* protoplasts with polyethylene glycol, PEG, 6000 (50% w/v) and the regeneration frequency ranged from  $1 \times 10^{-8}$  to  $3 \times 10^{-8}$ . Cocconcelli *et al* (1986) performed intraspecific protoplast fusion between *Streptococcus lactis* and *L. reuteri* with PEG 6000. Reed (1987) used 35% PEG 3350 to fuse *L. acidophilus* and *Streptococcus lactis* and

observed a regeneration frequency of  $1.1 \times 10^{-7}$  and  $7.5 \times 10^{-8}$  per cellular input. Kanatani *et al* (1989) reported protoplast fusion in *L. plantarum*. Using 50% of PEG 6000 with different cultures of lactobacilli, Prasad *et al* (1990), Aftabbudin (1997) and Anuradha (2000) observed regeneration frequencies of  $10 \times 10^{-8}$  to  $8 \times 10^{-10}$ ,  $0.3 \times 10^{-8}$  and  $1.2 \times 10^{-8}$ , respectively.

### **2.3            *In vivo* plasma cholesterol lowering effect**

In 1974, Mann and Spoerry serendipitously discovered that consumption of large quantities of fermented milk by Massai tribesmen in Africa actually lowered their serum cholesterol level and countered the hypercholesterolemic action of Tween 20 (Mann and Spoerry, 1974). Since this initial investigation, many studies have also been conducted to evaluate the cholesterol lowering effect of cultured milks in animals and human. These are reviewed under two subheadings:

1. Animals

2. Humans

#### **2.3.1            Animals**

Tortuero *et al* (1975) used normal and cecectomized laying hens to study the influence of intestinal flora and the implantation of *L. acidophilus* on the levels of serum and egg yolk cholesterol. Serum cholesterol levels of cecectomized laying hens were reported to be higher than those of the normal birds. *L. acidophilus* implantation resulted in a significant decrease in serum cholesterol levels in both normal and cecectomized birds. Egg yolk cholesterol levels were higher for cecectomized birds.

Grunewald (1982) found significantly lower serum cholesterol levels in 10% fermented milk (fermented by *L. acidophilus*) fed rats (serum cholesterol of 65 mg/dl) than those of either 10% water fed or 10% skim milk fed rats (serum cholesterol levels of 78 and 89 mg/dl, respectively).

Gilliland *et al* (1985) reported that consumption of a strain of *L. acidophilus*, which was selected for its ability to grow well in the presence of bile and assimilate cholesterol from the laboratory medium, significantly ( $p < 0.05$ ) inhibited increase in serum cholesterol levels of pigs fed a high cholesterol diet. Consumption of another strain of *L. acidophilus*, which grew well in bile but was lacking in its ability to remove cholesterol from the growth medium, failed to have a similar effect. Thus, they suggested that certain strains of *L. acidophilus* act directly on cholesterol in the gastro-intestinal tract and thereby reduce serum cholesterol levels.

Ishida and Kubo (1985) carried out experiments to test hypocholesterolemic effect of cultured milks in rats, whose feed included 15% of commercial yoghurt. They found that the concentrations of plasma total cholesterol, HDL cholesterol, triglycerides and phospholipids were significantly lower in the experimental group than in the control group. Other cultured products like kefir and buttermilk had no effect on the serum lipids.

Danielson *et al* (1989) used a specific strains of *L. acidophilus* to prepare yoghurt, which was fed to mature boars whose serum total cholesterol and LDL cholesterol concentrations were significantly reduced by intake of the fermented milk product. However, there was no significant

decrease in the concentrations of serum triglycerides or HDL cholesterol.

Imaizumi *et al* (1992) examined whey prepared from cultured milk fermented by 19 *Lactobacillus* and 20 *Bifidobacterium* strains for their effects on secretion and synthesis of bile acids in primary cultured rat hepatocytes. The stimulating effect of whey preparation on bile acid secretion depended on the species as well as the strain used to ferment the milk. *L. casei* and *B. longum* produced whey which stimulated both the secretion of bile acid and the activity of cholesterol 7- $\alpha$  hydroxylase, a rate limiting enzyme for bile acid synthesis. When the cultured products fermented by these two strains were given to rats for 14 days, the products of one strain of *L. casei* were found to stimulate the biliary secretion of bile acids. These results suggested that primary cultured hepatocytes were a useful system as an initial screening for an active principle modulating cholesterol metabolism.

Fukushima and Nakano (1995) reported the effect of a probiotic mixture of cultures composed of *Bacillus*, *Lactobacillus*, *Streptococcus*, *Saccharomyces* and *Candida* species (each at  $10^{7-8}$  cfu/g rice bran), given at a level of 150g/kg diet for 6 weeks, on lipid metabolism in liver of the male rats. Liver weight decreased 35% in the rats fed high-fat, high-cholesterol diet containing the probiotic mixture. Total cholesterol concentration in the serum was significantly lower in the probiotic mixture supplemented group than in the control group throughout the experimental period in rats fed on the high-fat, high-cholesterol diet. HDL cholesterol concentration was significantly higher ( $p < 0.05$ ) in the probiotic mixture supplemented group than in the control

group which was fed for the 6 week experimental period on a basal diet. The serum, VLDL+IDL+LDL cholesterol concentrations in the probiotic mixture supplemented groups were reduced compared with those on the corresponding control groups. Hydroxy methylglutaryl coenzyme A reductase activity in the liver was lower in rats fed on the high-fat, high-cholesterol diet with the probiotic mixture. *E. coli* decreased and *Bifidobacterium* and *Eubacterium* increased in the faecal microflora of rats fed on the dietary probiotic mixture of cultures. This investigation showed serum and liver cholesterol lowering and faecal *Bifidobacterium* and *Eubacterium* increasing effect by the probiotic mixture.

Mousa *et al* (1995) assigned 30 mature male albino rats to six groups with each group receiving one of these diets: dry diet, fresh cow milk, fresh yoghurt, acidophilus yoghurt, acidophilus bifidus yoghurt or biograde. Blood samples taken from the rats at the end of feeding experimental diets, were tested for the concentrations of serum total cholesterol, LDL and HDL cholesterol and total lipids. With the exception of HDL cholesterol, serum of rats receiving fresh cow milk, had higher levels of all the lipids fractions than those of the rats fed with dry diet alone, whereas the feeding of any of the cultured milks reduced the levels of the serum lipids. The reduction in the concentrations of serum cholesterol, triglycerides, total lipids and LDL cholesterol varied between the type of cultured milks; the overall effect was greatest with acidophilus bifidus yoghurt.

Haddadin *et al* (1996) fed laying hens with a basal diet supplemented with a selected strain of *L. acidophilus*, at the rate of  $4 \times 10^6$  cells/g of feed, for a period of 48 weeks. Levels of egg production and feed conversion were significantly

higher (8 and 14.8% respectively) than in the control flock, while as cholesterol values in yolks were decreased by 18.8%. They suggested that the latter effect was a reflection of lower serum cholesterol concentration in treated birds; a maximum reduction of over 55% followed incorporation of the cultures into the feed.

De Rodas *et al* (1996) studied cholesterol-lowering effect of *L. acidophilus* strain and calcium in swine with hypercholesterolemia induced by diet. It was found that the pigs fed with *L. acidophilus* had 11.8% lower serum cholesterol than the pigs fed without the organism. Also, pigs fed with 1.4% calcium had significantly lower total cholesterol than those fed with 0.7% of calcium. It was suggested that both *L. acidophilus* and calcium could enhance the reduction of cholesterol in pigs fed with high cholesterol diet; probably through alteration in the enterohepatic bile circulation.

Akalin *et al* (1997) observed the effects of yoghurt and acidophilus yoghurt on weight gain, on concentrations of serum cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and the number of faecal lactobacilli and coliforms in mice assigned to three dietary treatments for 56 days: (I) commercial rodent chow and water (control), (II) commercial rodent chow and yoghurt made from milk inoculated with a 3% (vol/vol) liquid culture of *Streptococcus thermophilus* and *L. delbreuckii* ssp. *bulgaricus* (yoghurt) and (III) commercial rodent chow plus yoghurt made from milk inoculated with a 0.01% (w/v) lyophilized culture of *L. thermophilus* plus *L. acidophilus*. The weight gains of mice receiving yoghurt or acidophilus yoghurt were higher than those of the mice in the control group. The mean values for serum cholesterol concentrations and LDL cholesterol

concentrations were significantly decreased when acidophilus yoghurt was fed. HDL cholesterol and triglycerides were not affected by yoghurt or acidophilus yoghurt. The highest number of faecal lactobacilli was found in mice receiving acidophilus yoghurt and the numbers of faecal coliform of that group was also lower than other two groups.

De Smelt *et al* (1998) reported that the feeding of live *L. reuteri* cells, containing active bile salt hydrolase, to pigs, reduced their serum cholesterol and LDL cholesterol levels without any effect on HDL cholesterol concentration.

Taranto *et al* (1998) fed Swiss Albino mice with a diet enriched with fat to produce hypercholesterolemia. Administration of *L. reuteri* to the hypercholesterolemic mice for 7 days decreased cholesterol by 38%, producing serum cholesterol concentrations similar to that of control group (67.4 mg/dl). There was a 40% reduction in triglycerides and 20% increase in the ratio of HDL to LDL.

Bomba *et al* (1998) reported that the feeding of *L. casei* and *L. acidophilus* to gnotobiotic piglets reduced their serum cholesterol concentrations by 2.4 and 3.2%, respectively.

Hashimoto *et al* (1999) reported that serum cholesterol concentration was decreased by 36% in hypercholesterolemic rats fed lyophilized *L. casei* cells. Triglyceride levels were also decreased non-significantly and HDL cholesterol was increased significantly. No such significant effect was observed with rats fed fermented milk fermented by the same strain of *L. casei*.

Kawase *et al* (2000) observed a significant reduction in serum total cholesterol concentrations of rats fed fermented milk fermented by *L. casei* and *S. thermophilus*.

Kikuchi-Hayakawa *et al* (2000) examined the effect of fermented skim milk fermented by *L. casei* on plasma lipids in hamsters. Hamsters fed on cholesterol free and enriched diets containing 30% fermented skim milk had lower levels of plasma triglycerides than those fed on control diet. In the experiment with the cholesterol-enriched diet fed hamsters, the plasma triglyceride level was suppressed by fermented skim milk at concentration of 10% and 30%.

Usman and Hosono (2000) examined the effects of milk and non-fermented milks containing *L. gasseri* on serum lipids, total bile acids, faecal steroids and microflora in rats fed cholesterol-enriched diets. Lactobacilli decreased and coliforms increased in faeces of the control group; however, the concentrations of faecal lactobacilli remained unchanged when rats were fed non-fermented milks. Faecal coliforms in rats receiving milk and non-fermented milk containing *L. gasseri* SBTO 270 increased, while rats receiving non-fermented milk containing *L. gasseri* SBTO 274 had lower faecal coliform counts than did control group. Only non-fermented milk containing *L. gasseri* SBTO 270 significantly reduced serum total cholesterol, LDL cholesterol and bile acids. HDL cholesterol was significantly decreased when rats were given milk and non-fermented milk.

### **2.3.2 Humans**

Harrison and Peat (1975) found that bottle-fed infants had total serum cholesterol of 147 mg/dl on the fifth day of birth and their stools contained more *E. coli* than lactobacilli. Adding bicarbonate or lactobacilli to feeds reversed the ratio of these organisms. This change was associated with a decrease in total cholesterol to a mean 199 mg/dl over the

next 3 days. Lactobacilli predominated in the stools when serum cholesterol was low, and might be playing a role in the metabolism of cholesterol.

Hepner *et al* (1979) studied the effect on serum cholesterol concentrations of 54 volunteers whose diet was supplemented for varying periods with non-pasteurized yoghurt, pasteurized yoghurt and 2% butterfat milk. It was observed that serum cholesterol was significantly reduced by 5 to 10% after one week supplementation of either non-pasteurized or pasteurized yoghurt; 2% butterfat milk reduced serum cholesterol to a smaller and less significant effect. Serum triglycerides were unaffected by the diet.

Kaul and Mathur (1982) reported hypocholesterolemic effect of *L. acidophilus*, supplemented with ice cream, in individuals with more than 250 mg/dl of blood cholesterol.

Bazzare *et al* (1983) compared the effects of yoghurt and calcium supplementation in 16 females and five males. Mean total cholesterol decreased significantly for females following yoghurt supplementation. Mean HDL cholesterol levels, and HDL cholesterol to total cholesterol ratios were significantly higher following yoghurt and calcium supplementation than baseline values. For males, no significant difference could be observed.

Gorbach *et al* (1988) observed reduction in serum cholesterol concentration of 35 healthy volunteers fed a fermented product containing *Lactobacillus* GG, strain isolated from human faeces.

Mohan *et al* (1990) reported short term hypolipidemic effects of oral *L. sporogenes* therapy (360 million spores/day in tablet form) in 17 patients with type II hyperlipidemia in an

open label fixed dose trial. Total serum cholesterol ( $330 \pm 55$  mg/dl vs  $226 \pm 46$  mg/dl,  $p < 0.001$ ), LDL cholesterol ( $267 \pm 58$  mg/dl vs  $173 \pm 54$  mg/dl) and total cholesterol to HDL cholesterol ratios were reduced significantly ( $p < 0.001$ ) over a period of three months. HDL cholesterol was marginally increased, however there was no change in serum triglyceride levels.

Hruby et al (1992) studied the effect of two different low fat fermented milks in 25 woman and 18 men in the age group of 26 to 60 years. One of the fermented milks contained cultures of *Bifidobacterium bifidum*, *Streptococcus faecium* and *Enterococcus hirae* and the other one contained *S. thermophilus*, *S. faecium*, *L. acidophilus* and various *Bifidobacterium* species. In most of these subjects, there was a decrease in total cholesterol and LDL cholesterol concentration with a simultaneous moderate increase in HDL cholesterol concentration and frequently, a decrease in triglyceride levels. This favorable effect persisted in many cases even after 15 days of consumption of fermented milk products. They concluded that the consumption of such fermented milk products might decrease the risk of arteriosclerosis.

Agerbek et al (1995) studied cholesterol lowering effect of a cultured milk product, Gaio, in 58 middle-aged male participates. The study was a randomized, double blinded and placebo controlled one. Daily diet of the subjects was supplemented with 200 ml of either Gaio (containing *Enterococcus faecium* and *S. thermophilus*) or a placebo product upto six weeks. Blood samples were drawn before and after three and six weeks of the feeding of the products and were analyzed for serum cholesterol, HDL cholesterol and

triacylglycerol concentrations. They found a significant reduction of 0.37 mmol/ litre in serum cholesterol levels in the group receiving cultured milk, whereas no change was observed in the placebo group. The reduction in total cholesterol was ascribed to a 10% fall in LDL cholesterol levels. HDL cholesterol and triglyceride levels remained unchanged in both the groups.

Anderson and Gilliland (1999) observed a reduction of 2.95 % in serum cholesterol concentration of hypercholesterolemic humans fed fermented milk fermented by *L. acidophilus*.

Ashar and Prajapati (2000) examined the possible hypocholesterolemic effect of acidophilus milk on 27 human subjects having different levels of serum cholesterol, viz, < 2.0 (group C<sub>1</sub>), 2.0-2.2 (C<sub>2</sub>), 2.2-2.5 (C<sub>3</sub>) and >2.5 g/l (C<sub>4</sub>). The acidophilus milk was prepared by fermentation of low fat milk with *L. acidophilus* and was fed to each volunteer at the rate of 2000 ml/day for 20 days. Blood samples from the volunteers were collected and analyzed for lipid profile twice prior to, during and after feeding, keeping a gap of 10 days between two collections. A significant decrease ( $p < 0.05$ ) in average total cholesterol was found in the C<sub>2</sub> and C<sub>3</sub> groups amounting to 21 and 12%, respectively. The average LDL cholesterol decreased in C<sub>2</sub>, C<sub>3</sub> and C<sub>4</sub> groups by 0.54, 0.26 and 0.46 g/l respectively. In the C<sub>2</sub> group the LDL/HDL and total/HDL cholesterol ratio was also reduced by 1.4 and 1.3, respectively. However, in the C<sub>1</sub> group, the average total and LDL cholesterol level did not show any significant change but serum triacylglycerols and VLDL cholesterol showed a significant ( $p < 0.05$ ) increase of 0.53 and 0.11 g/L, respectively. Overall, the feeding had the best effect in the

subjects with lipidemic status of borderline cholesterol level (2.0-2.2 g.L) group.

Kawase et al (2000) reported a significant increase as compared to control group, in HDL cholesterol, after a period of four weeks, in men fed fermented milk fermented by a strain of *L. casei*; also, there was a significant decline in serum triglycerides concentrations and atherogenic index as well.

Agerholm-Larsen et al (2000) studied cholesterol-lowering effect of CAUSIDO, a fermented milk product containing one strain of *Enterococcus faecium* and two strains of *S. thermophilus*. They observed that the product amounted to 8.4% reduction in LDL-cholesterol, which would correspond to a decrease in cardiovascular disease by 20 to 30%.

While all the above quoted studies have yielded positive results with respect to hypocholesterolemic effect of fermented milks, some contradictory reports have also been reported. These include Rossouw *et al* (1981), Thompson et al (1982), Massay (1984), Rajala et al (1988), Lin *et al* (1989). The contradiction might be because of the difference in strains used, lipidemic status of the subjects, diet exercise, etc.

### 3. MATERIALS AND METHODS

To achieve the objectives mentioned earlier, the investigation was carried out in three phases. In the first phase, screening of different *Lactobacillus* spp. was carried out for their gut colonizing and cholesterol lowering potential (3.1 below). In the second phase, strains with maximum probiotic potential were selected, and in an attempt to enhance their probiotic potential, were subjected to protoplast fusion (3.2 below). In the third phase, the selected strains were fed, along with different experimental diets, to rats to assess their effect on different plasma cholesterol fractions (3.3 below). The following materials and methods were used during the different phases of the research.

#### **3.1 Screening of *Lactobacillus* cultures for gut colonizing and plasma cholesterol lowering potential**

This involved the screening of different strains of lactobacilli for *in vitro* assessment of their gut colonizing and plasma cholesterol lowering potential.

##### **3.1.1 Cultures and their source**

Twenty-six strains of the following cultures were obtained from National Collection of Dairy Cultures (NCDC)\* and 24 were from our laboratory stock\*\*:

*L. acidophilus* strain 11, 13, 14, 15, 16, L1, L2, L3, L4, L5 and L6; *L. casei* ssp. *casei* strain 17, 19 and 63, *L. casei* strain L7, L8 and L9; *L. collinoides* 2, *L. delbreuckii* ssp. *bulgaricus* strain 4, 8, 9 and 26; *L. delbreuckii* ssp. *lactis* strain 3, 10 and 27; *L. fermentum* strain 141, 155 156, L10

and L11; *L. helveticus* strain 5 and 6; *L. plantarum* strain 20, 22, 24, 25 and L15; *L. rhamnosus* 18; and *Lactobacillus* spp. strain B4, F1, F2, F3, F4, F6, F7, L12, L13, L14 and L16.

**Note:**

\*Strains with numbers with no preceding letter.

\*\*Strains with numbers preceded by a letter

**3.1.1.1 Maintenance of the cultures**

All the cultures were maintained by sub culturing in MRS (de Man, Rogosa and Sharpe) broth using 1% inoculum and 18 to 20 hours of incubation at 37<sup>o</sup> C. The cultures were stored under refrigeration between transfers. Each culture was sub cultured at least thrice prior to experimental use.

**3.1.1.1.1 Composition of MRS broth** (de Man *et al*, 1960)

|   |         |
|---|---------|
| Peptone   | 10.00 g |
| Meat Extract  | 10.00 g |
| Yeast Extract   | 5.00 g  |
| Dextrose  | 20.00 g |
| Sodium acetate  | 5.00 g  |
| Dipotassium hydrogen orthophosphates (K <sub>2</sub> HPO <sub>4</sub> ) | 2.00 g  |
| Magnesium sulphate (MgSO <sub>4</sub> . 7 H <sub>2</sub> O)             | 0.20 g  |
| Manganese sulphate (MnSO <sub>4</sub> . 5 H <sub>2</sub> O)             | 0.05 g  |
| Tri ammonium citrate  | 2.00 g  |
| L- cysteine hydrochloride   | 0.50 g  |
| Tween – 80  | 1 ml    |
| Distilled water to make the volume to                                   | 1000 ml |
| pH  | 6.8     |

### 3.1.2 Screening of the cultures for different probiotic properties

#### 3.1.2.1 Acid tolerance of the cultures in growth medium

A modified method of Conway et al (1987) was followed. Each culture was inoculated at the rate of 1% in MRS broths of pH 1, 2, 3, 4 and 6.8 and incubated at 37° C for 24 hours. After incubation, optical density (OD) at 620 nm was read before and after incubation to calculate the coefficient of inhibition ( $C_{inh}$ ) of growth in the medium, at the different pH levels.

$$C_{inh} = 1 - \frac{OD_{control}}{OD_{pH\ level}}$$

Where:

$OD_{control}$  is the optical density, after 24 hours of incubation of the culture broth with normal (6.8) broth pH at the time of inoculation

$OD_{pH\ level}$  is the optical density after 24 hours incubation of the culture broth with pH level of either 1,2,3 or 4 at the time of inoculation.

### 3.1.2.2 Bile tolerance of the cultures in growth medium

A modified method of Gilliland *et al* (1984) was followed.

Each culture was inoculated at the rate of 1% in MRS broths of 0.0, 0.1, 0.2, 0.3 and 0.5% of Ox bile (Himedia Laboratories Ltd., Bombay, India) concentrations and incubated at 37°C for 24 hours. After inoculation, OD was read at 620nm before and after incubation to calculate the coefficient of inhibition ( $C_{inh}$ ) of growth in the medium, at the different Ox bile concentrations.

$$C_{inh} = 1 - \frac{OD_{control}}{OD_{Ox bile}}$$

Where:

$OD_{control}$  is the optical density after 24 hours of incubation of the culture broth without Ox bile.

$OD_{Ox bile}$  is the optical density, after 24 hours incubation of the culture broth with Ox-bile concentration of 0.1, 0.2, 0.3 or 0.5% at the time of inoculation.

### 3.1.2.3 Cell Surface hydrophobicity of the cultures

To evaluate cell surface hydrophobicity, the method of Rosenberg *et al* (1981) for adherence of bacteria to hexadecane was adopted with some modifications.

After growing the cultures in MRS broth at 37°C for 24 hours, bacteria were harvested by centrifugation at 10,000 × g for 10 minutes at 2 to 3°C and were washed twice and then resuspended in phosphate urea magnesium sulphate (PUM)

buffer. 2.4 ml of the turbid bacterial suspension (with O.D. of 0.90 to 1.00 at 400 nm) was taken in acid washed test tube to which 0.4<sup>ml</sup> of the hexadecane was added. The mixture was incubated at 30°C for 10 minutes and then the two phases were mixed by vortexing for 120 seconds and then allowed to stand for 15 minutes for phase separation at room temperature. The lower aqueous phase was carefully removed and its O.D. was measured at 400 nm. The hydrophobicity was expressed as the coefficient of the applied cell suspension O.D. that has been excluded from the aqueous phase.

#### **3.1.2.3.1 Phosphate urea magnesium sulphate buffer**

|   |         |
|---|---------|
| Dipotassium hydrogen phosphate ( $K_2HPO_4 \cdot 3H_2O$ ) | 22.2 g  |
| Potassium dihydrogen phosphat ( $KH_2PO_4$ )              | 7.26 g  |
| Urea  | 1.80 g  |
| Magnesium sulphate ( $MgSO_4 \cdot 7H_2O$ )               | 0.20 g  |
| Distilled water to make the volume to                     | 1000 ml |
| pH  | 7.10    |

#### **3.1.2.4 Cholesterol removal from growth medium** (Gilliland and Walker, 1990)

Freshly prepared MRS thio-broth (MRS broth with 0.2% of sodium thioglycollate) was supplemented with sodium taurocholate and cow plasma, to give a concentration of 0.2% sodium taurocholate and 0.24% of cholesterol. The broth was inoculated with 1% of each culture for 24 hours at 37°C and cholesterol content in the growth medium was determined using the following procedure based on the method described by Rudel and Morris (1973).

After 24 hours of incubation, the medium was centrifuged, to separate the cells, at 10,000 X g at 2 to 3°C for 10 minutes. To 0.1 ml of the supernatant, ethanol (3.0 ml of 95% solution) and potassium hydroxide (0.3 ml of 33% solution) were added and mixed thoroughly. The mixture was heated in a water bath maintained at 60°C for 15 minutes and then cooled to add 10.0 ml of hexane and then mixed. Distilled water (30 ml) was added and the mixture was shaken for one minute to ensure complete mixing.

One millilitre of hexane layer was taken and the solvent was evaporated under a flow of nitrogen gas. After evaporation, 2 ml of O- phthalaldehyde reagent (0.5mg/ml of glacial acetic acid) were added and the solution was thoroughly mixed to dissolve the sample completely. About 10 minutes after, 1.0 ml of concentrated sulphuric acid was added and the solution was immediately mixed on a tube vibrator. Absorbance at 550 nm was recorded to estimate the cholesterol content. The coefficient of cholesterol removal from the spent broth was estimated as follows:

Cholesterol in the supernatant of the broth inoculated with the  
organism

1-

---

Cholesterol in the control (broth without any organism)

### 3.1.2.5 Bile salt (sodium taurocholate) deconjugation (Walker and Gilliland, 1993)

To determine the bile salt deconjugating ability of various cultures, a Walker and Gilliland's modification of Irvin *et al* (1944) technique was used. Cultures were inoculated in MRS-thio-broth, with 0.2% sodium taurocholate and incubated at 37°C for 24 hours. After incubation 10 ml of spent broth from a culture were adjusted to pH 7.0 with 1 N NaOH. The volume was made upto 12.5 ml with distilled water, and the cells were removed by centrifugation for 10 minutes at 10,000×g at 2 to 3°C. After this, 7.5 ml of the resulting supernatant fluid was adjusted to pH 1 with 10 N HCl and the volume was made upto 12 ml with distilled water. Then, 3 ml of this diluted spent broth was taken and to this, 9 ml of ethyl acetate was added. The contents were mixed and phases were allowed to separate. Three millilitres of ethyl acetate layer was taken and evaporated to dryness at 60°C under a flow of nitrogen gas. One millilitre of 0.01 N NaOH was added to dissolve the residue. Six millilitres of 16N H<sub>2</sub>SO<sub>4</sub> were then added followed by the addition of 1 ml of 1% furfuraldehyde. After mixing, the solution was heated for 13 minutes in a water bath, maintained at 65°C. After cooling to room temperature, 5ml of glacial acetic was added to the solution and the absorbance was read at 660 nm to estimate the free cholic acid content. The results were expressed as the coefficient of taurocholate deconjugation.

### **3.1.2.6 Acid tolerance of the culture to the simulated pH of human stomachs**

For this, the method of Clark and Martin (1993) was adopted with some modifications.

Solution of 37% HCl in double distilled water was adjusted to pH levels of 2 and 3. Sterile double distilled water served as control. The solutions were sterilized and stored till needed.

Cultures of *Lactobacillus* were grown in MRS broth at 37°C for 18 hours. The stored pH solutions were thoroughly mixed and 10 ml of each pH solution were transferred into sterile test tubes. One millilitre of the culture of each *Lactobacillus* strain was then transferred separately into each of the three-pH solutions (pH 2.0, 3.0 and control), separate for each strain. This procedure was repeated for each species of lactobacilli used. Cultures were then plated immediately (0 hour) with MRS agar on petri plates using pour plates method. Serial dilutions were made using distilled water. The pH-solutions containing lactobacilli were then incubated at 37°C, followed by plating after 3 hours of incubation. The plates were allowed to solidify, inverted and incubated at 37°C for 72 hours. Following incubation colony forming units (cfu) were counted and recorded.

### **3.1.2.7 Bile tolerance of the cultures to the simulated bile concentration of human small intestines**

For this, the method of Clark and Martin (1994) was adopted with some modifications.

A solution of Oxgall (Difco) was prepared using 10g dry powder base in 90 ml distilled water (this was equivalent to

bile as such). This solution was then used to prepare 2.0% (maximum) and 4.0% (twice the maximum) concentrations of bile. Distilled water without Ox gall was used as the control. All solutions were sterilized at 121°C for 15 minutes. After sterilization, Ox gall solutions and distilled water controls were stored at refrigeration temperature until needed.

The cultures of lactobacilli were grown in MRS broth at 37°C for 18-20 hours. Ten millilitres of each Oxgall concentration and distilled water were transferred into sterile test tubes and the cultures of individual strains of lactobacilli were inoculated at the rate of 10%. The mixtures were then plated with MRS agar for initial count (0 h). After plating for initial counts, mixtures were incubated for 12 hours at 37°C. Lactobacilli were then enumerated again to test for survival rates after 12 hours of incubation. All the pour plates were allowed to solidify inverted and incubated at 37°C for 72 hours.

### **3.1.2.8 Antimicrobial activity**

The Agar spot assay (Fleming et al, 1975) was followed to evaluate the antimicrobial activity of the *Lactobacillus* cultures.

The indicator organisms used were: *Bacillus cereus* NCDC 66, *Staphylococcus aureus* NCDC 109, *Enterococcus faecalis*, *Leuconostoc mesenteroides* LY and *Escherichia coli* V517. Brain Heart Infusion (BHI) broth was used for culturing *B. cereus*, *S. aureus*, *E. faecalis* and *E. coli*. Tryptone glucose yeast extract (TGE) broth was used for growing *L. mesenteroides*.

Ten microlitres of *Lactobacillus* cultures were spotted on MRS agar plates. The plates were incubated overnight at

37°C. The indicator organisms were also grown for overnight in respective broth media. Five micro litres of indicator culture were added to 5 ml of soft agar, which was spread over the agar plates. The plates were incubated at 37°C for 48 hours. The zones of inhibition were measured for each culture.

#### **3.1.2.8.1 MRS agar**

To the MRS broth, agar powder was added at the rate of 1.5% and then autoclaved.

#### **3.1.2.8.2 BHI broth**

BHI broth was prepared by dissolving 37 g of BHI powder (Hi-media) in 1000 ml of distilled water, as per the instructions of the manufacturer.

#### **3.1.2.8.3 TGE broth (Biswas *et al*, 1991)**

|  |          |
|--|----------|
| Tryptone   | 10.00 g  |
| Glucose  | 10.00 g  |
| Yeast extract  | 10.00 g  |
| Magnesium sulphate (MgSO <sub>4</sub> . 7H <sub>2</sub> O) | 0.05 g   |
| Manganese sulphate (MnSO <sub>4</sub> . 5H <sub>2</sub> O) | 0.05 g   |
| Distilled water to make the volume to                      | 1000ml   |
| pH   | 6.5± 0.2 |

#### **3.1.2.8.4 Soft agar**

To the TGE broth, agar powder was added at the rate of 0.7% and then autoclaved.

## **3.2 Protoplast fusion of selected lactobacilli and screening of the fusants**

### **3.2.1 Protoplast fusion**

In an attempt to enhance the probiotic potential, the strains with the maximum values of different probiotic properties, were subjected to protoplast fusion. The technique involved the following methodology:

1. Determination of antibiotic sensitivity of the cultures
2. Protoplast formation
3. Protoplast regeneration
4. Protoplast fusion and regeneration of the fusants

#### **3.2.1.1 Determination of the antibiotic sensitivity of the cultures**

Disc assay (Baur *et al*, 1965) and Tube method were followed for the determination of antibiotic sensitivity of the cultures:

##### **3.2.1.1.1 Disc assay**

The cultures were grown in MRS broth at 37° C for 24 hours. One millilitre of each culture was taken in sterile petri plates. About 20 ml of lukewarm MRS agar was poured into each plate and the contents were thoroughly mixed. After solidification, about six antibiotic discs were equidistantly placed on each plate. The plates were incubated at 37° C for 18 hours. Diameter of each zone of inhibition around the antibiotic discs was measured and compared with the standard table (Himedia) to assess antibiotic sensitivity or resistance of test isolates.

### 3.2.1.1.2 Tube method

The cultures were grown overnight in MRS broth at 37° C and were then inoculated at the rate of 2% into tubes containing 5 ml of MRS broth with varying concentrations of different antibiotics. The cultures were again incubated for 20 hours at 37° C. Absorbance was read at 600 nm, against MRS broth as a blank, to assess the growth of the cultures under the influence of varying concentrations of different antibiotics.

### 3.2.1.2 Protoplast formation

The method described by Lee-Wicknerr and Chassy (1984) was followed:

The selected cultures were incubated overnight at 37 ° C in 10 ml of MRS broth. Cells were harvested by centrifugation at 10,000 × g for 10 minutes and were washed with protoplast formation buffer and then suspended in 5ml of it. Lysozyme (10mg/ml) and mutanolysin (50µg/ml) were added. The mixture was incubated at 37°C for 120 minutes with occasional agitation and checked for protoplast formation under phase contrast microscope. The enumeration of osmotically fragile cells (the difference between the number of osmotically resistance colonies before and after protoplast formation) was used as an index of protoplast formation.

#### 3.2.1.2.1 Protoplast formation buffer

|                          |          |
|--------------------------|----------|
| HEPES                    | 0.5206 g |
| MgCl <sub>2</sub> (15mM) | 0.3044 g |
| Gelatin (0.5%)           | 0.5000 g |

|                                       |           |
|---------------------------------------|-----------|
| Lactose (0.5M)                        | 18.0150 g |
| Distilled water to make the Volume to | 100ml     |

### 3.2.1.3 Protoplast regeneration

At different intervals during protoplast formation, the cell suspension was diluted in protoplast formation buffer and plated on regeneration medium. Similarly, the cell suspension was also diluted in distilled water and plated on MRS agar to enumerate osmotically insensitive cells. All the plates were incubated at 37 °C. The numbers of osmotically insensitive cells were counted after two days and the colonies on regeneration medium were counted after 3 days. Percent regeneration was expressed at the ratio of the net regenerated cells (cfu on regeneration medium- cfu on MRS agar) to initial cell count (cfu's on MRS agar before protoplast formation) multiplied by 100.

$$\% \text{ Regeneration} = \frac{\text{Cfu on regeneration medium} - \text{Cfu on MRS agar}}{\text{Initial cell count}} \times 100$$

#### 3.2.1.3.1. Protoplast regeneration medium (Reed, 1987)

|   |         |
|---|---------|
| Bovine serum albumin  | 0.500 g |
| Tryptone  | 1.0 g   |
| Yeast extract   | 0.500 g |
| Dipotassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> ) | 0.250 g |
| Sodium acetate  | 0.250 g |
| Triammonium acetate   | 0.150 g |

|   |          |
|---|----------|
| Manganese sulphate (MnSO <sub>4</sub> ) | 0.005 g  |
| Magnesium chloride (MgCl <sub>2</sub> ) | 0.020 g  |
| Calcium chloride (CaCl <sub>2</sub> )   | 0.020 g  |
| Dextrose                                | 1.000 g  |
| Gelatin                                 | 2.500 g  |
| Lactose (0.45M)                         | 16.240 g |
| Agar                                    | 1.500 g  |
| Distilled water to make the Volume to   | 100 ml   |

#### **3.2.1.4 Protoplast fusion and the regeneration of the fusants**

For this, the method described by Iwata *et al* (1986) was adopted.

Fifty micro litres of protoplast suspension of each parent strain were mixed together. 1.5 ml polyethylene glycol solution was added and mixed immediately. After 2 minutes at room temperature, the protoplasts were recovered by centrifugation at 2000xg for 5 minutes and were resuspended in 2 ml of protoplast formation buffer. One millilitre of a 10 fold dilution in protoplast formation buffer was pour plated with 20 ml of regeneration medium containing the selective antibiotics. The plates were then incubated at 37°C for 3 days. After 3 days, the colonies were picked up and subcultured in MRS broth at least 10 times before carrying out other experiments.

#### **3.2.1.4.1 Polyethylene glycol (PEG) solution** (Iwata *et al*, 1986)

|                                   |             |
|-----------------------------------|-------------|
| Polyethylene glycol               | 1 50.0000 g |
| Lactose (0.45M)                   | 16.2140 g   |
| HEPES (20mM)                      | 0.5206 g    |
| MgCl <sub>2</sub> (15mM; pH, 6.5) | 0.3044 g    |

#### **3.2.2 Screening of fusants for different probiotic properties**

Fusants were assessed for acid and bile tolerance, and cell surface hydrophobicity as per the methods described under section 3.1.

#### **3.3 Assessment of the effects of feeding of selected cultures on various parameters estimated in rats**

Assessment of the effects of feeding of selected cultures with different experimental diets was made on body weight, on concentrations of plasma triacylglycerols and plasma cholesterol fractions, and on counts of faecal lactobacilli and coliforms of rats.

The following materials and methods were utilized in this phase:

##### **3.3.1 Animals**

Male albino rats of Wistar strain used in the experiment were from small Animal House of National Dairy Research Institute (NDRI), Karnal, Haryana, India. The animals were about 8 weeks old and were approximately of similar body weight. The animals were housed in cages in an air-conditioned room at  $24 \pm 1^{\circ}\text{C}$ .

### 3.3.1.1 Grouping of animals

For each strain of microorganisms to be tested for its effect on body weight, concentrations of plasma triacylglycerols and various plasma cholesterol fractions, rats were divided, as per the diet fed to them, into three groups of eight rats each.

### 3.3.1.2 Diets

Among the three groups of rats one received basal, one cholesterol-enriched and one cream-enriched diet.

#### 3.3.1.2.1 Composition (g) of experimental diets

| Constituent                    | <i>Basal diet</i>                    |                                    | <i>Cholesterol-enriched diet</i>     |                                   | <i>Cream-enriched diet</i>           |                                   |
|--------------------------------|--------------------------------------|------------------------------------|--------------------------------------|-----------------------------------|--------------------------------------|-----------------------------------|
|                                | Without <i>Lactobacillus</i> culture | With <i>Lactobacillus</i> culture* | Without <i>Lactobacillus</i> culture | With <i>Lactobacillus</i> culture | Without <i>Lactobacillus</i> culture | With <i>Lactobacillus</i> culture |
| Wheat                          | 15.000                               | 15.000                             | 15.000                               | 15.000                            | 15.000                               | 15.000                            |
| Bengal gram<br>(Kala chana)    | 58.000                               | 58.000                             | 58.000                               | 58.000                            | 58.000                               | 58.000                            |
| Groundnut<br>cake              | 10.000                               | 10.000                             | 10.000                               | 10.000                            | 10.000                               | 10.000                            |
| Refined oil<br>(Sunflower oil) | 3.600                                | 3.600                              | 3.600                                | 3.600                             | 3.600                                | 3.600                             |
| Casein                         | 9.000                                | 9.000                              | 4.000                                | 4.000                             | 4.000                                | 4.000                             |
| Mineral<br>mixture             | 4.000                                | 4.000                              | 4.000                                | 4.000                             | 4.000                                | 4.000                             |

Continued next page

|                             |       |       |                    |                    |                    |                    |
|-----------------------------|-------|-------|--------------------|--------------------|--------------------|--------------------|
| Vitamin mixture             | 0.200 | 0.200 | 0.200              | 0.200              | 0.200              | 0.200              |
| Choline chloride            | 0.200 | 0.200 | 0.200              | 0.200              | 0.200              | 0.200              |
| Cholesterol                 | 0.000 | 0.000 | 0.500              | 0.500              | 0.000              | 0.000              |
| Sodium cholate              | 0.125 | 0.125 | 0.125              | 0.125              | 0.125              | 0.125              |
| Skim milk                   | 0.000 | 0.000 | 5.000 <sup>1</sup> | 0.000              | 5.000 <sup>2</sup> | 0.000              |
| Cream**                     | 0.000 | 0.000 | 0.000              | 0.000              | 10.000             | 10.000             |
| Fermented milk with culture | 0.000 | 0.000 | 0.000              | 5.000 <sup>3</sup> | 0.000              | 5.000 <sup>4</sup> |

\* About  $10^9$  cells of culture was supplemented as such in water

\*\* Cream contained about 70 to 80% fat.

<sup>1,2,3,4</sup> Dry matter basis.

### 3.3.1.2.1.1 Composition of mineral mixture

|                    |           |
|--------------------|-----------|
| Starch             | 3.000 kg  |
| Sodium fluoride    | 1.500 g   |
| Copper sulphate    | 1.000 g   |
| Manganese sulphate | 0.500 g   |
| Alum               | 0.250 g   |
| Table salt         | 250.000 g |
| Calcium carbonate  | 30.000 g  |

|                         |          |
|-------------------------|----------|
| Tricalcium phosphate    | 25.000 g |
| Magnesium sulphate      | 50.000 g |
| Ferric ammonium citrate | 42.500 g |
| Potassium iodide        | 0.125 g  |

### 3.3.1.2.1.2 Composition of vitamin (vit.) mixture

|  |            |
|--|------------|
| Biotin                                 | 0.0150g    |
| Folic acid                             | 0.0750 g   |
| Cyanocobalomin (vit. B <sub>12</sub> ) | 0.0015 g   |
| Menadion (vit. k)                      | 1.8420 g   |
| Para amino benzoic acid                | 3.7500 g   |
| Mesoinositol                           | 3.7500 g   |
| Thiamin (vit. B <sub>1</sub> )         | 0.0625 g   |
| Riboflavin (vit. B <sub>2</sub> )      | 0.2670 g   |
| Niacin                                 | 3.5380 g   |
| Pyridoxine                             | 0.0625 g   |
| Calcium pantothenate                   | 0.7080 g   |
| Vit. E                                 | 10,000 IU  |
| Calciferol (Vit. D)                    | 200,000 IU |

|                 |             |
|-----------------|-------------|
| Vit. A          | 2000,000 IU |
| Choline choride | 200.0000 g  |

### **3.3.1.2.1.3 Feeding schedule**

For two weeks, the rats in all the groups were fed basal diet. After this adaptation period, different groups were fed their respective diets viz. basal, cholesterol-enriched and cream-enriched diets, and the day on which the feeding of these diets was started was referred to as 0 day. After 15 days of the feeding of the diets, the diets were supplemented with the *Lactobacillus* culture to be tested for its effect on plasma cholesterol and triacylglycerol levels. The feeding of the diets along with the culture was continued for further 15 days.

### **3.3.1.3 Body weight**

Body weight was calculated on 0 day and after every fortnight, during the feeding of experimental diet, for a period of one month.

### **3.3.1.4 Blood collection and determination of concentrations of various plasma lipids**

#### **3.3.1.4.1 Blood collection**

For blood collection, overnight fasted rats were first anaesthetized by exposing them to chloroform in a glass jar for a brief period of time. Using a heparinized capillary tube, blood was then drawn from the orbital venous plexus and collected in storage vials wetted with heparin solution.

Blood was collected at day 0 and after every fortnight for a period of one month.

Immediately after collection, blood was centrifuged and plasma collected and stored at  $-20^{\circ}\text{C}$  till further analysis.

### **3.3.1.4.2 Determination of concentrations of various plasma lipids**

The concentrations of the following lipid fractions were determined in the blood:

1. Total cholesterol (through enzymatic commercial kits, Autopak, Bayer Diagnostics India Ltd. Gujarat, India).
2. High-density lipoprotein (HDL-) cholesterol (through enzymatic commercial kits, Autopak, Bayer Diagnostics India Ltd. Gujarat, India).
3. Triacylglycerols (through enzymatic commercial kits, Autopak, Bayer Diagnostics India Ltd. Gujarat, India).
4. Low-density lipoprotein (LDL) cholesterol (by Friedewald's equation).
5. Very low-density lipoprotein (VLDL) cholesterol (by Friedewald's equation).

#### **3.3.1.4.2.1 Friedewald's equation**

1.

$$\text{LDL cholesterol (mg/dl)} = \frac{\text{Total cholesterol} - \text{VLDL cholesterol}}{\text{HDL cholesterol}}$$

2.

$$\text{LDL-cholesterol (mg/dl)} = \frac{\text{Triacylglycerols}}{5}$$

### 3.3.1.4.2. 2 Athergenic index (AI)

$$\text{AI} = \frac{\text{LDL cholesterol}}{\text{HDL cholesterol}}$$

### 3.3.1.5 Counts of lactobacilli and coliforms in faeces

Faecal samples were collected at 0 day and after every fortnight for a period of one month by gently squeezing the rectal parts of the rats. Faecal samples were taken in sterile storage vials for determination of faecal *Lactobacillus* and coliform counts.

#### 3.3.1.5.1 Faecal *Lactobacillus* counts

For the determination of total lactobacilli, the obtained samples were homogenized in phosphate buffer saline (PBS), pH 6.8, on a vortex-mixer for 4 minutes. Then the homogenized samples were diluted and spread on *Lactobacillus* Selection Agar (Hi media). The plates were incubated at 37°C for 48 hours.

#### 3.3.1.5.2 Faecal coliform counts

The number of faecal coliforms was determined on Violet Red Bile Agar (Hi media). The plates were incubated at 37°C for 24 hours, and the colonies were counted.

### **3.4 Statistical analysis**

Wherever required, results were expressed as mean  $\pm$  standard error of mean. Significance was tested by employing analysis of variance (ANOVA) and comparison between means was made by either Duncan's multiple range test (DMRT) or Least significant difference (LSD) pair wise comparison, whichever was required. For adoption of different statistical methods for data analysis, Snedecor and Cochran (1967) was followed and for computation of the data, software application programmes like Microsoft Excel and Systat 7.0 were used.

## 4. RESULTS AND DISCUSSION

The study was undertaken to assess the hypocholesterolemic effect of the *Lactobacillus* strains exhibiting the best gut colonizing and cholesterol lowering potential among the fifty strains screened. The investigation involved three phases:

### Phase 1

The cultures were screened by various *in vitro* tests for their gut colonizing and cholesterol lowering potential, viz, acid and bile tolerance in growth medium, cell surface hydrophobicity, cholesterol removal from and taurocholate deconjugation in growth medium (Section 4.1). The results are presented in Table 4.1 to 4.10.

### Phase 2

Protoplast fusion was carried out between the strains with maximum values of different probiotic properties, in attempt to enhance their probiotic potential. The resultant fusants were screened for different probiotic properties in the same way as that for the parents. (Section 4.2). The results are presented in Table 4.11 to 4.14.

### Phase 3

The selected strains were assessed for their *in vivo* hypocholesterolemic effect in rats fed basal, cholesterol-enriched and cream-enriched diets. (Section 4.3). The results are presented in Table 4.15 to 4.32 and Fig. 1 to 6.

The results of the investigation are described and discussed as under:

#### 4.1 Screening of different *Lactobacillus* cultures

If a bacterial culture is able to remove cholesterol from or deconjugate bile acid in an artificial growth medium, it may lower blood cholesterol when fed to animals, provided it successfully survives harsh environment of gut and is able to colonize it. Thus for these reasons, the cultures were screened by various *in vitro* tests for both gut colonizing and cholesterol lowering potential.

##### 4.1.1 Acid tolerance in growth medium

Different regions of the gastrointestinal tract have varying pH levels. The stomach and the regions immediately following the stomach have the highest acidity, and the pH of these region may fall as low as 1.5. For a *Lactobacillus* to be used as a dietary adjunct for a particular probiotic property, the first hostile condition is that it should sustain the low pH.

For each strain, the mean of coefficients of inhibition of growth at various pH levels of the growth medium (MRS broth) are depicted in Table 4.1. For comparison of the means, analysis of variance (ANOVA) was used, and the means were assigned to groups after applying Duncan's multiple range test (DMRT) at  $p < 0.01$ . Superscript of a mean designates the group to which the strain with the mean belongs. There is no significant ( $p < 0.01$ ) difference between the means within a group.

*L. acidophilus* 14, *L. casei* ssp. *casei* 19 and *L. plantarum* 20 belong to group 'a' of the highest acid tolerance

**Table 4.1** Acid tolerance, measured as the mean of coefficients of inhibition (AT) of growth at 1, 2, 3, and 4 pH levels of growth medium, of different *Lactobacillus* spp.

| Strain  | AT                | Strain                        | AT                |
|---|-------------------|-------------------------------|-------------------|
| <i>L. acidophilus</i> 11                        | 0.82 <sup>c</sup> | <i>L. fermentum</i> 141       | 0.74 <sup>b</sup> |
| <i>L. acidophilus</i> 13                        | 0.95 <sup>c</sup> | <i>L. fermentum</i> 155       | 0.87 <sup>c</sup> |
| <i>L. acidophilus</i> 14                        | 0.66 <sup>a</sup> | <i>L. fermentum</i> 156       | 0.74 <sup>b</sup> |
| <i>L. acidophilus</i> 15                        | 0.95 <sup>c</sup> | <i>L. fermentum</i> L10       | 0.94 <sup>c</sup> |
| <i>L. acidophilus</i> 16                        | 0.86 <sup>c</sup> | <i>L. fermentum</i> L11       | 0.93 <sup>c</sup> |
| <i>L. acidophilus</i> L1                        | 0.93 <sup>c</sup> | <i>L. helveticus</i> 5        | 0.85 <sup>c</sup> |
| <i>L. acidophilus</i> L2                        | 0.92 <sup>c</sup> | <i>L. helveticus</i> 6        | 0.91 <sup>c</sup> |
| <i>L. acidophilus</i> L3                        | 0.95 <sup>c</sup> | <i>L. plantarum</i> 20        | 0.63 <sup>a</sup> |
| <i>L. acidophilus</i> L4                        | 0.91 <sup>c</sup> | <i>L. plantarum</i> 22        | 0.95 <sup>c</sup> |
| <i>L. acidophilus</i> L5                        | 0.86 <sup>c</sup> | <i>L. plantarum</i> 24        | 0.95 <sup>c</sup> |
| <i>L. acidophilus</i> L6                        | 0.89 <sup>c</sup> | <i>L. plantarum</i> 25        | 0.93 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 17            | 0.96 <sup>c</sup> | <i>L. plantarum</i> L15       | 0.88 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 19            | 0.64 <sup>a</sup> | <i>L. rhamnosus</i> 18        | 0.95 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 63            | 0.97 <sup>c</sup> | <i>Lactobacillus</i> spp. B4  | 0.93 <sup>c</sup> |
| <i>L. casei</i> L7                              | 0.94 <sup>c</sup> | <i>Lactobacillus</i> spp. F1  | 0.91 <sup>c</sup> |
| <i>L. casei</i> L8                              | 0.83 <sup>c</sup> | <i>Lactobacillus</i> spp. F2  | 0.92 <sup>c</sup> |
| <i>L. casei</i> L9                              | 0.90 <sup>c</sup> | <i>Lactobacillus</i> spp. F3  | 0.88 <sup>c</sup> |
| <i>L. collinoides</i> 2                         | 0.93 <sup>c</sup> | <i>Lactobacillus</i> spp. F4  | 0.94 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 4  | 0.97 <sup>c</sup> | <i>Lactobacillus</i> spp. F5  | 0.90 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 8  | 0.88 <sup>c</sup> | <i>Lactobacillus</i> spp. F6  | 0.92 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 9  | 0.75 <sup>b</sup> | <i>Lactobacillus</i> spp. F7  | 0.91 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 26 | 0.89 <sup>c</sup> | <i>Lactobacillus</i> spp. L12 | 0.93 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 3      | 0.92 <sup>c</sup> | <i>Lactobacillus</i> spp. L13 | 0.92 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 10     | 0.92 <sup>c</sup> | <i>Lactobacillus</i> spp. L14 | 0.91 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 27     | 0.82              | <i>Lactobacillus</i> spp. L16 | 0.92 <sup>c</sup> |

Means with no common superscripts are significantly different,  $p < 0.01$  (Duncan's multiple range test)

with coefficients of inhibition of 0.63 to 0.66. This is followed by group 'b' organisms (*L. delbrueckii* ssp. *bulgaricus* 9, *L. fermentum* 141 and *L. fermentum* 156) of lower acid tolerance with coefficients of inhibition of 0.74 to 0.75. The organisms with the coefficients of more than 0.82 belong to 'c' group and are least acid tolerant ones.

Many workers have reported similar results of acid tolerance of different lactobacilli at various pH levels in growth medium, in mixtures simulating to gastric juice, or in acid solutions as such, and this tolerance tended to be strain specific (Conway et al, 1987; Hood and Zottota, 1988; Lankaputra and Shah, 1995; Plockova et al, 1996; Charteris et al 1998; Chou and Weimmer, 1999; Anuradha, 2000; De Angelis et al 2001, Haller et al, 2001).

The acid tolerance of bacteria is being ascribed to adaptation of bacteria to acid (Haller et al, 2001) by production of new and over expression of some already existing proteins (De Angelis et al 2001, and Lorca and Valdez, 2001).

#### **4.1.2 Bile tolerance in growth medium**

Lactobacilli, which are to be used as dietary adjuncts, must survive the varying concentrations of bile in the small intestine. Depending mainly on the type of food consumed, the rate of secretion of bile and its concentration in different regions of the intestine vary, and during the first hour of digestion, bile concentration can range between 0.5 to 2.0 % and then the levels start decreasing (Davenport, 1977).

For each strain, Table 4.2 shows the mean of coefficient of inhibition of growth at various Ox bile concentrations of growth medium. For comparison of the means, ANOVA was

used, and the means were assigned to groups after applying DMRT at  $p < 0.01$ . Superscript of mean designates the group to which the strain with the mean belongs. There is no significant ( $p < 0.01$ ) difference between the means within a group.

Amongst all organisms tested, *L. casei* ssp. *casei* 19 in group 'a' with the highest bile tolerance and the lowest coefficient of inhibition of growth of 0.41. *L. acidophilus* 14, *L. casei* ssp. *casei* 63, *L. casei* L9, *L. plantarum* 20, *L. acidophilus* L3, *L. fermentum* 155 and *Lactobacillus* spp. L13 belong to group 'b' with coefficients of inhibition of growth ranging between 0.63 to 0.68. Group 'c' organisms were the least bile tolerant ones with coefficient of inhibition of more than 0.70.

Similar to the results of this study several workers have reported strain specific bile tolerance or resistance of lactobacilli in growth medium, in mixtures simulating gastric environment, or in bile solution as such (Gilliland *et al* 1984; Gilliland and Walker, 1990; Noh and Gilliland, 1993; Clark and Martin, 1994; Gupta *et al*, 1996; Jin *et al*, 1998; Charteris *et al*, 1998, Chou and Weimer 1999; Jacobson *et al*, 1999; Usman and Hosono, 1999; Annuradha, 2000; Haller *et al*, 2001).

The bile tolerance of bacteria may be because of their adaptation to bile salts (Haller *et al*, 2001) or because of their growth advantages over non-tolerant ones (Noh and Gilliland, 1993, and Chou and Weimer, 1999).

#### **4.1.3 Cell surface hydrophobicity**

Among the properties essential for lactobacilli to exert maximum probiotic effect, adhesion to enterocytes is a very

**Table 4.2** Bile tolerance, measured as the mean of coefficients of inhibition (BT) of growth at 0.1, 0.2, 0.3 and 0.5% Ox bile concentrations of growth medium, of different *Lactobacillus* spp.

| Strain  | BT                | Strain                        | BT                |
|---|-------------------|-------------------------------|-------------------|
| <i>L. acidophilus</i> 11                        | 0.88 <sup>c</sup> | <i>L. fermentum</i> 141       | 0.85 <sup>c</sup> |
| <i>L. acidophilus</i> 13                        | 0.75 <sup>c</sup> | <i>L. fermentum</i> 155       | 0.64 <sup>b</sup> |
| <i>L. acidophilus</i> 14                        | 0.66 <sup>b</sup> | <i>L. fermentum</i> 156       | 0.95 <sup>c</sup> |
| <i>L. acidophilus</i> 15                        | 0.78 <sup>c</sup> | <i>L. fermentum</i> L10       | 0.83 <sup>c</sup> |
| <i>L. acidophilus</i> 16                        | 0.88 <sup>c</sup> | <i>L. fermentum</i> L11       | 0.82 <sup>c</sup> |
| <i>L. acidophilus</i> L1                        | 0.70 <sup>c</sup> | <i>L. helveticus</i> 5        | 0.95 <sup>c</sup> |
| <i>L. acidophilus</i> L2                        | 0.89 <sup>c</sup> | <i>L. helveticus</i> 6        | 0.95 <sup>c</sup> |
| <i>L. acidophilus</i> L3                        | 0.59 <sup>b</sup> | <i>L. plantarum</i> 20        | 0.68 <sup>b</sup> |
| <i>L. acidophilus</i> L4                        | 0.72 <sup>c</sup> | <i>L. plantarum</i> 22        | 0.70 <sup>c</sup> |
| <i>L. acidophilus</i> L5                        | 0.79 <sup>c</sup> | <i>L. plantarum</i> 24        | 0.78 <sup>c</sup> |
| <i>L. acidophilus</i> L6                        | 0.94 <sup>c</sup> | <i>L. plantarum</i> 25        | 0.89 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 17            | 0.94 <sup>c</sup> | <i>L. plantarum</i> L15       | 0.8 <sup>c</sup>  |
| <i>L. casei</i> ssp. <i>casei</i> 19            | 0.41 <sup>a</sup> | <i>L. rhamnosus</i> 18        | 0.96 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 63            | 0.63 <sup>b</sup> | <i>Lactobacillus</i> spp. B4  | 0.74 <sup>c</sup> |
| <i>L. casei</i> L7                              | 0.75 <sup>c</sup> | <i>Lactobacillus</i> spp. F1  | 0.79 <sup>c</sup> |
| <i>L. casei</i> L8                              | 0.89 <sup>c</sup> | <i>Lactobacillus</i> spp. F2  | 0.84 <sup>c</sup> |
| <i>L. casei</i> L9                              | 0.65 <sup>b</sup> | <i>Lactobacillus</i> spp. F3  | 0.90 <sup>c</sup> |
| <i>L. collinoides</i> 2                         | 0.83 <sup>c</sup> | <i>Lactobacillus</i> spp. F4  | 0.79 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 4  | 0.97 <sup>c</sup> | <i>Lactobacillus</i> spp. F5  | 0.76 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 8  | 0.96 <sup>c</sup> | <i>Lactobacillus</i> spp. F6  | 0.94 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 9  | 0.96 <sup>c</sup> | <i>Lactobacillus</i> spp. F7  | 0.82 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 26 | 0.94 <sup>c</sup> | <i>Lactobacillus</i> spp. L12 | 0.95 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 3      | 0.73 <sup>c</sup> | <i>Lactobacillus</i> spp. L13 | 0.64 <sup>b</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 10     | 0.78 <sup>c</sup> | <i>Lactobacillus</i> spp. L14 | 0.78 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 27     | 0.92 <sup>c</sup> | <i>Lactobacillus</i> spp. L16 | 0.96 <sup>c</sup> |

Means with no common superscripts are significantly different,  $p < 0.01$  (Duncan's multiple range test)

**Table 4.3 Coefficient of cell surface hydrophobicity (CSH) of the *Lactobacillus* strains**

| Strain  | CSH               | Strain                        | CSH               |
|---|-------------------|-------------------------------|-------------------|
| <i>L. acidophilus</i> 11                        | 0.18 <sup>e</sup> | <i>L. fermentum</i> 141       | 0.08 <sup>f</sup> |
| <i>L. acidophilus</i> 13                        | 0.27 <sup>e</sup> | <i>L. fermentum</i> 155       | 0.48 <sup>c</sup> |
| <i>L. acidophilus</i> 14                        | 0.84 <sup>a</sup> | <i>L. fermentum</i> 156       | 0.13 <sup>e</sup> |
| <i>L. acidophilus</i> 15                        | 0.46 <sup>c</sup> | <i>L. fermentum</i> L10       | 0.14 <sup>e</sup> |
| <i>L. acidophilus</i> 16                        | 0.20 <sup>e</sup> | <i>L. fermentum</i> L11       | 0.26 <sup>c</sup> |
| <i>L. acidophilus</i> L1                        | 0.08 <sup>f</sup> | <i>L. helveticus</i> 5        | 0.05 <sup>f</sup> |
| <i>L. acidophilus</i> L2                        | 0.14 <sup>e</sup> | <i>L. helveticus</i> 6        | 0.12 <sup>e</sup> |
| <i>L. acidophilus</i> L3                        | 0.15 <sup>c</sup> | <i>L. plantarum</i> 20        | 0.75 <sup>b</sup> |
| <i>L. acidophilus</i> L4                        | 0.39 <sup>d</sup> | <i>L. plantarum</i> 22        | 0.12 <sup>e</sup> |
| <i>L. acidophilus</i> L5                        | 0.25 <sup>e</sup> | <i>L. plantarum</i> 24        | 0.23 <sup>e</sup> |
| <i>L. acidophilus</i> L6                        | 0.10 <sup>e</sup> | <i>L. plantarum</i> 25        | 0.18 <sup>e</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 17            | 0.03 <sup>f</sup> | <i>L. plantarum</i> L15       | 0.29 <sup>e</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 19            | 0.74 <sup>b</sup> | <i>L. rhamnosus</i> 18        | 0.10 <sup>e</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 63            | 0.06 <sup>f</sup> | <i>Lactobacillus</i> spp. B4  | 0.16 <sup>e</sup> |
| <i>L. casei</i> L7                              | 0.26 <sup>c</sup> | <i>Lactobacillus</i> spp. F1  | 0.08 <sup>f</sup> |
| <i>L. casei</i> L8                              | 0.18 <sup>e</sup> | <i>Lactobacillus</i> spp. F2  | 0.28 <sup>c</sup> |
| <i>L. casei</i> L9                              | 0.19 <sup>e</sup> | <i>Lactobacillus</i> spp. F3  | 0.21 <sup>e</sup> |
| <i>L. collinoides</i> 2                         | 0.04 <sup>f</sup> | <i>Lactobacillus</i> spp. F4  | 0.19 <sup>e</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 4  | 0.52 <sup>c</sup> | <i>Lactobacillus</i> spp. F5  | 0.27 <sup>e</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 8  | 0.17 <sup>e</sup> | <i>Lactobacillus</i> spp. F6  | 0.08 <sup>f</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 9  | 0.19 <sup>e</sup> | <i>Lactobacillus</i> spp. F7  | 0.26 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 26 | 0.07 <sup>f</sup> | <i>Lactobacillus</i> spp. L12 | 0.32 <sup>e</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 3      | 0.20 <sup>e</sup> | <i>Lactobacillus</i> spp. L13 | 0.16 <sup>e</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 10     | 0.05 <sup>f</sup> | <i>Lactobacillus</i> spp. L14 | 0.22 <sup>e</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 27     | 0.02 <sup>f</sup> | <i>Lactobacillus</i> spp. L16 | 0.06 <sup>f</sup> |

Means with no common superscripts are significantly different,  $p < 0.01$  (Duncan's multiple range test)

important one. This property creates a transient barrier effect, which prevents rapid elimination of the probiotic bacteria by peristalsis, and thus represents an ecological competitive advantage for the bacteria in the gastrointestinal tract (Alander *et al*, 1997; Johansson *et al*, 1993)

Greater surface hydrophobicity of bacterial cells results in greater attractive forces and higher levels of adhesion, whereas smaller electrokinetic potentials of cells and lower levels of ionic strength result in greater repulsive electrostatic interactions and lower levels of adhesion (Prasad *et al*, 1990)

Table 4.3 depicts the coefficients of cell surface hydrophobicity of various *Lactobacillus* strains. For comparison of the means, ANOVA was used, and the means were assigned to groups after DMRT at  $p < 0.01$ . Superscript of a mean designates the group to which the strain with the mean belongs. There was no significant ( $p < 0.01$ ) difference between the means within a group.

*L. acidophilus* 14 with highest cell surface hydrophobicity of 0.84 is the only organism in group 'a'. Next is the group 'b' with *L. casei* ssp. *casei* 19 and *L. plantarum* 20 with values of cell surface hydrophobicity of 0.74 and 0.75, respectively. *L. acidophilus* 15, *L. delbrueckii* ssp. *bulgaricus* 4 and *L. fermentum* 155 belong to group 'c' with cell surface hydrophobicity of 0.46 to 0.52. The organisms with cell surface hydrophobicity below 0.52 have been allotted to 'd', 'e', and 'f' groups (groups from 'a' to 'f' are in descending order of coefficient of cell surface hydrophobicity). Group 'f' organisms have the least cell surface hydrophobicity of 0.02 to 0.08.

Similar to the above results, Prakash *et al* (1997) reported 0.4 to 40.06% of cell surface hydrophobicity in various *Lactobacillus* isolates from rat intestine, while Aftabuddin (1997) and Anuradha (2000), 3.73 to 94.02% and 6.00 to 81.54% ,respectively.

Variation in cell surface hydrophobicity may be due to presence of lipoteichoic acid-protein complex (Christensen *et al*, 1985 and Sherman and Savage, 1986) or due to surface proteinaceous substance (Bhowmik *et al*, 1985 and Henriksson *et al*, 1991) or due to difference in configuration or structure of the lipopolymers present in the bacterial cell wall (Parkash, 1995).

In general, *Lactobacillus* isolates having higher cell surface hydrophobicity also exhibited higher adhesive index, indicating the involvement of hydrophobic interactions in the adhesion to epithelial cells (Wodstrom *et al*, 1987; Rijnaarts *et al*, 1993; Prakash *et al*, 1997).

#### **4.1.4 Cholesterol removal**

One of the important beneficial health effects attributed to various lactobacilli is their capability to lower plasma cholesterol (Fukushima and Nakano, 1995; Hashimoto *et al*, 1999; Kawase *et al*, 2000; Kikuchi-Hayakawa *et al*, 2000; Taranto *et al*, 2000), possibly through binding or assimilation (uptake) of the dietary cholesterol by the bacterial cells in the small intestine before it can be absorbed into the body (Gilliland *et al*, 1985; Hosono and Tono-Oka, 1995; Gopal *et al*, 1996). There is a great variation in *in vitro* cholesterol removal among different strains of lactobacilli (Gilliland *et al*, 1985; Usman and Hossono, 1999) and this variation must be

**Table 4.4** Coefficient of cholesterol removal (CR) by *Lactobacillus* spp. at 37°C after 24 h of incubation

| Strain  | CR                | Strain                        | CR                |
|---|-------------------|-------------------------------|-------------------|
| <i>L. acidophilus</i> 11                        | 0.13 <sup>d</sup> | <i>L. fermentum</i> 141       | 0.24 <sup>d</sup> |
| <i>L. acidophilus</i> 13                        | 0.26 <sup>d</sup> | <i>L. fermentum</i> 155       | 0.36 <sup>d</sup> |
| <i>L. acidophilus</i> 14                        | 0.66 <sup>a</sup> | <i>L. fermentum</i> 156       | 0.31 <sup>d</sup> |
| <i>L. acidophilus</i> 15                        | 0.28 <sup>d</sup> | <i>L. fermentum</i> L10       | 0.14 <sup>d</sup> |
| <i>L. acidophilus</i> 16                        | 0.15 <sup>d</sup> | <i>L. fermentum</i> L11       | 0.26 <sup>d</sup> |
| <i>L. acidophilus</i> L1                        | 0.08 <sup>d</sup> | <i>L. helveticus</i> 5        | 0.26 <sup>d</sup> |
| <i>L. acidophilus</i> L2                        | 0.14 <sup>d</sup> | <i>L. helveticus</i> 6        | 0.14 <sup>d</sup> |
| <i>L. acidophilus</i> L3                        | 0.15 <sup>d</sup> | <i>L. plantarum</i> 20        | 0.59 <sup>b</sup> |
| <i>L. acidophilus</i> L4                        | 0.39 <sup>d</sup> | <i>L. plantarum</i> 22        | 0.34 <sup>d</sup> |
| <i>L. acidophilus</i> L5                        | 0.25 <sup>d</sup> | <i>L. plantarum</i> 24        | 0.23 <sup>d</sup> |
| <i>L. acidophilus</i> L6                        | 0.10 <sup>d</sup> | <i>L. plantarum</i> 25        | 0.18 <sup>d</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 17            | 0.22 <sup>d</sup> | <i>L. plantarum</i> L15       | 0.29 <sup>d</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 19            | 0.52 <sup>c</sup> | <i>L. rhamnosus</i> 18        | 0.10 <sup>d</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 63            | 0.05 <sup>d</sup> | <i>Lactobacillus</i> spp. B4  | 0.16 <sup>d</sup> |
| <i>L. casei</i> L7                              | 0.26 <sup>d</sup> | <i>Lactobacillus</i> spp. F1  | 0.08 <sup>d</sup> |
| <i>L. casei</i> L8                              | 0.18 <sup>d</sup> | <i>Lactobacillus</i> spp. F2  | 0.28 <sup>d</sup> |
| <i>L. casei</i> L9                              | 0.19 <sup>d</sup> | <i>Lactobacillus</i> spp. F3  | 0.21 <sup>d</sup> |
| <i>L. collinoides</i> 2                         | 0.04 <sup>d</sup> | <i>Lactobacillus</i> spp. F4  | 0.11 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 4  | 0.42 <sup>d</sup> | <i>Lactobacillus</i> spp. F5  | 0.27 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 8  | 0.11 <sup>d</sup> | <i>Lactobacillus</i> spp. F6  | 0.08 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 9  | 0.07 <sup>d</sup> | <i>Lactobacillus</i> spp. F7  | 0.26 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 26 | 0.05 <sup>d</sup> | <i>Lactobacillus</i> spp. L12 | 0.32 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 3      | 0.18 <sup>d</sup> | <i>Lactobacillus</i> spp. L13 | 0.16 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 10     | 0.17 <sup>d</sup> | <i>Lactobacillus</i> spp. L14 | 0.22 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>Lactis</i> 27     | 0.15 <sup>d</sup> | <i>Lactobacillus</i> spp. L16 | 0.17 <sup>d</sup> |

Means with no common superscripts are significantly different,  $p < 0.01$  (Duncan's multiple range test)

considered when cultures are being selected for probiotic or food adjunct use.

For fifty different strains of lactobacilli, Table 4.4 depicts *in vitro* cholesterol removal and the results are expressed as means of coefficients of cholesterol removal. For comparison of means, ANOVA was used and means were assigned to groups after DMRT at  $p < 0.01$ . Superscript of mean designates the group to which the strain with the mean belongs. There is no significant ( $p < 0.01$ ) difference between the means within a group.

*L. acidophilus* 14 reported the highest cholesterol removing capability followed by *L. plantarum* 20 and *L. casei* ssp. *casei* 19 with coefficients of cholesterol removal of 0.66, 0.59 and 0.52, respectively. The rest of the strains belong to group 'd' and are the least cholesterol removers from the growth medium.

Several workers have also reported *in vitro* reduction of cholesterol from growth medium (Gilliland *et al*, 1984, 1985; Danilson, 1987; Lin *et al*, 1989; Gilliland and Walker, 1990; Vujicic *et al*, 1992; Walker and Gilliland, 1993; Brashears *et al*, 1998; Usman and Hosono, 1999).

Danielson *et al* (1987) found that the three test strains of *L. acidophilus* could reduce cholesterol levels by 30 to 80 % in MRS broth supplemented with 0.2 or 0.4% bile salts. Lin *et al* (1989) have reported a 26% decrease in cholesterol concentration by a tablet containing  $10^8$  viable lactobacilli from double strength MRS broth at 37°C after 45 hours of incubation.

Wide variations in cholesterol removal have been found among different cultures of lactobacilli. Vujicic *et al* (1992)

reported 28 to 65 and 41 to 84 % cholesterol assimilation during 24 and 48 hours of incubation, respectively, by six Kefir cultures.

Gilliland and Walker (1990) also observed significant ( $p < 0.05$ ) variations of 27.5 to 103.9  $\mu\text{g/ml}$  of cholesterol assimilation from the growth medium by 12 cultures of *L. acidophilus* of human origin. In yet another similar study by Walker and Gilliland (1993) cholesterol assimilation by individual strains varied between 0 to 50 mg/ml of broth.

Brashears *et al.* (1998) observed a pH (of growth medium) independent assimilation of cholesterol by the two strains of *L. acidophilus* from growth medium after 20 hours of incubation while as the strains of *L. casei* showed a pH dependent assimilation of cholesterol. Thus, cholesterol removal may be because of assimilation of cholesterol by bacterial cells or by precipitation of cholesterol at low pH.

Usman and Hosono (1999) reported that *L. gasseri* bound more cholesterol after 12 hours of incubation than after 48 hours of incubation at 37°C.

#### **4.1.5 Taurocholate deconjugation**

Many workers (Buck and Gilliland, 1994; Gopal *et al.*, 1996; Usman and Hosono, 1999) have reported deconjugation of bile acids by lactobacilli. The deconjugation of bile acids may help reduce serum cholesterol in humans as deconjugated bile acids are excreted more rapidly than conjugated ones (Chikai *et al.*, 1987); as a consequence, more cholesterol is needed to synthesize new bile acids, which in turn reduce the total cholesterol in the body (Driessen and Boer, 1989). Chikai *et al.* (1987) also reported that deconjugated bile acids adhere to bacteria or dietary fibre

**Table 4.5 Coefficient of Taurocholate deconjugation (TD) by *Lactobacillus* spp. at 37°C after 24 h of incubation.**

| Strain  | TD                | Strain                        | TD                |
|---|-------------------|-------------------------------|-------------------|
| <i>L. acidophilus</i> 11                        | 0.02 <sup>d</sup> | <i>L. fermentum</i> 141       | 0.28 <sup>c</sup> |
| <i>L. acidophilus</i> 13                        | 0.35 <sup>c</sup> | <i>L. fermentum</i> 155       | 0.34 <sup>c</sup> |
| <i>L. acidophilus</i> 14                        | 0.35 <sup>c</sup> | <i>L. fermentum</i> 156       | 0.43 <sup>b</sup> |
| <i>L. acidophilus</i> 15                        | 0.27 <sup>c</sup> | <i>L. fermentum</i> L10       | 0.18 <sup>c</sup> |
| <i>L. acidophilus</i> 16                        | 0.36 <sup>c</sup> | <i>L. fermentum</i> L11       | 0.26 <sup>c</sup> |
| <i>L. acidophilus</i> L1                        | 0.21 <sup>c</sup> | <i>L. helveticus</i> 5        | 0.29 <sup>c</sup> |
| <i>L. acidophilus</i> L2                        | 0.20 <sup>c</sup> | <i>L. helveticus</i> 6        | 0.46 <sup>b</sup> |
| <i>L. acidophilus</i> L3                        | 0.20 <sup>c</sup> | <i>L. plantarum</i> 20        | 0.56 <sup>a</sup> |
| <i>L. acidophilus</i> L4                        | 0.13 <sup>c</sup> | <i>L. plantarum</i> 22        | 0.14 <sup>c</sup> |
| <i>L. acidophilus</i> L5                        | 0.24 <sup>c</sup> | <i>L. plantarum</i> 24        | 0.04 <sup>b</sup> |
| <i>L. acidophilus</i> L6                        | 0.33 <sup>c</sup> | <i>L. plantarum</i> 25        | 0.20 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 17            | 0.28 <sup>c</sup> | <i>L. plantarum</i> L15       | 0.34 <sup>c</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 19            | 0.48 <sup>b</sup> | <i>L. rhamnosus</i> 18        | 0.06 <sup>d</sup> |
| <i>L. casei</i> ssp. <i>casei</i> 63            | 0.10 <sup>c</sup> | <i>Lactobacillus</i> spp. B4  | 0.18 <sup>c</sup> |
| <i>L. casei</i> L7                              | 0.22 <sup>c</sup> | <i>Lactobacillus</i> spp. F1  | 0.23 <sup>c</sup> |
| <i>L. casei</i> L8                              | 0.05 <sup>d</sup> | <i>Lactobacillus</i> spp. F2  | 0.38 <sup>c</sup> |
| <i>L. casei</i> L9                              | 0.26 <sup>c</sup> | <i>Lactobacillus</i> spp. F3  | 0.17 <sup>c</sup> |
| <i>L. collinoides</i> 2                         | 0.06 <sup>d</sup> | <i>Lactobacillus</i> spp. F4  | 0.18 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 4  | 0.28 <sup>c</sup> | <i>Lactobacillus</i> spp. F5  | 0.06 <sup>d</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 8  | 0.34 <sup>c</sup> | <i>Lactobacillus</i> spp. F6  | 0.13 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 9  | 0.19 <sup>c</sup> | <i>Lactobacillus</i> spp. F7  | 0.23 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>bulgaricus</i> 26 | 0.25 <sup>c</sup> | <i>Lactobacillus</i> spp. L12 | 0.30 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 3      | 0.37 <sup>c</sup> | <i>Lactobacillus</i> spp. L13 | 0.22 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 10     | 0.04 <sup>d</sup> | <i>Lactobacillus</i> spp. L14 | 0.21 <sup>c</sup> |
| <i>L. delbreuckii</i> ssp. <i>lactis</i> 27     | 0.30 <sup>c</sup> | <i>Lactobacillus</i> spp. L16 | 0.13 <sup>c</sup> |

Means with no common superscripts are significantly different,  $p < 0.01$  (Duncan's multiple range test)

more than conjugated bile acids, and this may increase excreted bile acids in faeces. Furthermore, deconjugated bile acids do not function as well as conjugated bile acids in the intestinal absorption of cholesterol (Eyssen, 1973). Thus, in assessing *in vivo* cholesterol lowering potential of probiotic bacteria, *in vitro* bile salt deconjugating capability of the bacteria needs to be taken into consideration.

Table 4.5 depicts *in vitro* sodium taurocholate deconjugation of various *Lactobacillus* strains, and the results are expressed as means of coefficients of the taurocholate deconjugation. For comparison of means, ANOVA was used, and means were assigned to groups after DMRT at  $p < 0.01$ . Superscript of a mean designates the group to which the strain with the mean belongs. There was no significant ( $p < 0.01$ ) difference between the means within a group.

*L. plantarum* 20 showed the highest taurocholate deconjugating capability followed by *L. casei* ssp. *casei* 19 and *L. acidophilus* 14 with coefficients of deconjugation of 0.56, 0.48 and 0.35, respectively. Lower taurocholate deconjugators belong to groups 'c' and 'd'.

Strain specific variation in deconjugation of bile salts was also reported by Walker and Gilliland (1993) and Gopal *et al* (1996). Walker and Gilliland (1993) reported 1.40 to 4.30 mmol deconjugated bile acid per ml of growth medium by 19 strains of lactobacilli, after 10 hours of incubation, while as Gopal *et al* (1996) observed 0.647 to 0.976  $\mu$ moles of cholic acid/ ml of broth for six strains of *L. acidophilus* after 24 hours of incubation. Brashears *et al* (1998) reported 60 to 70% bile salt deconjugation in different strains of *L. acidophilus* and *L. casei*. Usman and Hosono (1999) observed

1.24 to 1.94  $\mu\text{mol}$  of cholic acid released per ml of growth medium for 28 strains of *L. gasseri*, after 24 hours of incubation. They also reported that maximum deconjugation of sodium taurocholate was achieved with the cells during the stationary phase of growth.

#### **4.1.6 Selection of *Lactobacillus* strains with maximum probiotic potential**

As already described, means, within a probiotic property, were ascribed to groups after DMRT of data at 1% level of significance, and within a specified probiotic property, superscript designates the group to which a mean belongs. Mean of all the means within a group was calculated for all the probiotic properties studied and for each property, the group with the maximum value was given a score of 20. Within a particular probiotic property, this maximum score and the mean of means of each group were used to calculate the scores for the rest of the groups (Table 4.6). These scores were then used to evaluate the *Lactobacillus* cultures for overall probiotic potential.

Among the 50 strains tested, *L. acidophilus* 14 has the highest score of 92 followed by *L. casei* ssp. *casei* 19 and *L. plantarum* 20 with scores of 89.50 and 87.50, respectively (Table 4.7). These have the maximum of gut colonizing and cholesterol-lowering potential under various *in vitro* tests that were carried out.

The organisms were further tested for their survival in acid solutions of varying pH levels and also in the solutions of varying bile concentrations. Antimicrobial activity of the three microorganisms were also tested against various indicator organisms to check their competitive ability in colonizing the

**Table 4.6** Scores used to evaluate the gut colonizing and cholesterol lowering potential of the organisms

| Probiotic property                    | Group  |       |       |       |      |      |
|---------------------------------------|--------|-------|-------|-------|------|------|
|                                       | a      | b     | c     | d     | e    | f    |
| Acid tolerance                        | 20.00  | 14.50 | 5.00  | 0.00  | 0.00 | 0.00 |
| Bile tolerance                        | 20.00  | 12.00 | 5.00  | 0.00  | 0.00 | 0.00 |
| Cell surface hydrophobicity           | 20.00  | 17.50 | 11.50 | 9.00  | 5.00 | 1.50 |
| Cholesterol removing capability       | 20.00  | 18.00 | 16.00 | 6.00  | 0.00 | 0.00 |
| Taurocholate deconjugating capability | 20.00  | 16.00 | 8.50  | 2.00  | 0.00 | 0.00 |
| Total score                           | 100.00 | 78.00 | 46.00 | 17.00 | 5.00 | 1.50 |

**Table 4.7** Various scores of the organisms with maximum probiotic potential

| Strain                               | Acid tolerance | Bile tolerance | Cell surface hydrophobicity | Cholesterol removing capability | Taurocholate deconjugating capability | Total score |
|--------------------------------------|----------------|----------------|-----------------------------|---------------------------------|---------------------------------------|-------------|
| <i>L. acidophilus</i> 14             | 20.00          | 12.00          | 20.00                       | 20.00                           | 20.00                                 | 92.00       |
| <i>L. casei</i> ssp. <i>casei</i> 19 | 20.00          | 20.00          | 17.50                       | 16.0                            | 16.00                                 | 89.50       |
| <i>L. plantarum</i> 20               | 20.00          | 12.00          | 17.50                       | 18.00                           | 20.00                                 | 87.50       |

gut over other organisms. The results are presented in Table 4.8 to 4.10.

#### 4.1.6.1 Acid tolerance in acid solutions

To be used as dietary adjuncts, lactobacilli must be able to survive extreme low pH of stomach.

Table 4.8 shows the results of the three selected organisms, *L.casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20, for their survival at pH 2 and 3. Results are expressed as mean log cfu/ml of mixture. After ANOVA of the data, comparison between the means was made by Least significant difference (LSD) pairwise comparison.

Though all the three cultures survived the pH of 2.0 and 3.0, there was a significant ( $p < 0.05$ ) decline of three log cycles in comparison to control at these pH levels after three hours of incubation in all the strains tested. Individually for each strain, there was no significant difference between the means at pH 2 and 3, after 3 hours of incubation.

Several reports have indicated the effect of pH on survival of lactic acid bacteria (Conway *et al*, 1987; Hood and Zottola, 1988; Plockova *et al*, 1996). Conway *et al* (1987) observed a rapid decline in numbers of *L. acidophilus*, *L. bulgaricus* and *Streptococcus thermophilus* at pH 1.0 and a slower fall at pH 3.0 and 5.0 in phosphate buffer saline. Similar results were obtained when the organisms were incubated in gastric juice at pH 1.0 and 3.0. Using MRS broth, potassium chloride buffer and potassium phthalate buffer, Hood and Zottola (1988) examined the response of *L. acidophilus* to pH levels of 2.0, 3.0 and 4.0. In both the buffers and in the broth, bacterial numbers rapidly declined

to zero at pH 2.0 after 45 minutes, from an initial cell number of  $10^7$  to  $10^8$  cfu/ml. In MRS broth cell numbers remained relatively constant at pH levels of 3.0 and 4.0 over 2 hours of incubation. Similar studies were carried out by Plockova *et al* (1996) on the acid tolerance of several strains of lactobacilli that grew well in milk and found that survival of the lactobacilli at low pH was better in MRS broth than in potassium chloride or phosphate buffer.

#### **4.1.6.2 Bile tolerance in bile solutions**

To be used as dietary adjuncts, lactobacilli must be able to survive different concentration of bile in human small intestines.

Table 4.9 depicts the results of the three selected organisms *L.casei* ssp.*casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 for their survival in bile concentration of 2.0 and 4.0%. Results are expressed as mean log cfu/ml of mixture. After ANOVA of the data, comparison between the means was made by LSD pair wise comparison.

Though all the three cultures survived the bile concentration of 2 and 4%, there was a significant ( $p < 0.05$ ) decline in comparison to control in cell numbers of *L. casei* ssp.*casei* 19 at 4% of bile concentration after 12 hours of incubation. Similarly, *L. plantarum* 20 showed a significant ( $p < 0.05$ ) decline of about two log cycles in cell numbers at bile concentration of 2% after 12 hours of incubation. There was no significant ( $p < 0.05$ ) difference in survival of *L. acidophilus* 14 and *L. plantarum* 20 at 2.0 and 4.0% bile concentrations.

Several reports have indicated the effect of various bile salt concentrations on lactic acid bacteria (Conway *et al*, 1987; Walker and Gilliland, 1993; Clark and Martin, 1994;

Table 4.8

Survival (log cfu/ml) of different *Lactobacillus* spp. after 3h of incubation at 37° C, at various pH levels.

| Strain                                     | Period (h) of incubation |                         |                        |                        |                        |                        |
|--|--------------------------|-------------------------|------------------------|------------------------|------------------------|------------------------|
|  | 0 h                      |                         |                        | 3 h                    |                        |                        |
|  | Control<br>(water)       | pH2                     | pH3                    | Control<br>(water)     | pH2                    | pH3                    |
| <i>L. casei</i><br>ssp. <i>casei</i><br>19 | 8.57±0.59 <sup>a</sup>   | 7.67±0.77 <sup>ab</sup> | 7.03±0.35 <sup>b</sup> | 6.94±0.32 <sup>b</sup> | 4.16±0.61 <sup>c</sup> | 4.13±0.30 <sup>c</sup> |
| <i>L. acidophilus</i><br>14                | 9.25±0.57 <sup>a</sup>   | 7.31±0.40 <sup>b</sup>  | 6.76±0.70 <sup>b</sup> | 6.14±0.33 <sup>b</sup> | 3.01±0.64 <sup>c</sup> | 3.02±0.17 <sup>c</sup> |
| <i>L. palmarum</i><br>20                   | 8.68±0.42 <sup>a</sup>   | 6.95±0.26 <sup>b</sup>  | 6.58±0.19 <sup>b</sup> | 6.51±0.55 <sup>b</sup> | 4.03±0.13 <sup>c</sup> | 4.09±0.07 <sup>c</sup> |

The values (mean±SEM) in the same row with different superscripts are significantly (p<0.05) different by Least significant difference pair wise comparison

**Table 4.9** Survival (log cfu/ml) of different *Lactobacillus* spp. after 12h of incubation at 37° C, at various bile concentrations.

| Strain                               | Period (h) of incubation |                         |                         |                                     |                         |                        |
|--------------------------------------|--------------------------|-------------------------|-------------------------|-------------------------------------|-------------------------|------------------------|
|                                      | 0 h                      |                         |                         | 12 h                                |                         |                        |
|                                      | Bile concentration       |                         |                         | Bile concentration                  |                         |                        |
|                                      | 0%                       | 2%                      | 4%                      | 0%                                  | 2%                      | 4%                     |
| <i>L. casei</i> ssp. <i>casei</i> 19 | 9.98±0.37 <sup>a</sup>   | 9.24±0.38 <sup>a</sup>  | 9.14±0.15 <sup>a</sup>  | 8.82±0.30 <sup>a</sup>              | 7.17±0.46 <sup>a</sup>  | 4.94±0.42 <sup>b</sup> |
| <i>L. acidophilus</i> 14             | 8.89±0.41 <sup>a</sup>   | 8.10±0.53 <sup>ab</sup> | 6.83±0.66 <sup>ab</sup> | 7.43±0.45 <sup>a</sup> <sub>b</sub> | 5.38±0.31 <sup>bc</sup> | 3.68±0.58 <sup>c</sup> |
| <i>L. palantarum</i> 20              | 8.71±0.45 <sup>a</sup>   | 6.95±0.26 <sup>ab</sup> | 6.58±0.19 <sup>ab</sup> | 6.51±0.55 <sup>a</sup> <sub>b</sub> | 4.03±0.13 <sup>b</sup>  | 4.09±0.07 <sup>b</sup> |

The values (mean±SEM) in the same row with different superscripts are significantly different by Least significant difference pair wise comparison. (p<0.05)

Lankaputhra and Shah, 1995; Gopal *et al*, 1996; Usman and Hosono, 1999). Gilliland *et al*, (1984) compared the ability of seven strains of lactobacilli when grown in MRS broth with and without 0.3% Ox gall (Difco). They reported that 0.3% Ox gall exerted inhibitory effect on all the strains tested. Similar work was carried out by Walker and Gilliland (1993) and they reported that all the 19 strains of *L. acidophilus* tested exhibited some degree of bile tolerance. Clark and Martin (1994) found that *B. bifidum* was very sensitive bile concentration of 2% and above.

Lankaputhra and Shah (1998) studied the tolerance of six strains of *L. acidophilus* to 1.0 and 1.5% bile concentrations for a period of three hours. They reported strain specific survival of the organisms. A similar finding was reported by Gopal *et al* (1996). They used 0.3% Ox gall in MRS-thio broth (broth supplemented with thioglycollate) and observed that the difference in tolerance to bile may have been partly due to the natural difference in the growth of individual strains. Usman and Hosono (1999) also observed a significant ( $p < 0.05$ ) log time difference, required to reach absorbance at 620 nm by 0.3 units in MRS broth supplements with 0.3% Oxgall, among various strains of *L. gasseri*.

Most of the previous studies (Gilliland *et al*, 1984; Conway *et al*, 1987; Lankaputhra and Shah, 1996; Usman and Hosono, 1999) used bile salts in some type of growth medium. In the present investigation and also that of Clark and Martin (1994), no growth medium or buffers were used during the exposure of bacteria to bile solutions. This was to ensure that only the bile and no other factors should interfere with the growth and survival of the bacteria.

In the present study, lactobacilli were subjected to bile concentration for a period of 12 hours, because this time period has been reported to be sufficient for most foods to pass through the small intestine (Brown, 1991).

#### **4.1.6.3 Antimicrobial activity**

The ability of lactic acid bacteria to inhibit various Gram-positive and Gram-negative bacteria is well known (Chateau *et al*, 1993). This inhibition may be related to the production of organic acids, hydrogen peroxide and bacteriocin like substance (Klaenhammer, 1993). This antagonistic property could be used by probiotic bacteria to gain advantage over other bacteria to colonize the gut or to prevent the pathogenic organisms from establishing into the gut.

The antimicrobial activity of *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 against five species of bacteria is given in Table 4.10. Though the diameter of the zones of inhibition by the three *Lactobacillus* cultures varied against five indicator organisms, all the strains showed antimicrobial activity against the indicator organisms used.

The antagonistic activity observed against various indicator organisms may be because of the cumulative effect of all the antimicrobial metabolites like lactic acid, hydrogen peroxide, organic acids and bacteriocins, secreted by the test cultures into the medium (Gilliland and Speck, 1977; Klaenhammer, 1993; Korasapati, 1998) that get concentrated and diffuse around the test cultures spot much before the indicator is added (Korasapati, 1998).

**Table 4.10** Antimicrobial activity of different *Lactobacillus* spp. against various indicator organisms

| Strain                                  | Indicator organism                |  |                                      |  |                                 |
|---|-----------------------------------|--|--------------------------------------|--|---------------------------------|
|   | <i>Bacillus cereus</i><br>NCDC 66 | <i>Staphylococcus aureus</i><br>NCDC 109 | <i>Enterococcus faecalis</i><br>NCDC | <i>Leuconostoc mesenteroides</i><br>NCDC | <i>Escherichia coli</i><br>V517 |
| <i>L. casei</i> ssp.<br><i>casei</i> 19 | ++                                | +  | ++                                   | +  | ++                              |
| <i>L. acidophilus</i> 14                | ++                                | +  | ++                                   | +  | ++                              |
| <i>L. plantarum</i> 20                  | ++                                | +  | ++                                   | ++                                       | ++                              |

+: 11-20 mm diametre inhibitory zone

++: 21-30 mm diametre inhibitory zone

## **4.2 Protoplast fusions and the screening of the fusants**

In an attempt to enhance the probiotic potential of the selected strains (*L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20, were subjected to protoplast fusion and the fusants were screened for the probiotic properties to assess whether these have been enhanced or not.

### **4.2.1 Protoplast fusion of the selected lactobacilli**

The production of scientifically valid probiotic preparation requires that a number of desirable characteristics at an optimum level be present in an ideal probiotic culture. A relatively non-specific gene transfer system such as protoplast fusion, where there is a possibility of transfer of both plasmid as well as chromosomal markers (Hopwood, 1981), can be used for incorporating optimum levels of different probiotic properties in a single strain.

In an attempt to enhance probiotic potential, two strains, *L. casei* ssp. *casei* 19 and *L. acidophilus* 14, with maximum values of different probiotic properties were selected for protoplast fusion, which involved:

#### **4.2.1.1 Antibiotic sensitivity**

As shown in Table 4.11, among the nine antibiotics tested, *L. casei* ssp. *casei* 19 was resistant to kanamycin, streptomycin and erythromycin and *L. acidophilus* 14 was resistant to tetracycline, streptomycin and chloramphenicol. Two antibiotics, erythromycin, with minimum inhibitory concentration (MIC) of 15 µg/ml against *L. casei* ssp. *casei* 19, and tetracycline, with MIC of 25 µg/ml against *L. acidophilus* 14 (Table 4.12), were selected for selection of fusants.

#### 4.2.1.2 Protoplast formation

The selected lactobacilli were subjected to lysozyme (10 mg/ml) and mutanolysin (50 µg/ml) treatment to form protoplasts.

The percent protoplast formation of *L. casei* ssp. *casei* 19 and *L. acidophilus* 14 is depicted in Table 4.13. *L. casei* ssp. *casei* 19 formed about 75% protoplast in 60 minutes, after which it slowly increased to 81% in 120 minutes. *L. acidophilus* 14 was less sensitive to muralytic enzymes and formed 56% protoplasts by the end of second hour.

Other workers have carried out similar studies. Reed (1987) reported 35% protoplast formation of *Lactococcus lactis* and *L. acidophilus*, using 0.5M lactose or sucrose as an osmotic stabilizer. Miyamoto *et al* (1990) reported protoplast formation in the range of 85 to 90% in *L. casei* var. *casei*, *L. casei* var. *rhamnosus*, *L. delbreuckii* var. *bulgaricus* and *L. lactis* in 10 minutes using 0.9M lactose. Prasad *et al* (1990) observed more than 99% protoplast formation in *L. casei* 300, *L. acidophilus* R and *Lactobacillus* isolate (L<sub>4</sub>) in 30 minutes. Anuradha (2000) found 51% to 80% protoplast formation in two isolates of lactobacilli.

#### 4.2.1.3 Protoplast regeneration

As a shown in Table 4.13, protoplast regeneration of the two strains, *L. casei* ssp. *casei* 19 and *L. acidophilus* 14 ranged between 0.30 to 30.09%, depending upon the duration of incubation of protoplast formation. For *L. casei* ssp. *casei* 19, maximum regeneration of 26.25% was reached in 30 minutes, after which there was a decline in regeneration with increasing time of incubation. In contrast, *L. acidophilus* 14 showed maximum regeneration of 30.09% after an incubation

**Table 4.11 Antibiotic sensitivity of *L. casei* ssp. *casei* 19 and *L. acidophilus* 14**

| Antibiotic      | Content (µg) | <i>L. casei</i> 19 | <i>L. acidophilus</i> 14 |
|-----------------|--------------|--------------------|--------------------------|
| Ampicillin      | 10           | S                  | S                        |
| Tetracyclin     | 30           | S                  | R                        |
| Kanamycin       | 30           | R                  | S                        |
| Streptomycin    | 10           | R                  | R                        |
| Chloramphenicol | 30           | S                  | R                        |
| Novobiocin      | 30           | S                  | S                        |
| Vancomycin      | 30           | S                  | S                        |
| Erythromycin    | 15           | R                  | S                        |

S: Sensitive

R: Resistant

**Table 4.12 Antibiotics used for the selective isolation of various fusants**

| Strain                               | Antibiotics used | Minimum inhibitory concentration (µg/ml) | Designation of the fusants |
|--------------------------------------|------------------|--|----------------------------|
| <i>L. casei</i> ssp. <i>casei</i> 19 | Erythromycin     | 15                                       | FL1, FL2, FL3              |
| <i>L. acidophilus</i> 14             | Tetracyclin      | 25                                       |                            |

**Table 4.13** Percent protoplast formation and regeneration of parent strains during incubation with lysozyme (10 mg/ml) and mutanolysin (50 µg/ml)

| Strain                               | Incubation (minutes) | Protoplast formation (%) | Regeneration(%) |
|--------------------------------------|----------------------|--------------------------|-----------------|
| <i>L. casei</i> ssp. <i>casei</i> 19 |                      |                          |                 |
|                                      | 30                   | 30.00                    | 26.25           |
|                                      | 60                   | 75.21                    | 18.19           |
|                                      | 90                   | 78.32                    | 10.06           |
|                                      | 120                  | 81.61                    | 5.25            |
| <i>L. acidophilus</i> 14             |                      |                          |                 |
|                                      | 30                   | 25.35                    | 18.06           |
|                                      | 60                   | 43.21                    | 25.00           |
|                                      | 90                   | 47.02                    | 30.09           |
|                                      | 120                  | 55.68                    | 0.30            |

**Table 4.14** Tolerance, measured as the coefficients of inhibition of growth, to acid (at pH levels of 1, 2, 3 and 4) and bile (at the concentrations of 0.1, 0.2, 0.3 and 0.5%), and the coefficients of cell surface hydrophobicity of the fusants.

| Fusant | Acid tolerance | Bile tolerance | Coefficient of cell surface hydrophobicity |
|--------|----------------|----------------|--|
| FL1    | 0.86±0.02      | 0.49±0.06      | 0.50±0.03                                  |
| FL2    | 0.75±0.00      | 0.75±0.08      | 0.74±0.07                                  |
| FL3    | 0.73±0.08      | 0.63±0.11      | 0.35±0.01                                  |

The values are the means±SEM

period of 90 minutes, thereafter, percent regeneration started declining with increasing duration of incubation for protoplast formation.

In a previous study, Prasad *et al* (1990) reported that protoplast regeneration reached maximum regeneration of 30% in 90 minutes and 28% in 60 minutes of incubation for protoplast formation of *L. acidophilus* R and *L. casei* 300, respectively.

In the present study, after 30 or 90 minutes (30 minutes for *L. casei* ssp. *casei* 19 and 90 minutes for *L. acidophilus* 14) of incubation for protoplast formation, the regeneration of protoplasts started decreasing. This may be due to loss of some vital components necessary for survival because of longer exposure of bacteria to murelytic enzymes (Vescova *et al*, 1984). The findings are also similar to those of Cocconelli *et al* (1986).

Strain specific variation in protoplast regeneration, as is the case in this study, was also reported by other workers (Lee-Wickner and Chassy, 1984; Prasad *et al*, 1990; Aftabuddin, 1997; Anuradha, 2000).

#### **4.2.1.4 Protoplasts fusion and regeneration of the fusants**

The protoplast of *L. casei* ssp. *casei* 19 and *L. acidophilus* 14 were fused by using polyethylene glycol (PEG) 6000, and three fusants FL<sub>1</sub>, FL<sub>2</sub> and FL<sub>3</sub> resistant to erythromycin and tetracycline were picked up from the regeneration medium.

The regeneration frequency of the fusants was  $1.5 \times 10^{-8}$  in the present study. Cocconcelli *et al* (1986); Reed (1987),

Prasad et al (1991) and Anuradha (2000) reported regeneration frequencies of  $8 \times 10^{-4}$  to  $1 \times 10^{-5}$ ,  $7.5 \times 10^{-8}$  to  $1.1 \times 10^{-7}$ ,  $8 \times 10^{-10}$  to  $10^{-8}$  and  $1.2 \times 10^{-8}$  respectively.

#### **4.2.2 Screening of the fusants**

The three fusants, FL1, FL2 and FL3, obtained were tested for acid tolerance, bile tolerance and cell surface hydrophobicity by the same methods as described in the first phase for screening of various lactobacilli.

##### **4.2.2.1 Acid tolerance**

All the three fusants FL<sub>1</sub>, FL<sub>2</sub> and FL<sub>3</sub> showed reduced acid tolerance in comparison to the parent strains (Table 4.14).

##### **4.2.2.2 Bile tolerance**

Only one fusant FL<sub>1</sub> exhibited bile tolerance comparable with parent strains. FL<sub>2</sub> and FL<sub>3</sub> were of low bile tolerance (Table 4.14).

##### **4.2.2.3 Cell surface hydrophobicity**

Fusant FL2 exhibited cell surface hydrophobicity comparable with that of the parent strain, *L. casei* ssp. *casei* 19. FL<sub>1</sub> and FL<sub>3</sub> had lower cell surface hydrophobicity (Table 4.14).

#### **4.2.3 Probiotic potential of the fusants**

The fusants, from the parent strains with maximum probiotic potential, showed no significant improvement over the parents in so far as above properties are concerned. Thus, the parents, instead of fusants, were used in the animal experiment, in the third phase of the research, to test *in vivo* hypocholesterolemic effect of the strains, if any.

### **4.3 Effect of feeding of the selected cultures on various parameters estimated in rats**

Hyperlipidemia is the major risk factor leading to arteriosclerosis (Kawase *et al*, 2000), and the incidence of the disease is increased in persons with increased levels of blood cholesterol (Akalin *et al*, 1997), particularly LDL (Grundy, 1997).

Several investigators have reported that fermented milk products with lactic acid bacteria decrease serum cholesterol concentration (Taranto *et al*, 1999; Kawase *et al*, 2000; Kikuchi-Hayakawa, 2000) and the ingestion of probiotic lactic acid bacteria possibly would be a more natural method to decrease serum cholesterol concentration. Thus, in the present investigation, the three cultures, *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 with maximum probiotic potential, were fed to the rats along with basal, cholesterol-enriched and cream-enriched diets to test *in vivo* hypocholesterolemic and gut colonizing effect of the strains, if any.

Effect of feeding of each selected strain with different experimental diets, was observed on body weight, on concentrations of plasma triacylglycerols and various plasma cholesterol fractions and on counts of faecal lactobacilli and coliforms in rats.

Body weight of the rats was calculated to check whether the microorganisms affect the body weight in relation to concentrations of various plasma lipid fractions estimated.

Atherogenic index was calculated as given in the third chapter, "Materials and Methods". The higher the value of atherogenic index, the more is the risk of the development of

atherosclerosis. Faecal *Lactobacillus* and coliform counts of the rats were estimated to assess the survival of the strains fed, through the gastro- intestinal tract and the possible colonization in the gut.

For each of the strain to be tested for its *in vivo* effect on various parameters already mentioned, rats were divided, as per the diets fed, into three groups of eight rats each. The details of the diets are given in the chapter, "Materials and Methods".

For the various parameters estimated, results were expressed as mean + SEM (standard error of the mean). ANOVA was used to test the significance of difference at  $p < 0.05$ . LSD procedure was adopted for pairwise comparison of the means. For each selected strain, results are discussed under separate sub-heading "Inference ". The effect of all the three strains on plasma cholesterol concentrations in relation to findings of other workers in lactic acid bacteria are discussed at the end of the chapter under Section 4.3.4.

#### **4.3.1 Effect of *L. casei* ssp. *casei* 19**

The effect of feeding of *L. casei* ssp. *casei* 19 on body weight, concentrations of plasma triacylglycerols and various plasma cholesterol fractions, and counts of faecal lactobacilli and coliforms of rats are described in relation to basal, cholesterol-enriched and cream-enriched diets fed to the animals (Table 4.15 to 4.20, Fig. 4.1).

##### **4.3.1.1 Body weight and plasma lipids in basal diet fed rats**

There was non-significant increase in the body weight but a decline, though non-significant, in atherogenic index

and the concentrations of the various plasma lipids (plasma triacylglycerol and various plasma cholesterol fractions) of the rats after the feeding of basal diet with the bacterial cells alone, without any carrier like milk, for a period of 15 days (Table 4.15).

#### **4.3.1.2 Body weight and plasma lipids in cholesterol-enriched diet fed rats**

Table 4.16 shows the results for various parameters in rats before and after the supplementation of cholesterol-enriched diet with *L. casei* ssp. *casei* 19. No significant change was observed in the parameters.

#### **4.3.1.3 Body weight and plasma lipids in cream-enriched diet fed rats**

As shown in Table 4.17, there was a non-significant increase in body weight and a non-significant decline in concentration of various plasma lipids after supplementation of cream-enriched diet with *L. casei* ssp. *casei* 19 for 15 days; there was no significant change in the HDL cholesterol, but AI was significantly reduced from  $1.22 \pm 0.08$  to  $0.97 \pm 0.10$  and was comparable with that of 0 day ( $0.56 \pm 0.10$ )

#### **4.3.1.4 Comparison in body weight and in concentrations of plasma lipids**

Except body weight, there was no significant difference in other parameters among the three groups (basal-diet fed, cholesterol-enriched diet-fed and cream-enriched diet-fed groups) of rats at 0 day (Fig. 4.1).

After 15 days of feeding of the experimental diets without *L. casei* ssp. *casei* 19, atherogenic index and the concentrations of total plasma and LDL cholesterol were

significantly higher in cholesterol-enriched diet fed as compared to cream-enriched diet fed group of rats. There was no significant difference in the concentrations of plasma triacylglycerols and VLDL cholesterol between the two groups. In all the three groups HDL-cholesterol concentrations were not significantly different. Except HDL cholesterol and body weight, the rest of the parameters (plasma triacylglycerols and various plasma cholesterol fractions), were significantly lower in basal diet as compared to cholesterol-enriched diet fed group (Fig. 4.1).

After 15 days of the feeding of experimental diets with *L. casei* ssp. *casei* 19, HDL-cholesterol concentrations were significantly increased in cholesterol-enriched diet and cream-enriched diet fed groups as compared to basal diet fed group. Atherogenic index in cream-enriched diet fed group was reduced significantly from  $1.22 \pm 0.08$  to  $0.97 \pm 0.10$  and was comparable statistically with basal diet fed group. In comparison to cholesterol-enriched diet fed group, the concentrations of various plasma lipids estimated were significantly lower in basal diet fed group (Fig. 4.1).

#### **4.3.1.5 Faecal *Lactobacillus* counts**

There were no significant differences in faecal *Lactobacillus* counts of rats before and after feeding of *L. casei* ssp. *casei* 19 with various experimental diets. The values are given in Table 4.18.

#### **4.3.1.6 Faecal coliform counts**

There was significant decline of about 0.5 to one log cycle in coliform counts of rats before and after supplementation of the *Lactobacillus* strain with basal and cream-enriched diets, whereas no significant difference was

**Table.4.15** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of the rats fed basal diet with and without *L. casei* ssp. *casei* 19

|                            | Days after feeding diet without <i>L. casei</i> ssp. <i>casei</i> 19 |             | Days after feeding diet with <i>L. casei</i> ssp. <i>casei</i> 19 organism |
|----------------------------|--|-------------|--|
|                            | 0 day  | 15 days     | 15 days  |
| Body weight (g)            | 204.37±4.26  | 209.50±4.40 | 219.50±3.50  |
| Total cholesterol (mmol/l) | 1.72±0.09  | 1.71±0.08   | 1.67±0.09  |
| HDL-cholesterol (mmol/l)   | 0.83±0.06  | 0.86±0.05   | 0.79±0.04  |
| LDL-cholesterol (mmol/l)   | 0.47±0.05  | 0.52±0.04   | 0.44±0.07  |
| VLDL-cholesterol (mmol/l)  | 0.17±0.00  | 0.13±0.01   | 0.13±0.00  |
| Triacylglycerols (mmol/l)  | 0.90±0.04  | 0.70±0.05   | 0.67±0.04  |
| Atherogenic index          | 0.60±0.09  | 0.61±0.05   | 0.56±0.08  |

The values are the mean±SEM

**Table 4.16** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of the rats fed cholesterol-enriched diet with and without *L. casei* ssp. *casei* 19

|                               | Days after feeding diet without<br><i>L. casei</i> ssp. <i>casei</i> 19 |                           | Days after<br>feeding diet<br>with <i>L. casei</i><br>ssp. <i>casei</i> 19 |
|-------------------------------|---|---------------------------|--|
|                               | 0 day   | 15 days                   | 15 days  |
| Body weight (g)               | 210.50±3042 <sup>a</sup>  | 220.37±3.48 <sup>ab</sup> | 235.87±5.09 <sup>b</sup>   |
| Total cholesterol<br>(mmol/l) | 1.89±0.10 <sup>a</sup>  | 3.76±0.23 <sup>b</sup>    | 3.81±0.17 <sup>b</sup>   |
| HDL-cholesterol<br>(mmol/l)   | 0.93±0.07 <sup>a</sup>  | 0.99±0.06 <sup>a</sup>    | 1.01±0.05 <sup>a</sup>   |
| LDL-cholesterol<br>(mmol/l)   | 0.51±0.05 <sup>a</sup>  | 1.98±0.15 <sup>b</sup>    | 2.07±0.11 <sup>b</sup>   |
| VLDL-cholesterol<br>(mmol/l)  | 0.18±0.00 <sup>a</sup>  | 0.34±0.01 <sup>b</sup>    | 0.31±0.01 <sup>b</sup>   |
| Triacylglycerols<br>(mmol/l)  | 0.97±0.30 <sup>a</sup>  | 1.72±0.08 <sup>b</sup>    | 1.59±0.06 <sup>b</sup>   |
| Atherogenic index             | 0.56±0.07 <sup>a</sup>  | 2.01±0.13 <sup>b</sup>    | 2.06±0.10 <sup>b</sup>   |

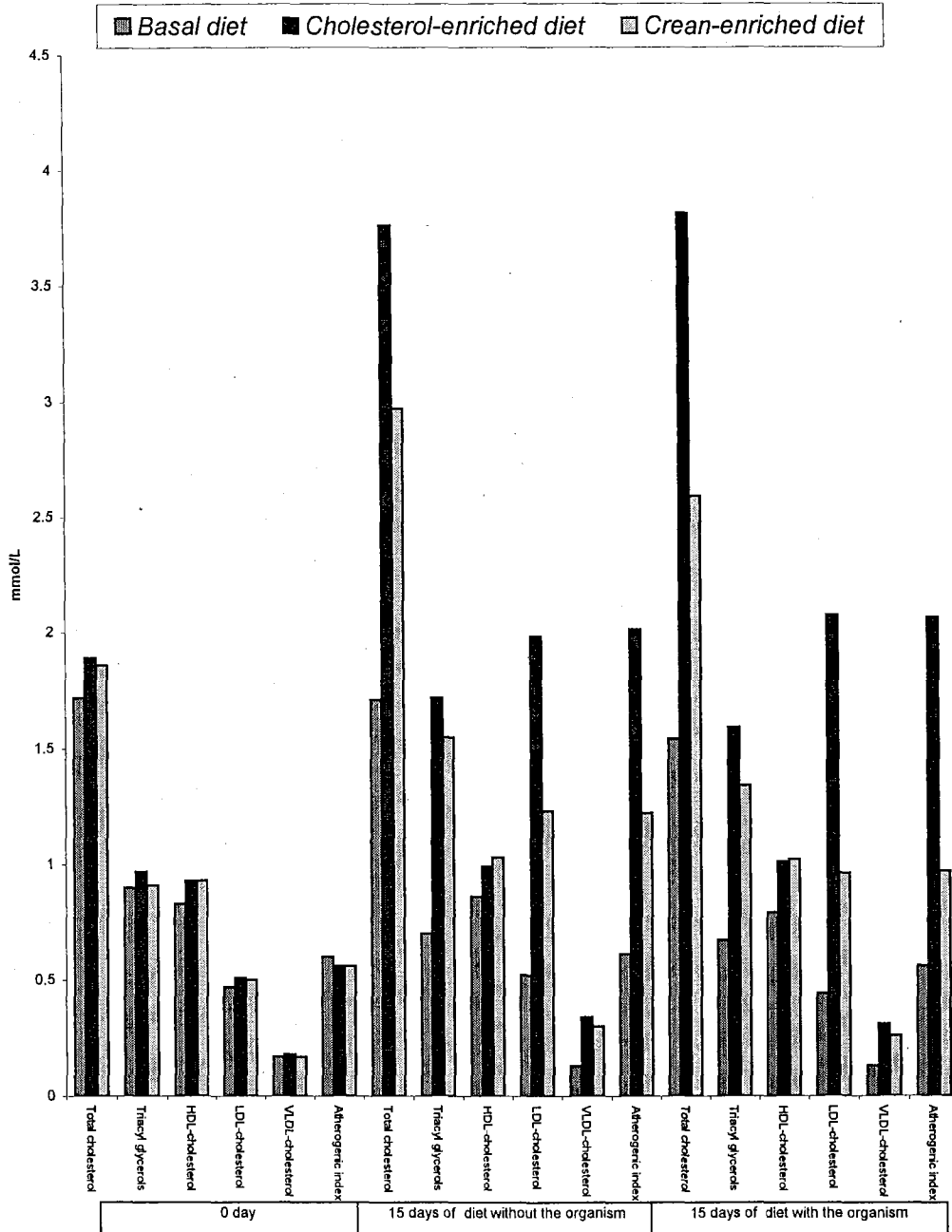
The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.17** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of the rats fed cream –enriched diet with and without *L. casei* ssp. *casei* 19

|                            | Days after feeding diet without <i>L. casei</i> ssp. <i>casei</i> 19 |                           | Days after feeding diet with <i>L. casei</i> ssp. <i>casei</i> 19 |
|----------------------------|--|---------------------------|---|
|                            | 0 day  | 15 days                   | 15 days   |
| Body weight (g)            | 221.25±3072 <sup>a</sup>   | 230.62±4.17 <sup>ab</sup> | 238.75±3.49 <sup>b</sup>  |
| Total cholesterol (mmol/l) | 1.86±0.10 <sup>a</sup>   | 2.97±0.09 <sup>b</sup>    | 2.59±0.04 <sup>b</sup>  |
| HDL-cholesterol (mmol/l)   | 0.93±0.07 <sup>a</sup>   | 1.03±0.06 <sup>a</sup>    | 1.02±0.05 <sup>a</sup>  |
| LDL-cholesterol (mmol/l)   | 0.50±0.07 <sup>a</sup>   | 1.23±0.03 <sup>b</sup>    | 0.96±0.04 <sup>b</sup>  |
| VLDL-cholesterol (mmol/l)  | 0.17±0.00 <sup>a</sup>   | 0.30±0.00 <sup>b</sup>    | 0.26±0.00 <sup>b</sup>  |
| Triacylglycerols (mmol/l)  | 0.91±0.04 <sup>a</sup>   | 1.55±0.04 <sup>b</sup>    | 1.34±0.04 <sup>b</sup>  |
| Atherogenic index          | 0.56±0.10 <sup>a</sup>   | 1.22±0.08 <sup>b</sup>    | 0.97±0.10 <sup>a</sup>  |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Fig. 4.1: Effect of *L. casei* ssp. *casei* 19 on concentrations of plasma triacylglycerols and cholesterol fractions of rats fed various experimental diets.**



**Plasma triacylglycerols and cholesterol fractions**

observed before and after feeding of the culture with cholesterol-enriched diet (Table 4.19).

#### **4.3.1.7 Comparison in counts of faecal lactobacilli and coliforms**

After 15 days of feeding of the experimental diets without *L. casei* ssp. *casei* 19, a significant decrease was observed in the faecal *Lactobacillus* counts in cholesterol-enriched diet fed and cream-enriched diet fed groups as compared to the basal diet fed group of rats. Cholesterol-enriched diet fed group showed the lowest faecal *Lactobacillus* and coliform counts.

After 15 days of the feeding of the experimental diets with the *Lactobacillus* culture, faecal *Lactobacillus* counts showed an overall increase in all the groups, but cholesterol-enriched diet fed group still showed the lower counts, which were not significantly different from cream-enriched diet fed group. Coliform counts exhibited a non-significant decline in basal and cream-enriched diet fed groups but were increased non-significantly in cholesterol-enriched diet fed group.

#### **4.3.1.8 Inference**

From the above results (Table 4.15 to 4.20, Fig. 4.1), it appears that *L. casei* ssp. *casei* 19 does not affect blood cholesterol through its action on dietary cholesterol (Table 4.16). This may be because of the poor survival or colonization of the gut by the organism when fed with cholesterol-enriched diet. Poor survival or colonization is indicated by lower faecal *Lactobacillus* counts and by an increase in coliform counts (Table 4.18 and 4.19).

The decrease in the concentrations of plasma total cholesterol and LDL-cholesterol and the values of atherogenic index observed in rats fed the organism with basal and cream-enriched diets (Table 4.15 and 4.17) may be because of inhibitory effect of the organism on cholesterol synthesis. Compared with the cholesterol-enriched diet fed rats, the faecal *Lactobacillus* counts were more in rats fed the organism with basal and cream-enriched diets and the rats also exhibited a significant decline in coliform counts after the feeding of the *Lactobacillus* culture (Table 4.18 and 4.19). This may be because of better survival or colonization of the organisms in the gut.

As can be seen from Fig. 4.1, feeding a diet containing 0.5% cholesterol is more hypocholesterolemic than a diet containing 10% cream. As can be gauged from the Figure, the value of atherogenic index in cream-enriched diet fed groups was reduced to as low as can be compared with that of basal diet fed groups of rats, when the cream-enriched diet is supplemented with the *Lactobacillus* culture.

Body weight does not appear to reflect any effect on the concentrations of various plasma cholesterol fractions and vice-versa.

#### **4.3.2 Effect of *L. acidophilus* 14**

The effect of feeding of *L. acidophilus* 14 on body weight, concentrations of plasma triacylglycerols and various plasma cholesterol fractions, and counts of faecal lactobacilli and coliforms of rats are described in relation to basal, cholesterol-enriched and cream-enriched diets fed to the animals (Table 4.21 to 4.26, Fig. 4.2) .

**Table 4.18** Faecal *Lactobacillus* counts (log cfu/g of faeces) of rats fed various diets with without *L.casei* ssp. *casei* 19

| Diet                 | Faecal <i>Lactobacillus</i> counts of the rats fed diet without <i>L.casei</i> ssp. <i>casei</i> 19 | Faecal <i>Lactobacillus</i> counts of the rats fed diet with <i>L.casei</i> ssp. <i>casei</i> 19 |
|----------------------|---|--|
| Basal                | 8.31 ± 0.41   | 8.62 ± 0.81  |
| Cholesterol-enriched | 6.54 ± 0.26   | 7.04 ± 0.26  |
| Cream-enriched       | 7.05 ± 0.23   | 7.79 ± 0.54  |

The values are mean ± SEM

**Table 4.19** Coliform counts (log cfu/g of faeces) of the rats fed various diets with and without *L. casei* ssp. *casei* 19

| Diet                 | Coliform counts of the rats fed the diet without <i>L.casei</i> ssp. <i>casei</i> 19 | Coliform counts of the rats fed the diet with <i>L.casei</i> ssp. <i>casei</i> 19 |
|----------------------|--|---|
| Basal                | 3.83±1.43 <sup>a</sup>   | 2.90±0.27 <sup>b</sup>  |
| Cholesterol-enriched | 2.95±0.20 <sup>a</sup>   | 3.06±0.24 <sup>a</sup>  |
| Cream-enriched       | 3.32±0.29 <sup>a</sup>   | 2.91±0.26 <sup>b</sup>  |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.20** Faecal *Lactobacillus* and coliform counts (log cfu/g of faeces) of rats fed various (basal, cholesterol- and fat-enriched) diets with and without *L. casei* ssp. *casei* 19

| Diet                 | Faecal <i>Lactobacillus</i> and coliform counts of rats fed diet without <i>L.casei</i> ssp. <i>casei</i> 19 |                         | Faecal <i>Lactobacillus</i> and coliform counts of rats fed diet with <i>L.casei</i> ssp. <i>casei</i> 19 |                        |
|----------------------|--|-------------------------|---|------------------------|
|                      | Lactobacilli   | Coliform                | Lactobacilli  | Coliform               |
| Basal                | 8.31±0.41 <sup>a</sup>   | 3.83±1.43 <sup>a</sup>  | 8.62±0.81 <sup>a</sup>  | 2.90±0.27 <sup>a</sup> |
| Cholesterol-enriched | 6.54±0.26 <sup>b</sup>   | 2.95±0.20 <sup>b</sup>  | 7.04±0.26 <sup>b</sup>  | 3.06±0.24 <sup>a</sup> |
| Cream-enriched       | 7.05±0.23 <sup>b</sup>   | 3.32±0.29 <sup>ab</sup> | 7.79±0.54 <sup>ab</sup>   | 2.91±0.26 <sup>a</sup> |

The values (mean±SEM) in the same coloumn with different superscripts are significantly ( $p<0.05$ ) different by Least significant difference pair wise comparison.

#### **4.3.2.1 Body weight and plasma lipids in basal diet fed rats**

There was no significant difference in body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions in rats before and after the supplementation of the bacterial cells with basal diet (Table 4.21).

#### **4.3.2.2 Body weight and plasma lipids in cholesterol-enriched diet fed rats**

Table 4.22 shows the results for various parameters in experimental rats before and after the supplementation of cholesterol-enriched diet with *L. acidophilus* 14. A non-significant increase in the body weight was observed after feeding of the diet with the organism for 15 days. There was a non-significant decline in the concentrations of total plasma cholesterol, LDL cholesterol and a non-significant increase in HDL cholesterol; atherogenic index also showed a non-significant decline. Notably, there was a significant decline in the concentrations of plasma triacylglycerols (declined from  $1.59 \pm 0.10$  to  $0.87 \pm 0.05$  mmol/l) and VLDL-cholesterol (declined from  $0.31 \pm 0.02$  to  $0.17 \pm 0.01$  mmol/l) after the supplementation of the diet with the *Lactobacillus* strain.

#### **4.3.2.3 Body weight and plasma lipids in cream-enriched diet fed rats**

As shown in Table 4.23, there was a non-significant increased in the body weight and a non-significant decline in atherogenic index and the concentrations of various plasma lipids after *L. acidophilus* 14 supplementation of cream-enriched diet for 15 days.

#### **4.3.2.4 Comparison in body weight and in concentrations of various plasma lipids**

Except body weight, there was no significant difference, in other parameters among the three groups of rats at 0 day (Fig. 4.2).

After 15 days of feeding of the experimental diets without *L. acidophilus* 14, atherogenic index and the concentrations of total plasma cholesterol and LDL cholesterol were significantly higher in cholesterol-enriched diet fed as compared to cream-enriched diet fed group of rats. There was no significant difference in the concentrations of plasma triacylglycerols, HDL cholesterol and VLDL cholesterol between the two groups. All the parameters were significantly lower in basal diet as compared to cholesterol-enriched diet fed group (Fig. 4.2).

After 15 days of the feeding of the experimental diets with the culture, there was a significant decline in the concentration of plasma total cholesterol, triacylglycerols, HDL-cholesterol and LDL cholesterol in cream-enriched diet fed as compared to cholesterol-enriched diet fed group. Except plasma triacylglycerols and VLDL cholesterol concentrations, the values of all the other parameters were higher in cholesterol-enriched diet fed than basal diet fed group (Fig. 4.2).

#### **4.3.2.5 Faecal *Lactobacillus* counts**

There were no significant differences in faecal *Lactobacillus* counts of the rats before and after feeding of *L. acidophilus* 14 with experimental diets. The values are given in Table 4.24.

**Table 4.21** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of the rats fed basal diet with and without *L. acidophilus* 14

|                            | Days after feeding diet without <i>L. acidophilus</i> 14 |             | Days after feeding diet with <i>L. acidophilus</i> 14 |
|----------------------------|--|-------------|---|
|                            | 0 day  | 15 days     | 15 days   |
| Body weight (g)            | 178.25±4.33  | 195.62±4.85 | 200.00±4.34   |
| Total cholesterol (mmol/l) | 1.94±0.10  | 1.96±0.13   | 1.86±0.13   |
| HDL-cholesterol (mmol/l)   | 0.95±0.06  | 0.91±0.06   | 0.87±0.05   |
| LDL-cholesterol (mmol/l)   | 0.56±0.04  | 0.54±0.07   | 0.46±0.05   |
| VLDL-cholesterol (mmol/l)  | 0.18±0.01  | 0.21±0.01   | 0.22±0.01   |
| Triacylglycerols (mmol/l)  | 0.94±0.05  | 1.10±0.05   | 1.14±0.06   |
| Atherogenic index          | 0.59±0.05  | 0.59±0.07   | 0.52±0.03   |

The values are mean±SEM

**Table 4.22** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of the rats fed cholesterol-enriched diet with and without *L. acidophilus* 14

|                            | Days after feeding diet without <i>L. acidophilus</i> 14 |                          | Days after feeding diet with <i>L. acidophilus</i> 14 |
|----------------------------|--|--------------------------|---|
|                            | 0 day  | 15 days                  | 15 days   |
| Body weight (g)            | 189.75±3.44 <sup>a</sup>                                 | 227.62±3.73 <sup>b</sup> | 239.75±3.59 <sup>b</sup>                              |
| Total cholesterol (mmol/l) | 2.05±0.13 <sup>a</sup>                                   | 3.95±0.25 <sup>b</sup>   | 3.66±0.22 <sup>b</sup>                                |
| HDL-cholesterol (mmol/l)   | 0.99±0.07 <sup>a</sup>                                   | 1.12±0.08 <sup>ab</sup>  | 1.30±0.08 <sup>b</sup>                                |
| LDL-cholesterol (mmol/l)   | 0.62±0.07 <sup>a</sup>                                   | 2.11±0.08 <sup>b</sup>   | 1.96±0.14 <sup>b</sup>                                |
| VLDL-cholesterol (mmol/l)  | 0.18±0.00 <sup>a</sup>                                   | 0.31±0.02 <sup>b</sup>   | 0.17±0.01 <sup>a</sup>                                |
| Triacylglycerols (mmol/l)  | 0.95±0.04 <sup>a</sup>                                   | 1.59±0.10 <sup>b</sup>   | 0.87±0.05 <sup>a</sup>                                |
| Atherogenic index          | 0.63±0.08 <sup>a</sup>                                   | 1.80±0.12 <sup>b</sup>   | 1.52±0.10 <sup>b</sup>                                |

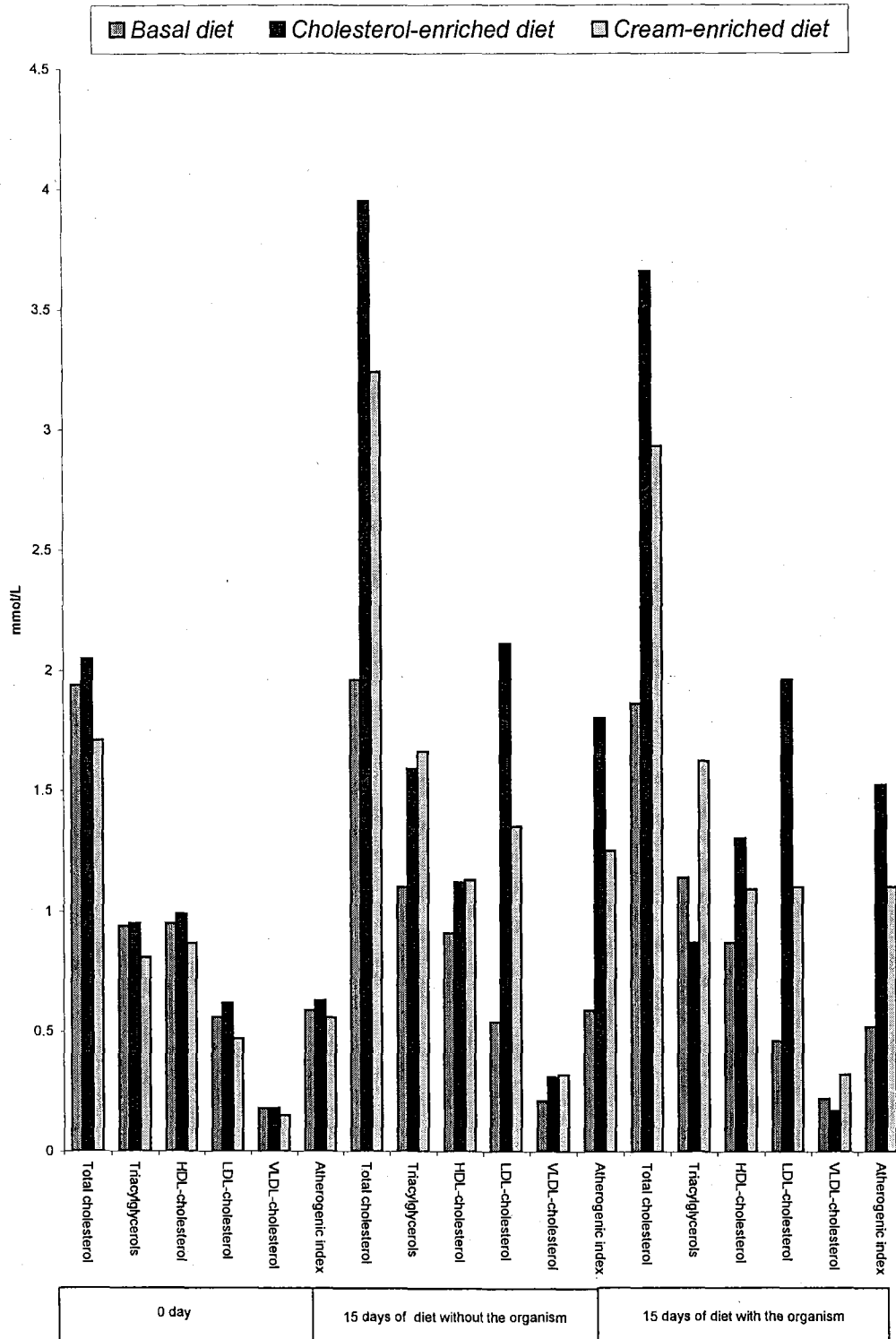
The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.23** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of the rats fed cream-enriched diet with and without *L. acidophilus* 14

|                            | Days after feeding diet without <i>L. acidophilus</i> 14 |                          | Days after feeding diet with <i>L. acidophilus</i> 14 |
|----------------------------|--|--------------------------|---|
|                            | 0 day  | 15 days                  | 15 days   |
| Body weight (g)            | 196.62±3.51 <sup>a</sup>                                 | 243.62±3.96 <sup>b</sup> | 254.50±5.33 <sup>b</sup>                              |
| Total cholesterol (mmol/l) | 1.71±0.10 <sup>a</sup>                                   | 3.24±0.14 <sup>b</sup>   | 2.93±0.14 <sup>b</sup>                                |
| HDL-cholesterol (mmol/l)   | 0.87±0.07 <sup>a</sup>                                   | 1.13±0.10 <sup>b</sup>   | 1.09±0.09 <sup>b</sup>                                |
| LDL-cholesterol (mmol/l)   | 0.47±0.03 <sup>a</sup>                                   | 1.35±0.05 <sup>b</sup>   | 1.10±0.11 <sup>b</sup>                                |
| VLDL-cholesterol (mmol/l)  | 0.15±0.00 <sup>a</sup>                                   | 0.32±0.01 <sup>b</sup>   | 0.32±0.01 <sup>b</sup>                                |
| Triacylglycerols (mmol/l)  | 0.81±0.04 <sup>a</sup>                                   | 1.66±0.06 <sup>b</sup>   | 1.62±0.05 <sup>b</sup>                                |
| Atherogenic index          | 0.56±0.06 <sup>a</sup>                                   | 1.25±0.11 <sup>b</sup>   | 1.10±0.18 <sup>b</sup>                                |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Fig. 4.2: Effect of *L. acidophilus* 14 on concentrations of plasma triacylglycerols and cholesterol fractions of rats fed varioul experimental diets.**



**Plasma triacylglycerols and cholesterol fractions**

#### **4.3.2.6 Faecal coliform counts**

There was a significant decline of about 1.5 log cycles in the coliform counts of the rats after supplementation of *L. acidophilus* 14 with cholesterol-enriched and cream-enriched diets, whereas no significant difference was observed in basal-diet fed group of rats (Table 4.25).

#### **4.3.2.7 Comparison in counts of faecal lactobacilli and coliforms**

After 15 days of feeding of the experimental diets without *L. acidophilus* 14, significant decrease was observed in faecal *Lactobacillus* counts of cholesterol-enriched diet fed group as compared to the rest of the two groups of rats, which showed no significant difference between each other (Table 4.26). After 15 days of the feeding of the experimental diets with the *Lactobacillus* culture cream-enriched diet fed group of rats exhibited significant increase in faecal *Lactobacillus* counts as compared to the other two groups of the rats. In comparison to basal diet fed group, the coliform counts were decreased significantly in cholesterol-enriched diet fed group of rats (Table 4.26).

#### **4.3.2.8 Inference**

From the above results (Table 4.21 to 4.26, Fig. 4.2), *L. acidophilus* 14 seems to exert its effect on lowering the atherogenic index and the concentrations of total plasma cholesterol, LDL and VLDL-cholesterol (Table 4.22 and 4.23) through its action on both dietary as well as endogenously synthesized cholesterol. This effect seems to be slightly more prominent in cholesterol-enriched diet fed group of rats, notably, on plasma triacylglycerols and VLDL cholesterol concentrations. The non-significance of some of the above

parameters in cholesterol-enriched diet fed and cream-enriched diet fed groups could be because of the shorter duration of the supplementation of the *Lactobacillus* strains with the diets. The supplementation of the *Lactobacillus* culture in the diets did not affect faecal *Lactobacillus* counts but the coliform counts were reduced (Table 4.24 and 4.25). This probably implies successful survival of the feed supplemented *Lactobacillus* through or colonization in the gut. It appears that cream-enriched diet helps the feed supplemented culture to survive through or colonize in the gastro-intestinal tract, whereas cholesterol-enriched diet does not (Table 4.26).

As can be gauged from Fig. 4.2, feeding a diet containing 0.5% cholesterol is more hypocholesterolemic than a diet containing 10% cream.

### **4.3.3 Effect of *L. plantarum* 20**

The effect of *L. plantarum* 20 on body weight, concentrations of plasma triacylglycerols and various plasma cholesterol fractions and counts of faecal lactobacilli and coliforms of rats are described in relation to basal, cholesterol-enriched and cream-enriched diets fed to the animals (Table 4.27 to 4.32, Fig. 4.3).

#### **4.3.3.1 Body weight and plasma lipids in basal diet fed rats**

When supplemented with the diet for 15 days, the organism non-significantly reduced the concentration of various plasma lipids in the rats, which also exhibited a non-significant increase in body weight as well (Table 4.27).

**Table 4.24** Faecal *Lactobacillus* counts (log cfu/g of faeces) of the rats fed various diets with and without *L. acidophilus* 14

| Diet                 | Faecal <i>Lactobacillus</i> counts of the rats fed diet without <i>L. acidophilus</i> 14 | Faecal <i>Lactobacillus</i> counts of the rats fed diet with <i>L. acidophilus</i> 14 |
|----------------------|--|---|
| Basal                | 7.45±0.43  | 7.95±0.49   |
| Cholesterol-enriched | 6.26±0.25  | 7.01±0.33   |
| Cream-enriched       | 7.48±0.38  | 8.15±0.43   |

The values are (mean±SEM)

**Table 4.25** Coliform counts (log cfu/g of faeces) of the rats fed various diets with and without *L. acidophilus* 14

| Diet                 | Coliform counts of the rats fed diet without <i>L. acidophilus</i> 14 | Coliform counts of the rats fed diet with <i>L. acidophilus</i> 14 |
|----------------------|---|--|
| Basal                | 4.38±0.33 <sup>a</sup>  | 3.62±0.21 <sup>a</sup>   |
| Cholesterol-enriched | 4.17±0.21 <sup>a</sup>  | 2.86±0.22 <sup>b</sup>   |
| Cream-enriched       | 4.06±0.23 <sup>a</sup>  | 2.52±0.19 <sup>b</sup>   |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.26** Faecal *Lactobacillus* and coliform counts (log cfu/g of faeces) of rats fed various (basal, cholesterol- and fat-enriched) diets with and without *L. acidophilus* 14

| Diet                 | Faecal <i>Lactobacillus</i> and coliform counts of rats fed diet without <i>L. acidophilus</i> 14 |                        | Faecal <i>Lactobacillus</i> and coliform counts of rats fed diet with <i>L. acidophilus</i> 14 |                         |
|----------------------|---|------------------------|--|-------------------------|
|                      | Lactobacilli  | Coliform               | Lactobacilli   | Coliform                |
| Basal                | 7.45±0.43 <sup>a</sup>  | 4.38±0.33 <sup>a</sup> | 7.95±0.43 <sup>a</sup>   | 3.62±0.21 <sup>a</sup>  |
| Cholesterol-enriched | 6.26±0.25 <sup>b</sup>  | 4.17±0.21 <sup>a</sup> | 7.01±0.33 <sup>a</sup>   | 2.86±0.22 <sup>ab</sup> |
| Cream-enriched       | 7.48±0.38 <sup>a</sup>  | 4.06±0.23 <sup>a</sup> | 8.15±0.49 <sup>b</sup>   | 2.52±0.19 <sup>b</sup>  |

The values (mean±SEM) in the same column with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison.

**Table 4.27** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of rats fed basal diet with and without *L. plantarum* 20

|                            | Days after feeding diet without <i>L. plantarum</i> 20 |                          | Days after feeding diet with the <i>L. plantarum</i> 20 |
|----------------------------|--|--------------------------|---|
|                            | 0 day  | 15 days                  | 15 days   |
| Body weight (g)            | 201.15±3.62 <sup>a</sup>                               | 212.37±4.01 <sup>b</sup> | 226.75±5.42 <sup>b</sup>                                |
| Total cholesterol (mmol/l) | 1.91±0.06 <sup>a</sup>                                 | 1.86±0.11 <sup>a</sup>   | 1.64±0.08 <sup>a</sup>                                  |
| HDL-cholesterol (mmol/l)   | 0.88±0.04 <sup>a</sup>                                 | 0.89±0.03 <sup>a</sup>   | 0.92±0.05 <sup>a</sup>                                  |
| LDL-cholesterol (mmol/l)   | 0.57±0.06 <sup>a</sup>                                 | 0.60±0.06 <sup>a</sup>   | 0.51±0.09 <sup>a</sup>                                  |
| VLDL-cholesterol (mmol/l)  | 0.19±0.01 <sup>a</sup>                                 | 0.16±0.01 <sup>a</sup>   | 0.10±0.01 <sup>a</sup>                                  |
| Triacylglycerols (mmol/l)  | 0.99±0.05 <sup>a</sup>                                 | 0.83±0.05 <sup>a</sup>   | 0.55±0.04 <sup>b</sup>                                  |
| Atherogenic index          | 0.66±0.08 <sup>a</sup>                                 | 0.66±0.06 <sup>a</sup>   | 0.57±0.12 <sup>a</sup>                                  |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p<0.05$ ) different by Least significant difference pair wise comparison

#### **4.3.3.2 Body weight and plasma lipids in cholesterol-enriched diet fed rats**

Table 4.28 shows the results for various parameters in rats before and after the supplementation of cholesterol-enriched diet with *L. plantarum* 20. After supplementation of cholesterol-enriched diet with the organism, there was a significant decline from  $3.66 \pm 0.16$  to  $2.38 \pm 0.13$ ,  $1.92 \pm 0.05$  to  $0.87 \pm 0.14$ ,  $0.29 \pm 0.01$  to  $0.25 \pm 0.01$  and  $1.48 \pm 0.07$  to  $1.27 \pm 0.05$  mmol/l in the concentrations of total plasma cholesterol, LDL and VLDL cholesterol and plasma triacylglycerols, respectively. The supplementation of the organism with the diet also exhibited significant fall from  $1.85 \pm 0.18$  to  $1.00 \pm 0.18$  in atherogenic index in rats.

#### **4.3.3.3 Body weight and plasma lipids in cream-enriched diet fed rats**

After *L. plantarum* 20 supplementation of cream-enriched diet for 15 days, there was a non-significant increase in body weight and a significant decline from  $3.10 \pm 0.13$  to  $2.22 \pm 0.11$ ,  $1.32 \pm 0.16$  to  $0.80 \pm 0.13$ ,  $0.30 \pm 0.01$  to  $0.21 \pm 0.01$  and  $1.65 \pm 0.06$  to  $1.07 \pm 0.06$  mmol/l in the concentrations of total plasma cholesterol, LDL and VLDL-cholesterol and plasma triacylglycerols, respectively. Atherogenic index was also reduced to as low as can be compared to that of basal diet fed group or rats (Table 4.29).

#### **4.3.3.4 Comparison in body weight and in concentration of various plasma lipids**

There was no significant difference in any parameter among the three groups of rats at 0 day (Fig. 4.3).

After 15 days of feeding of the experimental diets without *L. plantarum* 20, atherogenic index and the concentration of total plasma cholesterol and LDL cholesterol were higher in cholesterol-enriched diet fed as compared to cream-enriched diet fed group of rats. All the parameters were significantly lower in basal diet as compared to cholesterol enriched diet fed group (Fig. 4.3).

After 15 days of feeding of the experimental diets with the culture, all the parameters studied, were not significantly different between cholesterol-enriched diet and cream-enriched diet fed group of rats (Fig. 4.3).

#### **4.3.3.5 Faecal *Lactobacillus* counts**

After supplementation of the diets with *L. plantarum* 20, there was a significant increase of about 1.00 log cycle in faecal *Lactobacillus* counts of rats fed cholesterol-enriched and cream-enriched diets and a non-significant increase in basal diet fed groups of rats (Table 4.30).

#### **4.3.3.6 Faecal coliform counts**

After supplementation of the various experimental diets with the organism, there was a significant decrease of about 1.00 to 1.50 log cycles in coliform counts of all the three groups of rats (Table 4.31).

#### **4.3.3.7 Comparison in counts of faecal lactobacilli and coliforms**

After 15 days of the feeding of the experimental diets without *L. plantarum* 20, a significant decrease was observed in faecal *Lactobacillus* counts of cholesterol-enrich diet fed as compared to other two groups, which showed no significant difference between each other. There was no significant

**Table 4.28** Body weight and concentration of plasma triacylglycerols and various plasma cholesterol fractions of rats fed cholesterol-enriched diet with and without *L. plantarum* 20

|                            | Days after feeding diet without <i>L. plantarum</i> 20 |                          | Days after feeding diet with <i>L. plantarum</i> 20 |
|----------------------------|--|--------------------------|---|
|                            | 0 day  | 15 days                  | 15 days   |
| Body weight (g)            | 194.75±5.65 <sup>a</sup>                               | 220.62±6.71 <sup>b</sup> | 233.31±6.19 <sup>b</sup>                            |
| Total cholesterol (mmol/l) | 1.77±0.09 <sup>a</sup>                                 | 3.66±0.16 <sup>b</sup>   | 2.38±0.13 <sup>c</sup>                              |
| HDL-cholesterol (mmol/l)   | 0.86±0.04 <sup>a</sup>                                 | 1.05±0.06 <sup>b</sup>   | 0.92±0.06 <sup>a</sup>                              |
| LDL-cholesterol (mmol/l)   | 0.50±0.08 <sup>a</sup>                                 | 1.92±0.05 <sup>b</sup>   | 0.87±0.14 <sup>a</sup>                              |
| VLDL-cholesterol (mmol/l)  | 0.17±0.01 <sup>a</sup>                                 | 0.29±0.01 <sup>b</sup>   | 0.25±0.01 <sup>b</sup>                              |
| Triacylglycerols (mmol/l)  | 0.89±0.05 <sup>a</sup>                                 | 1.48±0.07 <sup>b</sup>   | 1.27±0.05 <sup>b</sup>                              |
| Atherogenic index          | 0.59±0.11 <sup>a</sup>                                 | 1.85±0.18 <sup>b</sup>   | 1.00±0.18 <sup>a</sup>                              |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.29** Body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions of rats fed cream-enriched diet with and without *L. plantarum* 20

|                            | Days after feeding diet without <i>L. plantarum</i> 20 |                          | Days after feeding diet with <i>L. plantarum</i> 20 |
|----------------------------|--|--------------------------|---|
|                            | 0 day  | 15 days                  | 15 days   |
| Body weight (g)            | 210.87±5.59 <sup>a</sup>                               | 239.50±5.81 <sup>b</sup> | 242.25±5.96 <sup>b</sup>                            |
| Total cholesterol (mmol/l) | 1.85±0.07 <sup>a</sup>                                 | 3.10±0.13 <sup>b</sup>   | 2.21±0.11 <sup>c</sup>                              |
| HDL-cholesterol (mmol/l)   | 0.81±0.03 <sup>a</sup>                                 | 1.02±0.04 <sup>b</sup>   | 0.92±0.05 <sup>b</sup>                              |
| LDL-cholesterol (mmol/l)   | 0.56±0.08 <sup>a</sup>                                 | 1.32±0.16 <sup>b</sup>   | 0.80±0.13 <sup>a</sup>                              |
| VLDL-cholesterol (mmol/l)  | 0.36±0.01 <sup>a</sup>                                 | 0.32±0.01 <sup>a</sup>   | 0.21±0.01 <sup>b</sup>                              |
| Triacylglycerols (mmol/l)  | 1.05±0.05 <sup>a</sup>                                 | 1.65±0.06 <sup>b</sup>   | 1.07±0.06 <sup>a</sup>                              |
| Atherogenic index          | 0.69±0.09 <sup>a</sup>                                 | 1.31±0.18 <sup>b</sup>   | 0.89±0.16 <sup>a</sup>                              |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.30** *Lactobacillus* counts (log cfu/g of faeces) of the rats fed various diets with and without *L. plantarum* 20

| Diet                 | Faecal <i>Lactobacillus</i> counts of rats fed diet without <i>L. plantarum</i> 20 | Faecal <i>Lactobacillus</i> counts of rat fed diet with <i>L. plantarum</i> 20 |
|----------------------|--|--|
| Basal                | 7.98±0.24 <sup>a</sup>   | 8.60±0.14 <sup>a</sup>   |
| Cholesterol-enriched | 6.78±0.26 <sup>a</sup>   | 7.99±0.40 <sup>b</sup>   |
| Cream-enriched       | 7.03±0.27 <sup>a</sup>   | 8.07±0.34 <sup>b</sup>   |

The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.31** Coliform counts (log cfu/g of faeces) of the rats fed various diets with and without *L. plantarum* 20

| Diet                 | Coliform counts of rats fed diet without <i>L. plantarum</i> 20 | Coliform counts of rats fed diet with <i>L. plantarum</i> 20 |
|----------------------|---|--|
| Basal                | 4.24±0.33 <sup>a</sup>  | 3.16±0.24 <sup>b</sup>                                       |
| Cholesterol-enriched | 3.74±0.31 <sup>a</sup>  | 2.80±0.26 <sup>b</sup>                                       |
| Cream-enriched       | 3.98±0.32 <sup>a</sup>  | 2.53±0.26 <sup>b</sup>                                       |

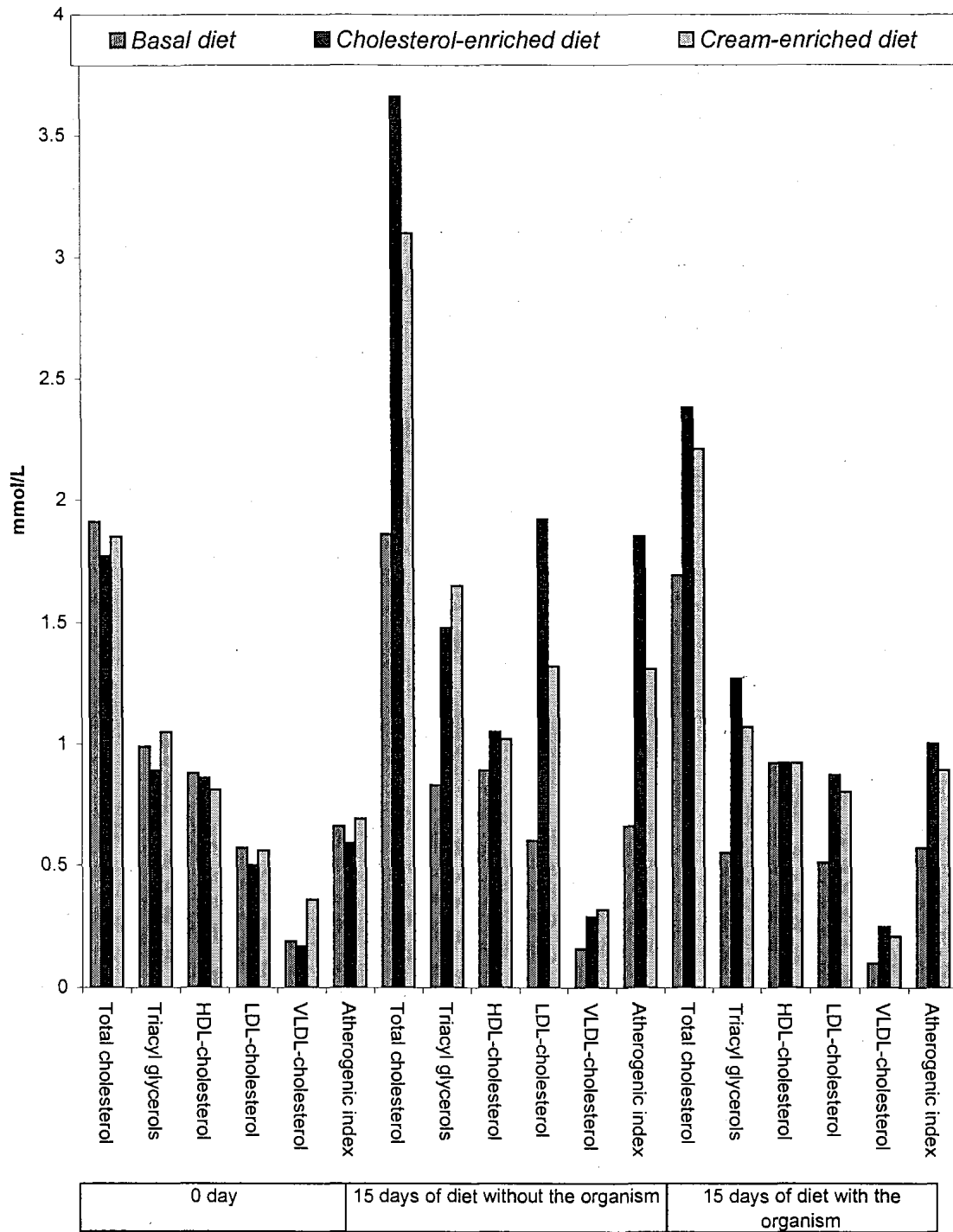
The values (mean±SEM) in the same row with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison

**Table 4.32** Faecal *Lactobacillus* and coliform counts (log cfu/g of faeces) of rats fed various (basal, cholesterol- and fat-enriched) diets with and without *L. plantarum* 20

| Diet                 | Faecal <i>Lactobacillus</i> and coliform counts of rats fed diet without <i>L. plantarum</i> 20 |                        | Faecal <i>Lactobacillus</i> and coliform counts of rats fed diet with <i>L. plantarum</i> 20 |                        |
|----------------------|---|------------------------|--|------------------------|
|                      | Mean  | SEM                    | Mean   | SEM                    |
| Basal                | 7.98±0.24 <sup>a</sup>  | 4.24±0.33 <sup>a</sup> | 8.60±0.14 <sup>a</sup>   | 3.16±0.24 <sup>a</sup> |
| Cholesterol-enriched | 6.78±0.26 <sup>b</sup>  | 3.74±0.31 <sup>a</sup> | 7.99±0.40 <sup>a</sup>   | 2.80±0.26 <sup>a</sup> |
| Cream-enriched       | 7.03±0.27 <sup>a</sup>  | 3.98±0.32 <sup>a</sup> | 8.07±0.34 <sup>a</sup>   | 2.53±0.20 <sup>a</sup> |

The values (mean±SEM) in the same column with different superscripts are significantly ( $p < 0.05$ ) different by Least significant difference pair wise comparison.

**Fig. 4.3: Effect of *L. plantarum* 20 on concentrations of plasma triacylglycerols and various cholesterol fractions of rats fed various experimental diets**



**Plasma triacylglycerols and cholesterol fractions**

difference among the three groups in coliform counts (Table 4.32).

After 15 days of feeding of the experimental diets with the *Lactobacillus* culture, there was no significant difference in either faecal *Lactobacillus* or coliform counts among the three groups of rats (Table 4.32).

#### **4.3.3.8 Inference**

From the above results (Table 4.27 to 4.32, Fig. 4.3), it appears that *L. plantarum* 20 exerts its hypocholesterolemic effect (Table 4.27, 4.28 and 4.29) through its equal action on both dietary as well as endogenously synthesized cholesterol. An increase in faecal *Lactobacillus* and a decrease in coliform counts of the rats in all the groups after the dietary supplementation of *L. plantarum* 20 probably implies a successful survival through or colonization in the gut by the feed supplemented culture.

#### **4.3.4 Hypocholesterolemic effect of the three selected strains**

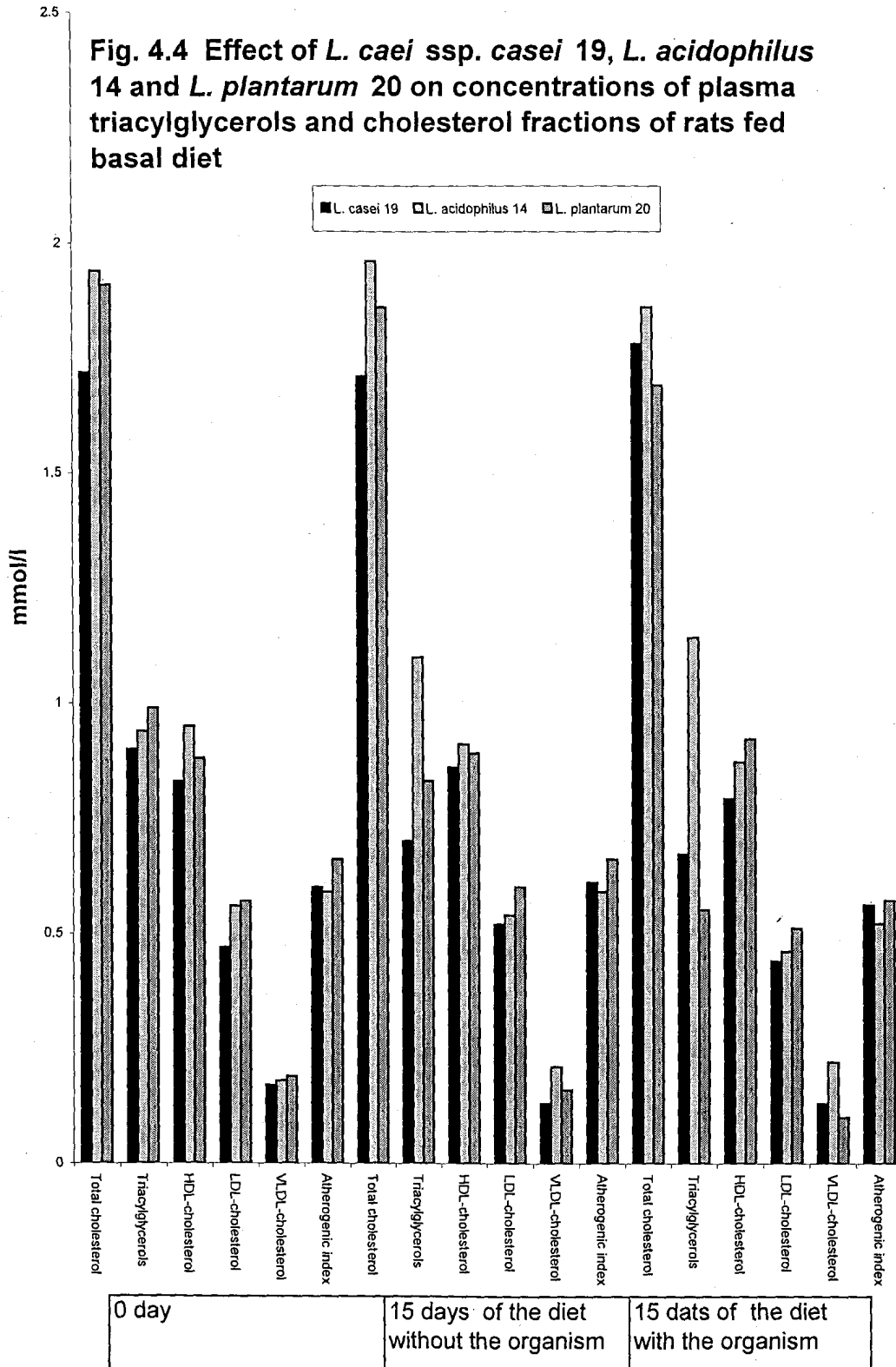
The effect of feeding of the three strains (Fig. 4.4 to 4.6), *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20, for 15 days on the concentration of various plasma lipids of rats fed basal, cholesterol-enriched and cream-enriched diets reveals that among the three strains, *L. plantarum* 20 is the best hypocholesterolemic one. In comparison to the rest of the two strains, the dietary supplementation of *L. plantarum* 20 has exhibited the lowest concentrations of total plasma cholesterol, plasma triacylglycerols and VLDL-cholesterol in basal diet fed rats (Fig. 4.4). In cholesterol-enriched diet fed rats, and the strain has caused significant lowering of atherogenic index and the concentrations of total plasma and

LDL cholesterol (Fig. 4.5). The dietary supplementation of the organism has also resulted in lowering of total plasma and LDL cholesterol concentration and atherogenic index in cream-enriched diet fed rats, and the values are lowest among the three strains (Fig. 4.6).

Similar hypocholesterolemic effects, as described in previous section for the three *Lactobacillus* strains, were also observed in different lactic acid bacteria by other workers (Rao *et al*, 1981; Grunewald, 1982; Chawla and Kansal, 1984; Hashimoto *et al*, 1999; Usman and Hosono, 2000)

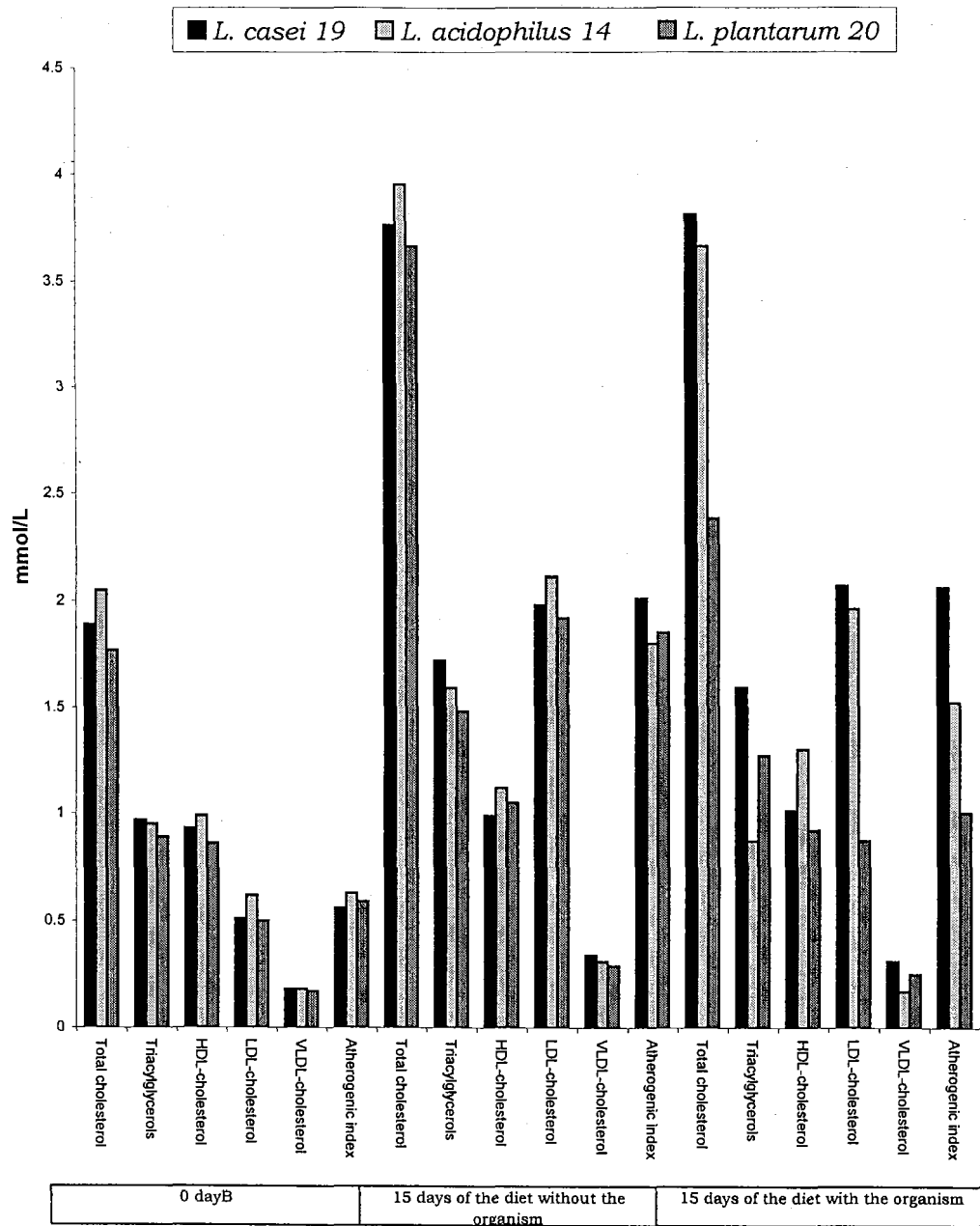
Rao *et al* (1981) reported significant ( $p < 0.05$ ) decline in plasma cholesterol of rats fed *Thermophilus* milk. Grunewald (1982) also reported lower serum cholesterol levels in rats fed fermented milk (fermented by *L. acidophilus*) than in rats fed milk or diet alone, and similar results of hypocholesterolemic effect of various cultured milks was also observed by Chawla and Kansal (1984) with milk fermented by *L. acidophilus*, by Zommará *et al* (1996) with whey obtained from cultured milk products of *Streptococcus thermophilus*, by Taranto *et al* (2000) with milk fermented by *L. reuteri*. Fukushima and Nakano (1995) reported that serum total cholesterol concentration of the rats fed on the mixture of organisms (*Bacillus*, *Lactobacillus*, *Streptococcus*, *Clostridium*, *Saccharomyces* and *Candida*) supplemented with fat and cholesterol-enriched diet was reduced compared with the groups not fed with the mixture of the organisms. Similarly Hashimoto *et al* (1999) found that increase of serum cholesterol was suppressed in rats fed *L. casei ssp. casei* (strain TMC 0409) supplemented with cholesterol-enriched diet. Kikuchi-Hayakawa (2000) observed 30% reduction in the concentration of plasma triacylglycerols in hamsters fed

**Fig. 4.4 Effect of *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 on concentrations of plasma triacylglycerols and cholesterol fractions of rats fed basal diet**



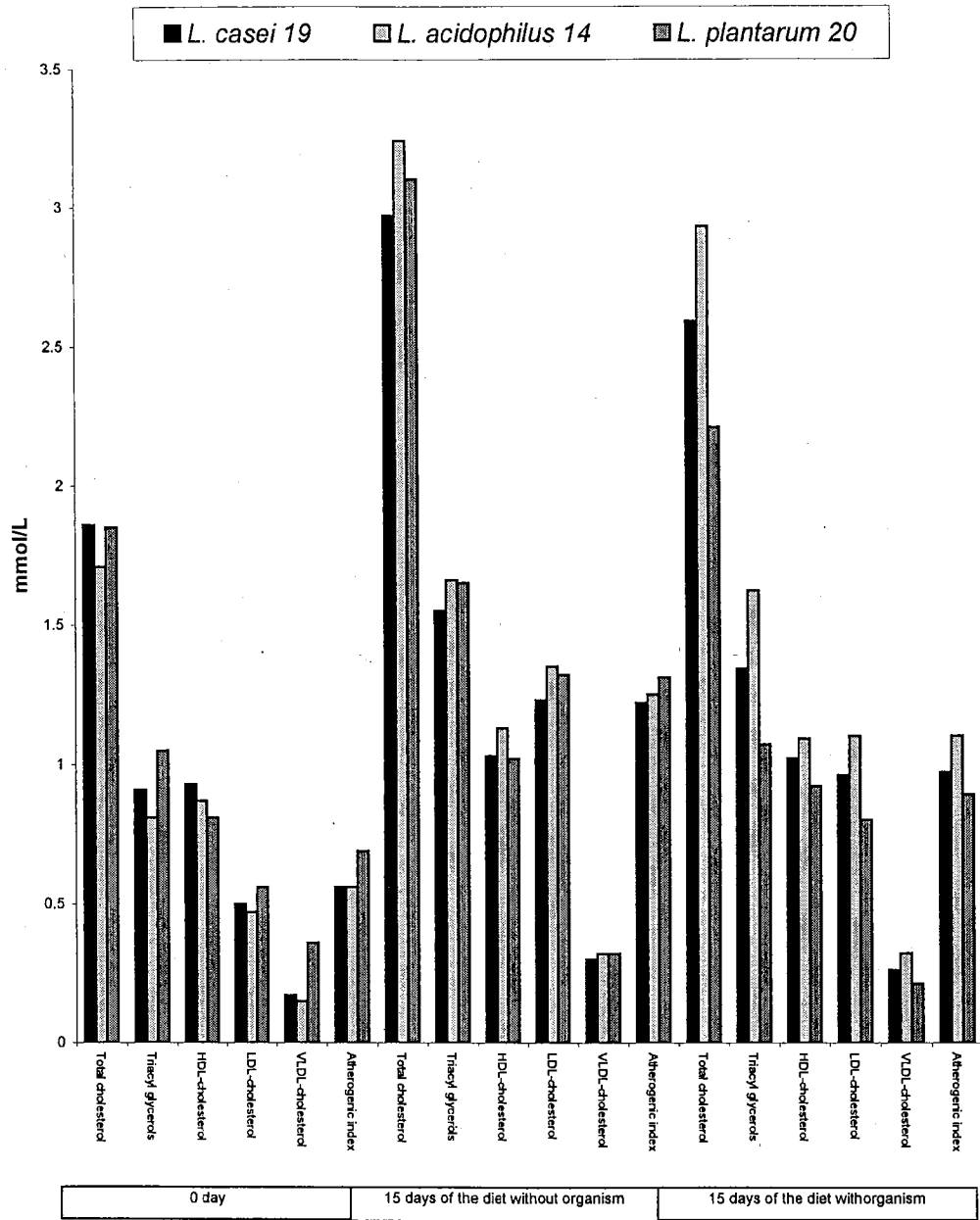
**Plasma triacylglycerols and cholesterol fractions**

**Fig. 4.5 Effect of *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 on concentrations of plasma triacylglycerols and cholesterol fractions of rats fed cholesterol-enriched diet**



**Plasma triacylglycerols and cholesterol fractions**

**Fig. 4.6: Effect of *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 on concentrations of plasma triacylglycerols and cholesterol fractions of rats fed cream-enriched diet**



**Plasma triacylglycerol and cholesterol fractions**

cholesterol-enriched diet supplemented with skim milk fermented by *L. casei* strain Shirota. Usman and Hosono (2000) found hypocholesterolemic effect in rats fed cholesterol-enriched diet supplemented with *L. gasseri*.

## 5. SUMMARY AND CONCLUSIONS

One key priority in dairying is to increase the development of dairy foods that promote health and well being. Dairy foods fit naturally with probiotics, which can offer a broad range of potential health benefits in preventing or reducing the risks of atherosclerotic cardiovascular diseases.

Because of cardiovascular diseases being one of the major causes of death in the world, and hypercholesterolemia being considered as a major risk factor for the disease, the present investigation was carried out to check hypocholesterolemic effect of fifty strains of lactobacilli. The most hypocholesterolemic strains can be used as dietary adjuncts to reduce the risk of the disease in population as a whole, which is more economical than any of the approaches adopted so far.

### 5.1 SUMMARY

The work was carried out in three phases (bold headings below) and the results are summarized as below:

- **Screening of different *Lactobacillus* cultures**

If a bacterial culture is able to remove cholesterol from or deconjugate bile acids in an artificial growth medium, it may be able to lower plasma cholesterol when fed to animals, provided it successfully colonizes it. Thus, a total of fifty strains of lactobacilli the cultures were screened for both gut colonizing and cholesterol lowering potential:

1. *L. acidophilus* 14, *L. casei ssp. casei* 19 and *L. plantarum* 20 are the highest acid tolerant ones with coefficients of inhibition of 0.63 to 0.66. This is

followed by *L. delbrueckii* ssp. *bulgaricus* 9, *L. fermentum* 141 and *L. fermentum* 156 of lower acid tolerance with coefficients of inhibition of 0.74 to 0.75. The organisms with the coefficients of more than 0.82 are the least acid tolerant ones.

2. *Lactobacillus. casei* ssp. *casei* 19 exhibited the highest bile tolerance and the lower coefficient of inhibition of growth of 0.41. *L. acidophilus* 14, *L. casei* ssp. *casei* 63, *L. casei* L9, *L. plantarum* 20, *L. acidophilus* L3, *L. fermentum* 155 and *Lactobacillus* spp. L13 have the coefficients of inhibition of growth ranging between 0.63 to 0.68. The rest were the least bile tolerant organisms with coefficient of inhibition of more than 0.70.
3. *L. acidophilus* 14 showed the highest cell surface hydrophobicity of 0.84 followed by *L. casei* ssp. *casei* 19 and *L. plantarum* 20 with values of cell surface hydrophobicity of 0.74 and 0.75, respectively. *L. acidophilus* 15, *L. delbrueckii* ssp. *bulgaricus* 4 and *L. fermentum* 155 possessed cell surface hydrophobicity of 0.46 to 0.52, respectively.
4. *L. acidophilus* 14 had the highest cholesterol removing capability followed by *L. plantarum* 20 and *L. casei* ssp. *casei* 19 with coefficients of cholesterol removal of 0.66, 0.59 and 0.52, respectively. The rest of the strains were the least cholesterol removers from the growth medium.
5. *L. plantarum* 20 showed the highest taurocholate deconjugating capability followed by *L. casei* ssp.

*casei* 19 and *L. acidophilus* 14 with coefficients of 0.56, 0.48 and 0.35, respectively.

6. Among the fifty strains tested, *L. acidophilus* 14, followed by *L. casei* ssp. *casei* 19 and then, *L. plantarum* 20 were the organisms with maximum probiotic potential in so far as various probiotic properties, (viz, acid and bile tolerance, cell surface hydrophobicity, cholesterol removal from and taurocholate deconjugation from growth medium), tested *in vitro*, were concerned.
7. Though all the three cultures survived the solutions of pH 2.0 and 3.0, there was a significant ( $p < 0.05$ ) decline of three log cycles in comparison to control at these pH levels after three hours of incubation.
8. Although all the three cultures survived the bile solutions of concentrations of 2 and 4%, there was a significant decline at 4% after 12 hours of incubation in *L. casei* ssp. *casei* 19. Similarly, *L. plantarum* 20 showed a significant decline of about 2 log cycles at bile concentrations of 2%.
9. When tested by Agar spot assay, all the three organisms showed antimicrobial activity against indicator organisms (*Bacillus cereus* NCDC 66, *Staphylococcus aureus* NCDC 109, *Enterococcus faecalis*, *Leuconostoc mesenteroides* LY and *Escherichia coli* V517), but the diameter of the zones of inhibition varied against the indicators.

- **Protoplast fusion of the selected *Lactobacillus* strains and the screening of the fusants**

10. The selected strains, *L. casei* ssp. *Casei* 19, *L. acidophilus* 14 and *L. plantarum* 20, with maximum values of different probiotic properties were subjected to protoplast fusion and the resultant fusants were screened for probiotic properties. The fusants showed no significant improvement over the parents in so far as above properties were concerned. Thus, parents were used in the animal experiment, in the third phase of the research, to test *in vivo* hypocholesterolemic effect of the strains.

- **Effect of feeding of the selected cultures on various parameters estimated in rats**

11. *L. casei* ssp. *Casei* 19, *L. acidophilus* 14 and *L. plantarum* 20, with maximum gut colonizing and cholesterol lowering potential, were fed to rats with various experimental diets to test *in vivo* hypocholesterolemic effect of the cultures.

12. A non-significant increase in body weight and a decline in atherogenic index and concentrations of various plasma lipids of the rats was observed after the feeding of basal diet with *L. casei* ssp. *Casei* 19 for a period of 15 days.

13. No significant change was observed in body weight and concentrations of plasma triacylglycerols and various plasma cholesterol fractions, viz, LDL-, VLDL- and HDL- cholesterol, of rats before and after

the supplementation of cholesterol-enriched diet with *L. casei* ssp. *casei* 19.

- 14.** There was a non-significant increase in body weight and a non-significant decline in concentrations of various plasma lipids (plasma triacylglycerols and various plasma cholesterol fractions) after supplementation of cream-enriched diet with *L. casei* ssp. *casei* 19 for 15 days, and there was also a significant decline from  $1.22 \pm 0.08$  to  $0.97 \pm 0.10$  in atherogenic index.
- 15.** After 15 days of the feeding of experimental diets with *L. casei* ssp. *casei* 19, HDL cholesterol concentrations were significantly increased in cholesterol-enriched diet and cream-enriched diet fed groups as compared to basal diet fed group. Atherogenic index in cream-enriched diet fed group was reduced significantly and was comparable to that of the basal diet fed group. In comparison to cholesterol-enriched diet fed group, all the variables were significantly lower in basal diet fed group.
- 16.** No significant difference was observed in faecal *Lactobacillus* counts of rats before and after feeding of *L. casei* ssp. *casei* 19 with various experimental diets.
- 17.** There was a significant decline of about 0.50 to 1.00 log cycles in coliform counts of rats before and after supplementation of *L. casei* ssp. *casei* 19 with basal and cream-enriched diets, whereas no significant difference was observed before and after feeding of

the culture in cholesterol enriched-diet fed group of rats.

18. After 15 days of the feeding of experimental diets *L. casei* ssp. *casei* 19, faecal *Lactobacillus* counts showed an overall increase in all the groups, but cholesterol-enriched diet fed group still showed the lowest counts, which were not significantly different from cream enriched diet fed group. Coliform counts exhibited a non-significant decline in basal and cream-enriched diet fed groups but were increased non-significantly in cholesterol-enriched diet fed group.
19. No significant difference was observed in body weight and concentrations of various plasma lipids estimated in rats before and after the supplementation of *L. acidophilus* 14 with basal diet.
20. There was a non-significant increase in body weight after feeding of cholesterol-enriched diet with *L. acidophilus* 14 for 15 days and a decline in the concentrations of total plasma and LDL cholesterol. Atherogenic index also showed a non-significant decline in rats fed the diet with the organism. Notably, there was a significant decline in the concentrations of plasma triacylglycerols (declined from  $1.59 \pm 0.10$  to  $0.87 \pm 0.05$  mmol/l) and VLDL cholesterol (declined from  $0.31 \pm 0.02$  to  $0.17 \pm 0.01$  mmol/l).
21. A non-significant increase in the body weight, and decline in atherogenic index and the concentrations of various plasma lipids was observed after *L.*

*acidophilus* 14 supplementation of cream-enriched diet for 15 days.

- 22.** After 15 days of the feeding of the experimental diets with *L. acidophilus* 14, there was a significant decline in the concentrations of total plasma and LDL cholesterol in the rats; atherogenic index also showed a non-significant decline. Notably, there was a significant decline in the concentrations of plasma triacylglycerols and VLDL cholesterol.
- 23.** No significant difference was observed in faecal *Lactobacillus* counts of the rats before and after the feeding of *L. acidophilus* 14 with various experimental diets.
- 24.** There was a significant decline of about 1.00 log cycle in coliform counts of the rats after supplementation of *L. acidophilus* 14 strain with cholesterol-enriched and cream-enriched diets, whereas no difference was observed in basal diet fed group of rats.
- 25.** After 15 days of the feeding of the experimental diets with *L. acidophilus* 14, cream-enriched diet fed group of rats exhibited significant increase in faecal *Lactobacillus* counts as compared to the other two groups of the rats. In comparison to basal diet fed group, coliform counts were decreased significantly in cholesterol-enriched diet fed group of rats.
- 26.** When supplemented with the diet for 15 days, *L. plantarum* 20 non-significantly reduced the concentrations of various plasma lipids in the rats,

which also exhibited a non-significant increase in body weight as well.

**27.** After supplementation of cholesterol-enriched diet with the organism, there was a significant decline from  $3.66 \pm 0.16$  to  $2.38 \pm 0.13$ ,  $1.92 \pm 0.05$  to  $0.87 \pm 0.14$ ,  $0.29 \pm 0.01$  to  $0.25 \pm 0.01$  and  $1.48 \pm 0.07$  to  $1.27 \pm 0.05$  mmol/l in the concentrations of total plasma cholesterol, LDL and VLDL cholesterol and plasma triacylglycerols, respectively. The supplementation of the organism with the diet also exhibited significant fall from  $1.85 \pm 0.18$  to  $1.00 \pm 0.18$  in atherogenic index in rats, which were non-significantly increased in body weight as well.

**28.** After *L. plantarum* 20 supplementation of cream-enriched diet for 15 days, a non-significant increase in body weight and a significant decline from  $3.10 \pm 0.13$  to  $2.22 \pm 0.11$ ,  $1.32 \pm 0.16$  to  $0.80 \pm 0.13$ ,  $0.30 \pm 0.01$  to  $0.21 \pm 0.01$  and  $1.65 \pm 0.06$  to  $1.07 \pm 0.06$  mmol/l in the concentrations of total plasma cholesterol, LDL and VLDL-cholesterol and plasma triacylglycerols, respectively. Atherogenic index was also reduced to as low as can be comparable to that of basal diet fed group of rats.

**29.** Supplementation of experimental diets with *L. plantarum* 20, caused a significant increase of about 1.00 log cycle in faecal *Lactobacillus* counts of rats fed cholesterol-enriched and cream-enriched diets where as a non-significant increase was observed in basal diet fed group of rats.

30. After supplementation of the various experimental diets with *L. plantarum* 20, there was a significant decrease of about 1.00 log cycle in coliform counts of all the groups of rats.

## 5.2 CONCLUSIONS

From the present investigation, carried out to study the cholesterol lowering effect of the selected lactobacilli, following conclusions can be drawn:

1. To assess the gut colonizing and cholesterol lowering potential of various *Lactobacillus* cultures, tests to check: tolerance to acid and bile, percent cell surface hydrophobicity, assimilation of cholesterol and deconjugation of bile salts, are important considerations.
2. Among the fifty strains of lactobacilli tested, *L. casei* ssp. *casei* 19, *L. acidophilus* 14 and *L. plantarum* 20 have the maximum probiotic potential.
3. Protoplast fusion, between the strains with maximum probiotic potential, does not seem to improve probiotic properties of fusants over parents.
4. Cholesterol-enriched diet appears to affect colonization of the cultures supplemented with diet, as indicated by altered counts of faecal lactobacilli and coliforms.
5. Compared to cholesterol-enriched diets, cream-enriched diets favour survival of lactobacilli.
6. Body weight does not seem to reflect any effect on the concentrations of various plasma cholesterol fractions of rats and vice-versa.

## 6. RECOMMENDATIONS

As lactobacilli are Generally Recognized as Safe (GRAS), *L. plantarum* 20 can be tested on humans for its plasma cholesterol lowering effect. Milk and its products can be used as vehicles for the delivery of the organism.

Search for hypocholesterolemic strains can be extended to other lactobacilli and lactic acid bacteria in general. Such strains could prove very economical in controlling cardiovascular diseases and most of gastro-intestinal disorders (if the strains are good gut colonizers) on population basis.

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