EFFECT OF LACTOSE HYDROLYSED CONDENSED WHEY AND *Bifidobacterium bifidum* IN YOGURT

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "Effect of Lactose Hydrolysed Condensed Whey and ***Bifidobacterium bifidum*** in yogurt" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled "Effect of Lactose Hydrolysed Condensed Whey and *Bifidobacterium bifidum* in yogurt" is a record of research work done independently by Smt. Beena, A.K., under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Introduction

INTRODUCTION

Fermented milk products which originated in Middle East before the Phoenician era have now become popular throughout the world. Fermented milk products now constitute an important component of diet in countries of Europe, Asia and Africa.

Belief in the beneficial aspects of yogurt for human health have existed in many civilizations for a long time. Yogurt is a cultured milk transformed into a smooth custard like consistency by controlled fermentation, using selected viable cultures of Streptococcus salivarius ssp thermophilus (S. salivarius ssp thermophilus) and Lactobacillus delbrueckii ssp bulgaricus (L. delbrueckii ssp bulgaricus). At the peak of its palatable perfection, acid production and microbial growth are arrested by refrigeration so that final product has rods and cocci in the ratio 1:1 and a viable count of 10^8 colony forming units per millilitre (cfu/ml).

Work of Metchnikoff (1910) in Paris on the beneficial effects of some lactobacilli represent a milestone in the search of truth of this topic. Yogurt has now been raised to a status that holds reputation as a highly nutritious food with therapeutic benefits.

Lactose intolerance is a condition resulting from deficiency of intestinal enzyme lactase. When people consume

lactose in excess of hydrolytic capacity of intestinal lactase, a portion of undigested lactose pass from small intestine to colon, increasing the osmolarity of intestinal fluid. As the osmotic equilibrium is disturbed, water is drawn from tissues into the intestine leading to diarrhoea. Symptoms vary from bloat, flatulence, abdominal pain, borborygmus, loss of appetite, nausea, vomiting and heartburn to headache. Thus lactose intolerant people are forced to withdraw milk, which is an excellent source of many nutrients such as calcium, phosphorus, riboflavin, vitamin B_{12} and animal protein, from their diet.

Several studies have suggested that yogurt and other fermented milk products are better tolerated than milk by lactose intolerant individuals. This increased tolerance is thought to be due to either a low lactose content or *in vivo* autodigestion of lactose by microbial β-galactosidase enzyme.

Fortification of yogurt mix with skim milk powder to increase total solids is an essential step in yogurt manufacture so as to get a product with desirable body and texture. This step also enhances the nutritive value of the product. Attempts are being made to use whey solids for the mix fortification instead of skim milk powder, with an aim to utilize one of the important by-product from dairy industry and also to reduce cost of production.

Whey is the liquid remaining after recovery of curd during cheese manufacture. This contains more than half the solids present in original milk including twenty per cent of the protein, most of lactose, vitamins and minerals.

Whey has a Biological oxygen demand (BOD) of 3500-4500 mg/litre. Hundred litres of whey is said to have a polluting strength equivalent to sewage produced by forty five people. The increasing pressure of antipollution regulations and cost of disposal have made traditional method of whey disposal impractical. The geometric increase in world population and arithmetic increase in food production have created a problem of protein caloric malnutrition. In such a situation, it is paradoxical to waste this nutritionally superior by-product, which has got excellent functional properties too.

Whey also possesses some disadvantages. Major limitations to whey utilization in human food systems are, (1) Compositional imbalance - high salt/lactose ratio, too low protein/salt and protein/energy ratio, low sweetening power of lactose, poor digestibility of lactose in certain population, too dilute for efficient transportation (2) saltiness and acidity and (3) threat of contamination.

As three fourth of whey total solids is lactose, problem of whey utilization and disposal is largely a problem of lactose

itself. Lactose as such has low sweetness and solubility making its food application all the more difficult. Bioconversion of lactose into sweeter and more soluble carbohydrates is an attractive avenue for whey utilization. Enzymic hydrolysis of lactose using β-galactosidase enzyme is preferred because of its specific reaction pathways with no side effect. Other technologically feasible processes for lactose hydrolysis in fluid dairy system include immobilized enzyme reactors and membrane reactors with soluble enzymes recycling.

Nowadays people are very much aware of the correlation between blood cholesterol and coronary heart disease. Milk being rich in saturated fatty acids, has got the adverse publicity of being hypercholesteraemic. Research conducted in this field have given convincing results that orotic acid and 3-hydroxy 3-methyl glutaric acid of milk is actually hypocholesteraemic. Various studies have revealed that fermented milk products are capable of reducing cholesterol, probably due to assimilation of cholesterol by starter organisms.

Eventhough conventional yogurt starter organisms possess many beneficial effects, desirable results need prolonged consumption or are not as rewarding as expected, because of their inability to colonise in intestine. Major limiting factor is the lack of acid and bile tolerance. Thus to enhance

beneficial effects, it is preferable to use a normal intestinal inhabitant as dietary adjunct in yogurt.

Bifidobacteria are indigenous microflora of human gastrointestinal tract and constitute 99 per cent of intestinal flora in breast fed infants (Ishibashi and Shimamura, 1993). Gilliland (1979) suggested that starter cultures of intestinal origin in fermented milk products are more beneficial, because of better adaptation to gastrointestinal environment. Considerable beneficial effects have been reported by Colombel et al. (1987) due to the incorporation of bifidobacteria in regular diet of human beings. Some of the potentially most useful media for administering bifidobacteria to human beings are yogurt and milk.

To harness the therapeutic benefits of probiotics, Fernades and Shahani (1989) have recommended to screen the cultures for lactase activity, its ability to grow in the presence of bile salts and biosynthesise lactase and its tendency to establish and proliferate in the gastrointestinal tract.

The present study is aimed at incorporation of Bifidobacterium bifidum (B. bifidum) in yogurt (Bifidus yogurt) fortified with lactose hydrolysed condensed whey solids, and evaluation of its beneficial effects.

The products were assessed for

1. β-galactosidase specific activity
2. Hypocholesteraemic effect on rats.

The bile tolerance and acid tolerance of yogurt organisms and B. bifidum were also tested. An attempt was also made to study the growth rate of rats fed with yogurt under different treatments.

Review of Literature

REVIEW OF LITERATURE

2.1 Fermented milk products and yogurt

Metchnikoff (1910) in his fascinating treatise "The prolongation of life" propounded that longevity of Bulgarians was in part due to their consumption of large quantities of milk fermented with lactobacilli. This observation has led to burgeoning activity on the elucidation of role of lactic acid cultures and cultured milk products in the alleviation of human and animal disorders.

According to International Dairy Federation (IDF) (1969), fermented milks are defined as products prepared from milks - whole, partially or fully skimmed, concentrated milk or milk substituted from partially or fully skimmed dried milk, homogenized or not, pasteurised or sterilised and fermented by means of specific organisms.

Kosikowsky (1977) has classified fermented milks into four types based on their acid content. These include acid-alcohol products like Kefir and Kumiss, high acid product like Bulgarian sour milk, medium acid products like Acidophilus milk and yogurt, low-acid products like cultured butter milk and cultured cream.

Yogurt is generally fermented with a mixed culture of S. salivarius ssp thermophilus and L. delbrueckii ssp. bulgaricus. In some products, an additional species Lactobacillus helveticus may be used. In recent years, yogurts have become very popular vehicles for incorporating the probiotic species like Lactobacillus acidophilus and Bifidobacterium species (Deeth, 1984).

2.2 Fortification of yogurt mix

Tamime and Deeth (1980) reported that the consistency and aroma of yogurt depend on the level of total solids in the product. They recommended 15-16 per cent total solids for the best quality yogurt.

Abrahamsen and Holmen (1980) found that yogurt prepared from ultrafiltered milk gave a firm coagulum with high viscosity than that fortified with skim milk powder.

Yogurt prepared from skim milk concentrated by reverse osmosis (RO), was more viscous with less syneresis and had the typical flavour and texture (Dixon, 1985).

The desired level of total solids in yogurt mix could be achieved by partial evaporation of milk (Norling, 1979), fortification with skim milk powder (Tamime and Robinson, 1988); or by fortification with condensed whey and whey protein

concentrate (WPC) (Abd-El-Salam et al., 1991, and Pearce and Marshall, 1991).

When yogurt prepared from milk fortified with ultrafiltered whey protein concentrate and that from whole buffalo milk was compared, Abd-El-Salam et al. (1991) found that fortification with WPC at a level of 20 per cent of the total mix improved the texture and mouth feel and reduced the syneresis in yogurt.

Opdahl and Baer (1991) after conducting a consumer's acceptance survey of frozen yogurt reported that 87.8 per cent consumers accepted the product wherein 100 per cent non-fat dry milk (NDM) was replaced by spray dried whey protein concentrate.

2.3 Whey solids in yogurt

2.3.1 Composition of cheese whey

Nutting (1970) reported that cheese whey contained seven per cent total solids which was about half the total solids of milk. This contained 4.9 per cent lactose, 0.9 per cent protein, 0.3 per cent fat and 0.6 per cent ash.

According to Palmer (1980), lysine content of whey protein was 10.9 per cent as against 7.6 per cent in casein. Percentage of cysteine in whey protein and casein was 3.15 and

0.4 respectively. The total essential amino acids in whey was higher (62.55 per cent) than in casein (49.45 per cent).

2.3.2 Preparation of whey protein concentrate

Vishweshwariah and Ramanathan (1991) developed a method of preparation of whey protein concentrate (WPC). Fresh cheese whey was concentrated to 60 per cent total solids and blended with coagulated whey proteins in a ratio of 1:1.5 by weight. Latter component was obtained by coagulating another batch of cheese whey with two per cent citric acid at 80°C. After cooling to 35°C, the whey was centrifuged and sediment of whey protein was collected. The mixture of two products was homogenised in a single stage homogenizer and dried in vacuum shelf drier at a temperature of 65°C with a vacuum of 28 inches of mercury. The dried WPC was having 36 per cent protein, 51 per cent lactose 5.2 per cent fat, 4.8 per cent ash and three per cent water.

Whey could be concentrated to 25 per cent total solids by membrane processing techniques like reverse osmosis and ultrafiltration. These techniques have a low energy requirement and do not cause any chemical changes in different whey components (Marshall et al., 1982; Nanjudaswamy, 1992).

2.3.3 Incorporation of whey solids in yogurt

McDonough et al (1976) conducted a feeding trial in rats and concluded that bioavailability of dried WPC was higher than that of casein and skim milk.

Broome et al. (1982) reported that replacement of non-fat dry milk (NDM) upto 25 per cent with ultrafiltered cheddar cheese whey did not affect the taste and textural properties of yogurt.

Guirguis et al. (1984) observed that fortifying the yogurt with spray dried WPC to replace 50 per cent of NDM improved viscosity and reduced syneresis.

Addition of WPC did not have any adverse effect on quality of yogurt, on the contrary, it imparted a rich and smooth taste (Mohammed et al., 1991).

Baig and Prasad (1995) have reported that 100 per cent replacement of skim milk powder with condensed whey solids did not affect the organoleptic quality of yogurt.

2.3.4 Effect of whey solids on starter organisms

Streptococcus thermophilus (S. thermophilus) showed an increase in growth rate when condensed whey protein concentrate was added in the growth media. The presence of small peptides

such as basic peptides derived from $\alpha S_1 / \beta$ casein and glycomacropeptide from the action of renin on K-casein were responsible for this change (Hill et al., 1974).

Increasing the concentration of whey proteins in milk has been shown to stimulate the growth of S. thermophilus TS2 and Lactobacillus helveticus LB, (Broome et al., 1982).

Pahwa and Mathur (1983) found that inclusion of higher amounts of whey in commercial infant formula increased Bifidus activity by 50 per cent.

Mohammed et al. (1991) observed that more acidity developed in yogurt with WPC compared with control ($P<0.01$) suggesting that WPC encouraged the growth and activity of culture.

Baig and Prasad (1995) have reported that incorporation whey solids in yogurt was stimulatory to the growth of S. salivarius ssp. thermophilus and B. bifidum.

2.4.1 Lactose hydrolysis

Gyuricsek and Thompson (1976) observed that as percentage of lactose hydrolysis increased, time required for curd setting decreased.

Williams and Macdonald (1982) fed diets containing 80 per cent by weight of hydrolysed lactose to male and female baboons over a 10 week period. During the experimental period, an increase in serum triglycerides and decrease in serum cholesterol was observed.

The rate and extend of lactose hydrolysis achieved was independent of protein concentration in whey preparation used (Sheth et al., 1988).

Partial replacement of sucrose and non fat milk solids by lactose hydrolysed whey in the manufacture of ice cream was studied by Mitchell (1991). Replacement upto 50 per cent level resulted in an acceptable ice cream with less body defects like lactose crystallisation.

Linear increases in contact time between lactose and lactase did not bring about similar increases in degree of hydrolysis, particularly above 50 per cent level (Mitchell and Hourigan, 1993).

According to Paul and Mathur (1993), the available lysine content decreased proportionally with increase in lactose hydrolysis. Loss of available lysine and increase in Hydroxy methyl furfural (HMF) resulted in a decrease in nutritive value of lactose hydrolysed infant formula.

2.4.2 Bifidus factors

According to Burvall et al. (1979) higher the initial lactose concentration, greater was the proportion of oligosaccharides and longer was the chain length.

If initial lactose concentration in the substrate was high, about 40 per cent of activity of enzymes derived from Kluyveromyces fragilis and S. thermophilus was reported to be diverted for oligosaccharide formation (Roberts and Pettinati, 1957; Smart, 1991).

In vitro studies have shown that galacto-oligosaccharides were well utilised by bifidobacteria for their growth (Smart et al., 1992) and that bifidobacteria have unique and highly efficient metabolic mechanism for utilisation of galactooligosaccharides (Smart, 1992).

Smart (1993) reported that β-galactosidases were able to calatyse a series of transferase reactions involving both lactose and its hydrolytic products, particularly galactose, to form a family of galactose rich oligosaccharides, termed galactooligosaccharides.

The fact that oligosaccharides are likely to reach lower intestine undigested and the direct link to natural bifidus factors in human milk suggest that lactose derived compounds may

have competitive advantage over other artificial compounds as commercial bifidus factors (Smart, 1993).

2.5.1 Growth behaviour of bifidobacteria

Scardovi (1986) reported that optimum growth temperature for majority of bifidobacteria species was 37-41°C. They can grow at 25-28°C and could tolerate a temperature upto 45°C. Optimum pH for initial growth was 6.5-7.

Robinson (1990) studied the survival of B. bifidum in yogurt prepared using 1.5 per cent B. bifidum and 0.5 per cent yogurt culture and incubated to a pH of 4 to 4.5. B. bifidum count after 21 days of refrigerated storage was $1x10^9$ cfu/gram.

Martin and Chou (1992) observed that population of bifidobacteria declined rapidly in yogurt having higher acidity. They reported that survival rate of bifidobacteria in yogurt was strain and species dependent.

Murti et al. (1992) compared the growth of bifidobacteria in soya extract, in cow milk supplemented with yeast extract and in unsupplemented cow milk. They found that organism did not grow in unsupplemented milk. The growth was more rapid in supplemented milk than in soya extract. The population of bifidobacteria in supplemented milk was $1x10^{10}$ cfu/ml and in soya extract, count was $2.5x10^9$ cfu/ml.

Ishibashi and Shimamura (1993) suggested that cultivation of S. salivarius ssp thermophilus with bifidobacteria would be beneficial. The release of carbondioxide by the thermophilus would be stimulatory for the growth of bifidobacteria.

Klaver et al. (1993) had reported that when S. salivarius ssp thermophilus and B. bifidum were grown together, rapid acidity produced by S. salivarius ssp thermophilus prevented the lowering of redox potential, thus preventing bifidobacteria from growing.

Kaneko et al. (1994) reported that Propionibacterium freudenreichii 7025 released a water soluble Bifidus growth stimulator (BGS) and this was present in cell free extract and methanol extract fraction of cells. They also reported that several intestinal bacteria such as Bacteroides, Enterobactor and Enterococcus stimulated growth of bifidobacteria.

2.5.2 Whey as a growth promoter of bifidobacteria

Cheng and Sandine (1989) reported that a whey based medium (seven per cent sweet whey, 0.05 per cent cysteine and 0.3 per cent yeast extract) would be satisfactory for the growth of a variety of Bifidobacterium species, without use of anaerobic conditions.

Anita et al. (1989) suggested that rennet whey which contains the glycopeptide released by K-casein can be considered as a source of Bifidus growth stimulating factor.

Higher amount of proteins present in skim milk retentate stimulated growth of B. bifidum. Addition of whey retentate to milk retentate resulted in better growth of B. bifidum and shorter fermentation time (Magdalenic and Krdev, 1990).

Gorre et al (1992) in their attempt to produce a low cost medium for production of concentrated B. bifidum, found out that a fifteen fold improvement on batch productivity could be achieved using a whey based medium.

Modler and Villa-Garcia (1993) developed an inexpensive whey based medium for large scale production of bifidobacteria. This medium contained 11 per cent whey solids, 0.05 per cent cysteine and 0.23 per cent yeast extract and gave a count of 10^{10} cfu/millilitre.

2.5.3 Other growth supplementers of bifidobacteria

Evog (1965) suggested that addition of glucose at a rate of 1-5 per cent in conjunction with yeast extract at a level of 0.1-0.5 per cent may further shorten the coagulation time required by bifidobacteria.

Jao et al. (1977) found out that stimulatory effect of aminosugars on B. bifidum was in the order of

N-acetylglucosamine > N-acetylgalactosamine > N-acetylmannosamine > N-acetylmuramic acid

For satisfactory growth of B. bifidum in milk, Anand et al. (1984) suggested the addition of one per cent dextrose and 0.1 per cent yeast extract, whereas Collins and Hall (1984) advocated supplementation with 0.05 per cent cysteine plus either pyruvic acid at a level of 0.05 per cent or ascorbic acid at 0.2 per cent level.

Goh et al. (1986) found out that fortification of skim milk with 0.05 per cent L-cysteine and 0.2 per cent yeast extract and increasing the inoculation rate from two to five per cent increased the B. bifidum count from 8.2×10^8 to 6.7×10^9 cfu/ml.

Zbikowsky and Zikjka (1986) reported that enrichment of reconstituted skim milk with five per cent carrot juice improved acid production by B. bifidum.

Bovine casein digest and yeast extract were found to have maximum growth promoting effect on Bifidobacterium species (Poch and Anatoly, 1988).

Klaver et al. (1993) observed that growth of Bifidobacterium in milk needs the presence of peptides and aminoacids derived from casein degradation.

2.5.4 Cultured dairy products containing bifidobacteria

Schular et al. (1968) advocated the following method for the preparation of cultured milk product containing B. bifidum, S. thermophilus and L. acidophilus. Single cultures of these were incubated for seven, four and twenty four hours respectively at 42°C. A bulk starter containing these in the proportion 1:1:1 was incubated at 42°C for four hours. Bulk culture was inoculated and incubated for three hours at 42°C to get a product with 10^6-10^8 cfu of bifidobacteria/ml.

Marshall et al. (1982) prepared a fermented milk with B. bifidum using ultrafiltered skim milk fortified with ultrafiltered cheese whey and threonine. Product obtained after 24 hours of incubation at 37°C had $3x10^9$ cfu/ml of viable counts. After 21 days of storage at 4°C count was reduced to $5.2x10^7$ cfu/ml.

Misra and Kuila (1991) used ten per cent inoculum of B. bifidum for preparation of cultured bifidus milk from cow and buffalo milk. Incubation for 18 hours at 37°C gave a product with 0.78-0.86 per cent developed acidity and a viable count of $4x10^9$ cfu/ml.

Klaver et al. (1993) suggested that for the manufacture of cultured dairy products containing bifidobacteria, an inoculum containing final number of cells required for the product, was needed.

Maintenance of an anaerobic environment and pH at a level of $\geq$ 5.5 is important to have viable cells of bifidobacteria at a concentration of 10^6 cfu/ml in the final product (Ventling and Mistry, 1993).

2.5.5 Effects of consumption of Bifidobacteria on intestine

According to Bouhnik et al. (1992), under physiological conditions, exogenously administered Bifidobacterium species does not colonize human intestine. However, high faecal concentration of exogenous bifidobacteria are compatible with metabolic probiotic activities. When ingestion stops exogenous bifidobacteria gradually decrease, and will no longer be detectable eight days after cessation of ingestion.

In a study by Pochart et al. (1992), it was observed that only 23.5 per cent of the ingested bifidobacteria were present in terminal ileum after eight hours of ingestion.

Grimaud et al. (1993) reported that milk containing bifidobacteria reduced intestinal transit time ($P<0.01$)

primarily due to an increase in transit through pelvic colon, the reduction in transit time being related to the stage of development of bifidobacteria.

Marteau et al. (1993) reported that almost 30 per cent of ingested bifidobacteria remained viable on arrival at the end of small intestine and all of these were found in stools.

2.6.1 Lactose intolerance - incidence

Simmons (1973) observed that all the population groups in traditional non-dairying areas had a high incidence of lactose intolerance.

Newcomer and McGill (1984) advocated that nearly all newborn full term infants possessed sufficient lactase activity to digest milk, which however declined with age, and by the age of six, only five to ten per cent of the activity remained.

It had been estimated that 70 per cent of the world's population experiences reduced intestinal lactase activity after early childhood (National Dairy Council 1985).

Savaiano and Levitt (1987) hypothesised that lactose intolerance is a genetically linked trait.

2.6.2 Mechanism of lactose intolerance

Lactose is the main carbohydrate present in milk. It is a disaccharide composed of glucose and galactose joined by glycosïdic 1-4 β linkage. Humans cannot absorb disaccharide and so lactose must be hydrolysed into its component monosaccharides by lactase which is a membrane bound enzyme present in the brush border of intestinal epithelial cells (Asp and Dahlquist, 1972).

Hourigan and Rand (1977) reported that if the amount of lactose ingested exceeded the hydrolytic capacity of available intestinal lactase, a portion of lactose remained undigested and was transported into intestine, increasing the osmolarity of intestinal fluid and thus drawing water from tissues into intestine. Undigested lactose may also be fermented by bacteria in colon thus generating organic acids, carbondioxide and hydrogen. These fermentation products together with large amount of water drawn into intestine are largely responsible for various symptoms.

The term lactose malabsorption refers to physiological effects like low blood sugar rise following lactose malabsorption test (Newcomer and McGill, 1984), whereas lactose intolerance refers to clinical signs (diarrhoea, bloating, flatulence) or subjective symptoms (abdominal gas, pain) following the same test (Renner, 1986).

Savaiano and Levitt (1987) classified lactase deficiency as that due to congenital and acquired causes. Acquired causes were further divided into primary cause and secondary cause. Primary cause is the genetically programmed loss of lactase following weaning. Secondary deficiency results from a disease process that involve the small bowel mucosa and reduced lactase level or that which causes insufficient exposure of mucosa to ingested lactose as in infectious diarrhoea, short gut, cetiac sprue, Crohn's disease, tropical sprue, malnutrition, blind loop syndrome, giardia, subintestinal gastrectomy and immunological deficiency syndrome.

2.6.3 Lactase of yogurt cultures and B. bifidum

Citti et al. (1965) studied the β-galactosidase specific activities of Streptococcus lactis 7962 and reported that its activity differed depending on the buffer solution used for assay. The highest specific activity of 0.75 units was found when 0.05 M sodium phosphate buffer was used.

Kilara and Shahani (1974) reported that yogurt, unless pasteurised prior to sale, contained substantial amount of lactase bound in the cells of microbial culture and that this lactase might contribute to intestinal hydrolysis of lactose after consumption of yogurt.

Various studies have revealed that lactose malabsorbing humans digested lactose from yogurt much more efficiently than lactose from any other dairy product (Kilara and Shahani, 1976; Savaiano and Levitt, 1987 and Onwulata et al., 1989).

Alm (1982) reported that microorganisms used for fermented milk products reduced the lactose content of milk considerably. It has been shown that 500 millilitres of low fat milk caused abdominal distress and diarrhoea in lactose intolerant individuals whereas the same quantity of yogurt or acidophilus milk did not result in any palpable symptoms.

Kolars et al. (1984) reported that yogurt was well tolerated by lactose intolerant individuals even when unusually large quantities of lactose in yogurt were ingested.

Nicolai and Ziliken (1984) reported that β-D galactosidase enzyme of B. bifidum ssp pensylvanicum had a molecular weight of 23000 and an optimum pH 6-7, similar to the pH of intestine of breast fed infants. N-acetyl glucosamine was cleaved only half as fast as lactose by the enzyme while lactulose was hardly affected.

Yogurt possessed considerable lactase activity due to the presence of lactase in yogurt organisms. S. thermophilus contained approximately three times more lactase activity than did L. bulgaricus. The lactase activity was 1.5, 2.4 and 3.8

units/gram for L. bulgaricus, S. thermophilus and combined culture respectively (Savaiano and Levitt, 1987).

Lin et al. (1989a) demonstrated a method for determining β-galactosidase activity of yogurt culture in skim milk. The specific activity was found to be 4.5 units under optimal assay conditions.

Lin et al. (1991) estimated β-galactosidase activities of various culture organisms. Mixed yogurt strain had a maximum activity (2.8 units) followed by single strains of L. bulgaricus (2.4 units) and S. thermophilus (1.8 units).

Desjardins et al. (1991) observed that when B. bifidum ATCC 15696, B. breve ATCC 15698, B. longum ATCC 15707 and B. infantis ATCC 27920 were grown in ten per cent sterile reconstituted skim milk, organisms differed in their pattern of growth and induction of β-galactosidase. Specific activity of B. bifidum increased during the exponential phase and was maintained during the initial period of stationary phase. B. infantis reached peak during mid log phase. B. infantis and B. longum which had the lowest generation time showed the highest β-galactosidase activity. Growth performance of Bifidobacteria appeared to be associated with β-galactosidase activity.

2.7.1 Hypercholesteraemia

Cholesterol is the prime suspect in coronary heart disease (CHD) because formation of atherosclerotic lesion is an inflammatory response to this substance. Spain (1966) conducted studies on 6000 men and found that cholesterol levels in blood and blood pressure, had a positive correlation with atherosclerosis.

Kruski and Narayana (1976) reported that the chickens fed with feed containing cholesterol showed an increase in HDL-level from 65.5 ± 16.4 mg/100 ml to 77.1 ± 13.9 mg/100 ml. They also found that LDL-level raised to 152.3 ± 41.3 from 25.6 ± 6.7 mg/100 ml.

The lipids in plasma circulate with lipoproteins, namely chylomicrones, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). About 70 per cent of total plasma cholesterol in normal human beings is contained in LDL, the lipoprotein most strongly correlated with atherosclerosis. LDL carries cholesterol into blood vessels whereas HDL takes plasma cholesterol to liver for metabolism. There is an inverse relationship between levels of HDL and development of atherosclerosis (Robbins and Cotran, 1981).

Brown and Goldstein (1984) advocated that cholesterol being hydrophobic in nature does not circulate freely in the blood. They circulate only in association with lipoproteins, LDL and HDL. High levels of LDL in blood causes atherosclerosis to develop. The level of LDL particles in blood is affected by specialised proteins called LDL receptors. These receptors bind LDL particles and extract them from the fluid that bathes the cells. The LDL is broken down in the cells, and cholesterol is used for biological functions. When the need is low, cell makes fewer LDL receptors, thus LDL level in blood rises, accumulating excess cholesterol in arteries, which accelerates atherosclerosis. The inadequacy in LDL receptors has been attributed to both genetic and environmental factors.

Kansal (1990) reported that elevated serum cholesterol levels clearly increased risk of cardiovascular diseases. It was predicted that a two per cent reduction in cardiovascular disease would be there for every one per cent reduction in serum cholesterol.

2.7.2 Milk and hypocholesteraemia

Orotic acid, a pyrimidine intermediate in nucleic acid synthesis generally exist in milk at a concentration ranging from 72-122 mg/litre (Hallanger et al., 1953).

Windmuller (1963) observed that plasma lipids, particularly triglycerides, were depressed in experimental rats as early as 16 hours after orotic acid was introduced in the diet. He also found that LDL fraction almost disappeared from plasma and there was a reduction of 60 per cent in HDL fraction in orotic acid fed rats. Roheim et al. (1965) stated that orotic acid depressed the formation or release of VLDL.

About one third of the fatty acids of milk are monounsaturated, which neither raises nor lowers blood cholesterol levels. Polyunsaturated fatty acids account for approximately four per cent and have been reported to lower blood cholesterol levels. Of the remaining (63 per cent) saturated fatty acids, nearly 13 per cent are short chains fatty acids which are metabolised in a manner that has no effect on blood cholesterol and are not deposited in adipose tissue. Stearic acid constituting 13-14 per cent probably is not involved in increasing plasma cholesterol content. Therefore only about one third of milk fatty acids are of the kind suspected of elevating the blood cholesterol (Kahn, 1970, Gurr, 1984 and Hornstra, 1989).

Payens et al. (1976) did not find lowering of serum cholesterol levels in human subjects who consumed large quantities of yogurt as part of their diet.

Ahmed et al. (1979) reported that in all yogurts, prepared using different strains of S. thermophilus and L. bulgaricus, orotic acid decreased 15-53 per cent after fermentation, whereas uric acid level did not change.

Rossouw et al. (1981) did not observe a serum cholesterol lowering effect when adolescent boys were administered two litres of skim milk yogurt or 20 per cent cream.

The triglycerides (lipids) are sparingly hydrolysed by lipases of lactic acid bacteria (LAB). These lipases are more active towards lower but not higher molecular weight tri-glycerides (Morales and Chandan, 1982).

Chawla and Kansal (1984) conducted experiments with rats and reported that milk feeding in rats reduced deposition of cholesterol in liver and blood vessels.

Srinivasan and Kansal (1986) reported that buffalo milk, despite its high saturated fatty acid content, induced hypocholesteraemia in rats. Increase in HDL and decrease in LDL in experimental rats were significant.

Srinivasan and Kansal (1988) suggested that hypocholesteraemic effect of milk was partly attributed to enhanced excretion of bile acids in faeces.

A recent epidemiological study have shown that people who regularly consumed whole milk were much less likely to suffer a heart attack than those who consumed skim milk. Of the people included in the survey, 9.9 per cent experienced major Ischemic Heart Disease (IHD) in non-milk drinking group. The incidence was 6.3, 5.8 and 1.2 per cent in half pint, half to one pint and more than one pint milk drinking groups respectively (Medical Research Council, 1991).

2.7.3 Hypocholesteraemic effect of fermented milks

Role of microorganisms in the cholesterol destruction or degradation in rats had been reported by Danielsson and Gustafsson (1959).

Wostmann et al. (1966) reported an accelerating effect of normal intestinal microflora on systemic cholesterol catabolism and elimination in rats. This conclusion was based on lower systemic cholesterol in a normal environment when compared with systemic cholesterol in rats in a sterile microbe free environment.

Bifodobacteria can bring about deconjugation of bile salts. This is important in controlling serum cholesterol concentrations since this cannot function as well as conjugated bile acids in solubilisation and absorption of lipids (Eyssen, 1973).

In a study conducted by Beena and Prasad (1995) regarding the bile salt deconjugation capacity, it was seen that L. delbrueckii ssp. bulgaricus had the maximum deconjugating capacity, followed by B. bifidum and S. salivarius ssp. thermophilus.

Mann and Spoerry (1974) studied a group of Massai tribesmen and reported that consumption of large quantities of fermented milk, actually lowered their serum cholesterol levels and cardiac risk factor. This inverse relationship between serum cholesterol level and consumption of milk fermented with a wild strain of lactobacilli, suggested that there must be a factor in fermented milk that somehow inhibited the biosynthesis of cholesterol.

Harrison and Peat (1975) reported that orotic acid content of milk did not decrease during the manufacture of fermented milk products.

Mann (1977a) conducted an experiment on human volunteers wherein the subjects were fed whole milk yogurt, skim milk yogurt or fresh milk daily for 12 days. In general there was a reduction in serum cholesterol during the feeding period, with a slow return towards normality upon cessation of yogurt diet. Fresh milk at an intake of two litres daily did not statistically affect cholesteraemia and he concluded that factors affecting serum cholesterol were produced or enhanced

in milk by microbial action. In the same experiment by administering radioactive acetate to human volunteers, it was observed that incorporation of acetate into cholesterol was inhibited during consumption of yogurt, resulting in decreased cholesterol biosynthesis. He postulated that 3-hydroxy 3-methyl glutaric acid (HMG) in fermented milk inhibited the rate limiting enzyme in cholesterol biosynthesis, HMG CoA reductase. Mann also suggested that in the biosynthesis of cholesterol from acetate, the acetate would be activated by acetyl CoA synthetase and a decrease in cholesterol biosynthesis occurred by inhibition of this enzyme. He also claimed that the milk factor (MF) which was responsible for hypocholesteraemia was slightly more in fermented milk. He also stated that MF is a non-protein, dialyzable, heat and acid stable and polar.

Hepner et al. (1979) confirmed the ability of yogurt in reducing serum cholesterol levels in human volunteers.

Thakur and Jha (1981) conducted a research on rabbits in which they were fed stock diet, milk, yogurt or calcium carbonate. Milk reduced the effects of cholesterol but yogurt and calcium carbonate were similar and had more marked effects. Atherosclerotic lesions and aortic sudanophilia was maximum in control group. The groups receiving yogurt and calcium carbonate showed an intermediate degree of sudanophilia. It was

suggested that calcium was responsible for the cholesterol lowering effect of yogurt, but that other hypocholesteraemic agents might also be present.

Rats fed with milk fermented by S. thermophilus exhibited a reduction in plasma cholesterol levels (Rao et al., 1981).

In another experiment, Pulusani and Rao (1983) compared the effect of water, skim milk and skim milk, fermented by S. thermophilus, L. bulgaricus or L. acidophilus. After the feeding trials plasma cholesterol levels (mg/dl) and whole body lipids (mg/g dry matter) for the treatments one to five were 61.3, 54.7, 56.0, 57.1, 58.1 and 3.68, 3.58, 3.27, 3.18, 3.00 respectively. It was postulated that the hypocholesteraemia of fermented milks might be due to an increased excretion of cholesterol or its metabolites, and inhibition of cholesterol biosynthesis by metabolites produced by lactic cultures.

Jaspers et al. (1984) fed adult human volunteers with yogurt and found that there was a significant reduction in total serum cholesterol by 10 to 12 per cent in the initial period, but returned to the level of controls with continued yogurt consumption. Differences in concentration of uric acid, orotic acid and HMG in yogurt were insufficient to account for the differences in temporary hypocholesteraemia seen between trials.

The ability of L. acidophilus of human origin was tested for cholesterol assimilation in an in vitro study by Nelson and Gilliland (1984). They reported that the strains exhibiting low bile tolerance were less active in removing cholesterol from the growth media, while strains exhibiting high bile tolerance varied in their ability to remove cholesterol.

Chikai et al. (1987) inoculated germ free rats with human intestinal bacteria and found that bile acid excretion was significantly higher in rats inoculated with intestinal microorganism than in gnotobiotic rats and most of these bile acids were deconjugated. They suggested that free bile acids adhered to bacteria or dietary fibres, thus enhancing excretion of bile acids. This action might trigger the feed back mechanism that regulates the hepatic cholesterol synthesis and subsequent transformation into bile acids, which might reduce cholesterol concentration.

Homma (1988) reported a lowering of serum cholesterol in rats fed orally with yogurt containing Bifidobacteria. He also suggested that reduction in serum cholesterol was due to inhibition of hydroxymethylglutaryl CoA reductase.

Lin et al. (1989a) explored the effect of tablets containing L. acidophilus and L. bulgaricus on cholesterol. In vitro tests revealed that the organisms significantly reduced the cholesterol in growth medium. Bacteria assimilated

cholesterol only when they were alive and at numbers above 10^8 cfu/millilitre. Oxgall inhibited the growth of bacteria, especially L. bulgaricus, thus reducing its ability to assimilate cholesterol.

During in vivo trials, human subjects were either fed normal diet as control or normal diet with lactobacillus tablet. In all subjects treated with tablet, mean concentration of total cholesterol (TC) and low density lipoproteins (LDL) reduced to statistically significant level. High density lipoproteins (HDL) increased 1.8 to three mg/dl. The cardiac risk factor (TC/HDL) was unchanged in control group (4.45-4.43) but decreased with time in lactobacillus treated group significantly.

In the same experiment, a commercial hypolipidemic tablet placebo and lactobacillus tablet were tested for their hypocholesteraemic effect on human subjects. The cardiac risk factor (TC/HDL) did not change significantly neither in Placebo group (4.39 to 3.68) nor in lactobacillus treated group (4.22 to 3.45).

Rasic et al. (1992) reported that S. thermophilus assimilated less cholesterol than that of L. bulgaricus. They also observed that B. bifidum can assimilate cholesterol actively than S. thermophilus and L. bulgaricus.

2.8 Physiological values of rats

Harkness and Wagner (1989) reported the physiological values of laboratory rat. Serum proteins were around 5.6-7.6 g/dl, lipids 70-415 mg/dl, phospholipids 36-130 mg/dl, triglycerides 26-145 mg/dl and total cholesterol 40-130 mg/dl.

2.9 Growth promoting effects of yogurt

Breslaw and Kleyn (1973) concluded from an *in vitro* study that yogurt was more digestible than the raw mixture from which it was made. They observed that yogurt protein was twice as digestible as milk proteins since for yogurt only three hours were required to attain more than 70 per cent digestion compared with six hours for milk.

Reddy *et al*. (1975) reported significant increases of folic acid in yogurt.

The increased digestibility of yogurt protein when compared with milk protein has been attributed to several factors. These include softer curd resulting from high heat treatment (Jay, 1975), high acidity and smaller casein curd content (Hiv *et al*., 1963), the increased secretion of digestive enzymes by salivary glands when stimulated by curd particles (Halden, 1964) and the increased peptide and free aminoacid

content resulting from heat treatment and from proteolysis by yogurt bacteria (Pien, 1964).

Incorporation of B. bifidum with yogurt culture in the ratio of 2:1 resulted in greater changes in nitrogen compounds than with yogurt cultures alone. This was attributed to the proteolytic activity of B. bifidum in milk and this confirmed improved protein digestibility (Goodenough and Kleyn, 1976).

Hargrove and Alford (1978) for the first time reported that yogurt diet induced a better growth than milk did in rats. They also suggested that the improved feed efficiency of rats fed low fat yogurt might be related to improved bioavailability of protein.

According to Renner (1986), consumption of yogurt promotes growth as a result of improved lactose digestion and mineral absorption, besides providing thiamine, riboflavin, niacin and folic acid.

2.10 Acid tolerance of yogurt cultures and B. bifidum

Franklin and Skornya (1971) have reported that for a microorganism to reach intestine it must first pass through the hostile environment of stomach containing hydrochloric acid and enzymes. It is likely that ingested organisms would come in contact with pH values ranging from two to eight.

Acott and Labuza (1972) have shown that yogurt microflora were capable of surviving simulated gastric digestion. S. thermophilus was rapidly destroyed whereas small proportion of Lactobacilli survived after 3.5 hours at pH 2.

Salvadori and Salvadori (1974) and Rocchietta (1975) have found that significant number of yogurt organisms can survive passage through gastrointestinal tract.

Hargrove and Alford (1978) reported that eventhough L. bulgaricus was frequently isolated in the intestinal tract during feeding trials, it disappeared three days after discontinuation of yogurt feeding. S. thermophilus never was isolated below the upper small intestine.

By conducting studies in rats, Goodenough and Kleyn (1976) concluded that viable cell population increased in direct proportion to that in natural yogurt and that counts remained elevated two to three hours after ingestion of yogurt, thereby demonstrating significant survival and potential metabolic activity in the upper gastrointestinal tract of rats.

Deeth and Tamime (1981) reported that neither L. bulgaricus nor S. thermophilus used in modern yogurt making were native to gastrointestinal tract and stated that prolonged beneficial effects could only be obtained if adherent strains

of starter organisms like Lactobacillus acidophilus (L. acidophilus) were used.

Resistance to gastric acidity has not been demonstrated for bifidobacteria (Rasic and Kurmann, 1983).

In vitro studies by Petterson et al (1983) showed that L. acidophilus survived better than L. bulgaricus in gastric juice.

Chomakov and Boicheva (1984) found that there was no difference in L. bulgaricus and S. thermophilus count of Bulgarian sour milk starters kept in gastric juice (pH 3.48 to 4.26) and bile (pH 5.09-6.75) for three hours in vitro. Endurance of both were similar.

Starter culture bacteria, L. bulgaricus and S. thermophilus used for manufacture of yogurt did not survive and grow in gastrointestinal tract. Therefore the benefits received from yogurt were derived from contents of culture rather than the viability of culture in intestinal tract (Speck, 1977; Gilliland and Kim, 1984).

Kolars et al. (1984) and Savaiano et al. (1984) reported that S. thermophilus and L. bulgaricus were resistant to gastric acidity and consequently are alive and active in human intestine though they are not natural inhabitants.

Gilliland (1985) studied the viability of yogurt culture organisms in the intestine and found that they did not survive or grow in the intestinal tract, thereby indicating that they only served as a source of B-galactosidase in alleviating lactose maldigestion.

Yogurt is reported to be an excellent buffer. Fifty millilitres of yogurt require 6.2 m mol of hydrochloric acid to reduce pH from 4.1 to 2.0 which was nearly three times the acid required to reduce the pH of the same quantity of acidified milk (Martini et al., 1987). Same authors also reported that pH of gastric samples following yogurt ingestion remained >2.7 for three hours following the yogurts meal.

Tomar and Prasad (1989) compared the ability of strains of L. acidophilus and L. bulgaricus to implant in intestine. It was observed that L. bulgaricus had poor ability to implant when compared to L. acidophilus.

It had been shown by Berrada et al. (1991) that resistance to gastric acidity varied between strains of bifidobacteria.

Hoier (1992) compared acid tolerance of L. acidophilus La-5 and bifidobacteria Bb-12 by incubating in MRS nutrient solution adjusted to pH 1-4 with hydrochloric acid. Both species were capable of 100 per cent survival at pH 3 and 4.

Bifidobacteria Bb-12 had a higher tolerance to acid than L. acidophilus at lower pH.

2.11 Bile tolerance of yogurt cultures and B. bifidum

Lembke's work (1964) showed that S. thermophilus was very susceptible whereas some of the lactobacillus organisms survived a short time in the lowest concentration of bile salt (0.01 per cent deoxycholic acid).

Catteau et al. (1971) found that 0.2 N, 0.5 per cent sodium deoxycholate was bacteriostatic for twenty two strains of B. bifidum and B. breve.

Moore and Holdeman (1972) isolated the common lactobacilli from human intestine. They were identified as L. acidophilus, L. bifidus, L. plantarum, L. casei and L. fermentum. Of these only L. acidophilus and L. bifidus were present in sufficient quantities.

Eventhough many lactobacilli survive selective pressures of gastrointestinal environment, flow rates of digesta through the small intestine washes out any organism which is unable to multiply rapidly enough to avoid dilution or to maintain their residence by physical attachment to intestinal epithelium (Robins-Browne and Levine, 1981).

Lindwall and Fonden (1984) studied the viability of various lactic acid bacteria in the presence of bile and gastric juice. Both in vitro and in vivo experiments showed a better survival of L. acidophilus as compared to L. bulgaricus.

Conway et al. (1987) compared the ability of two L. acidophilus strains, L. bulgaricus and S. thermophilus to adhere to human and pig ileal caecum or colon cells in vitro. Both strains of L. acidophilus survived and adhered better than L. bulgaricus and S. thermophilus.

Khattab and Abour-Donia (1987) reported that out of six strains of lactic acid bacteria tested for their ability to grow in the presence of 0.3 per cent bile salt, S. thermophilus and S. lactis failed to grow in the presence of bile salt at any concentration (0.15, 0.2 and 0.3 per cent); L. bulgaricus eventhough grew in low concentration, failed to show any growth at 0.3 per cent.

Hoier (1992) compared the bile tolerance of L. acidophilus La-5 and bifidobacteria Bb-12 by growing in milk yeast medium containing oxbile (0.5-2 per cent) at 37°C for 24 hours. Both were not inhibited except at high concentrations which was unlikely to be found under normal intestinal conditions.

Clark et al. (1994) found that several species of bifidobacteria were capable of surviving pH of human stomach. In an attempt to determine the effect of bile acids on the pH resistant species, B. longum, B. infantis, B. bifidum and B. adolescentis were allowed to grow at different levels of bile acids reported in human gastrointestinal tract. Oxgall was mixed in sterile distilled water at levels of zero, two and four per cent and bifidobacteria were added. Samples were plated at zero time (control) and after twelve hours to simulate the time necessary for passage through the digestive system. Results showed that B. adolescentis and B. infantis survived in two per cent oxgall but at a lessser extent than B. longum. B. adolescentis and B. longum survived well during 12 hours at both oxgall concentrations. B. adolescentis decreased substantially in four per cent oxgall while B. bifidum did not survive in either two or four per cent oxgall during 12 hours of incubation. Therefore B. longum was considered as the species of choice as a dietary adjunct in cultured dairy products.

Materials and Methods

MATERIALS AND METHODS

3.1.1 Starter cultures

The following pure freeze dried cultures were used for the experiment.

(i) Streptococcus salivarius ssp. thermophilus YH-5

(ii) Lactobacillus delbrueckii ssp. bulgaricus YH-L
(Procured from National Dairy Research Institute (NDRI) Karnal)

(iii) Bifidobacterium bifidum 2715
(Obtained as a gift from Institute of Food Research, Reading Laboratory, Reading RG6 2EF, UK)

3.1.2 Maintenance of starter cultures

Lyophilised yogurt cultures were aseptically transferred separately into sterile skim milk and incubated at 37°C until curdling. Three consecutive transfers were done daily for maximum activation of culture.

Bifidobacterium bifidum was activated by transferring the lyophilised culture into sterile skim milk containing one per cent dextrose and 0.1 per cent yeast extract and incubating at 37°C under carbondioxide tension till it got

curdled. Further activation was achieved by three consecutive transfers in the same media.

Routine maintenance of all these cultures were carried out by fortnightly transfer in sterile skim milk. In between the transfers, cultures were kept at 4°C.

3.1.3 Preparation of cottage cheese whey

Fresh skim milk was procured from KAU Dairy Plant. It was pasteurised by holder method and cooled to 30°C in a cheese vat. An active culture of Lactococcus lactis spp. cremoris was added at one per cent level to skim milk. After the development of sufficient acidity, (an increase of 0.02 per cent from initial level) rennet (Rennilase 150 L - Novo Nordisk diluted to 1:100) was added at the rate of 10 ml/litre. The enzyme was thoroughly mixed with skim milk and kept undisturbed till a curd firm enough to cut was got.

The curd so got was cut first horizontally and then vertically to get small cubes of uniform size. Temperature of curd was then raised to 40°C and left for five minutes before drainage of whey. Whey was then drained and collected in a stainless steel bucket after filtering through a muslin cloth. It was then heated to 85°C for 10 minutes to inactivate residual rennet and to destroy starters.

3.1.4 Condensation of cheese whey

Cheese whey was condensed to approximately 8:1 concentration using a vacuum evaporator 'Anhydro Type Lab E.W.O. 1688' at 50°C with a vacuum of 70 centimetres of mercury. Slightly over condensed whey so obtained was standardised to get 50 per cent total solids by adding sufficient quantity of distilled water. It was then immediately cooled and stored at -20°C till it was utilised for fortification of yogurt mix. The standardised condensed whey was having the following percentage composition (on dry matter basis).

Protein	-	10.32
Fat	-	0
Lactose	-	78.62
Ash	-	11.06

3.1.5 Preparation of lactose hydrolysed condensed whey

To 120 ml condensed whey, 7.5 ml of β-galactosidase enzyme (LACTOZYM - Novo Nordisk) was added, and allowed it to act for one hour at room temperature. The lactose content in hydrolysed whey was then estimated as per the procedure described by Nickerson *et al*. (1975).

3.1.5.1 Estimation of lactose

Reagents

a. Zinc acetate phosphotungstic acid (ZAPT) - Prepared by dissolving 25.0 g Zinc acetate and 12.5 g phosphotungstic acid in water. Then 20 ml of glacial acetic acid was added and this was diluted to 100 ml.

b. Glycine - Sodium hydroxide buffer. By mixing 150 ml of glycine solution containing 2.4768 g glycine and 1.935 g sodium chloride with 850 ml of 0.385 N sodium hydroxide (pH-12.8)

c. Methylamine solution - Five per cent of methylamine hydrochloride in distilled water stored in refrigerator.

d. Sodium sulfite solution - Freshly prepared by dissolving one gram of sodium sulfite in distilled water and diluting to 100 ml.

e. Lactose standard solutions

   1. Stock solution - Prepared by dissolving 2.6315 g lactose monohydrate USP grade and diluting to 200 ml with 0.1 per cent Benzoic acid. Stored in refrigerator.

2. Working solutions with 0.5, 0.75, 1.00, 1.25 and 1.50 mg lactose/ml were prepared by diluting 10, 15, 20, 25 and 30 ml stock solution to 250 ml respectively.

Preparation of sample

a. To eight ml of whey, added one ml of ZAPT reagent, diluted to 10 ml and mixed. After 10 minutes it was filtered through whatman No.1 filter paper (Corrected for volume of fat and proteins using the formula given by Grimbleby , 1956).

$$\text{Corrected reading} = \text{Observed reading} \times \frac{100 - (P \times 0.84 + F \times 1.07)}{100}$$

where P and F are the percentages of protein and fat in the sample.

b. To 0.5 ml filtrate, added 0.5 ml IN NaOH, diluted to 10 ml and filtered.

c. Diluted 5 ml of filtrate to 10 ml which becomes the sample.

Procedure

Pipetted 5 ml each of standard, unknown and water (blank) into 25 ml test tubes. To this the following solutions were added.

a. 5 ml glycine-sodium hydroxide buffer.

b. 0.5 ml methylamine solution.

c. 0.5 ml sodium sulfite solution and mixed thoroughly.

d. Heated the tubes in a thermostatically controlled water bath at 65°C for exactly 25 minutes and cooled immediately in ice water bath for two minutes to stop the reaction.

e. Read absorbance against blank at 540 nm in UV-Vis 118 spectrophotometer. Lactose concentration was then obtained from standard curve (A standard curve was prepared by plotting absorbance against lactose concentration in standard solutions).

3.1.6 Preparation of yogurt and Bifidus yogurt

Fresh good quality cow milk, was procured from KAU Dairy plant. The fat content was standardised to 3.5 per cent. This milk was divided into six portions for different treatments.

The following materials were used for fortifying the solids in yogurt and Bifidus yogurt mix.

1. Non-fat dry milk (NDM)
2. Condensed whey
3. Lactose hydrolysed condensed whey

Depending upon the method of fortification of milk solids in the product, the following treatment groupings were done - A_1, A_2, A_3 (Yogurt); B_1, B_2 and B_3 (Bifidus yogurt). The groups A_1 and B_1 were fortified with NDM to get 16 per cent total solids in the mix and these acted as controls for the respective groups. For A_2 and B_2, fortification was done with condensed cottage cheese whey to get 16 per cent solids. The lactose hydrolysed condensed whey was used for fortifying the total solids in groups A_3 and B_3. The calculated amount of lactose hydrolysed condensed whey was added to the mix so as to get a total solids of 16 per cent and a lactose content of four per cent.

After fortification, the mixes under all groups were heated to 60°C and homogenised at 2000-2500 psi. This was followed by heat treatment of mix for 30 minutes at 85°C. The mix was then cooled to room temperature for inoculation.

The mixes under A_1, A_2 and A_3 were inoculated separately with yogurt cultures namely S. salivarius ssp. thermophilus and L. delbrueckii ssp. bulgaricus each at one per cent level to prepare yogurt. The mixes under B_1, B_2 and B_3 were inoculated with S. salivarius ssp. thermophilus. L. delbrueckii ssp. bulgaricus and B. bifidum at 1:1:10 per cent level respectively to prepare bifidus yogurt. Various treatment groupings are depicted in Fig.1.

After inoculation, mixes were thoroughly mixed and incubated at room temperature till a pH of 4.8 was attained. Both yogurt and bifidus yogurt under different treatments were then transferred to refrigerator for cooling and for further analysis.

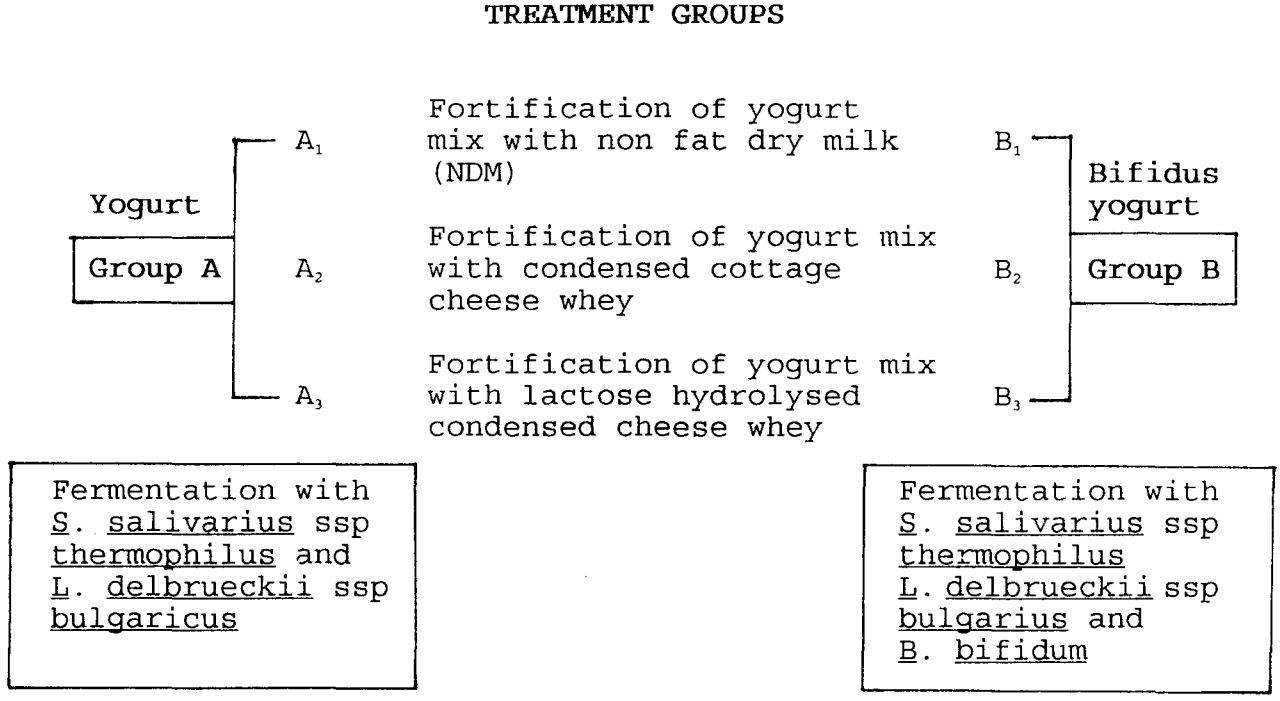
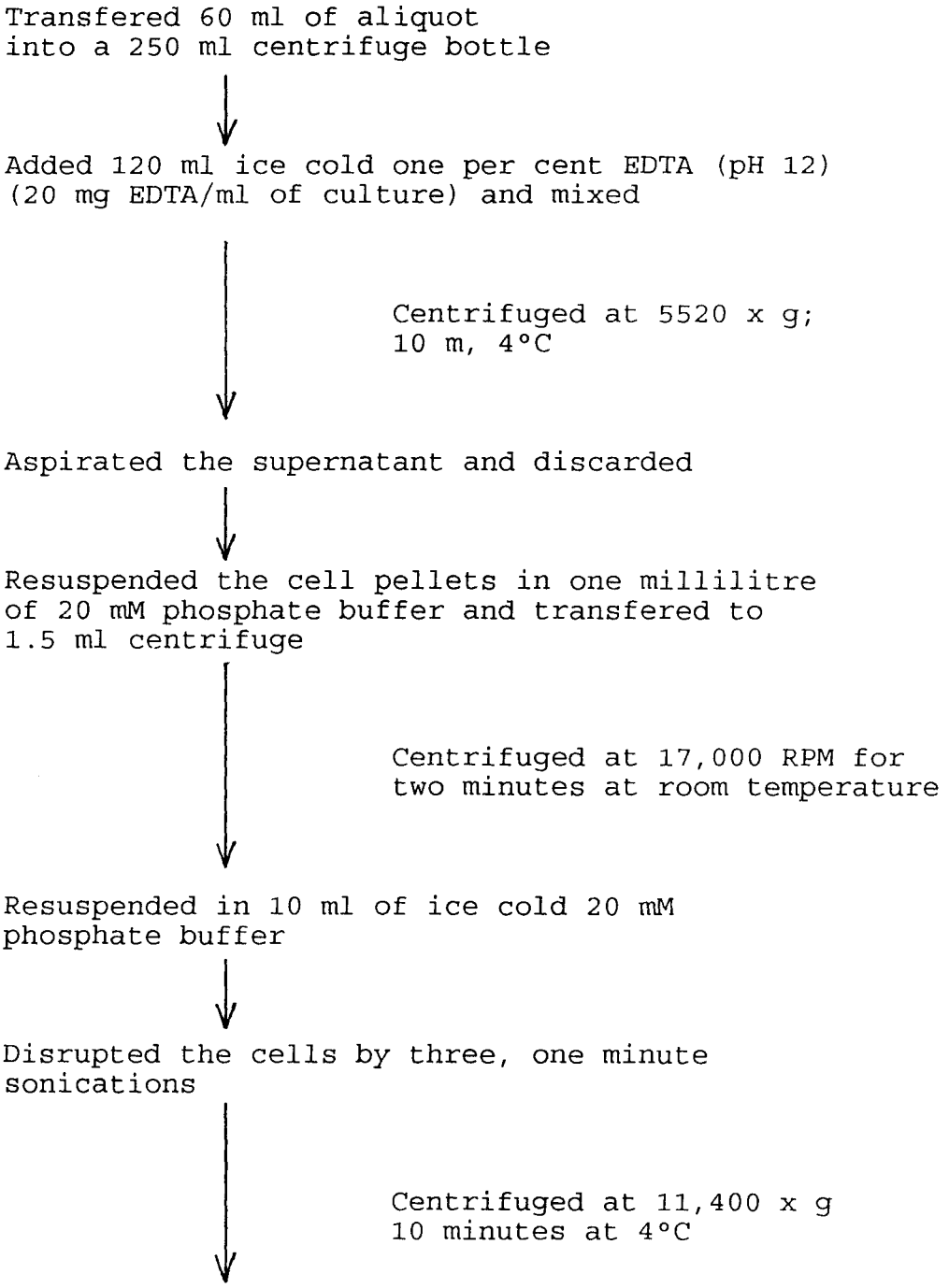


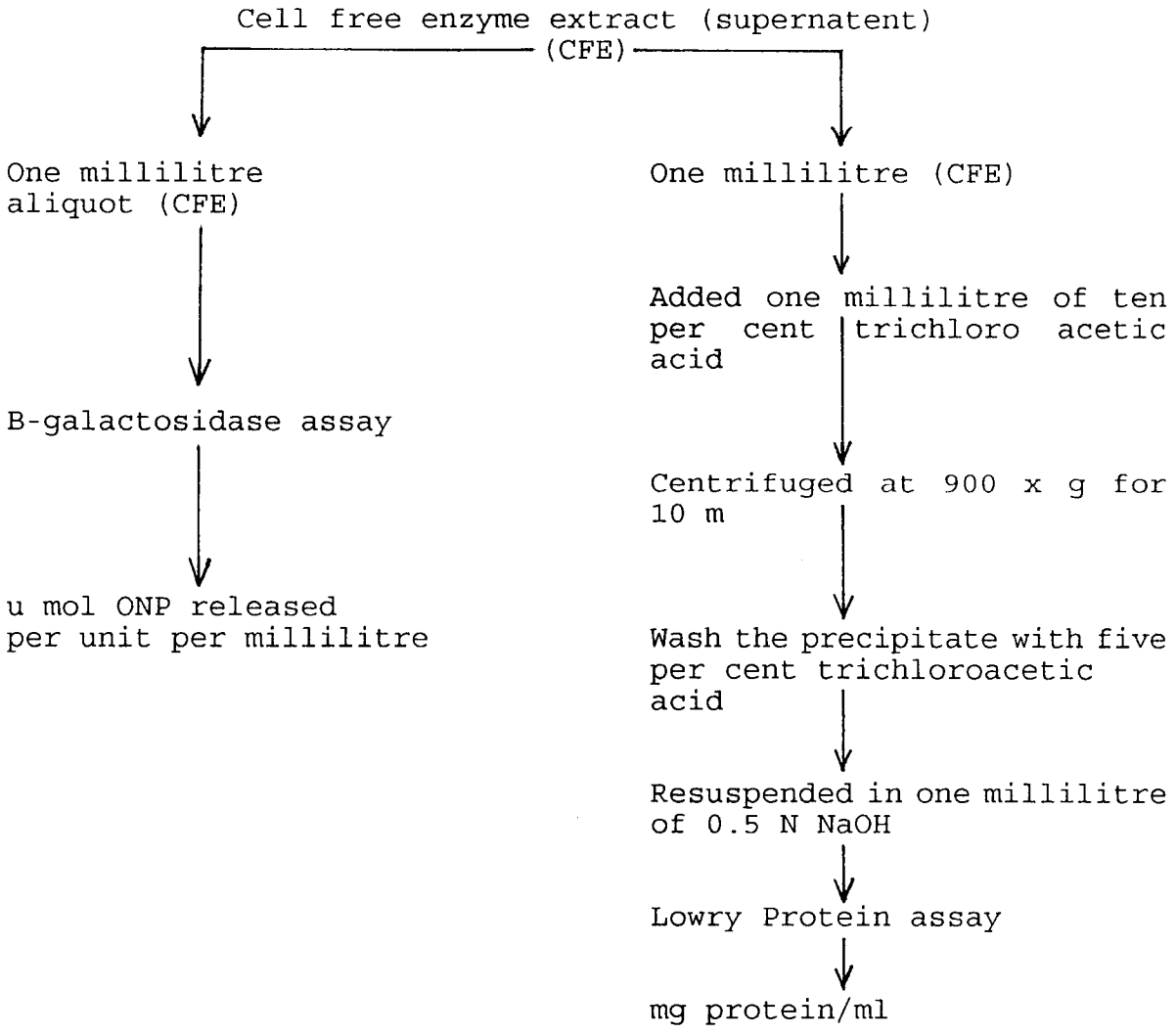
Fig.1 PLAN OF EXPERIMENT

3.2.1 B-galactosidase specific activity

B-galactosidase specific activity was measured in A_1, B_1, A_2, B_2, A_3 and B_3 as per the procedure developed by Lin *et al*. (1989b). Detailed procedure is as follows.

Flow diagram





$$\text{B-galactosidase specific activity} = \frac{\text{u mol ONP released}}{\text{mg protein}}$$

3.2.2 Preparation of cell free enzyme extract

Sixty millilitres of the sample was dissolved in 120 ml ice cold, one per cent Ethylene diamine tetrachloro acetic acid (EDTA) (pH 12) to solubilise milk proteins and centrifuged at 5520 x g at 4°C for ten minutes. Discarded the supernatant and

repeated the procedure eight times so as to get clear cell pellets.

Washed the cell pellets twice with one millilitre of potassium phosphate buffer (20 mM containing 5 mM magnesium sulphate pH 7). Resuspended the cell pellets in 10 ml phosphate buffer and sonicated for three times, one minute each, with sufficient intervals using Imeco ultrasonic sonifier. Samples were maintained on ice throughout the procedure to prevent enzyme denaturation by the heat generated during sonication. Then the cell suspension was centrifuged at 11400 g for 10 minutes to remove the cell debris and whole cells. The supernatant cell free enzyme extract was immediately assayed for β-galactosidase activity and protein.

3.2.3 β-galactosidase assay

β-galactosidase activity was measured using a chromogenic substance O-nitrophenyl-β-D-galactopyranoside (ONPG). Reaction mixtures were prepared by mixing four millilitres of thirty two micromoles of ONPG solution and one millilitre of cell free enzyme extract. This was then incubated at 37°C in a waterbath for 30 minutes. Colour development at the end of incubation was measured at 420 nm using a Spectronic-20 colorimeter. Total ONP released was calculated from the standard curve. (Standard curve was prepared by

dissolving O-Nitrophenol in minimum quantity of alcohol and making up the volume by phosphate buffer so as to give concentrations ranging from two to 48 micromoles/ml. Optical density of each concentration was measured at 420 nm. The readings were plotted in a graph).

3.2.4 Protein assay

The protein content of the cell free enzyme extract was estimated using the procedure described by Lowry et al. (1951).

Reagents

(i) Four per cent sodium carbonate in distilled water (Reagent I).

(ii) 0.5 per cent copper sulphate in one per cent potassium sodium tartarate in distilled water (Reagent II).

(iii) Alkaline copper reagent was prepared by mixing 30 millilitres of Reagent I with two millilitres of Reagent II.

(iv) 0.1 N sodium hydroxide

(v) Diluted Folin reagent (Folin reagent was diluted with equal volume of 0.1 N sodium hydroxide).

(vi) Standard protein solution (Bovine serum albumen 100 mg/ml).

One millilitre of cell free enzyme extract (CFEE) was added to one millilitre of 10 per cent trichloroacetic acid (TCA) and centrifuged at 900 x g for 10 minutes at 4°C. Discarded the supernatant. Washed the protein precipitate twice with five per cent trichloroacetic acid and resuspended in one millilitre of 0.5 N sodium hydroxide. To this added 1.5 millilitre of alkaline copper reagent. Mixed it thoroughly and allowed it to stand for ten minutes. Then transferred exactly 0.15 millilitre of diluted folins reagent using a micropipette with continuous shaking. Allowed it to stand for 30 minutes. The colour development was then read at a wavelength of 500 nm using a spectronic-20 colorimeter. Protein value was then obtained from standard curve.

(A standard curve was prepared by dissolving bovine serum albumen in distilled water to get concentrations ranging from 25 to 400 microgram per millilitre. Optical density of each concentration was measured at 500 nm. The values were plotted in a graph).

3.3.1 Hypocholesteraemic effect

A biological experiment was carried out using albino rats to find out the hypocholesteraemic effect of different treatment viz. A_1, B_1, A_2, B_2, A_3 and B_3, and whole milk.

Fifty four albino rats of uniform weight and age were selected from Small Animal Breeding Station (SABS) of the University. They were divided into nine groups of six rats each. Each group was allotted equal number of male and female rats to eliminate influence of sex.

The rats were caged individually. Clean good quality drinking water was made available all the time using dripping bottles.

3.3.2 Feeding pattern

The basal ration was having the following composition.

Groundnut cake	40 per cent
Gingely cake	10 per cent
Wheat	20 per cent
Wheat bran	28 per cent
Mineral mixture	one per cent
Multivitamin mix	one per cent

Ration required for each rat was calculated as ten per cent of the body weight and fed daily in the morning. An adaptation period of one week was given before the actual feeding trial started. The following feeding pattern was carried out. Yogurt was replaced on dry matter basis.

Group I Basal rat ration

Group II Basal rat ration + 0.5 per cent cholesterol

Group III 50 per cent basal rat ration + 50 per cent yogurt A_1 (fortification with NDM) + 0.5 per cent cholesterol

Group IV 50 per cent basal rat ration + 50 per cent Bifidus yogurt B_1 (fortification by NDM) + 0.5 per cent cholesterol

Group V 50 per cent basal rat ration + 50 per cent yogurt A_2 (fortification by condensed whey) + 0.5 per cent cholesterol

Group VI 50 per cent basal rat ration + 50 per cent Bifidus yogurt B_2 (fortification by condensed whey) + 0.5 per cent cholesterol

Group VII 50 per cent basal rat ration + 50 per cent yogurt A_3 (fortification by lactose hydrolysed condensed whey) + 0.5 per cent cholesterol

Group VIII 50 per cent basal rat ration + 50 per cent Bifidus yogurt B_3 (fortification by lactose hydrolysed condensed whey) + 0.5 per cent cholesterol

Group IX 50 per cent basal rat ration + 50 per cent whole milk (replaced on dry matter basis) + 0.5 per cent cholesterol

Groups	Type of ration	Cholesterol
I	Basal rat ration	-
II	Basal rat ration	+
III	Basal rat ration + yogurt A_1	+
IV	Basal rat ration + Bifidus yogurt B_1	+
V	Basal rat ration + yogurt A_2	+
VI	Basal rat ration + Bifidus yogurt B_2	+
VII	Basal rat ration + yogurt A_3	+
VIII	Basal rat ration + Bifidus yogurt B_3	+
IX	Basal rat ration + whole milk	+

Group I was to know the influence of basal rat ration used in this experiment on the normal cholesterol level of rats. Group II was used to measure the hypercholesteraemic effect got by the addition of cholesterol in diet. Group IX was to know effect of whole milk on cholesterol level of rats fed with 0.5 per cent cholesterol. The hypocholesteraemic effect of yogurts and bifidus yogurts under treatment groups (A_1), (B_1), (A_2), (B_2), (A_3) and (B_3) were determined from group III, IV, V, VI, VII and VIII respectively. One pre

experimental group was used to estimate normal blood cholesterol levels of rats. Weekly weight gain of rats was also recorded. Feeding trial was continued for 30 days. Experimental rats were also checked for symptoms of lactose intolerance daily.

3.3.3 Blood collection

At the end of feeding trial, rats were starved overnight. By retrobulbar puncture using heparinised capillary tube, blood from each rat was collected separately into clean dry test tubes. Bleeding was done after anaesthetising the rats with chloroform. Serum was collected and stored in a freezer till use.

Following estimations were done with the serum samples collected from rats.

(i) Total serum cholesterol

(ii) Serum triglycerides

(iii) High density lipoprotein cholesterol (HDL-cholesterol)

(iv) Low density lipoprotein cholesterol (LDL-cholesterol)

3.3.4 Estimation of total serum cholesterol

This was done based on Zak's procedure (1957).

Reagents

(i) Stock ferric chloride solution

Ferric chloride (840 mg) was dissolved in glacial acetic acid and made upto 100 millilitres with the same and stored in refrigerator.

(ii) Ferric chloride precipitating agent

The stock ferric chloride was diluted to one in ten with glacial acetic acid.

(iii) Ferric chloride blank

With the help of a clean pipette, 1.7 millilitres of stock ferric chloride was diluted to 20 ml with glacial acetic acid.

(iv) Cholesterol stock standard

100 milligram of cholesterol was accurately weighed and dissolved in 100 millilitres of glacial acetic acid and stored in freezer.

(v) Working standard

Two millilitres of stock cholesterol standard was mixed with 1.7 millilitre of stock ferric chloride solution and diluted to 20 ml with glacial acetic acid.

(vi) Final standard

This was always prepared just before estimation by mixing two millilitres of working standard and four millilitres of ferric chloride blank.

Procedure

Using a clean pipette, 0.1 ml serum was added to six millilitres of ferric chloride precipitating agent. After thorough mixing, this was filtered through a whatman No.42 filter paper. Collected the filtrate in a clean dry test tube. Three millilitres each of the filtrate final standard and ferric chloride blank were taken in separate test tubes. Two millilitres of concentrated sulphuric acid was added and mixed by gentle shaking. After cooling, colour was read in UV-Vis 118 spectrophotometer at 420 nm against blank.

3.3.7 Triglyceride estimation

Serum triglycerides were estimated by making use of commercially available enzymatic Kit (Orthodiagnostic lab).

Principle

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3 phosphate} + O_2 \xrightarrow{\text{glycerol-3-phosphate oxidase}} \text{Dihydroxy acetone phosphate} + \text{Hydrogen peroxide}$$

$$\text{Hydrogen peroxide} + \text{4-Aminoantipyrine} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoeimine (red colored)} + \text{water}$$

Triglycerides are hydrolysed by lipase and the liberated glycerol is phosphorylated in the presence of ATP to glycerol-3-phosphate. Glycerol-3-phosphate is then oxidised in the presence of glycerol phosphate oxidase (GPO) to Dihydroxyacetone phosphate and Hydrogen peroxide. Phenol and 4 aminoantipyrine then combines with hydrogen peroxide by oxidative condensation in the presence of peroxidase to produce red colored quinoeimine which shows maximum absorbance at 500 nm. Intensity of colour thus produced is directly proportional to triglyceride concentration.

Procedure

Using a micropipette, 40 microlitres of serum was added to 0.5 ml of reconstituted reagent I and 0.5 millilitre of reagent II. A standard was prepared similarly using the standard available in the kit, in place of serum. Mixed the contents thoroughly and incubated at 37°C for ten minutes. Then added two millilitres of distilled water. Colour so developed was read at 500 nm against blank.

Calculation

$$\text{Serum triglycerides (mg/decilitre)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 300$$

3.3.6 Estimation of HDL - cholesterol

Serum HDL-cholesterol was estimated by use of commercially available HDL cholesterol kit (GLAx0).

Principle

The very low density lipoprotein (VLDL) and low density lipoprotein (LDL) fractions of serum sample are precipitated using buffered polyethelene glycol (PEG-6000) and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the ester cholesterol. Then cholesterol is oxidised by cholesterol oxidase (CHO) to cholesterol-4-en-30-one and hydrogenperoxide. Hydrogen peroxide in the presence of enzyme peroxidase (POD) reacts with 4-aminoantipyrine and phenol to produce a red colored complex, whose absorbance is proportional to HDL cholesterol concentration.

Procedure

Step 1. Precipitation of VLDL and LDL

In a clean dry centrifuge tube 0.1 ml of serum and 0.1 ml of precipitating reagent I were taken. Mixed well and kept it at room temperature for five minutes. centrifuged at 2000-3000 revolutions per minute (rpm) for ten minutes to get a clear supernatent.

Step 2. Assay of HDL-cholesterol in supernatent

Fifty microlitres of water standard and serum were taken separately in test tubes. One millilitre of enzyme reagent was added to this to get blank standard and test solution respectively. Mixed well and incubated at 37°C for five minutes. Measured the absorbance at 505 nm in a UV-Vis spectrophotometr-118.

Calculation

$$\text{HDL-cholesterol (milligram percentage)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 50$$

3.3.7 LDL cholesterol

The LDL cholesterol was calculated by the following formula

$$\text{LDL cholesterol (milligrams per decilitre)} = \text{Total cholesterol} - \left[\text{HDL-cholesterol} + \frac{\text{Triglycerides}}{5}\right]$$

(Friedewald, W.J., 1972)

3.3.8 Cardiac risk factor

Cardiac risk factor was calculated by the formula put forward by Lin *et al*. (1989a).

$$\text{Cardiac risk factor} = \frac{\text{Total cholesterol}}{\text{HDL-cholesterol}}$$

3.3.9 Weight gain of rats

The daily weight gain of the rats of all experimental groups were calculated.

$$\text{Weight gain} = \frac{\text{Increase in weight in grams}}{\text{Experimentation period in days}}$$

3.4.1 Acid tolerance

As the ability to survive in human stomach and digestive system (and take up residence in intestine) is an important criteria for the selection of dietary adjuncts, an attempt was made to study the acid tolerance of the cultures used in this work as suggested by Clark *et al*. (1994).

3.4.2 Maintenance of viable cells

Pure cultures of S. salivarius ssp thermophilus, L. delbrueckii ssp bulgaricus and B. bifidum maintained in sterilised skim milk were used for the study. Cultures were made active by two consecutive transfers before each trial.

3.4.3 Preparation of solutions to simulate pH of human stomach

Solutions simulating pH of human stomach were prepared by making a 37 per cent hydrochloric acid solution whose pH was adjusted to two using 0.1 N sodium hydroxide. The solutions were dispensed in nine millilitre volumes and sterilised by autoclaving at 121°C, 15 lb pressure for 15 minutes.

3.4.4 Enumeration of organisms in pH solution

One millilitre of active stock culture containing 10^9 cfu/ml was transferred into hydrochloric acid solution having pH two. A control was maintained in distilled water. After inoculation, tubes were incubated at 37°C.

At zero, half, one and one and a half hours, serial dilutions of these were prepared in normal saline. Enumeration

of S. salivarius ssp thermophilus and L. delbrueckii ssp bulgaricus were accomplished by plating in yogurt lactic agar and incubating at 37°C for forty eight hours.

Composition of yogurt lactic agar is as follows.

Tryptone	20 g
Yeast extract	5 g
Gelatin	2 g
Glucose	5 g
Sucrose	5 g
Lactose	5 g
Sodium chloride	5 g
Sodium acetate	1.5 g
Ascorbic acid	0.5 g
Agar	15 g

Distilled water to make up 1000 ml (pH 6.8)

Enumeration of B. bifidum was achieved by incubating at 37°C under carbondioxide tension for forty eight hours in Yoshioka agar having the following composition.

Yeast extract	-	5.5 g
Peptone	-	12.5 g
Glucose	-	11 g
Potassium dihydrogen phosphate	-	0.25 g

Dipotassium hydrogen phosphate	-	0.25 g
Sodium acetate	-	10 g
Magnesium sulphate 7 H_2O	-	0.1 g
Manganese sulphate	-	5 mg
Ferrous sulphate	-	5 mg
N-acetyl-D-glucosamine	-	1 g
Sodium thioglycolate	-	1 g
Agar	-	20 g
Distilled water	-	1000 ml (pH 7.2)

3.5.1 Bile tolerance

In order to know the ability of bacteria used in this study to grow in the presence of bile, a test was done as suggested by Gilliland et al. (1984).

3.5.2 Preparation of inoculum

The cultures of all lactic acid bacteria were propagated in ten millilitres of MRS broth for twenty four hours.

Composition of MRS broth

Peptone	-	10 g
Beef extract	-	10 g
Yeast extract	-	5 g
Glucose	-	20 g

Tween-80	-	1 ml
Dipotassium hydrogen phosphate	-	2 g
Sodium acetate $3H_2O$	-	5 g
Triammonium citrate	-	2 g
Magnesium sulphate $7H_2O$	-	0.2 g
Manganese sulphate $4H_2O$	-	0.05 g

Distilled water to make up 1000 ml

The propagated cultures were centrifuged at 3000 g at 4°C for ten minutes. The supernatant was discarded and the cell pellets were resuspended in two millilitres of fresh MRS broth. This suspension was used to adjust ten millilitres of MRS broth to an optical density of 0.62-0.64 at 650 nm.

3.5.3 Preparation of test broth

MRS broth was prepared with and without 0.3 per cent oxgall. Ten millilitres were dispensed in test tubes and sterilised by autoclaving at 121°C for 15 minutes. To conduct the test one tube of each media was inoculated with 0.1 millilitre of readjusted inoculum.

After inoculation, media was incubated at 37°C in a water bath. Growth was monitored by measuring the increase in optical density at 600 nm with a spectronic-20 colorimeter. Time required to reach an optical density of 0.3 was determined.

Both treatment and control were incubated at 37°C for five hours and change in optical density for both were measured at regular intervals.

The data obtained from the experiment were tabulated and subjected to stastical analysis as per the standard procedure described by Snedecor and Cochran (1967). Completely randomised design (CRD) was used for the analysis of data regarding β-galactosidase specific activity of different treatments, total cholesterol, Triglyceride, HDL cholesterol, LDL cholesterol, cardiac risk factor and daily weight gain of rats.

Students 't' test was used to know the significance between treatments for the parameters acid tolerance and bile tolerance.

Results

Legends for the treatments are indicated on page 136a

RESULTS

The beneficial effect of lactose hydrolysed condensed whey and B. bifidum in yogurt was studied in the present experiment. The results obtained are given below.

Totally six different types of yogurt were prepared using the three cultures - S. salivarius ssp thermophilus, L. delbrueckii ssp bulgaricus and B. bifidum and using three methods for fortification of total solids in the mix, viz., skim milk powder (SMP), condensed whey and lactose hydrolysed condensed whey. A_1, A_2 and A_3 were yogurts fortified with SMP, condensed whey and lactose hydrolysed condensed whey respectively. B_1, B_2 and B_3 were bifidus yogurts fortified with SMP, condensed whey and lactose hydrolysed condensed whey respectively. The products were prepared by adopting standard procedures. These were then analysed for various parameters mentioned below:

1. β-galactosidase specific activity
2. Hypocholesteraemic effect in rats
3. Growth rate in experimental rats
4. Acid tolerance and
5. Bile tolerance of starter cultures used

4.1 β-galactosidase specific activity

The β-galactosidase specific activity of all treatment yogurts were measured in terms of number of units. One unit is defined as the number of u moles of orthonitrophenol (ONP) released/millilitre/minute. The specific activities of different treatments are given in Table 1a.

The mean specific activity of yogurt A_1 was 3.44 ± 0.08. The values ranged between 3.21 and 3.72 in this group. A highly significant decrease in the specific activity was noticed in the bifidus yogurt B_1 when compared to yogurt A_1. Mean value of this group (B_1) was 2.40 ± 0.08 with a minimum of 2.24 and a maximum of 2.65.

Yogurt A_2 and bifidus yogurt B_2 (fortified with condensed whey) showed a mean specific activity of 5.17 ± 0.05 and 2.79 ± 0.04 respectively with values ranging from 4.96 to 5.35 (A_2) and 2.64 to 2.88 (B_2). The decrease in specific activity of bifidus yogurt B_2 was highly significant when compared to A_2.

The mean specific activity of yogurt A_3 and bifidus yogurt B_3 was 3.65 ± 0.06 and 5.24 ± 0.07 with minimum and maximum values ranging from 3.50 to 3.91 and 5.01 to 5.49 respectively. When compared to A_3, increase in specific activity of B_3 was highly significant.

Table 1a. β-galactosidase specific activities of different treatments (units)

Treatment replication	Fortification with skim milk powder		Fortification with condensed whey		Fortification with lactose hydrolysed condensed whey	
	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	3.72	2.24	4.96	2.64	3.62	5.01
2	3.21	2.29	5.20	2.88	3.50	5.21
3	3.63	2.26	5.35	2.83	3.91	5.49
4	3.36	2.30	5.17	2.84	3.60	5.21
5	3.42	2.63	5.24	2.69	3.56	5.36
6	3.28	2.65	5.12	2.85	3.72	5.13
Mean	3.44	2.40	5.17	2.79	3.65	5.24
SE±	0.08	0.08	0.05	0.04	0.06	0.07

Table 1b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	5	42.57	8.51	33.71**
Within treatment	30	0.77	0.03	
Total	35	43.33		

** Highly significant

Pairwise comparison was done to find out the treatment effects (method of fortification). Yogurt cultures exhibited maximum specific activity when mix fortification was done with condensed whey (5.17 ± 0.05). When yogurt was fortified with SMP, specific activity reduced to 3.44 ± 0.08. However, specific activity increased to 3.65 ± 0.06 when lactose hydrolysed condensed whey was used for fortification. The differences observed between these treatments were highly significant.

When fortification was done with lactose hydrolysed condensed whey the increase in specific activity of bifidus yogurt (5.24 ± 0.07) was highly significant when compared to B_1 (2.40 ± 0.08) and B_2 (2.79 ± 0.04). For bifidus yogurt also differences between treatments were found to be highly significant, indicating the influence of fortification method on B-galactosidase specific activity.

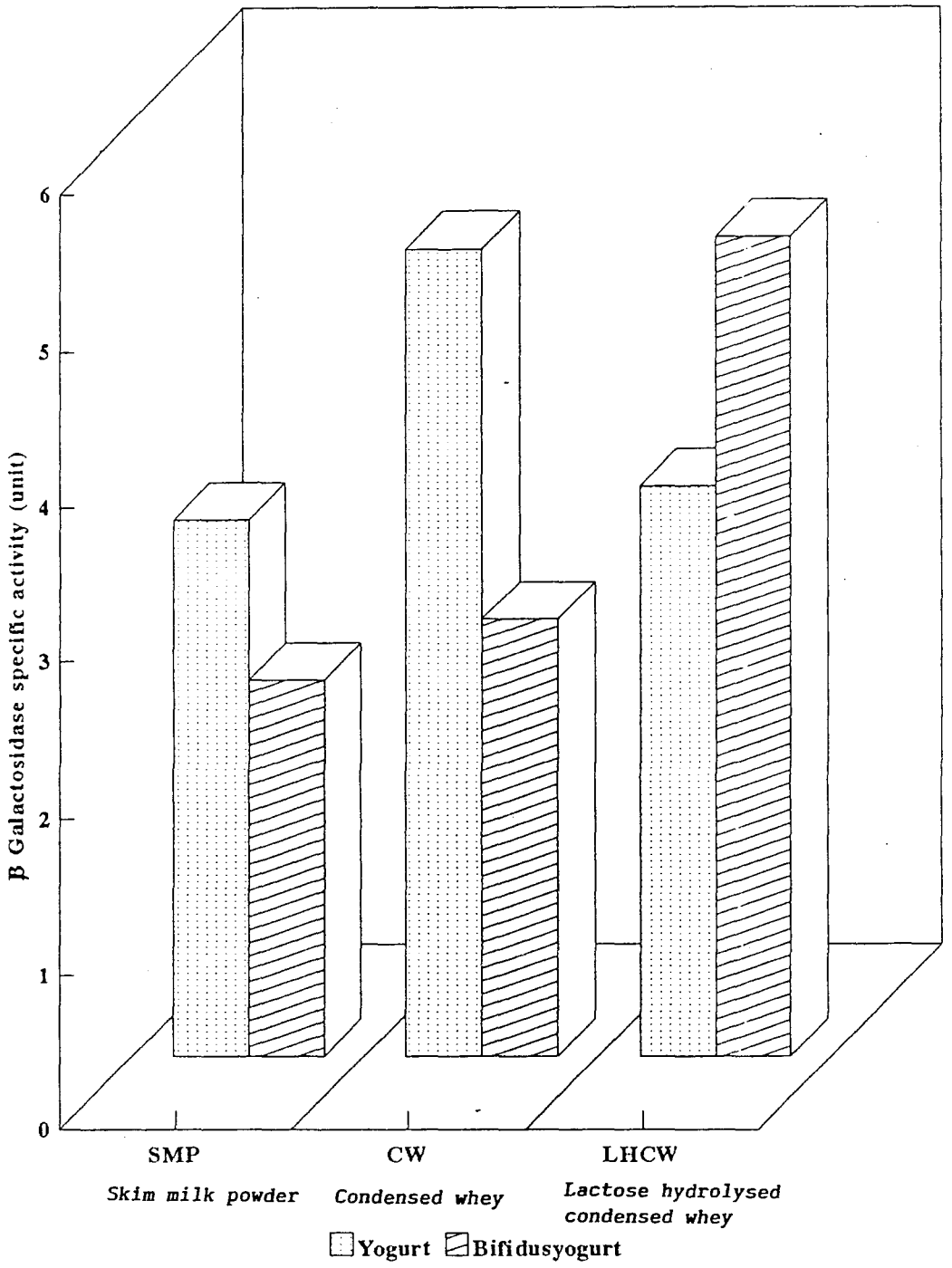


Fig.2 β GALACTOSIDASE SPECIFIC ACTIVITY OF DIFFERENT TREATMENTS (UNITS)

4.2 Hypocholesteraemic effect

The hypocholesteraemic effect of yogurt and bifidus yogurt fortified with SMP, condensed whey and lactose hydrolysed condensed whey was studied in a biological test using albino rats. One group was maintained exclusively with normal feed and another group with normal feed ± 0.5 per cent cholesterol (NFC). All other groups were fed with feed plus yogurt/bifidus yogurt prepared under different treatments plus 0.5 per cent cholesterol. As milk is also said to have some hypocholesteraemic effect, a group fed with milk in place of yogurt was also maintained. After a feeding trial of 30 days the blood collected from individual rats were analysed for the following parameters.

1. Serum total cholesterol
2. Serum triglycerides
3. Serum HDL-cholesterol

From the above values following parameters were calculated.

1. Serum LDL-cholesterol
2. Cardiac risk factor

Daily weight gain of rats in grams was also calculated.

Statistical analysis showed that no significant differences existed between the milk fed group and the group fed with feed and cholesterol, in all the parameters tested. Hence, all the treatment groups were compared with milk fed group and not with NFC group.

4.2.1 Serum total cholesterol

The mean serum total cholesterol level of rats fed with different treatment groups are given in Table 2a.

The mean total cholesterol level of rats fed with normal feed was 149.35 ± 8.64 mg/100 ml. The values ranged between 116.39 and 170.90. When cholesterol was supplemented in the feed, the mean total cholesterol level showed a highly significant increase to 172.01 ± 9.26. The maximum value obtained for this group was 196.89. This showed that a definite increase in cholesterol level was achieved when it was added in the ration. In the case of milk fed group, a reduction in cholesterol level (153.21 ± 3.72) was noticed when compared to NFC group. The values for milk fed group ranged between 140.58 and 163.52. The reduction noticed here was not significant statistically.

Table 2a. Serum total cholesterol levels of rats under different treatments (mg/100 ml)

Treatment Replication	Normal feed (NF)	Normal feed with cholesterol (NFC)	Normal feed with cholesterol plus						
			Milk	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	170.90	184.42	163.11	165.98	138.93	137.30	74.18	125.40	87.70
2	159.40	190.57	150.00	192.62	121.14	135.65	95.49	92.62	92.62
3	160.00	196.89	147.13	181.14	100.40	143.85	84.84	124.59	85.65
4	130.00	145.02	163.52	144.67	88.93	143.03	120.49	95.08	90.16
5	159.40	170.08	140.58	137.29	163.11	140.16	102.86	128.68	85.24
6	116.39	145.08	154.91	178.69	110.60	158.19	73.77	112.27	81.97
Mean	149.35	172.01	153.21	166.73	120.50	143.03	91.94	113.11	87.22
SE±	8.64	9.26	3.72	8.90	10.04	3.30	7.40	6.51	1.55

Table 2b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	8	49637.035	6204.629	20.027**
Within treatment	45	13941.551	309.812	
Total	53	63578.586		

** Highly significant

When rats were fed with yogurt A_1, the mean value obtained was 166.73 $\pm$ 8.90 and the values ranged from 137.29 to 192.62. When comparison was made with milk fed group, differences shown was not significant. The mean total cholesterol level of rats fed with bifidus yogurt B_1 showed a highly significant reduction when compared to milk fed group. Mean value of this group was 120.50 $\pm$ 10.04 with values ranging from 88.93 to 163.11.

When condensed whey was used for fortification, yogurt A_2 fed group showed a mean value of 143.03 $\pm$ 3.30 with values ranging between 135.65 and 158.19. The differences noticed when compared to milk fed group was not significant. When B_2 was fed, mean total cholesterol level obtained was 91.94 $\pm$ 7.40 ranging from 73.77 to 120.49. This group showed a highly

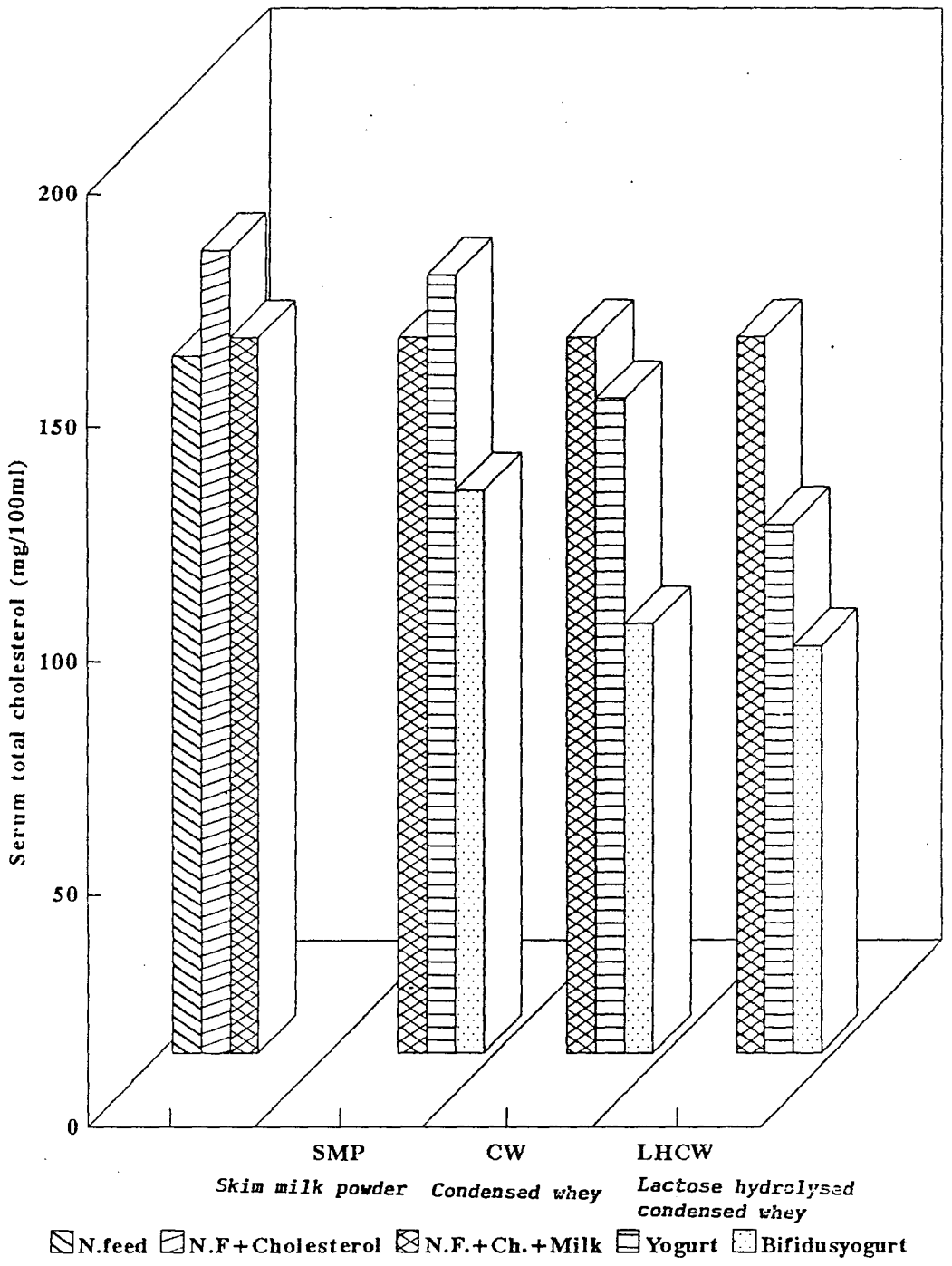


Fig.3 SERUM TOTAL CHOLESTEROL OF RATS UNDER DIFFERENT TREATMENTS (mg/100ml)

significant reduction in total cholesterol when compared to milk fed group.

The mean total cholesterol level of group fed yogurt A_3 (fortification with lactose hydrolysed condensed whey) was 113.11 ± 6.51. The values ranged between 92.62 and 128.68. A highly significant reduction was noticed in this group when compared to the group fed milk along with feed and cholesterol. The mean total cholesterol value of group fed with bifidus yogurt B_3 was 87.22 ± 1.55 with values ranging between 81.97 and 92.62. In this group also the reduction in total cholesterol noticed was highly significant when compared to milk fed group.

A pairwise comparison was done to know the effects of addition of B. bifidum in yogurt prepared by three methods of fortification. In all methods of fortification, addition of B. bifidum resulted in a significant reduction in total cholesterol level.

4.2.2 Serum triglycerides

The serum triglycerides levels of rats fed with different treatment rations are given in Table 3a.

The mean triglyceride level of rats fed with normal feed was 46.50 ± 3.00 with minimum and maximum values 39.89 and

Table 3a. Serum triglyceride level of rats under different treatments (mg/100 ml)

Treatment Replication	Normal feed (NF)	Normal feed with cholesterol (NFC)	Normal feed with cholesterol plus						
			Milk	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	52.02	65.89	47.80	78.03	112.70	108.33	75.00	90.18	84.60
2	39.89	60.69	58.00	90.17	104.50	80.50	137.50	44.17	64.42
3	39.89	45.08	68.10	78.03	65.28	95.83	81.94	53.40	73.62
4	55.30	60.69	53.30	88.44	61.11	108.33	159.70	46.01	82.82
5	52.02	65.89	69.93	90.17	75.00	99.90	124.90	99.38	58.89
6	39.89	69.30	47.80	100.50	58.00	115.28	49.90	64.42	69.93
Mean	46.50	61.26	57.49	87.56	79.43	101.36	104.82	66.26	72.38
SE±	3.00	3.51	3.97	3.48	9.57	5.03	17.24	9.55	4.13

Table 3b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	8	18912.91	2364.11	6.21**
Within treatment	45	17143.76	380.97	
Total	53	36056.67		

** Highly significant

55.30 respectively. When cholesterol was incorporated in the diet, triglyceride level elevated to 61.26 ± 3.51; with values ranging between 45.08 and 69.30. However, increase noticed in NFC group was not significant when compared to normal feed fed group. When milk was added in the diet along with feed and cholesterol, triglyceride level observed was 57.49 ± 3.97, with values ranging between 47.80 and 69.93. This value showed no significant difference, when compared to NFC group.

In group fed with yogurt A_1 (fortification with SMP), mean triglyceride level observed was 87.56 ± 3.48 with values ranging from 78.03 to 100.50. When compared to milk fed group, this group showed a highly significant increase in triglyceride level. Mean triglyceride level of rats fed with bifidus yogurt B_1 was 79.43 ± 9.57. Values of this group ranged between 58.00

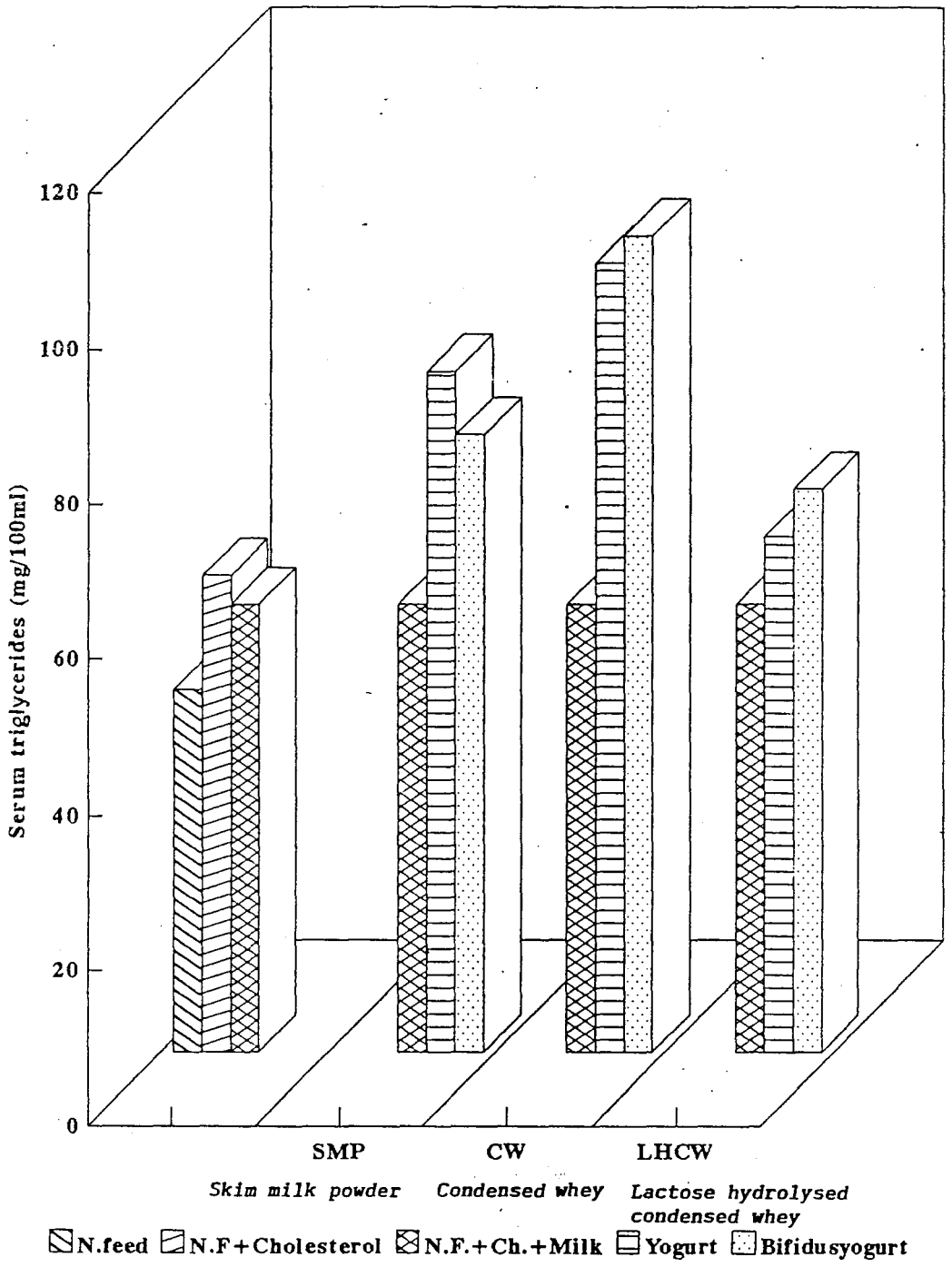


Fig.4 SERUM TRIGLYCERIDES OF RATS UNDER DIFFERENT TREATMENTS (mg/100ml)

and 112.70. When compared to milk fed group, this group showed a non-significant elevation in triglyceride level.

The mean triglyceride level of rats fed A_2 and B_2 (fortification with condensed whey) was 101.36 $\pm$ 5.03 and 104.82 $\pm$ 17.24 respectively with values ranging from 80.50 to 115.28 (A_2) and 49.90 to 159.70 (B_2). When compared to milk fed group (57.49 $\pm$ 3.97) both these groups (those groups fed A_2 and B_2) showed a highly significant increase in triglyceride level.

When compared to milk fed group rats fed yogurt A_3 and bifidus yogurt B_3 (fortification with lactose hydrolysed condensed whey) did not show significant difference. Mean triglyceride level of rats fed A_3 and B_3 were 66.26 $\pm$ 9.55 and 72.38 $\pm$ 4.13, with values ranging from 44.17 to 99.38 and 58.89 to 84.60 respectively.

Pair-wise comparison done to find out culture effects showed that, in all methods of fortification yogurt and bifidus yogurt had a similar effect on serum triglyceride level.

4.2.3 HDL-cholesterol

The serum HDL-cholesterol level of rats fed with different treatments were estimated. The values are given in Table 4a.

The mean serum HDL-cholesterol level of rats fed with normal feed was 49.91 ± 2.17 with values ranging from 41.02 to 56.92. When compared to this group, the rats fed cholesterol along with feed showed a highly significant increase in HDL-cholesterol level. Mean value of this group was 62.84 ± 4.46 with minimum and maximum values 53.07 and 84.30. When milk was added along with feed and cholesterol, mean HDL-cholesterol level was 57.93 ± 2.98, with values ranging between 54.35 and 62.90. Mean value of this group did not differ significantly, when compared to the group fed feed and cholesterol.

The mean HDL cholesterol level of group fed yogurt A_1 was 45.72 ± 4.06. Values of this group ranged between 31.28 and 56.15. When compared to milk fed group, this group showed a significant lowering of HDL-cholesterol. The mean HDL level of the group fed bifidus yogurt B_1 was 74.04 ± 8.25 with minimum and maximum values 60.10 and 111.40 respectively.

The mean HDL cholesterol level of groups fed yogurt A_2 and bifidus yogurt B_2 were 60.12 ± 5.24 and 53.96 ± 1.16

Table 4a. Serum HDL-cholesterol level of rats under different treatments (mg/100 ml)

Treatment Replication	Normal feed (NF)	Normal feed with cholesterol (NFC)	Normal feed with cholesterol plus						
			Milk	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	56.92	59.74	59.52	56.15	61.60	54.56	51.26	52.20	59.52
2	51.40	84.30	54.35	50.76	60.10	82.99	51.02	41.41	62.54
3	47.30	57.90	56.94	55.38	70.80	52.79	57.87	41.84	56.07
4	51.40	61.02	62.90	39.96	58.85	48.98	56.85	42.20	56.90
5	51.40	53.07	57.80	31.28	111.40	53.90	53.29	43.13	59.50
6	41.02	61.02	56.07	40.77	81.47	67.50	53.45	39.60	51.76
Mean	49.91	62.84	57.93	45.72	74.04	60.12	53.96	43.40	57.72
SE±	2.17	4.46	2.98	4.06	8.25	5.24	1.16	1.82	1.51

Table 4b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	8	4208.24	526.03	5.44**
Within treatment	45	4351.31	96.70	
Total	53	8559.55		

** Highly significant

respectively with values ranging from 48.98 to 82.99 (A_2) and 51.02 to 57.87 (B_2). When compared to milk fed group (57.93 $\pm$ 2.98), HDL levels of A_2 and B_2 fed groups showed no difference.

When rats were fed with yogurt, fortified with lactose hydrolysed whey (A_3), a highly significant decrease in HDL-cholesterol level was noticed when compared to milk fed group. Mean value of this group was 43.40 $\pm$ 1.82 with values ranging between 39.60 and 52.20. In group fed with B_3, mean HDL level observed was 57.72 $\pm$ 1.51 with minimum and maximum values 51.76 and 62.54 respectively. This value did not differ significantly when compared to milk fed group.

Pair-wise comparison done to find out the effects of B. bifidum revealed that, HDL level of rats fed bifidus yogurt B_1 was significantly higher when compared with the group fed

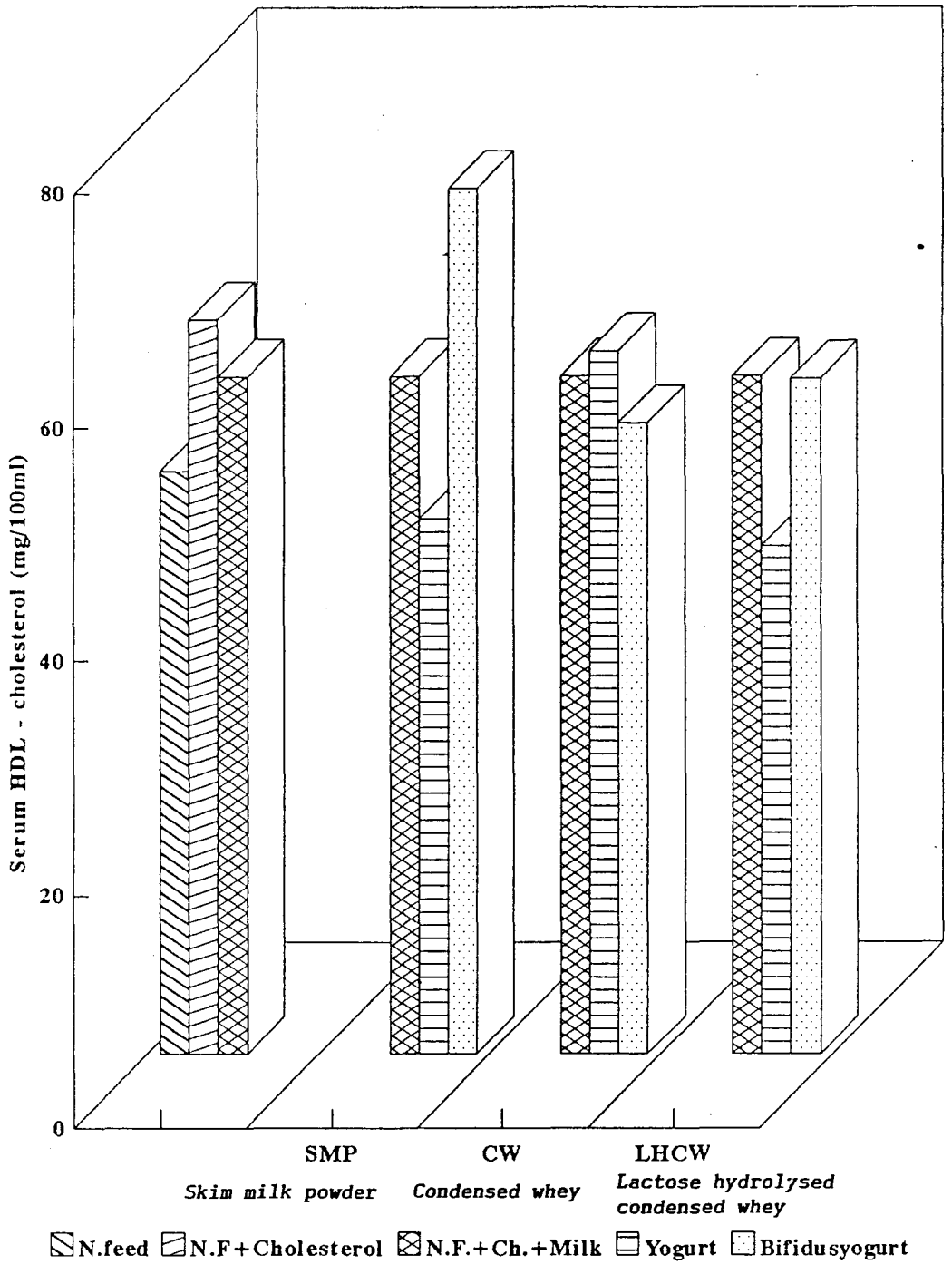


Fig.5 SERUM HDL - CHOLESTEROL OF RATS UNDER DIFFERENT TREATMENTS (mg/100ml)

with A_1. But no significant difference existed in the HDL cholesterol levels of groups fed A_2 and B_2 (fortification with condensed whey) However, HDL level of group fed bifidus yogurt B_3 was significantly higher than those fed yogurt A_3 (fortification with lactose hydrolysed condensed whey).

4.2.4 LDL-cholesterol

The serum LDL-cholesterol level of rats fed with different treatment groups were calculated. Values are presented in Table 5a.

The rats fed normal feed had a mean LDL-cholesterol level of 95.08 ± 9.57 with values ranging between 67.54 and 127.21. When cholesterol was added in the diet, LDL increased to a non-significant level of 96.92 ± 9.49 with minimum and maximum values 70.20 and 129.97. When milk was added in the diet along with feed and cholesterol, mean LDL level observed was 83.79 ± 3.87. Values of this group ranged between 68.82 and 94.03. This value did not differ significantly from the NFC group.

In group fed yogurt A_1, mean LDL-level observed was 103.50 ± 6.48 with values ranging from 87.02 to 123.83. When compared to milk fed group, LDL level of rats fed A_1 (skim milk powder) was significantly higher. LDL-cholesterol level

Table 5a. Serum LDL-cholesterol level of rats under different treatments (mg/100 ml)

Treatment Replication	Normal feed (NF)	Normal feed with cholesterol (NFC)	Normal feed with cholesterol plus						
			Milk	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	103.58	111.50	94.03	94.22	26.23	67.07	7.92	55.16	11.26
2	100.02	94.13	84.04	123.83	16.90	54.56	16.67	55.17	17.19
3	104.72	129.97	76.57	110.15	16.54	71.89	10.59	42.38	14.86
4	67.54	71.86	89.96	87.02	17.86	72.38	28.92	72.07	16.70
5	127.21	103.83	68.82	87.98	36.70	66.28	24.59	43.62	13.96
6	67.89	70.20	89.28	117.82	18.46	67.63	10.34	65.67	16.04
Mean	95.08	96.92	83.79	103.50	22.12	66.64	16.51	55.68	15.00
SE±	9.57	9.49	3.87	6.48	3.26	2.63	3.49	4.80	0.89

Table 5b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	8	62641.78	7830.22	40.18**
Within treatment	45	8768.82	194.86	
Total	53	7140.59		

** Highly significant

of group fed with bifidus yogurt B_1 showed a highly significant decrease when compared to milk fed group. Mean value of this group was 22.12 ± 3.26 with minimum and maximum values 16.54 and 36.70 respectively.

Mean LDL-cholesterol level of rats fed with yogurt A_2 and bifidus yogurt B_2 were 66.64 ± 2.63 and 16.51 ± 3.49 respectively with values ranging from 54.56 to 72.38 (A_2) and 7.92 to 28.92 (B_2). When compared to milk fed group (83.79 ± 3.87) both A_2 and B_2 fed groups showed a highly significant lowering of LDL-cholesterol level.

When yogurt and bifidus yogurt fortified with lactose hydrolysed condensed whey (A_3 and B_3) were given, a highly significant reduction in LDL-cholesterol level was evident, when compared to milk fed group (83.79 ± 3.87). Mean LDL level of

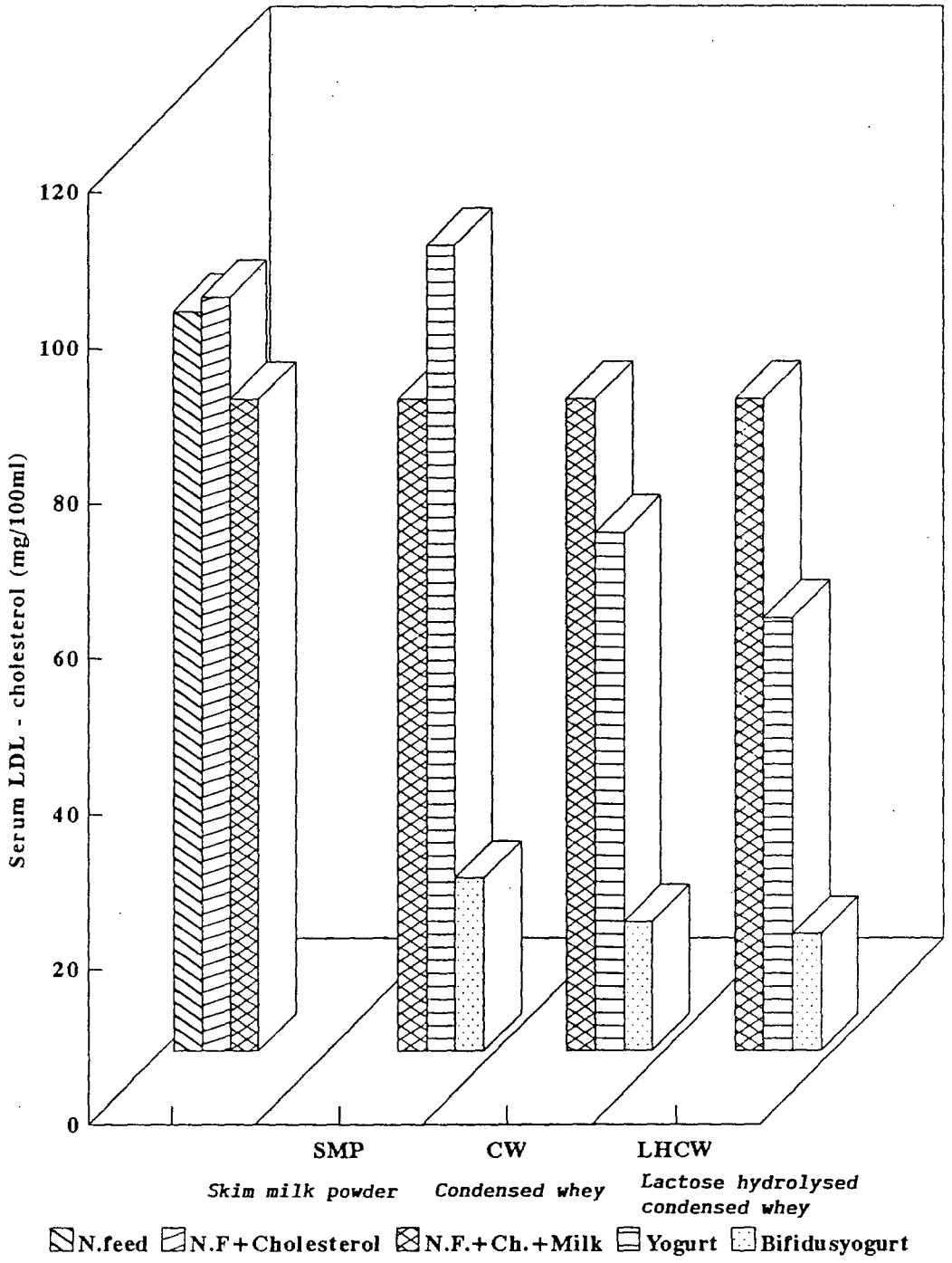


Fig.6 SERUM LDL - CHOLESTEROL OF RATS UNDER DIFFERENT TREATMENTS (mg/100ml)

groups fed A_3 and B_3 were 55.68 ± 4.8 and 15.00 ± 0.89 respectively with values ranging from 42.38 to 72.07 (A_3) and 11.26 to 17.19 (B_3).

Pair-wise comparison done to find out the effects of addition of B. bifidum to yogurt cultures showed that LDL level of rats fed with bifidus yogurts. B_1, B_2 and B_3 showed a highly significant decrease when compared to groups fed with A_1, A_2 and A_3 respectively.

4.2.5 Cardiac risk factor

The cardiac risk factor (CRF) calculated for groups fed different treatment yogurt and bifidus yogurt are given in Table 6a.

The mean CRF of rats fed with normal feed was 3.09 ± 0.07 with minimum and maximum values 2.84 and 3.38 respectively. When cholesterol was supplemented in the feed, the mean cardiac risk factor obtained was 2.78 ± 0.21. When this groups was compared to normal feed fed group, the difference was found to be statistically not significant. When milk was added in the diet along with feed and cholsesterol the mean CRF value was 2.64 ± 0.05. When compared to NFC group the difference noticed in the CRF value of milk fed group was not significant statistically.

Table 6a. Cardiac risk factor of rats under different treatments

Treatment Replication	Normal feed (NF)	Normal feed with cholesterol (NFC)	Normal feed with cholesterol plus						
			Milk	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	3.00	3.08	2.74	2.95	2.25	2.51	1.45	2.40	1.47
2	3.10	2.26	2.75	3.79	2.61	1.63	1.87	2.24	1.48
3	3.38	3.40	2.58	3.27	1.42	2.72	1.47	2.97	1.52
4	3.10	2.37	2.59	3.62	1.51	2.92	2.11	2.25	2.87
5	3.10	3.20	2.43	4.38	1.46	2.60	1.93	2.98	1.58
6	2.84	2.37	2.76	4.38	1.35	2.34	1.38	2.84	1.58
Mean	3.09	2.78	2.64	3.73	1.77	2.45	1.70	2.61	1.75
SE±	0.07	0.21	0.05	0.24	0.22	0.18	0.13	0.15	0.23

Table 6b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	8	22.27	2.78	15.29**
Within treatment	45	8.20	0.18	
Total	53	30.47		

** Highly significant

When rats were fed with yogurt A_1, highly significant increase in the CRF value was noticed when compared to milk fed group. Mean value obtained was 3.73 $\pm$ 0.24. In B_1 fed group, mean value obtained was 1.77 $\pm$ 0.22 and was found to be significantly lower than the milk fed groups.

When compared to milk fed group, mean CRF value of A_2 (fortification with condensed whey) fed group decreased to 2.45 $\pm$ 0.18. However, this decrease was found to be statistically not significant. When the rats were fed with bifidus yogurt B_2, mean CRF value observed was 1.70 $\pm$ 0.13. A highly significant reduction in CRF value of B_2 fed group was evident when compared to the group fed milk along with feed and cholesterol.

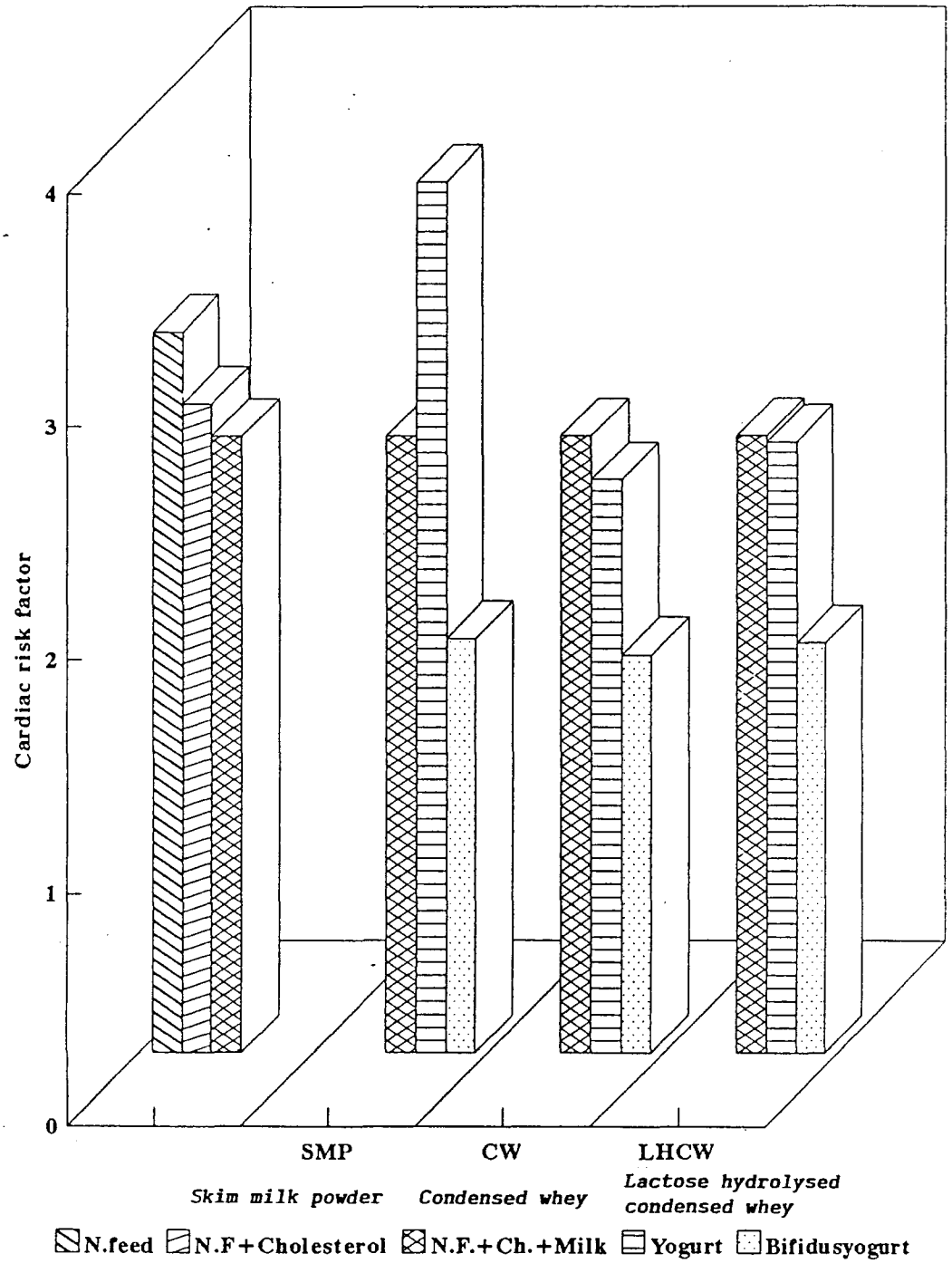


Fig.7 CARDIAC RISK FACTOR OF RATS UNDER DIFFERENT TREATMENTS

In group fed with yogurt fortified with lactose hydrolysed condensed whey A_3, the mean CRF value was 2.61 ± 0.15 and this showed no significant difference when compared with milk fed group (2.64 ± 0.05). The mean CRF value of group fed bifidus yogurt B_3 was 1.75 ± 0.23. The value observed in this group was significantly lower when compared to CRF value of milk fed group.

Pairwise comparison done to know the effects of B. bifidum as a dietary adjunct showed that, irrespective of the method of fortification, all groups fed bifidus yogurt showed a highly significant reduction in the CRF value when compared to groups fed with respective yogurts.

4.2.6 Daily weight gain

The daily weight gain of rats under different feeding groups were calculated and the values got are given in Table 7a.

The mean weight gain of rats belonging to normal feed fed group was 0.79 ± 0.27 g/day, the values ranged between 0.17 and 1.53. When cholesterol was added in the feed, a slight reduction in weight gain was seen when compared to the group under normal feed. The mean weight gain of NFC group was 0.70 ± 0.33 with values ranging between 0.13 and 1.93. Reduction in weight gain noticed in this group was statistically not

Table 7a. Daily weight gain of rats under different treatments (g/day)

Period in weeks	Normal feed (NF)	Normal feed with cholesterol (NFC)	Normal feed with cholesterol plus						
			Milk	Yogurt A_1	Bifidus yogurt B_1	Yogurt A_2	Bifidus yogurt B_2	Yogurt A_3	Bifidus yogurt B_3
1	1.53	1.93	1.12	2.92*	3.75	2.35	4.63	3.88	2.68
2	1.31	0.71	2.22	2.14	1.19	1.67	1.19	1.53	1.80
3	0.17	0.57	0.77	4.42	2.37	2.50	3.98	0.81	1.10
4	0.43	0.14	0.94	1.33	2.61	1.07	0.76	0.87	1.80
5	0.50	0.13	0.48	2.09	2.50	3.34	3.23	1.50	1.50
Mean	0.79	0.70	1.11	2.58	2.48	2.19	2.76	1.72	1.78
SE±	0.27	0.33	0.30	0.52	0.41	0.39	0.76	0.56	0.34

* Each value depicted in the table is the average of six rats

Table 7b. Analysis of variance

Source	DF	SS	MS	F value
Between treatment	8	24.37	3.05	3.01*
Within treatment	36	36.45	1.01	
Total	44	60.82		

* significant

significant. When milk was added in the diet along with feed and cholesterol, weight gain increased to 1.11 ± 0.30 with minimum and maximum values 0.48 and 2.22 respectively. However, the increase in weight gain shown in this group was found to be not significant when compared to group fed cholesterol along with feed.

The mean weight gain of rats fed with yogurt A_1 and bifidus yogurt B_1 were 2.58 ± 0.52 and 2.48 ± 0.41 g/day, with the respective values ranging from 1.33 to 4.42 and 1.19 to 3.75. When compared to milk fed group, both these groups (A_1 and B_1) showed a significant increase in weight gain.

In group fed yogurt A_2 (fortification with condensed whey) mean weight gain observed was 2.19 ± 0.39 with values ranging between 1.07 and 3.34. When compared to milk fed group,

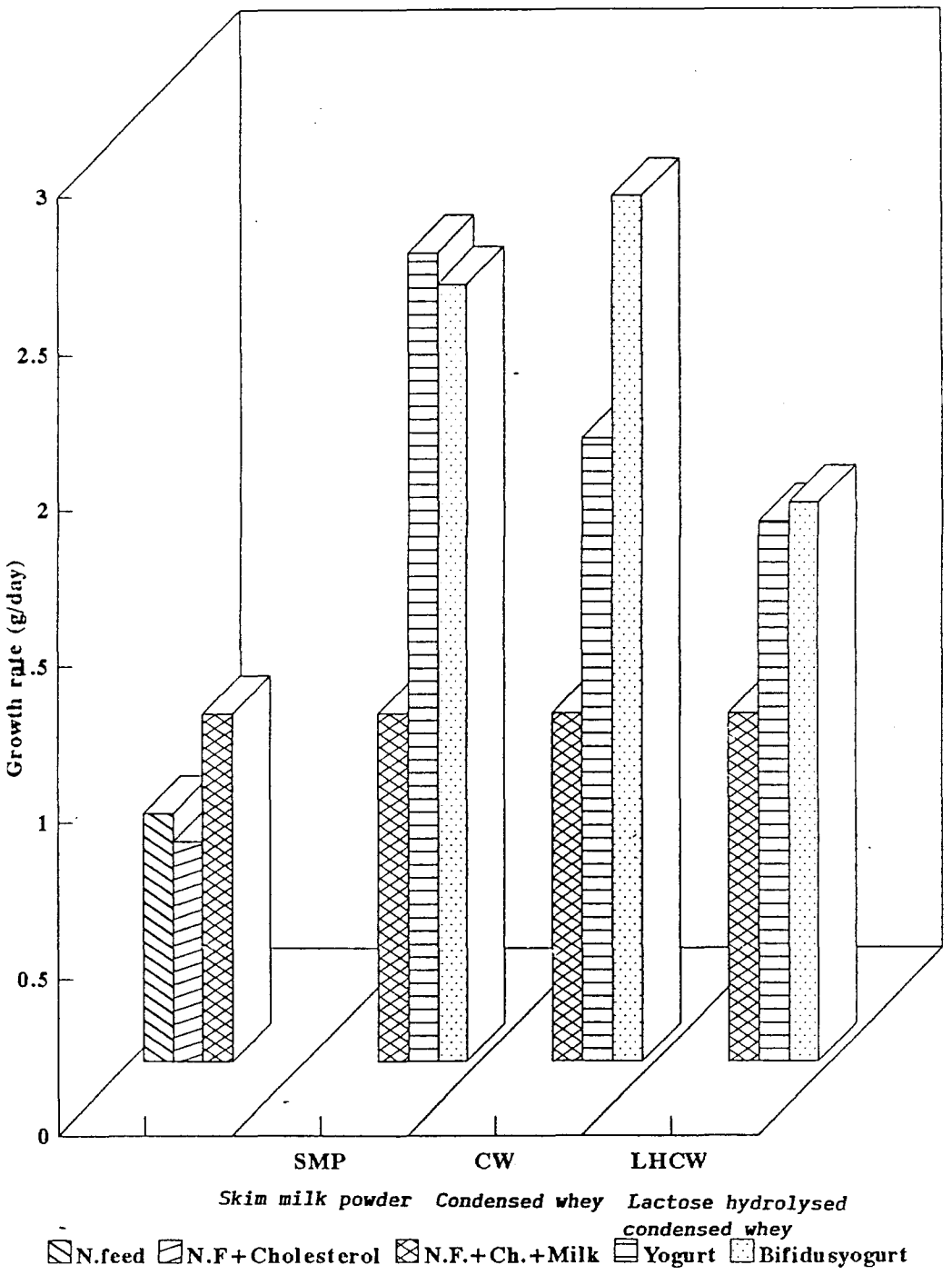


Fig.8 GROWTH RATE OF RATS UNDER DIFFERENT TREATMENTS (g/day)

increase noticed was not statistically significant. When compared to milk fed group (1.11 ± 0.30), the group fed bifidus yogurt B_2 showed a significant increase in weight gain. Mean growth rate of B_2 fed group was 2.76 ± 0.76 with values ranging from 0.76 to 4.63.

The mean weight gain of rats fed A_3 and B_3 was 1.72 ± 0.56 and 1.78 ± 0.34 respectively with values ranging from 0.81 to 3.88 (A_3) and 1.10 to 2.68 (B_3). When compared to milk fed group, both these groups (A_3 and B_3 fed groups) showed a slight increase in weight gain. But the increase was found to be statistically not significant.

A pairwise comparison was made to know the effects of addition of B. bifidum to yogurt culture. In all methods of fortification, addition of B. bifidum did not cause any significant difference in weight gain, when compared to the respective yogurt fed groups.

4.3 Acid tolerance

Acid tolerance of starter cultures used in the present study was assessed. All the three cultures were initially standardised to get 10^8 cells/ml. The standardised pure active cultures were then put into hydrochloric acid solution of pH 2. At 0 h, 30 min, 1 h, 1 h 30 min samples were taken, serially diluted and plated in a suitable selective media.

Table 8. Acid tolerance of starter bacteria after exposure to hydrochloric acid solution of pH 2

(a) S. Salvaricus ssp thermophilus cfu/ml x 10^3

Replication	Duration of exposure			
	0 h	30 min	1 h	1 h 30 min
1	N	8	2	0.05
2	"	9	2.6	0.04
3	"	9.6	2.1	0.04
4	"	7.6	4.0	0.06
5	"	8.2	3.2	0.03
6	"	9.2	3.6	0.04
7	"	8.8	4.2	0.06
8	"	8.6	4.4	0.05
Mean		8.63	3.26	0.06
SE±		0.23	0.33	0.01

Exposure time comparison - 't' values

30 min vs 1 h -- 13.16*
1 h vs 1 h 30 min -- 9.61*
30 min vs 1 h 30 min -- 36.54*

* Significant

b. L. delbrueckii ssp bulgaricus (cfu/ml) x 10^3

Replication	Duration of exposure			
	0 h	30 min	1 h	1 h 30 min
1	N	N	N	N
2	"	"	"	"
3	"	"	"	"
4	"	"	"	"
5	"	"	"	"
6	"	"	"	"
7	"	"	"	"
8	"	"	"	"
Mean	-	-	-	-
SE±				

N = No growth

c. B. bifidum (cfu/ml) x 10^3

Replication	Duration of exposure (hours)			
	0 h	30 min	1 h	1 h 30 min
1	N	40	20	12.0
2	"	60	18	9.2
3	"	50	15	13.0
4	"	38	16.2	14.0
5	"	36	15.4	11.8
6	"	35	18.2	13.6
7	"	46	15.0	10.2
8	"	42	14.0	11.2
Mean		43.38	16.48	11.88
SE±		2.97	0.73	0.59

N - No growth

Exposure time comparison - 't' values

30 min vs 1 h -- 8.80*
1 h vs 1 h 30 min -- 4.94*
30 min vs 1 h 30 min -- 10.41*

Thermophilus vs Bifidobacterium - 't' values

30 min -- 11.67*
1 h -- 16.56*
1h 30 min -- 20.18*

* Significant

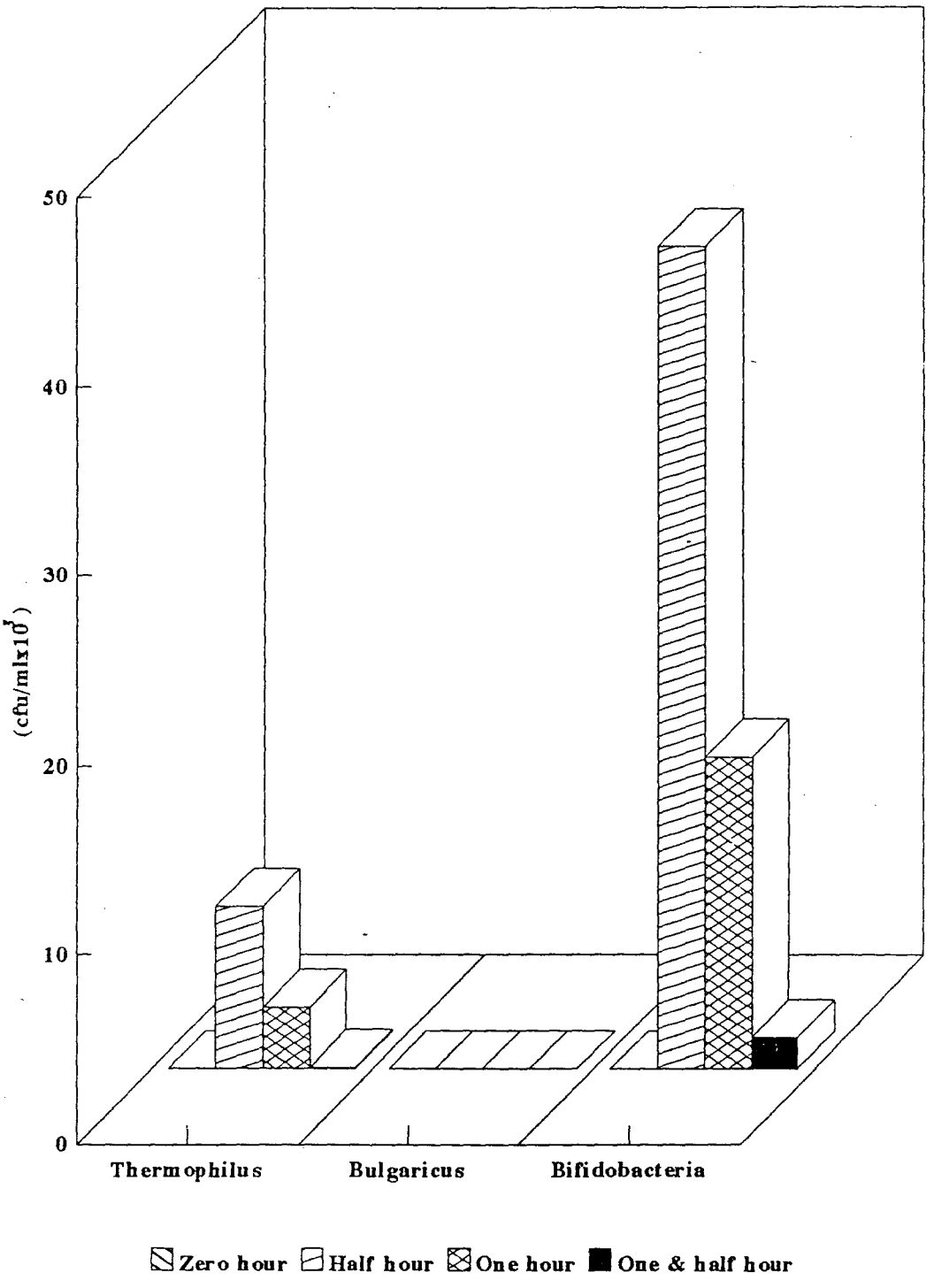


Fig.9 ACID TOLERANCE OF STARTER CULTURES USED (cfu/mlx10^3)

When plating was done at 0 hour, none of the tested organisms showed growth Mean growth shown by S. salivarius ssp thermophilus at 30 min, 1 h and 1 h 30 min were $8.63 \times 10^3 \pm 0.23$, $3.26 \times 10^3 \pm 0.33$ and $0.06 \times 10^3 \pm 0.01$ cfu/ml (Table 8a). L. delbrueckii ssp bulgaricus did not show any growth at 0 h, 30 min, 1 h and 1 h 30 min (Table 8b) whereas B. bifidum showed acid tolerance which was highly significant when compared to S. salivarius ssp thermophilus at 30 min, 1 h and 1 h 30 min. Mean growth shown by B. bifidum at 30 min, 1 h and 1 h 30 min were $43.38 \times 10^3 \pm 2.97$, $16.48 \times 10^3 \pm 0.73$ and $11.88 \times 10^3 \pm 0.59$ cfu/ml (Table 8c).

The present study revealed that, acid tolerance of B. bifidum was significantly higher than thermophilus and that L. delbrueckii ssp bulgaricus was not at all acid tolerant. Another observation made was that, irrespective of the culture used, there was a significant decrease in the number of surviving organisms as duration of exposure to pH 2 increased.

4.4 Bile tolerance

The bile tolerance of active cultures of S. salivarius ssp thermophilus, L delbrueckii ssp bulgaricus and B. bifidum were assessed by comparing their ability to grow in MRS broth with and without 0.3 per cent oxgall. Growth capacity of

Table 9. Growth of starter bacteria in MRS broth with and without 0.3 per cent oxgall

Replication	Time taken to reach an optical density of 0.3 at 600 nm (Hours minutes)					
	S. Salivarius ssp *thermophilus*		*L. delbrueckii* ssp *bulgaricus*		*B. bifidum*	
	MRS	MRS + Oxgall	MRS	MRS + Oxgall	MRS	MRS + Oxgall
1	2.25	*	2.50	-	3.00	x
2	2.30	*	2.40	-	2.45	x
3	2.20	*	2.40	-	3.05	x
4	2.30	*	2.55	-	2.55	x
5	2.20	*	2.50	-	2.50	x
6	2.25	*	2.40	-	2.45	x
7	2.20	*	2.50	-	2.50	x
8	2.20	*	2.45	-	2.55	x
	2.23		2.46		2.52	

* Optical density increased to 0.16 after five hours of incubation

\- Optical density increased to 0.14 after five hours of incubation

x Optical density increased to 0.13 after five hours of incubation

cultures were assessed by noting the time required to reach an optical density (OD) of 0.3 at 600 nm after inoculation with a standardised inoculum.

In MRS broth without oxgall, S. salivarius ssp thermophilus took a mean time of 2 h 23 min to attain an OD of 0.3 (Table 9). The time required for growth ranged between 2 h 20 min and 2 h 30 min. Mean time taken by L. delbrueckii ssp bulgaricus and B. bifidum to reach an optical density of 0.3 in MRS broth were 2 h 46 min and 2 h 52 min respectively (Table 9).

In MRS broth containing 0.3 per cent oxgall, none of the tested organisms were able to attain the standard OD of 0.3 even after five hours of incubation. At the same time, none of these organisms were completely inhibited, as shown by the slight increase in OD after five hours of incubation. Mean OD values attained at the end of five hours of incubation for S. salivarius ssp thermophilus, L. delbrueckii ssp bulgaricus and B. bifidum were 0.16, 0.14 and 0.13 respectively.

Discussion

DISCUSSION

5.1 β-galactosidase specific activity

β-galactosidase specific activity of different treatments are given in Table 1a.

The results have shown that normal yogurt (A_1) produced a specific activity of 3.44 ± 0.08 which is in agreement with the values reported by of Savaiano and Levitt, 1987 for yogurt cultures. When B. bifidum was incorporated as an additional culture the specific activity reduced to 2.40 ± 0.08. This reduction in activity could be due to competition between cultures for the limiting nutrients. When a third organism was introduced, competition between cultures might have resulted in a reduction in total cell biomass and consequently a reduction in enzyme activity. It could also be due to some inhibitory effect on yogurt cultures by B. bifidum, eventhough pairing of the organisms were done before selecting the strains. Baig and Prasad (1995) have reported that count of L. delbrueckii ssp bulgaricus decreased in yogurt in the presence of B. bifidum. Decrease in the bulgaricus count probably may be the reason for the reduced enzyme activity in bifidus yogurt.

When condensed whey was used for fortification, an abrupt increase in specific activity (5.17 ± 0.05) was noticed when compared to A_1. This indicates that the components present in condensed whey like amino acids would have stimulated the growth of both S. salivarius ssp thermophilus and L. delbrueckii ssp bulgaricus. This observation is supported by Baig and Prasad (1995), who have reported that condensed whey in yogurt can actually increase the viable count of thermophilus and bulgaricus. McDonough et al. (1976) have reported that bioavailability of nutrients in whey protein concentrate was higher. Broome et al. (1982) observed that, incorporation of whey proteins in yogurt stimulated S. thermophilus more then L. bulgaricus. Savaiano and Levitt (1987) reported that S. thermophilus had approximately three times more lactase activity than L. bulgaricus. All these observations support the enhanced enzyme activity of yogurt A_2.

When bifidus yogurt was prepared with condensed whey (B_2) a highly significant increase in specific activity was noticed (2.79 ± 0.04) when compared to B_1 (2.40 ± 0.08). This clearly shows the effect of addition of condensed whey. Some of the limiting aminoacids available in condensed whey might have contributed to the growth of all organisms. Baig and Prasad (1995) have shown that presence of condensed whey stimulated growth of B. bifidum. They also reported that inhibitary effect

of B. bifidum on L. delbrueckii ssp bulgaricus could be alleviated by supplementation of condensed whey. All these explains the reasons for the increased specific activity of B_2 when compared to B_1.

However, when compared to A_2, B_2 showed less activity. The reports of Broome et al. (1982) have shown that condensed whey stimulated S. thermophilus more than L. bulgaricus. Report of Baig and Prasad (1995) have shown that it is stimulatory to B. bifidum also. So it can be presumed that thermophilus and B. bifidum might have multiplied at a faster rate dominating L. bulgaricus. Thus increased specific activity in A_2 is presumed to be due to more number of thermophilus cells in the total cell mass.

In treatment 3, where fortification was done with lactose hydrolysed condensed whey bifidus yogurt B_3 showed significantly higher specific activity (5.24 $\pm$ 0.07) than A_3 (3.65 $\pm$ 0.06). The results have also shown, a substantial increase in activity in A_3 B_3 when compared to A_1 A_2 and B_1 B_2 respectively. This trend in B-galactosidase specific activity is depicted in Fig.2. This indicates that incorporation of lactose hydrolysed condensed whey has got a beneficial effect in increasing B-galactosidase activity of bifidus yogurt.

The increased activity in B_3 when compared to A_3 may be due to combined effect of B. bifidum and yogurt cultures. This could also be explained by the fact that lactose hydrolysis results in production of bifidus factors as reported by Smart et al. (1992). In addition, hydrolysis would have resulted in easily available nutrients.

5.2.1 Serum total cholesterol

The mean serum total cholesterol level of rats under different treatments are given in Table 2a.

When cholesterol was added in the diet, increase in serum cholesterol (172.01 ± 9.26) was highly significant when compared to group fed with normal feed (149.35 ± 8.64). This has shown that incorporation of cholesterol in diet definitely elevated total serum cholesterol level. Similar report has been given by Eleven (1995). He noticed a rise in serum cholesterol level in rats when cholesterol was added along with feed.

Addition of milk along with feed and cholesterol, reduced the serum total cholesterol level (153.21 ± 3.72) when compared to normal feed plus cholesterol group, but reduction was not significant, indicating that whole milk is not hypocholesteraemic. This observation is supported by Mann (1977) who reported that fresh whole milk at the rate of two

litres daily did not statistically affect cholesteraemia in human volunteers.

When compared to milk fed group (153.21 ± 3.72), the group fed yogurt A_1, showed a slight increase in total cholesterol level (166.73 ± 8.90). However, this increase was not significant statistically suggesting that yogurt A_1 is not having any hypocholesteraemic effect. This observation is in line with the reports of Payens et al. (1976). They did not find hypocholesteraemic effects in human subjects when large quantities of yogurt was introduced as a part of their diet. Similar reports have been published by Rossouw et al. (1981).

When compared to milk fed group, serum total cholesterol level of rats fed with yogurt fortified with condensed whey (A_2) reduced to 143.03 ± 3.30. But this reduction was statistically not significant.

A significant reduction in total cholesterol level (113.11 ± 6.51) was evident in group fed yogurt A_3 when compared to milk fed group. As the fortification of A_3 was done by lactose hydrolysed condensed whey, yogurt cultures would have multiplied more rapidly presumably due to the presence of easily digestible form of carbohydrates. Role of microorganisms in cholesterol destruction had been reported by Danielsson and Gustafsson (1959). So decreased cholesterol level in A_3 fed

groups may be due to increased number of thermophilus and bulgaricus in yogurt A_3. The bile salt deconjugating ability of yogurt cultures might have also played a role in lowering serum total cholesterol level (Beena and Prasad, 1995).

Bifidus yogurt, B_1 B_2 and B_3, irrespective of method of fortification of solids, when fed to rats showed a highly significant decrease in total cholesterol when compared to milk fed group. This observation is in par with Homma (1988), who reported a lowering of serum cholesterol levels in rats fed orally with bifidobacteria presumably due to inhibition of hydroxymethyl glutaryl CoA reductase.

The hypocholesteraemic effect of Bifidus yogurt was found to be superior when compared to yogurt under all treatments. The effect on serum total cholestrol of rats fed with yogurts/bifidus yogurts is depicted in Fig.3. The pronounced decrease in serum cholesterol level of rats when fed with bifidus yogurt may be due to the combined effects of yogurt culture and B. bifidum. Eyssen (1973) has reported that bifidobacteria were capable of deconjugating bile salts making it more important in controlling serum cholesterol levels. Similar observations for B. bifidum was made by Beena and Prasad (1995). Reports of Chikai et al. (1987) also supports this observation. They suggested that deconjugated free bile acids

would adhere to bacteria or dietary fibres therby enhancing excretion of bile acids. This action might trigger the feed back mechanism that regulate the hepatic cholesterol synthesis and subsequent transformation into bile acids, thus reducing cholesterol concentration.

All the rats given yogurt and bifidusyogurt except those fed with yogurt A_1 (fortification with SMP) showed a reduction in serum total cholesterol level when compared to milk fed group. This observation is in par with the reports of Pulusami and Rao (1983) who postulated that hypocholesteraemic effect of fermented milks might be due to an increased excretion of cholesterol or its metabolites and inhibition of cholesterol biosynthesis by metabolites produced by lactic cultures.

To know the effect of different methods of fortification, a comparison was made. It was observed that those rats fed with A_2, B_2, A_3 and B_3 had a lower level of serum total cholesterol when compared to their respective control (A_1 and B_1) Yogurt prepared using lactose hydrolysed condensed whey had a more pronounced effect in lowering total cholesterol. This could be due to the faster multiplication and consequent increase in count of all the organisms used for its preparation. This can be correlated with the result of B-galactosidase specific activity wherein yogurt and bifidus yogurt under

treatment 2 and 3 (condensed whey and lactose hydrolysed condensed whey) showed elevated enzyme activity.

Beena and Prasad (1995) observed that L. dilbrueckii ssp bulgaricus, B. bifidum and S. salivarius ssp thermophilus have the ability to deconjugate bile salts. So it can be assumed that, due to the larger number of organisms, more bile salt would have deconjugated making the cholesterol unavailable for absorption, in group fed yogurt and bifidus yogurt prepared using condensed whey and lactose hydrolysed condensed whey.

5.2.2 Serum triglycerides

The mean serum triglyceride level of rats under different treatments is shown in Table 3a.

Feeding normal ration to rats resulted in a mean triglyceride level of 46.50 ± 3 mg/100 ml. When cholesterol was added in the diet, triglyceride level shot up to 61.26 ± 3.51 but this increase was found to be statistically not significant. When compared to NFC group, triglyceride level of milk fed group was not affected significantly.

In general, an increase in triglyceride level was noticed in all groups fed with yogurt and bifidus yogurt. This trend in triglyceride level is depicted in Fig.4.

In rats fed with yogurt fortified with SMP and condensed whey (A_1 and A_2), there was a significant increase in triglyceride level (87.56 ± 3.48 and 101.36 ± 5.03) when compared to milk fed group (57.49 ± 3.97). In group fed with yogurt A_3 (fortification with hydrolysed condensed whey), the increase in triglyceride level was not significant when compared to milk fed group.

Rossouw et al. (1994) have reported that even low fat yogurt can raise the serum lipids and lipoproteins temporarily in school boys. Windmuller (1963) has reported that plasma triglycerides were depressed in rats fed with orotic acid showing that orotic acid is capable of reducing serum triglyceride level. Ahmed et al. (1979) observed that orotic acid decreased by 15-53 per cent in yogurt prepared using different strains of S. thermophilus and L. bulgaricus. Thus high level of triglycerides in yogurt fed group could be due to low level of orotic acid in the product. According to Morales and Chandan (1982) triglycerides are sparingly soluble by lipases of lactic acid bacteria so that these are absorbed as such elevating serum triglycerides. All these factors might have contributed to the elevated level of triglycerides in the serum of experimental rats fed with yogurt.

Though an increase in triglyceride level was noticed in all rats fed with bifidus yogurt, a significant increase when

compared to milk fed group was seen only in rats given bifidus yogurt B_2 fortified with condensed whey (104.82 ± 17.24).

Same trend was noticed in group fed with yogurt A_2 (fortification with condensed whey). This could probably be due to the presence of condensed whey in the product. The orotic acid metabolism by bifidobacteria might have also contributed to this effect in group fed with bifidus yogurt B_2.

An increase in serum triglyceride level with a concomitant decrease in total cholesterol was observed by Williams and McDonald (1982) in female baboons fed with a diet containing 80 per cent by weight of hydrolysed lactose. Similar observation was made in the present study in rate fed with yogurt and bifidus yogurt and bifidus yogurt fotified with lactos hydrolysed condensed whey (A_3 and B_3).

From the pair-wise comparison done, it was seen that in all methods of fortification, influence of yogurt and bifidus yogurt cultures on serum triglycerides were not different.

5.2.3 Serum HDL-cholesterol

The serum HDL-cholesterol level of rats under different treatments is given in Table 4a.

The rats fed with normal feed and cholesterol showed a significant elevation in HDL-cholesterol level when compared to the group fed with normal feed. This is in agreement with the result obtained by Eleven (1995) who also noticed an elevation in HDL-cholesterol level in serum of rats, when cholesterol was added in the feed.

Kruski and Narayana (1976) have also reported that when chickens were fed with cholesterol along with feed, HDL-cholesterol increased when compared to control. When milk was given along with feed and cholesterol, HDL-level showed no significant change when compared to NFC group.

In rats fed with yogurt A_1 and A_3 a significant decrease in HDL cholesterol level was noticed when compared to milk fed group. However, in rats fed with yogurt A_2, HDL-cholesterol level was almost similar to that of milk fed group. The lower HDL-cholesterol level of group fed with yogurt fortified with lactose hydrolysed condensed whey could be attributed to the lower level of total cholesterol in this group. This is in agreement with the findings of Eleven (1995). However, the lower HDL-cholesterol level inspite of higher serum total cholesterol in group fed with yogurt A_1 could not be explained.

A significant increase in the HDL-cholesterol level was noticed in rats fed with bifidus yogurt B_1 (74.04 ± 8.25) when compared to milk fed group. The HDL-cholesterol level of groups fed with B_2 and B_3 did not differed significantly from that of milk fed group. This could be due to lower serum total cholesterol in these groups. The observations made by Eleven (1995) supports this. The HDL level of B_2 and B_3 fed groups did not differ from that of milk fed group. Hence a highly significant increase in HDL-cholesterol level of B_1 fed group is thought to be due to comparatively higher serum total cholesterol.

From the pairwise comparison done to find out the effect of addition of B. bifidum, it was seen that, a significant increase in HDL level was seen only in groups of rats which consumed bifidus yogurt B_1. This is clearly presented in Fig.5.

5.2.4 Serum LDL-cholesterol

The serum LDL-cholesterol level of rats under different treatments is presented in Table 5a.

Elevation of LDL-cholesterol level in group fed with feed and cholesterol was not significant when compared to normal feed fed group. When milk was supplemented in the diet along

with feed and cholesterol, LDL-level did not change significantly when compared to NFC group.

The mean LDL-cholesterol level of groups fed with yogurt A_1 (103.50 $\pm$ 6.48) showed a highly significant increase when compared to milk fed group (83.79 $\pm$ 3.87). In this group, cholesterol added in the ration might not have metabolised and hence serum total cholesterol level remained elevated. Robbins and Cotran (1981) reported that 70 per cent of total cholesterol is contained in LDL. So the increased LDL-level in A_1 fed group may be assumed to be due to higher level of total cholesterol in this group.

The rats fed with yogurts A_2 and A_3 showed a highly significant reduction in serum LDL-cholesterol (66.64 $\pm$ 2.63 and 55.68 $\pm$ 4.80) when compared to milk fed group.

Rasic et al. (1992) have reported that yogurt cultures could assimilate good amount of cholesterol. In the presence of whey proteins, yogurt cultures would have multiplied more (Baig and Prasad, 1995), thereby enhancing their bile salt deconjugation capacity (Beena and Prasad, 1995) and further lowering the serum total cholesterol. As 70 per cent of total cholesterol is contained in LDL (Robbins and Cotran, 1982), the lower LDL-cholesterol level of A_2 and A_3 fed group could be due to its lower serum total cholesterol.

Irrespective of the method of fortification, all bifidus yogurt fed group showed a highly significant decrease in LDL-cholesterol level when compared to milk fed group. The drastic lowering of LDL level could be due to the combined action of yogurt cultures and B. bifidum.

Homma (1988) reported the serum cholesterol lowering ability of orally fed bifidobacteria in rats. Bile salt deconjugation capacity of B. bifidum (Eyssen, 1973) added as dietary adjunct would have further raised the total bile salt deconjugation capacity of cultures, thereby causing a pronounced lowering of serum total cholesterol.

From the pairwise comparison done to find out the effect of addition of B. bifidum, it was observed that, all the bifidus yogurt fed group had a significantly lower LDL-cholesterol when compared to groups fed with respective yogurt as shown in Fig.6. This observation clearly indicate the LDL lowering capacity of B. bifidum. Serum total cholesterol lowering capacity (Homma, 1988) and bile salt deconjugation capacity of B. bifidum (Eyssen, 1993; Beena and Prasad, 1995) supports this finding.

5.2.5 Cardiac risk factor

Cardiac risk factor is the ratio between total cholesterol and HDL-cholesterol. It gives an idea about how

likely a person is going to suffer from cardiac problems due to cholesterol. Higher the CRF value, greater are the chances for heart ailments. Mean CRF value of rats under different treatments is given in Table 6a.

The mean CRF value of rats fed with normal feed was 3.09 ± 0.07 whereas in group fed cholesterol along with feed mean value was 2.78 ± 0.21. Though a little decrease was noticed in the latter group when compared to former, difference was found to be statistically not significant. When the milk fed group was compared with NFC group, CRF value showed no change. The reduction in CRF value of NFC and milk fed group is actually due to the increase in HDL-cholesterol level, eventhough hypercholesteraemia was achieved in both groups.

In the group fed with yogurt A_1, a highly significant increase in CRF value was evident when compared to milk fed group. This increase in CRF value is actually due to higher total cholesterol level in group fed yogurt A_1 (166.73 ± 8.90). In A_2 and A_3 fed groups, though a reduction in CRF value was noticed, it was found to be statistically not significant.

Irrespective of the method of fortification, all bifidus yogurt fed group showed a highly significant decrease in CRF value when compared to milk fed group (2.64 ± 0.05). The CRF

value of groups fed with bifidus yogurt B_1, B_2 and B_3 were 1.77 $\pm$ 0.22, 1.70 $\pm$ 0.13 and 1.75 $\pm$ 0.23 respectively. From the pairwise comparison, it was observed that, addition of B. bifidum to yogurt cultures had a definite influence in reducing CRF value. This trend is clearly depicted in Fig.7.

The drastic reduction in CRF values of bifidus yogurt fed groups is assumed to be due to the combined effect of yogurt cultures and B. bifidum. Homma (1988) had reported the ability of orally administered bifidobacteria to lower serum cholesterol in rats. Walker and Gilliland (1993) found a positive correlation between bile salt deconjugation and cholesterol assimilation. The bile salt deconjugating capacity of bifidobacteria make this organism more important in controlling serum cholesterol. All these supports the present finding of reduced CRF value in bifidus yogurt fed groups.

The presence of condensed whey and lactose hydrolysed condensed whey also might have contributed to the reduction of CRF value by stimulating the growth of yogurt cultures, thereby increasing the deconjugation ability. Hence A_2 and A_3 fed group had a significantly low CRF value when compared to A_1 fed group. However, between treatments all bifidus yogurts had a similar effect on CRF value.

5.2.6 Daily weight gain

The daily weight gain of rats under different treatments is presented in Table 7a.

Rats given normal feed showed an average weight gain of 0.79 ± 0.27 g/day. The decrease in weight gain noticed when cholesterol was incorporated in the diet, was not significant when compared to normal feed fed group. Weight gain in milk fed group was in par with that of NFC group.

Eventhough all groups fed with yogurt and bifidus yogurt showed an increased weight gain when compared to milk fed group, significant increase was noticed only in rats fed with yogurt A_1, bifidus yogurt B_1 and bifidus yogurt B_2.

Daily weight gain of rats fed with yogurt A_1 was significantly higher (2.58 ± 0.52) when compared to group fed with milk. This observation is in line with the findings of Eleven (1995) who also observed an increased daily weight gain in yogurt fed group when compared to control. The present finding is also supported by Renner (1986) who reported that yogurt consumption promoted growth rate as a result of improved lactose digestion and mineral absorption besides providing thiamine riboflavin niacin and folic acid.

A significant increase in weight gain was noticed in groups fed with bifidus yogurt B_1 and B_2 as shown in Fig.8. Incorporation of B. bifidum to yogurt culture was found to be beneficial. This observation is supported by Goodenough and Kleyn (1976). They suggested that addition of B. bifidum to yogurt culture resulted in greater changes in nitrogen compounds and the increased proteolytic activity improved protein digestibility. The better growth performance of groups fed with B_1 and B_2 may be assumed to be due to increased bioavailability in bifidus yogurt. Weight gain in B_3 fed group did not show significant difference when compared to milk fed group.

Daily weight gain noticed in rats given yogurt and bifidus yogurt fortified with lactose hydrolysed condensed whey (A_3 and B_3) was less when compared to respective treatments. This could be due to the influence of lactose hydrolysed condensed whey. According to Paul and Mathur (1993), loss of available lysine and increase in hydroxy methyl furfural occurring during lactose hydrolysis reduced the nutritive value of lactose hydrolysed formulas. This may be the reason for comparatively lower weight gain in rats fed with A_3 and B_3.

Pair-wise comparison done showed that neither the addition of B. bifidum nor the different methods of fortification affected the growth performance of rats significantly.

5.3 Acid tolerance

Acid tolerance of lactic cultures used in the present study was assessed by plating the pure and active cultures in their selective media, after serial dilution in normal saline at 0 h, 30 min, 1 h and 1 h 30 min hours of exposure to hydrochloric acid of pH 2. The results are depicted in Fig.9.

None of the organisms showed growth at 0 hour. This may be due to the acid shock caused to the organisms when they were suddenly exposed to an extreme environment of pH 2. However, after an adaptation period of 30 min, S. salivarius ssp thermophilus and B. bifidum showed a significant growth $8.63 \times 10^3 \pm 0.23$ and $43.38 \times 10^3 \pm 2.97$ respectively. Both thermophilus and bifidobacteria showed growth even at 1 h 30 min of exposure.

Kolars et al. (1984) and Savaiano et al. (1984) reported that S. thermophilus and L. bulgaricus are resistant to gastric acidity and consequently are alive and active in human intestine though they are not natural inhabitants of it. In vitro study conducted here showed that S. salivarius ssp. thermophilus was acid tolerant since considerable number of organisms survived even after 1h 30 min of exposure to pH 2 ($0.06 \times 10^3 \pm 0.01$).

Gilliland (1985) reported that yogurt culture cannot survive or grow in intestinal tract presumably because of acid sensitivity and bile intolerance. However, in the present study S. thermophilus showed some amount of resistance to acid probably due to the differences in the strains of organisms used in the study. The acid sensitivity of L. bulgaricus noticed in this study is agreeing with the report of Gilliland (1985).

B. bifidum used here was found to be acid tolerant. Though Ventling and Mistry (1993) reported that a pH 5.5 is the lowest pH B. bifidum could withstand, present study revealed that considerable member of B. bifidum could survive even at 1 h 30 min of exposure to pH 2. Reason may be strain variation. Berrada et al. (1991) reported that resistance to gastric acidity vary between strains of bifidobacteria.

Martini et al. (1985) reported that gastric pH remained >2.7 for three hours following a yogurt meal. It was also reported that gastric transit time was 90 minutes (Berrada et al., 1991). Reports of Grimaud et al. (1993) suggested that milk containing bifidobacteria can reduce intestinal transit time.

Under in vivo conditions, gastric pH immediately after product (yogurt) consumption would be higher. Due to this

organisms would be expected to be exposed to actual gastric acidity very slowly. In *in vitro* study conducted here organisms were exposed all on a sudden to the extreme pH of 2 for 1 h 30 min. Since the product was expected to remain in the stomach for a shorter period, chances of exposure to extreme pH were meagre.

Thus in the present study organisms were exposed to a much harsher extreme environment than is expected normally in *in vivo*. Even then considerable number of *S. salivarius* ssp. *thermophilus* and *B. bifidum* survived at 1 h 30 min. *In vivo* conditions being much milder, it can be assumed that sufficient number of thermophilus and bifidobacteria could withstand acid conditions in stomach and survive gastrointestinal tract.

5.4 Bile tolerance

Bile tolerance is an important pre-requisite for an organism which is expected to colonise in intestine. Bile tolerance of all the cultures used in the present study were measured. An optical density of 0.3 at 600 nm was taken as standard. Ability of cultures to grow in MRS broth with and without 0.3 per cent Oxgall was assessed by monitoring the time required to reach the standard optical density of 0.3.

None of the organisms used in this study were completely inhibited by bile as shown by the slight increase in optical density when grown in MRS broth with 0.3 per cent oxgall. Though the standard optical density of 0.3 was not attained, it could be assumed that all tested cultures had some degree of tolerance to bile environment.

As some increase in optical density was seen we could infer that, these organisms would have survived and multiplied even in the presence of bile. Goodenough and Kleyn (1981) after conducting studies in rats concluded that increase in the viable cell population in intestine is directly proportional to the viable count in yogurt consumed, showing a significant survival and potential metabolic activity of thermophilus and bulgaricus in the upper gastro-intestinal tract of rats.

According to Eleven (1995) L. delbrueckii ssp. bulgaricus showed only a mild growth in MRS broth containing 0.3 per cent oxgall. Petterson et al. (1983) and Abour-Donia (1984) have reported poor survival of L. bulgaricus in bile. In this study also, similar observations were made.

However, for B. bifidum, increase in optical density was minimum when compared to other two organisms. This shows that among the three cultures B. bifidum was least bile tolerant.

Catteau et al. (1971) reported that 0.2 or 0.5 per cent sodium deoxycholate is bacteriostatic for twenty two strains of B. bifidum and B. brevi.

Bile sensitivity of these may be beneficial in products geared to lactose maldigesting individuals (McDonough et al., 1971 and Lin et al., 1991). Since the tested organisms showed some amount of bile tolerance, we could presume that some organisms could survive upper gastrointestinal tract. It can be assumed that, survived organisms reaching the lower tract would extend beneficial effects to the consumers.

From the foregoing discussions, it can therefore be concluded that,

1. Yogurt cultures possess significant β-galactosidase specific activity. However, when B. bifidum was added to yogurt cultures specific activity reduced. The incorporation of lactose hydrolysed condensed whey in bifidus yogurt was found to increase B-galactosidase specific activity.

2. From the biological study conducted in rats, it was found that whole milk has no hyocholesteraemic effect. When compared to milk fed group those rats fed with yogurt A_1 and A_2 showed no significant change in serum total cholesterol. However, when yogurt fortified with lactose hydrolysed

condensed whey (A_3) was given, a highly significant reduction in serum total cholesterol was evident. All bifidus yogurts particularly B_3 (fortified with lactose hydrolysed condensed whey) showed a highly significant hypocholesteraemic effect when compared to milk fed group.

3. Serum triglyceride levels of rats under different treatment groups showed an increase when compared to milk fed group. However, the difference was significant only in groups fed with yogurt A_1, A_2 and Bifibdus yogurt B_2.

4. Serum HDL-cholesterol level either decreased or remained unchanged in groups fed with yogurt and bifidus yogurt when compared to milk fed group. However, a significant increase in HDL-cholesterol was observed in group fed with bifidus yogurt B_1.

5. When compared to milk fed group serum LDL-cholesterol level of rats fed with yogurt A_1 showed a significant increase. When rats were given yogurt fortified with condensed whey and lactose hydrolysed condensed whey (A_2 and A_3), a significant reduction in LDL cholestrol was noticed indicating the hypocholesteraemic effect of yogurt supplemented with whey proteins. In all the rats fed with bifidus yogurt the serum LDL level reduced drastically indicating that bifidus yogurt is having a profound effect in lowering LDL level when compared to yogurt.

6. Incorporation of bifidus yogurt in diet could reduce cardiac risk factor even when a higher level of chlestrol was introduced in the diet.

7. Rats fed with yogurt and bifidus yogurt showed an increased daily weight gain when compared to milk fed group.

8. B. bifidum was found to be significantly more acid tolerant than S. salivarius ssp. thermophilus. However, L. delbrueckii spp. bulgaricus was found to be acid sensitive.

9. All the tested cultures showed some degree of bile tolerance.

Future prospects

Many of the sociological and nutritional impacts that affect today's diet suggest that replenishment of intestinal microbes will have beneficial effects. As B. bifidum isolated from natural environment is expected to have better acid and bile tolerance, further research studies can be done in products prepared using such strains.

Reason for the comparatively lower weight gain in rats fed with products fortified with lactose hydrolysed condensed whey should be further investigated.

Though the hypocholesteraemic benefits of B. bifidum is assured from the biological study conducted here, it is desirable to conduct more controlled experiments in human volunteers, since metabolism of rats differ from that of human beings. Additional work in collaboration with the medical profession will be rewarding. Such an effort will yield a cost effective reliable remedy for hypercholesteraemia without any side effect.

A_1 Yogurt fortified with skim milk powder

A_2 Yogurt fortified with condensed whey

A_3 Yogurt fortified with lactose hydrolysed condensed whey

B_1 Bifidus yogurt fortified with skim milk powder

B_2 Bifidus yogurt fortified with condensed whey

B_3 Bifidus yogurt fortified with lactose hydrolysed condensed whey

Summary

SUMMARY

An experiment was undertaken to study the effect of lactose hydrolysed condensed whey and B. bifidum in yogurt. Yogurt and bifidus yogurt were prepared using three methods of fortification viz. skim milk powder, condensed whey and lactose hydrolysed condensed whey. A_1, A_2 and A_3 are yogurts and B_1, B_2 and B_3 are bifidus yogurts fortified in this way. The products prepared were analysed for

1. β-galactosidase specific activity
2. Hypocholesteraemic effect in rats
3. Weight gain in rats
4. Acid tolerance and
5. Bile tolerance of starter cultures used.

From the results obtained, following conclusions were made

1. Significant level of β-galactosidase specific activity was observed in all yogurts. Bifidus yogurts showed a reduced specific activity. However, introduction of lactose hydrolysed condensed whey in bifidus yogurt was found to be a method to increase specific activity. Thus it can be concluded that bifidus yogurt fortified with lactose hydrolysed condensed whey can be given to patients suffering

from lactose intolerance since it contain more lactose splitting enzyme.

2. From the biological study conducted in rats, following observations were made:

a. Whole milk has no significant hypocholesteraemic effect.

b. Addition of lactose hydrolysed condensed whey in yogurt resulted in a significant reduction in serum total cholesterol level of rats.

c. All bifidus yogurts particularly B_3 (fortified with lactose hydrolysed condensed whey) showed a highly significant hypocholesteraemic effect when compared to respective yogurts and whole milk, indicating the increased advantage of using lactose hydrolysed condensed whey for fortification and B. bifidum as a dietary adjunct.

d. Serum triglyceride level increased in rats fed with yogurt and bifidus yogurt. However, HDL-cholesterol level either remained unchanged or decreased in groups fed with yogurts and bifidus yogurts except B_1.

e. Yogurt supplemented with whey proteins when given to rats showed a drastic reduction in serum LDL-cholesterol level. LDL lowering effect was profound in yogurt especially with whey proteins.

f. Incorporation of bifidus yogurt in diet could reduce cardiac risk factor even when a higher level of cholesterol was introduced in the diet.

g. Satisfactory weight gain in rats were noticed, when fed with yogurt and bifidus yogurt supplemented with whey solids.

3. From the acid tolerance study it was observed that B. bifidum was more acid tolerant than S. salivarius ssp thermophilus. L. delbrueckii ssp bulgaricus was found to be acid sensitive.

4. All the tested cultures showed some degree of bile tolerance.

The present study proved that, lactose hydrolysed condensed whey and B. bifidum can be successfully incorporated in yogurt for improving therapeutic benefits, in terms of increased β-galactosidase specific activity and hypocholesteraemic effect. Bifidus yogurt particularly fortified with lactose hydrolysed condensed whey, can be recommended for patients with high cardiac risk factor arising from high serum total cholesterol. LDL-cholesterol, a lipoprotein strongly correlated with atherosclerosis was found to be significantly low in rats fed with bifidus yogurt.

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EFFECT OF LACTOSE HYDROLYSED CONDENSED WHEY AND *Bifidobacterium bifidum* IN YOGURT

By

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ABSTRACT OF A THESIS

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ABSTRACT

An experiment was conducted to assess the possibilities of utilising whey solids in the form of condensed whey or lactose hydrolysed condensed whey as a substitute for NDM. Their effect was also studied in conjunction with B. bifidum as a dietary adjunct.

A detailed review of literature has been presented on the issues of lactose intolerance, hypercholesteraemia, beneficial effects of lactic acid bacteria in alleviating these conditions and also on acid tolerance and bile tolerance of cultures used in the present study. Methodology for the condensation of cheese whey, estimation of lactose in whey, β-galactosidase specific activity in the products, total cholesterol, HDL-cholesterol and triglycerides in serum, assessment of acid tolerance and bile tolerance of lactic cultures used here have been detailed.

The experiment comprised of preparation of yogurt and bifidus yogurt using three methods of fortification viz. skim milk powder, condensed whey and lactose hydrolysed condensed whey. The products prepared were then analysed for β-galactosidase specific activity. Hypocholesteraemic and growth promoting effects of these products were assessed in a biological study using rats. Hypocholesteraemic and growth

promoting effects of whole milk was also assessed in the biological study. Acid tolerance and bile tolerance of lactic cultures used in this study were also determined, in vitro.

From the above study, following conclusions were made.

1. β-galactosidase specific activity was noticed in substantial amount, in yogurt under different treatments. Bifidus yogurt showed a reduction in β-galactosidase specific activity, however, the activity was found to be enhanced when fortification was done with lactose hydrolysed condensed whey indicating that bifidus yogurt fortified with lactose hydrolysed condensed whey is superior.

2. No significant hypocholesteraemic effect was noticed in rats due to consumption of milk. All the rats fed with yogurt and bifidus yogurt except that given yogurt A_1 showed a substantial reduction in serum LDL-cholesterol level and cardiac risk factor. However, bifidus yogurt supplemented with whey proteins showed maximum hypocholesteraemic effect and lowest cardiac risk factor showing the superiority of bifidus yogurt with whey proteins.

3. All rats given yogurt and bifidus yogurt showed a better daily weight gain when compared to the group fed whole milk along with feed and cholesterol.

4. Evaluation of acid tolerance of lactic cultures showed that, among the three cultures tested, B. bifidum exhibited maximum acid tolerance followed by S. salivarius ssp. thermophilus. L. delbrueckii spp. bulgaricus was found to be acid sensitive.

5. Bile tolerance study of pure and active cultures revealed that none of the tested cultures were completely inhibited by bile indicating theses cultures were bile tolerant to some extent.