2. REVIEW OF LITERATURE

2.1. History of fermented products

The practice of fermenting foods has its origin since ancient times and in many countries. Fermentation continues to provide a simple household procedure for the production and preservation of traditionally accepted foods. Fermented dairy products have been playing a prominent role in the diet of mankind irrespective of their sects and cultures. The advent of modern techniques for food preparation and preservation has resulted in slight reduction in the consumption of such products in some western countries.

Metchnikoff (1908) first advocated the consumption of milk fermented with lactobacilli that are capable of implanting in the intestinal tract for health benefits. The scientific basis for this recommendation has been a matter of immense scientific interest. Yuguchi et al. (1987) briefly reviewed the history of research on intestinal flora, and the characteristics of the flora and their interaction with the body. The beneficial effects of cultured milks, and their capacity to improve bowel regularity in the elderly, to decrease counts of pathogenic bacteria in the bowels, and to enhance resistance to cancer and liver function, have been clearly established.

Various nutritional and therapeutic attributes of fermented foods have the potential to reduce the role of medicine in maintenance of normal health. Some of the reported nutritional and physiological benefits of fermented milks are the promotion of growth and digestion, setting effect on the gastro-intestinal tract by decreasing population of harmful bacteria, suppression of
The term probiotics was first used by Lilly and Stillwell in 1965 to describe the growth-promoting effect of one microorganism on another. According to Parker (1974) probiotics are "organisms and substances which contribute to intestinal microbial balance". More recently, Fuller (1989) redefined the word "probiotics" as a "live microbial food or feed supplement which beneficially affects the animals and humans by improving their intestinal microbial balance".

Probiotics is mono or mixed culture of live microorganisms which, when applied to animal or man, affects beneficially the host by improving properties of the indigenous microflora (Hanevaar and Huisin't Veld, 1992). An optional balance of microbial organisms in the intestine is suggested to be an important aspect of maintaining good health. The health-driven foods are commonly referred to as functional foods, designer foods, pharma foods, bio-foods or neutraceuticals. Besides the therapeutic properties that they possess, probiotics also stimulate natural activities of the body cells, protect against infections, break down carcinogenic substances, protect against pathogenic organisms, lower serum cholesterol level, inhibit the mutagenicity of the intestinal tumours, metabolize lactose and reduce lactose intolerance, improve absorption of calcium, aid in treatment of peptic ulcer and synthesize vitamins and predigest proteins.
Hull et al. (1992) discussed the use of probiotic bacteria in foods with reference to probiotic organisms (mainly derived from normal inhabitants of the gut); probiotic foods e.g. yoghurt, milk, cheese and butter, and attributes of good probiotic organisms. A functional food is defined as any food that has positive impact on human health, physical performance or state of mind in addition to its nutritive values (Goldberg, 1994).

Bottazzi (1992) reviewed the results of research over the last decade on the probiotic and therapeutic activities, anti-carcinogenic activity, and activation of immune system, hypocholesteremic effect, protection against intestinal infections, deconjugation of bile acids, inactivation of toxic compounds, reduction of hydrogen emission, and reduction of incorrect lactose digestion. Anon (1995) gave a brief account on the current position of functional foods in Japan. Hull (1995) discussed the current knowledge concerning probiotics and types of probiotic foods.

2.1.2. Beneficial effects of probiotic and functional foods

Bottazzi (1987) discussed the sources of probiotic organisms including cultured milks and special yoghurts. He briefly reviewed the human intestinal microflora with emphasis on the *Bifidobacterium* and lactobacilli and their characteristics and probiotic functions such as lowering of cholesterol content of blood. Gilliland (1989) reported that the consumption of the milk products containing *L. acidophilus* has the potential for preventing intestinal infections, helping serum cholesterol levels and exerting anti-carcinogenic activity.

Sellars and Robinson (1991) reported that the intestinal microflora involving *Lactobacillus acidophilus* promote growth, lactose digestion and
mineral absorption, contains antimicrobial and carcinogenic factors that stimulate the immune system and reduces blood cholesterol. Kurman et al. (1991) gave a brief account on therapeutic properties of cultured milk products containing viable bifidobacteria. Peitersen et al. (1992) studied the antagonistic activity of probiotic cultures against intestinal pathogens.

Fuller (1993) studied the scientific basis for the use of microorganisms that inhabit the gastrointestinal tract (probiotics) in foods, e.g. bio yoghurts. Bio yoghurts are cultured with starter strains of lactic acid bacteria, e.g. Lactobacillus sp. and Bifidobacterium spp. Ishibashi and Shimamura (1993) focused on the beneficial role of bifidobacteria in the human intestinal microflora, including anti-diarrhoeal, infection-preventing, immunity-activating and anti-tumour effects. Milk products containing bifidobacteria that have recently been developed in Japan and problems that have had to be overcome to maintain the viability of bifidobacteria during bifidus yoghurt manufacture have been focused.

Assche and Van (1994) gave a brief account on the nutritional and therapeutic properties of cultured milks. These include greater digestibility particularly by lactose intolerant subjects than non-cultured milks, because of the partial digestion of lactose during processing. Krasheninin and Shamanova (1994) revealed that the cultured milk products prepared by using specially selected strains of lactobacilli and bifidobacteria play an important role in the nutrition of children of 5-8 years of age.

Spork (1994) outlined the health benefits of probiotic cultured milk products. Gonc and Akalin (1996) reported that the cultured milk products might have a significant role in reducing the blood cholesterol levels and
hence reduce the rise of coronary heart disease. Kresv (1996) revealed that the cultured milk products containing active strains of lactic acid bacteria have beneficial effects on human health through their role in the development of the intestinal microflora. Lemoine (1996) discussed the role of probiotics, the well documented advantages of lactic cultures, the current emphasis on health foods and the advantages of using mixtures of bacterial strains in dairy cultures. Jung (1996) explained the concept of ‘functional food’ described about the food products which combine healthy eating with taste.

Wenoe (1996) discussed the dairy application of functional foods and revealed that probiotic product is added to the diet in order to increase the health-promoting attributes of gut microflora. Chandan (1999) reported the potential for the Indian dairy industry in the market for functional foods, particularly probiotics and therapeutic immunoglobulin preparations.

Jurcic and Oberiter (1996) presented the current knowledge regarding the role of cultured milk products in preventing and treating diarrhea in children. The greater tolerance to lactose associated with milk containing live bacterial cultures is related to its lower lactose content and also the action of bacterial β-galactosidase in the intestinal lumen. The low pH of cultured milk also plays a role in inhibiting the growth of pathogens in the intestine and concluded that future research needs to focus on the effects of different doses of cultured milk products, the specific effects on growth of bifidobacteria and lactobacilli on the immune response, and the formation of substances that inhibit pathogens. Buttriss (1997) attributed health benefits to the consumption of fermented milk products, particularly yoghurt.
2.1.3. *Bifidobacterium* spp. in probiotic foods

Marshall et al. (1982) reported that *Bifidobacterium bifidum* GSNE grew better in milk under anaerobic conditions than did standard strains of *B. longum, B. bifidum, B. infantis* and *B. adolescentis*. This strain gave best results when added at 5-10% to milk and when stored at pH 5.0-5.3, storage at lower pH adversely affected growth and acid production. Kanbe (1986) reviewed the characteristics of *Bifidobacterium* spp. growth factors, and evidence for beneficial effects on protein metabolism, vitamin metabolism, and inhibition of putrefactive and pathogenic bacteria, faecal excretion, kidney problems and the immune response.

Fernandes *et al.* (1987) mentioned that *Lactobacillus* spp. along with *Bifidobacterium bifidum* and *Streptococcus faecium* constitute an integral part of the healthy gastrointestinal micro ecology and are involved in the host metabolism. They impart nutritional and therapeutic benefits to the consumer. The vitamins and enzymes produced by the lactic acid bacteria contribute to host metabolism. The antimicrobial substances produced by these bacteria control the proliferation of undesired pathogens. They also elaborate enzymes that aid host metabolism. This is particularly true in case of lactose-intolerant individuals. Their anticholesteralemic properties assist in lowering serum cholesterol. Hughes and Hoover (1991) discussed about bifidobacteria, a group of lactose-utilizing bacteria, with emphasis on classification, recent research on these organisms and benefits obtained from ingestion of bifid-containing food products.

Misra and Kuila (1991) summarized the biological activities, role of bifidobacteria, preparation, antimicrobial activity and therapeutic properties of
bifidus milk. Consumption of bifidus milk with high numbers (10^8 cfu/g) of this organism will provide L (+)-lactic acid, antibiotic factor and live bifidobacteria in addition to other nutritional components. The combined action of these factors will create favourable conditions for the proliferation of intestinal bifidobacteria and discourage the growth of harmful organisms.

Kurmann and Rasic (1991) in a review focused on the therapeutic properties of cultured milk products containing viable bifidobacteria, most commonly *Bifidobacterium bifidum, B. longum or B. breve*. Bifidobacteria in the intestines of infants and young children are claimed to protect against invading pathogens by competitive antagonism, produce organic acids and other antimicrobial substances, improve N retention and weight gain, and inhibit nitrate reduction. In the adult intestine they may inhibit tumour growth and stimulate the immune system. The strains selected for therapeutic use must be normal inhabitants of the intestinal tract, must remain viable in the carrier product before consumption, must survive the upper digestive tract and must be urease-negative.

Vijayendra and Gupta (1992) described the use of *Bifidobacterium* and *Lactobacillus acidophilus* in preparation of fermented dairy products such as dahi, yoghurt and lassi. Beneficial effects of fermented products containing these bacteria include antimicrobial activity, anti-carcinogenic activity, and cholesterol-lowering effects, alleviation of lactose intolerance and provision of group B vitamins. Gonc and Akalin (1996) in a review examined the history on use of bifidobacteria in milk products, their characteristics for growth and the therapeutic benefits associated with their ingestion by humans. These include antimicrobial and anti-carcinogenic
qualities, reduction in serum cholesterol level, synthesis of B-complex vitamins, and improved lactose tolerance.

David (1995) discussed the health benefits of probiotic microorganisms, with reference to the use of intestinal species such as *Bifidobacterium* and *Lactobacillus*. Gönç and Akalin (1995) suggested that *L. acidophilus* and *B. bifidus* have potential as beneficial dietary adjuncts and reported that these organisms are able to survive in the digestive tract and also have antagonistic activity towards enteric pathogens. While discussing the therapeutic role of bifidobacteria, Mishra *et al.* (1996) suggested that the success of bifidobacteria-based cultured milk products is due to their organoleptic properties than to their nutritional or therapeutic attributes.

Sanders *et al.* (1996) evaluated commercial strains of lactic acid bacteria (6 of *Lactobacillus acidophilus*, 5 of *Bifidobacterium* and 6 of *Streptococcus thermophilus*) for characteristics that are important to the activity and stability of uncultured, probiotic milk products, and selected strains were used to manufacture a probiotic milk product. All lactobacilli and most of the bifidobacteria were resistant to 1-3% bile, and all streptococci were sensitive to bile. The probiotic milk product manufactured contained $10^7$ cfu/ml of *L. acidophilus* LH1, $10^7$ cfu/ml of *Bifidobacterium* BG9 and $5 \times 10^7$ cfu/ml of *S. thermophilus* SG91.

Haneváar and Huisint Veld (1992) reviewed the ecology of *Bifidobacterium* and *Lactobacillus* in the digestive tract, antimicrobial effects of lactic probiotics, immunological properties of lactic probiotics, adhesion of lactic acid bacteria to human intestinal cells, *in vitro* antimicrobial properties
of lactic probiotics, secretion of antagonistic substances by lactic acid bacteria and in vivo antagonistic effects of lactic probiotics.

Horn (1999) discussed the potential of probiotic lactic acid bacteria (Lactobacillus and Bifidobacterium) with reference to: health-promoting effects- effects of stress on intestinal function, effects of antibiotics on intestinal function; effects of probiotics on intestinal function; mechanisms of effects of probiotic bacteria, safety requirements of probiotic products, bacterial resistance to gastric acids, and acceptable shelf-life.

2.2. Isolation of Bifidobacterium spp.

Teraguchi et al. (1978) developed an improved selective medium, for enumerating bifidobacteria in dairy products containing lactobacilli and streptococci, using BL agar. Villa and Modler (1990) indicated that lactic acid-producing bacteria can be differentiated from bifidobacteria on the basis of morphology when grown on blood liver (BL) agar: Streptococcus thermophilus, small fat/round colonies with darker centre; Lactobacillus bulgaricus, large star-like colonies, Bifidobacterium longum, cream coloured/convex colonies with dark centers, which are larger than S. thermophilus and smaller than L. bulgaricus. Identification is slow and requires a well-trained eye.

Sozzi et al. (1990) studied the use of the antibiotic dicloxacillin for isolating and enumerating the bifidobacteria present in yoghurt and other cultured milks. The addition of 2 µg/ml of dicloxacillin to trypticase- phytone-yeast extract (TPY) medium was found to inhibit growth of lactobacilli and streptococci, whereas most bifidobacteria strains grew well. TPY medium
was found to be more suitable as a selective medium for bifidobacteria than the deMan-Rogosa-Sharpe (MRS) medium.

Samona and Robinson (1991) observed that there is much interest in the potential role of bifidobacteria as dietary inclusions, but their isolation from milk products, and subsequent identification, can cause problems. A range of selective and non-selective media were examined, and modified Rogosa agar was found to give the best recovery from yoghurt-like products. A morphological examination of typical isolates suggested that only bifidobacteria had grown on the medium, but confirmation by means of biochemical tests proved inconclusive, and it appears that the genus/species definition merits further attention.

Huang and Huang (1994) made studies on eight *Bifidobacterium* strains (4 of *B. longum* and 2 each of *B. breve* and one of *B. bifidum*) all of which showed good utilization of sugars used frequently (glucose, galactose, lactose, fructose and sucrose) and some oligosaccharides (galactooligosaccharides, fructooligosaccharides), but all strains except *B. breve* were unable to utilize sorbitol and mannitol. In anaerobic conditions, the best strains were *B. longum* CCRC 14605, *B. breve* CCRC 14632 and 11846 for acid production, and *B. longum* CCRC 14605 for consistency of quality.

enumeration of *Bifidobacterium* in cultured milks. They also described the steps taken to prepare a selective culture medium (incorporating modified Columbia Agar Base with glucose).

Chevalier *et al.* (1991) developed a new method using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-galactoside for differential enumeration of *Bifidobacterium* spp. and lactic acid bacteria used in preparation of fermented and non-fermented dairy products. X-α-gal-based medium was useful to identify bifidobacteria among *Lactobacillus* since action of α-galactosidase splits X-α-gal substrate and releases indole which imparts a blue colour to bifidobacterial colonies on agar plates. Blue colonies can also be assigned to the genus *Bifidobacterium* using a simple permeabilization procedure with Triton X-100 which allows reliable determination of fructose-6-phosphate phosphoketolase activity. They also performed physiological characterization of *Bifidobacterium* spp. based on phenotypic characteristics, carbohydrate fermentation patterns, enzyme profiles, fructose-6-phosphate phosphoketolase (F6PPK) assays and antibiotic sensitivities.

2.3. **Selection criteria for probiotic cultures**

Kurmann (1998) outlined biotechnical functions and selection criteria such as acid and gas production, aroma substances, ropiness, consistency and proteolytic activity of selected intestinal bacteria such as *Bifidobacterium bifidum, B. infantis*, etc. Sanders *et al.* (1996) evaluated commercial strains of lactic acid bacteria that are important to the activity and stability of uncultured, probiotic milk products, and selected strains were used to
manufacture a probiotic milk product. Speciation, strain relatedness, frozen concentrate stability, bile sensitivity and lactase activity were evaluated.

2.3.1. Antimicrobial activity of probiotic bacteria

Mantere-Alhonen et al. (1989) studied the antimicrobial activity of Bifidobacterium bifidum, B. longum, Lactobacillus acidophilus, L. delbrueckii var. bulgaricus and L. helveticus against pathogenic bacteria using the method of 'overlapping droplets'. The results showed that all bacteria studied possessed antimicrobial activity, but that of the bifidobacteria was high and their fractions had a stronger inhibitive effect.

Misra and Kuila (1991) examined three Bifidobacterium bifidum strains, isolated from the stools of infants given breast milk exclusively, and 4 standard strains of the same organism for their suitability in the manufacture of milks. Suitability was assessed on the basis of the technological criteria of titratable and volatile acid production, diacetyl and acetoin production, proteolytic activity, the dietetic criteria of lactic acid production, microbial growth and antibiotic activity against Shigella dysenteriae.

Ibrahim and Bezkorovainy (1993) investigated the ability of five species of Bifidobacterium to produce antimicrobial compounds by measuring the effects of spent bifidobacterial broths on growth of E. coli in thioglycollate medium. The broths were most inhibitory if their pH was not adjusted to neutrality. Once adjusted, the inhibition ranged from 30 to 43%. This level of inhibition could be duplicated by a 3:2 acetic acid: lactate mixture adjusted to neutral pH. It was concluded that no antibacterial substances other than lactic and acetic acids were produced by the bifidobacteria.
Gibson and Wang (1994) reported that bifidobacteria in the human intestine are considered to exert a range of biological activities related to host health. One aspect is the inhibitory effect of these bacteria on other species, possibly preventing long-term colonization by invasive pathogens. The mechanism of inhibition by bifidobacteria is possibly related to the fermentative production of acids such as acetate and lactate. *Bifidobacterium infantis* (NCFB 2205) was incubated with *E. coli* and *Clostridium perfringens*, in a variety of fermentation systems. Results indicated that the inhibitory effect was not necessarily related to acid production. Further studies showed that eight species of bifidobacteria excreted an antimicrobial substance with a broad spectrum of activity. Species belonging to the genera *Salmonella*, *Listeria*, *Campylobacter* and *Shigella*, as well as *Vibrio cholerae*, were all affected. Results show that bifidobacteria exert greater than a single mechanism of inhibition, which may be of some importance with regard to protection against gastroenteritis.

Misra and Kuila (1995) studied on *Bifidobacterium bifidum* which was inoculated into sterilized skim milk and incubated at 37°C for 48 h. The culture was centrifuged and the clear supernatant passed through a Seitz filter to produce a cell-free culture filtrate. Antibiotic substance(s) in the cell-free culture were obtained by methanol fractionation and acetone extraction. The filtrate showed maximum antagonistic action against *Shigella dysenteriae* between pH 4.8 and 5.5. The inhibitory substance was heat-stable (100°C/30 min) and could be stored at 4-5°C for >3 months in an active state.
Gönc and Akalin (1995) stated that *Lactobacillus acidophilus* and *L. bifidus* have potential as beneficial dietary adjuncts. They are able to survive in the digestive tract and also have antagonistic activity towards enteric pathogens. The health benefits of consuming cultured milk products (e.g. yoghurt) containing *L. acidophilus* and *L. bifidus* were discussed. Kim *et al.* (1998) investigated anti-microbial activity of 20 isolates of bifidobacteria isolated and reported that strains A1 and A2 showed antimicrobial activity against *Staphylococcus aureus*. Shah *et al.* (1997) studied the antagonistic relationship between yoghurt and probiotic bacteria and the nature of the inhibitory compound produced by the organisms. Eight strains isolated from eight commercial cultures (*L. acidophilus* and *Bifidobacterium* spp.) were screened for the production of bacteriocin against each of the eight isolates of *L. acidophilus* and *Bifidobacterium* spp. Twelve strains showed inhibitory activity against all the eight strains of *Bifidobacterium* spp. and five *L. acidophilus* isolates with the 'spot on lawn' assay. Of these, only one yoghurt bacterium, *S. thermophilus*, was a bacteriocin-producing organism.

2.3.2. Bile tolerance of probiotic bacteria

Kobayashi *et al.* (1974) compared growth at pH 6-7 and survival at pH 3.0 of streptococci, lactobacilli and bifidobacteria in artificial intestinal juice. The tolerance of these organisms to sodium deoxycholate in terms of 50% growth inhibitory concentration of sodium deoxycholate (μg/ml) was also considered. Hoier (1992) tested bile tolerance of *Lactobacillus acidophilus* La-5 and *Bifidobacterium* Bb-12 by incubation in milk-yeast medium containing ox bile (0.5-2.0%) at 37°C. These two strains were not inhibited by bile except at the
highest concentration which is unlikely to be found under normal intestinal conditions.

Clark and Martin (1994) evaluated the in vitro tolerance of four species of bifidobacteria (*Bifidobacterium adolescentis*, *B. bifidum*, *B. infantis* and *B. longum*) to concentrations of bile up to twice the maximum level reportedly produced in the human small intestine for determining the extent to which these species could be used successfully as probiotics in cultured milk products. Bifidobacteria were inoculated into solutions with 0, 2 and 4% bile concentrations, based on a solution of oxgall (Difco). These mixtures were then plated with modified BL agar for initial counts using a pour-plate method, then incubated anaerobically for 12 h at 37°C and enumerated again to determine survival rates after 12 h incubation. *B. longum* exhibited best tolerance to bile by surviving at both 2 and 4% concentrations after 12 h, whereas *B. infantis*, *B. adolescentis* and *B. bifidum* did not survive in 2% oxgall after 12 h incubation.

Lankaputhra and Shah (1995) studied the tolerance of six strains of *L. acidophilus* and nine strains of *Bifidobacterium* bile concentration similar to those encountered in the human intestine. Survival of bacteria in the presence of 1.0 and 1.5% bile was also evaluated for 3 h at 37°C. Tolerance of bacteria to bile varied; viable counts of *L. acidophilus* strains 2404 and 2415 and *B. longum*, *B. pseudolongum* and *B. infantis* remained close to initial levels, but viable counts of *L. acidophilus* 2400 and 2405 decreased from $10^8$ to $10^3$ cfu/ml.

Beena and Prasad (1996) examined the ability of yoghurt starter cultures of *Streptococcus salivarius* ssp. *thermophilus*, *Lactobacillus*
delbrueckii ssp. bulgaricus, and Bifidobacterium bifidum 2715 to grow in the presence of bile, using media with or without 0.3% oxgall. Growth was monitored in tubes at 600 nm and time required to reach the standard OD of 0.3 was determined. None of the organisms was completely inhibited by bile after 5 h incubation at 37°C; although the standard optical density was not achieved in any case. The corresponding OD values for the above bacteria were 0.16, 0.14 and 0.13, differences between organisms being highly significant.

Krsev (1996) observed in vitro studies that industrial strains of Lactobacillus bulgaricus and Streptococcus thermophilus used in yoghurt production are more sensitive to conditions of low pH and high bile concentrations than L. acidophilus, L. casei and Bifidobacterium spp. Sanders et al. (1996) reported that all lactobacilli and most of the bifidobacteria were resistant to 1-3% bile; all streptococci were sensitive to bile.

Martin (1996) examined the survivability of two strains of Bifidobacterium longum, two of B. adolescentis, two of B. infantis and one each of B. breve and B. bifidum under conditions of simulated gastric digest. B. bifidum, B. adolescentis and B. infantis had little resistance to either 2 or 4% bile salts after a 12 h contact period, but B. longum remained active. Strain differences can be significant and manufacturers must pay attention to culture selection. Gopal et al. (1996) observed that six strains of Lactobacillus acidophilus and nine strains of different species from the genus Bifidobacterium differed in their tolerance to bile.
Nam et al. (1998) reported that in the presence of bile salt (0.6% oxgall), *B. infantis* and *B. longum* showed 80 and 9% survival, respectively. Kim et al. (1998) investigated bile resistance of 20 isolates of bifidobacteria isolated from healthy Koreans and three commercial strains as controls. Strains A2, A5, A13, A14, A18 and A22 showed excellent bile resistances.

Chung et al. (1999) developed a method for the selection of acid and bile resistant bifidobacteria from human faecal samples. Colonies which passed screening were tested for the presence of fructose-6-phosphate phosphoketolase activity and acetate/lactate production. At pH 5 and 7, or when bile was not present, all strains tested grew well. Survival of two strains, designated SI 31 and HJ 30, was much greater than other strains following incubation in 50 mM phosphate buffer (pH 2 or 3) or in 50 mM phosphate buffer (pH 7) with 0.5 or 1% (w/v) bile salt.

Heo and Yoon (1995) studied four strains of *Bifidobacterium* spp. and three strains of *Lactobacillus acidophilus* isolated from commercial starters with respect to tolerance to bile. *L. acidophilus* exhibited bile tolerance by sustaining growth in MRS-thio broth containing 0.3% oxgall, whereas growth of *Bifidobacterium* strains was inhibited under these conditions. Ahn et al. (1999) evaluated the resistance to bile of various strains of lactobacilli and bifidobacteria. Most lactobacilli showed resistance to 1 and 2% oxgall, *L. acidophilus* being more resistant to bile acid than the other species; bifidobacteria also showed resistance to bile acid. Reilly and Gilliland (1999) also tested four strains of *Bifidobacterium longum* grown at pH 5.5, 6.0, 6.5 and 7.0 for survival and bile tolerance during frozen (28 days) and
subsequent refrigerated storage (up to 21 days) in milk. There were no reductions in cell numbers following initial freezing.

2.3.3. Acid tolerance of probiotic bacteria

Kobayashi et al. (1974) compared growth at pH 6-7 and survival at pH 3.0 of streptococci, lactobacilli and bifidobacteria in artificial intestinal juice. The tolerance of these organisms to sodium deoxycholate in terms of 50% growth inhibitory concentration of sodium deoxycholate (μg/ml) was also considered.

Hoier (1992) tested *Lactobacillus acidophilus* La-5 and *Bifidobacterium* Bb-12 inoculated into milk for acid by incubation in MRS nutrient solution adjusted to pH 1-4 with HCl, at 37°C for 12 or 24 h, respectively. Both the species were capable of 100% survival at pH 3 and 4; *Bifidobacterium* Bb-12 had a higher tolerance to acid than *L. acidophilus* at lower pH. The importance of high initial bacterial population and a minimal amount of processing in order to maximize probiotic effects during digestion of cultured milk products was focused. Direct inoculation with a high initial starter concentration was recommended.

Clark et al. (1993) evaluated *in vitro* tolerance of *Bifidobacterium infantis*, *B. adolescentis*, *B. longum* and *B. bifidum* to pH levels that commonly exist in the stomach. Cultures were maintained anaerobically at 37°C in modified NPNL broth. Approximately 10^9 cfu/ml of each *Bifidobacterium* was transferred into HCl solutions at pH 1, 2 and 3 and, after 0, 1, 2 and 3 h in acid, and aliquots were plated onto NPNL agar at pH 7.2. *B. infantis*, *B. adolescentis* and *B. longum* were unaffected at pH 2. At pH 1.0 no *B. infantis* and *B. adolescentis* survived at 2 h, but approximately 102 cfu/ml of *B. longum* survived at 3 h. *B. bifidum* did not survive at 2 h in pH 2.
solution. It was suggested that *B. longum* would be the most suitable dietary adjunct in cultured dairy foods.

Lankaputhra and Shah (1995) studied the suitability of strains of *Lactobacillus acidophilus* and *Bifidobacterium* for use as dietary adjuncts in fermented dairy products. The tolerance of six strains of *L. acidophilus* and nine strains of *Bifidobacterium* to acidic conditions similar to those encountered in the human stomach were assessed. During incubation at 37°C for 3 h in HCl solutions at pH 1.5, 2.0, 2.5 and 3.0, bacterial counts decreased, especially at pH less than 2.5. *L. acidophilus* strains 2409, 2415 and 2401, *B. longum* and *B. pseudolongum* showed the highest survival rates under acidic conditions. It was concluded that *L. acidophilus* strains 2401, 2409 and 2415, *B. longum* and *B. pseudolongum* were suitable for use as adjuncts in fermented dairy products due to their resistance to pH commonly existing in the human digestive system.

Heo and Yoon (1995) tested four *Bifidobacterium* and three *Lactobacillus acidophilus* strains, isolated from commercial starters, for tolerance to bile and HCl. Two of the *Bifidobacterium* strains, one of which was *B. infantis*, showed greater acid tolerance at pH 2.0 than did the *L. acidophilus* strains, which survived for >3 h at pH 3.0. Martin (1996) examined the survivability of two strains of *Bifidobacterium longum*, two of *B. adolescentis*, two of *B. infantis* and one each of *B. breve* and *B. bifidum* under conditions of simulated gastric digest (addition of $10^8$ viable cells to HCl solution at pH 1.0, 2.0 and 3.0 for 3 h), *B. adolescentis*, *B. longum* and *B. infantis* survived well (pH 2.0-3.0) for 1 h. *B. bifidum* showed poor
tolerance to these conditions, suggesting that strain differences could be significant and manufacturers must pay attention to culture selection.

Beena and Prasad (1997) reported that *B. bifidum* had the highest acid tolerance followed by *S. salivarius* ssp. *thermophilus*. *L. delbrueckii* ssp. *bulgaricus* was not acid-tolerant. Kim *et al.* (1998) investigated acid resistance of 20 isolates of bifidobacteria isolated from healthy Koreans and three commercial strains as controls. Strains A2 and A9 showed more acid resistance than other strains.

Ahn *et al.* (1999) tested the resistance of the six strains of *Lactobacillus acidophilus*, one of *L. casei* ssp. *rhamnosus*, four of *Bifidobacterium longum*, two of *B. infantis* and three of other *Bifidobacterium* spp. isolated from Korean cultured milk products to low pH and bile acids. More than 84% of *Bifidobacterium* cells from normal yoghurt products survived at a pH of 3.0 and 2.5, respectively.

Charteris *et al.* (1998) developed an in vitro method which mimics in vivo human upper gastrointestinal transit. The transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species was determined by exposing washed cell suspensions at 37°C to a simulated gastric juice (pH 2.0) and monitoring changes in total viable count periodically. The majority (14 of 15) of isolates lost >90% viability during simulated gastric transit. Two strains, *L. casei* 2123 and *Bifidobacterium infantis* 25962, exhibited 100% gastric transit tolerance in the presence of milk proteins.

2.3.4. β-Galactosidase activity of probiotic bacteria

Martini *et al.* (1991) stated that the lactose in yoghurt with live bacteria was better tolerated than lactose in other dairy foods, partly because of the
activity of microbial β-galactosidase (β-Gal), which digests lactose in vivo. Tianan-Jiang (1996) assessed the improvement of lactose digestion by consumption of an unfermented dairy product containing lactic acid bacteria. An in vivo study showed that consumption of nonfermented milk containing Bifidobacterium longum B6 or ATCC 15708 improved lactose digestion.

Khattab et al. (1986) experimented with Lactobacillus acidophilus and Bifidobacterium adolescentis which were grown in skim milk that had previously been incubated with β-galactosidase (β-Gal) at 20 mg/100 ml for (i) 0, (ii) 0.5, (iii) 1 or (iv) 2 h at 37°C to obtain about 0, 20, 40 or 60% hydrolysis of lactose. Addition of β-Gal increased growth and acid production of both organisms, and the order of effectiveness was (i) > (iv) > (iii) > (ii). Acid production in milk treated with β-Gal at the time of inoculation increased with increasing per cent inoculum. β-Gal treated milk coagulated after 16 h with 3, 5 or 10% L. acidophilus inoculum and after 20 h with 5 or 10% B. adolescentis. Control sample without β-gal coagulated within 24 h. Use of sterilized milk or milk that had been steamed for 30 min prior to addition of β-gal gave an acceptable cultured product.

Mustapha and Savaiano (1995) investigated the β-galactosidase activity, bile sensitivity and lactose uptake of 5 strains of (i) Lactobacillus delbrueckii ssp. bulgaricus, 6 strains of (ii) L. acidophilus and 5 strains of (iii) Streptococcus thermophilus were examined, while (i) produced higher levels of β-galactosidase than (iii), where as (ii) exhibited β-galactosidase activities of 1.9-3.0 U/mg protein. Also, Hughes and Hoover (1995) evaluated Bifidobacterium breve NCFB 2258, B. bifidum NCFB 2715, B. longum ATCC 15707, B. angulatum ATCC 27535, and Lactobacillus acidophilus N2e for
viability and β-galactosidase and α-galactosidase activities under conditions of refrigerated and frozen storage in reconstituted nonfat dried milk (NDM). β-and α-galactosidase activities varied between the bifidobacteria tested.

Ordonez and Jeon (1995) reported that sonicated samples showed higher amounts of lactose hydrolysis ($P<0.05$) than unsonicated samples. Initial lactose concentration decreased by 40% more in sonicated than in unsonicated samples. In sonicated samples, B. bifidum reduced lactose concentration by an average of 18%. The mixed culture showed the highest enzyme activity, causing a 40% reduction in lactose.

Sanders et al. (1996) reported that the lactase activities were highest for S. thermophilus strains, supporting the use of this species to aid lactose digestion in humans. Eleven and Prasad (1998) observed that β-galactosidase activity was higher in dahi samples inoculated with L. acidophilus than in those not containing L. acidophilus (3.075 and 2.179 units respectively). However, yoghurt samples containing L. acidophilus had lower β-galactosidase activity than samples containing no bacterium (3.629 and 4.281 units, respectively).

2.4. Associate action of bifidobacteria with yoghurt culture

Borisova and Slivko (1973) discussed about the biochemical activity of Lactobacillus bifidus [Bifidobacterium bifidum] during cultivation in sterilized milk and the possibility of using it in starters for the production of cultured milks was studied. Results reported for 6 high acid-producing strains isolated from the mouth and intestines of humans show that milk coagulation occurred in 18-28 h with corresponding decreases in pH to 4.38-4.58. In proteolytic activity, the bacteria were comparable to lactic streptococci.
Kisza et al. (1974) reported that, when 4 strains of *B. bifidum* were grown in semi-continuous culture at controlled pH 5.6-6.2, cell yields were highest on media containing pepsin-digested whey or skim-milk. Fermentative and proteolytic activity increased with cell numbers. Cell yields using semi-continuous culture were 5 to 10 x greater than those obtained using the same volume of medium in batch culture.

Cerna and Snaselova (1979) reported that *L. casei* and *B. bifidum* when grown in skim milk had shown the highest variability of the acidifying capacity of milk. Hrabova (1980) emphasized the variability in the properties of pure dairy cultures including *Bifidobacterium bifidum* and stressed the importance of continuous control of cultures used in dairy processing to ensure the manufacture of products of standard. Cheng and Nagasawa (1983) reported that the acid production by mixed cultures of *Bifidobacterium breve* or *B. infantis* with *Lactobacillus casei* or *L. acidophilus*, grown in skim milk medium, was greater than the sum of acidity produced by the individual cultures. During 18 h incubation at 37°C, counts of bifidobacteria increased more in the mixed cultures than in pure culture.

Yankovskii and Dyment (1990) described a method that involves joint culture in milk of bifidobacteria and microorganisms which stimulate their growth. To increase the viability of the bifidobacteria, the growth-stimulating organisms used were *Acetobacter aceti* and *A. pasteurianum*, selected to have a proteolytic activity of greater than 7.5 mg% in terms of milk protein. The bifidobacteria and acetic acid bacteria were in a 3:1 ratio.

Klaver et al. (1993) tested several *Bifidobacterium* species and strains for growth potential in pure milk or in milk supplemented with casein.
hydrolysate. Fifteen of 17 strains did not grow in pure milk as growth required the presence of peptides or amino acids derived from casein degradation. Manufacture of fermented milk products with bifidobacteria would therefore require the use of an inoculum containing the final number of cells of *Bifidobacterium* required.

Samona and Robinson (1994) examined the growth of *Bifidobacterium*, *B. bifidum*, *B. longum* and *B. adolescentis* in reconstituted skim milk (12% TS) either individually or in combination with one of 3 commercial yoghurt cultures. Total colony counts were recorded for the bifidobacteria over a growth period of 24 h, and for samples with culturing to pH 4.6, during storage at 5°C for 21 days. The results indicated that, while co-inoculation with the yoghurt organisms tended to inhibit growth of the bifidobacteria, subsequent storage in presence of yoghurt cultures did not lead to any significant decrease in numbers.

El-Sayed and Shafei (1996) in their study inoculated sterilized skim milk with 3% *Streptococcus salivarius* ssp. *thermophilus* CNRZ 385 (St), *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 369 (Lb) and *B. infantis* 4038 (Bi) either alone or in combination. At intervals between 0 and 24 h, titratable acidity, pH values and total acidity of volatile acids were determined. Growth of Bi with St or Lb increased the titratable acidity and total acidity of volatile acids produced. Addition of Bi filtrate stimulated the titratable acidity and acetaldehyde production by yoghurt bacteria. In addition, the counts of St and Lb on Elliker and MRS agar increased (by $10^7$ to $10^8$ cfu/ml over a 24-h period) with addition of Bi filtrate.
Huang and Huang (1996) studied the relationship between growth of *B. longum* in milk and *Streptococcus thermophilus* CCRC 14086, *Lactobacillus delbrueckii* ssp. *bulgaricus* CCRC 14009 and *L. acidophilus* CCRC 14072. Milk (12% SNF) was incubated with *B. longum* with or without the three test organisms at 37°C for 24 h. Titratable acidity, pH and viable counts were determined. When *B. longum* was cultured in milk with the test organisms, viable counts and acid production increased compared with *B. longum* alone. The greatest and smallest effects occurred in the presence of *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus*, respectively. The main amino acids that might have promoted growth of *B. longum* were aspartic acid, alanine, methionine and cysteine; arginine, glycine and proline produced only a small growth-promoting effect.

Samona *et al.* (1996) used *Bifidobacterium bifidum*, *B. longum* and *B. adolescentis* both in pure culture and in combination with B3 and SBI cultures (yoghurt bacteria) to produce cultured milks. *B. bifidum* and *B. longum* produced only low amounts of acids in pure culture, while *B. adolescentis* produced 10 times more acid (maximum of 290 mmol/litre) than the other two species; lactic acid being the main metabolic product. In mixed yoghurt and *Bifidobacterium* cultures, the levels of acid reflected the combination of yoghurt culture and species of *Bifidobacterium*, and this observation suggested that there was a certain amount of interference between the cultures. Due to higher levels of acid being obtained with yoghurt culture B3 than SBI, it appeared that the main pattern of acid production was regulated by the yoghurt bacteria. The lower quantities of
acid present in the samples of SBI culture were attributed to selected incompatibility between the constituent species of yoghurt bacteria.

Ahn et al. (1997) reported that final pH value of the fermented product produced by *B. longum* JR20 and *B. adolescentis* CN2 was 4.71 and 4.58, respectively, while titratable acidity value was 0.67 and 0.77%, respectively. The fermented dairy product produced by *B. longum* JR20 had a less irritating acid aroma than that produced by *B. adolescentis* CN2. Kim et al. (1998) investigated acid producing capacity of 20 isolates of bifidobacteria isolated from healthy Koreans and three commercial strains as controls. Strains A1, A2, A3, A4, A6, and A23 showed pH <4.5 and titratable acidity over 0.90 after 24 h of fermentation.

Gomes et al. (1998) investigated the growth and acidification rates of *Bifidobacterium lactis* Bo and *Lactobacillus acidophilus* Ki under conditions of low redox potential and addition of milk hydrolysates. The growth and acid production of *B. lactis* in milk were affected by the addition of proteinase-mediated hydrolysate and, to a lesser extent, by neutrase-mediated hydrolysate. Higher hydrolysis of either hydrolysate resulted in greater biomass increase and greater acid production, indicating that the poor growth of bifidobacteria in milk was due partially to the lack of small peptides and free amino acids. The rates of growth and acidification by *B. lactis* increased when co-cultured with *L. acidophilus* (1:1 inoculum ratio). Co-culture with *B. lactis* (1:1 inoculum ratio) increased the rates of growth and acidification compared with that of the single strain, an indication of some degree of symbiosis between the strains.
Kim et al. (1998) investigated the acid and bile resistance, fermentation properties, viability, cholesterol assimilation, anti-microbial activity, anti-mutagenicity, and immuno-activation of the strains of bifidobacteria isolated from healthy Koreans. Twenty strains of 200 isolates were used, while three commercial strains were included as controls. Strains A2 and A9 showed more acid resistance than other strains, whereas A2, A5, A13, A14, A18 and A22 showed excellent bile resistances. Strains A1, A2, A3, A4, A6, and A23 showed pH <4.5 and titratable acidity over 0.90 after 24 h of fermentation. A1 and A2 showed antimicrobial activity against Staphylococcus aureus.

Abu et al. (1998) evaluated four species of bifidobacteria for growth, viability and proteolytic activity in whole camel milk and cow milk. Growth of all species in both milks was characterized by the appearance of two logarithmic phases following anaerobic incubation at 37°C for 36 h. B. bifidum 2715 and B. breve 2258 showed the same trend as B. angulatum after 16 h of incubation. Viable counts of all species except B. bifidum increased in the cultured whole milks during the first three days of storage at 4°C. However, such counts did not change in non-cultured milk, except for B. longum, which increased after 12 days of storage. Viability of all species in cultured and non-cultured milks was not affected by refrigerated storage for 15 days.

Gregurek (1999) made samples of yoghurt with three commercial starter cultures (A1, A2 and A3) and were stored at 4°C for 35 days. Counts of Lactobacillus acidophilus and Bifidobacterium were determined immediately after the addition of inoculum to cooled yoghurt mix, after
overnight cooling and after every 5 days. The counts and the stability of bifidobacteria starter cultures used were higher than those of \textit{L. acidophilus}. Post-acidification was slightly higher in yoghurts prepared with lower amounts of inoculum. It was concluded that the associative yoghurt organisms and pH of yoghurt affect the viability of the probiotic bacteria and that the amount of inoculum and incubation temperature should be properly maintained to achieve maximum viability.

Shihata and Shah (2000) in their study on proteolytic profiles of yoghurt and probiotic bacteria concluded that the yoghurt bacteria appeared to be highly proteolytic as compared to the probiotic bacteria (\textit{L. acidophilus} and \textit{Bifidobacterium} spp.). The yoghurt bacteria released higher amounts of free amino acids and demonstrated greater amino peptidase and dipeptidyl activity than the probiotic bacteria. As a result, yoghurt bacteria grow faster in milk, whereas the probiotic bacteria grow slowly due to lack of proteolytic activity and require an exogenous supply of peptides and amino acids for optimum growth, in particular for starter cultures that do not contain \textit{L. delbrueckii} ssp. \textit{bulgaricus}.

2.5. Yoghurts as probiotic foods

Lang (1981) presented the current position in yoghurt consumption in major European countries and information presented on recent advances in drinking yoghurt in the Netherlands (where its current per capita consumption is about 3 l/yr). Kisza \textit{et al.} (1978) incorporated bifidobacteria into yoghurt starter which had effect on biochemical changes, and rheological properties of the final product, thereby enhancing its dietary value.
Ballester (1984) discussed technological developments that have taken place in the manufacture of yoghurt, and products fermented with *Lactobacillus acidophilus* and bifidobacteria, in the automation of yoghurt manufacture and in packaging. Rasic (1987) discussed various aspects of the nutritive value of yoghurt, its composition and digestibility and other beneficial effects such as in the treatment of gastrointestinal disorders. Sellars (1989) reviewed the health properties of yoghurt including promotion of growth, improved lactose digestion and mineral absorption, presence of antimicrobial factors, anticarcinogenic properties, acidophilus yoghurt factors, factors in yoghurt affecting the immunological responses and reduction of blood serum cholesterol concentration.

Gupta and Tiwari (1990) tested yoghurt starter bacteria for compatibility with *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, and a modified starter was developed and used for the manufacture of liquid yoghurt. Rogers (1991) reported the growing interest in bifidobacteria utilization in cultured milks and yoghurts. A number of difficulties involved in using the bacteria under standard processing conditions are listed; these include: cultivation problems due to oxygen sensitivity, lack of acid tolerance, weaker viscosity, milder taste and aroma of the final product.

Honer (1991) has given details of new milder-tasting yoghurt with a less acidic flavour. The difference in taste is due to the combination of microorganisms used to ferment the milk, namely *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *L. bulgaricus*. This culture combination produced yoghurt with a higher pH and a lower acidic flavour note. Yajima et al. (1992) described a method for producing a
cultured milk product such as yoghurt, in which a *Bifidobacterium* or a lactic acid bacterium, such as *Streptococcus thermophilus*, or a combination thereof, is inoculated into and cultured in a medium, composed mainly of milk. The bifidobacteria are selected from *B. breve*, *B. longum*, *B. bifidum* or *B. infantis*. The milk used may be fresh or reconstituted cow, goat or similar milk, or a mixture of these.

Assche and Van-Assche (1994) described the technology of yoghurt manufacture, and of the manufacture of cultured milks using starter cultures containing bifidobacteria or *Lactobacillus acidophilus*. The nutritive and therapeutic properties of cultured milks included greater digestibility, particularly by lactose-intolerant subjects, than non-cultured milks, because of the partial digestion of lactose during processing, positive effects on intestinal flora, positive effects on the HDL: LDL cholesterol ratio contributing to a reduction in blood cholesterol levels, and antitumoral effects.

Patel and Renz-Schauen (1997) discussed the nutritive attributes of yoghurt, e.g. its richness in Ca, P, K, easily absorbed carbohydrate, vitamins and free amino acids, and recent advances in the preparation of yoghurt cultures, i.e. the use of a single strain of lactic acid bacteria (e.g. *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) and the production of 'bio'-yoghurts (using *L. acidophilus* and *Bifidobacterium*) via a direct-vat inoculation system.

Kailasapathy and Rybka (1997) reported that *L. acidophilus* and *Bifidobacterium* provide excellent therapeutic benefits when incorporated into yoghurt, therefore, improve it as a therapeutic functional food. However, poor viability and survival remain a problem in commercial yoghurts. Malik *et al.*
(1998) reported the biochemical changes occurring during fermentation of yoghurt and its beneficial aspects. The benefits of the development of bio-yoghurts which contain bacteria such as Lactobacillus acidophilus and certain species of Bifidobacterium, which are capable of surviving in the intestinal tract, were highlighted. Sarkar and Mishra (1998) discussed the production of probiotic yoghurts containing beneficial cultures such as bifidobacteria and propionibacteria, for use in infant and child nutrition. They suggested an incubation temperature of 42 ± 1°C instead of 37 ± 1°C suggested for yoghurt manufacture in 4 h.

Kurmann (1998) reviewed outline biotechnical functions and selection criteria such as acid and gas production, aroma substances, ropiness, consistency and proteolytic activity of selected intestinal bacteria such as Bifidobacterium bifidum, B. infantis which may be used in fermented milk products such as yoghurt. Kisla and Unluturk (1998) made an attempt to produce a new type of fermented milk product containing Lactobacillus acidophilus, L. casei and Bifidobacterium longum. A starter inoculum combination of 2% L. acidophilus, 2% L. casei and 10% B. longum resulted in the best flavour in ranking tests ($P < 0.05$). Consistency and syneresis of the new type of fermented milk product compared well with traditional yoghurt produced with L. bulgaricus and Streptococcus thermophilus. Sensory evaluation showed that there was no significant difference between new fermented milk and the traditional yoghurt.

El-Nagar and Shenana (1998) used five starters to produce set-type probiotic yoghurt. Starters were added to recombined milk at 3 g/100 g in which bifidobacteria accounted for 50% of the starter blend. Initial counts of
Bifidobacterium were $10^7$-$10^8$/g. Samples were analyzed over 21 days of storage. No spoilage organisms were detected and yoghurt was of acceptable organoleptic quality, with some combinations of probiotic starter resulting in yoghurt of better quality than controls made using conventional starters.

Huang and Huang (1999) studied the optimum conditions for manufacturing frozen yoghurt. Best incubation temperature for acid production with mixed starter containing Bifidobacterium longum CCRC 14605: Streptococcus thermophilus CCRC 14086: Lactobacillus acidophilus CCRC 14072 (2:2:1) was 40°C for 5 h.

2.5.1. Changes in yoghurt on storage
Marshall et al. (1982) prepared probiotic products that had mean acetaldehyde contents in the range 27 to 39 ppm and pH in the range 4.65 to 5.14; the coagulum was similar to that of conventional yoghurt. Quality of the products did not change appreciably during storage for 21 days at 4°C.

Sakai et al. (1987) investigated the survival of several bacteria in boiled yoghurt incubated at 30°C, and in fresh refrigerated yoghurt. The most stable organism was Bifidobacterium breve 203, which maintained its initial viable count for greater than 5 days in boiled yoghurt; it was also stable in refrigerated yoghurt at 4 or 10°C. Robinson (1989) stated that the probiotic yoghurts that contain Lactobacillus acidophilus and/or bifidobacteria, in some cases in addition to normal yoghurt bacteria, must have either a pH remaining above 4.6-4.7 or contain a sufficient number of these special microorganisms for them to still be present at $10 \times 10^5$ viable cells/ml after storage at pH 4.1-4.2 for 2-3 weeks.
Robinson (1990) stated that if the 'health-promoting' reputation of bifidus yoghurts/desserts is to be maintained, then it is essential that the products always contain the 'therapeutic minimal' number of viable cells of *Bifidobacterium* spp. at the time of consumption. The effects and popular flavouring ingredients of other starter bacteria on the survival of *B. bifidum* were studied. Bifidus desserts were made containing 2% (v/v) *B. bifidum* incubated to pH 4.0 or 4.5. Fruit purees (banana, black cherry or strawberry) or a sugar/starch base flavoured with chocolate, mocha or toffee were then added. Bifidus yoghurts were made containing 1.5% *B. bifidum* + 0.5% yoghurt culture and were flavoured as above. Cartons containing 175-g of desserts or yoghurts were stored at refrigeration temperature for up to 21 days. Fruit purees or flavoured sugar/starch bases did not adversely affect survival of *B. bifidum* in the desserts; values were $>1 \times 10^9$ cfu/g after 21 days.

Laroia and Martin (1991) made studies on *Bifidobacterium bifidum* and *Lactobacillus acidophilus* which were incorporated into frozen cultured milk products in order to determine their survival during frozen storage. *B. bifidum* was enumerated using NPNL agar and *L. acidophilus* using MRS agar. *B. bifidum* did not survive in the frozen low-pH cultured milk product probably due to the pH being below that required by *B. bifidum* for survival. *L. acidophilus* survived in the low-pH frozen milk product. Both organisms survived in the high-pH cultured milk product and the high pH yoghurt.

Martin and Chou (1992) used 11 strains of *Bifidobacterium* as dietary adjuncts in low-pH (4.1-4.2) and high-pH (5.5-5.6) plain yoghurt. *Bifidobacterium adolescentis* ATCC 15703, *B. longum* C01, *B. infantis* C02
and *B. longum* C07 were good candidates as dietary adjuncts for yoghurt due to their higher survival rate in the low-pH product. The pH values in yoghurt containing bifidobacteria remained very stable during refrigerated storage, indicating that incorporating bifidobacteria did not affect the overall quality of yoghurt. Martin and Chou (1992) studied the use of bifidobacteria as dietary adjuncts in fermented dairy products that provide a number of advantages including mild sour taste, limited acidification during storage, and healthful and therapeutic properties. Bifidobacterial populations declined rapidly at pH less than 4.6.

Kneife *et al.* (1993) reported that counts of lactobacilli in fresh yoghurts varied between $5.5 \times 10^7$ and $6.5 \times 10^8$ cfu/ml; counts of streptococci varied from $3.5 \times 10^7$ to $1.2 \times 10^8$ cfu/ml. Approximately 80% of yoghurts had higher counts of cocci than rods. During storage of products for 2 weeks at 6°C stability of microflora differed markedly among the cultures. In fresh yoghurt-related products *L. acidophilus* counts ranged from $4.0 \times 10^6$ to $2.6 \times 10^8$ cfu/ml; bifidobacteria counts were between $4.0 \times 10^6$ and $2.6 \times 10^8$ cfu/ml. In most products a reduction in viable counts was observed after 2 weeks. Titratable acidity increased on an average by 22.3% in yoghurts and by 14.9% in yoghurt-related products during storage. In most products, a greater amount of L (+)-than D (-)-lactic acid was found.

Kim *et al.* (1992) studied the changes of various lactic acid bacteria in stirred yoghurts during delivery and storage. Two different mixed strain cultures were used for the sample yoghurts: ABT (*Lactobacillus acidophilus, Bifidobacterium bifidum* and *Streptococcus salivarius* var. *thermophilus*) and BT (*Lactobacillus delbrueckii* var. *bulgaricus* and *Streptococcus salivarius* var. *thermophilus*).
var. thermophilus). In ABT yoghurt after 15 days, titratable acidity slowly increased from 1.13 to 1.18% at 10°C and to 1.30% at 20°C and pH decreased from 4.18 to 4.16 and 3.98 at 10 and 20°C respectively. Numbers of B. bifidum were $7.9 \times 10^6$ cfu/ml at 10°C and $1.0 \times 10^6$ cfu/ml at 20°C after 15 days' storage. Titratable acidity of BT yoghurt rapidly increased from 1.24 to 1.38% at 10°C and to 1.60% at 20°C after 15 days' storage.

Brunner et al. (1993) studied survival of bifidobacteria in cultured milk made with selected strains of Bifidobacterium bifidum, B. breve and B. longum, and stored for 28 days at 4 and 8°C. All the three bifidobacteria examined showed comparable survival rates during storage, with pH exerting the greatest influence. D-values were 16.6 and 15.3 days respectively at 4 and 8°C at pH 4.9; 6.7 and 6.0 days at pH 4.5; and 1.5 and 1.2 days at pH 4.1. Modler and Villa-Garcia (1993) observed that B. longum had poor survival in acid-high yoghurt with a developed acidity of 0.45% (pH 4.47); however, incorporation of these organisms in low-acid yoghurt (developed acidity of 0.215% and pH of 5.85) resulted in only one log decline in bacterial counts during 11 weeks storage of yoghurt mix.

Klaver (1993) tested several Bifidobacterium species and strains for growth potential in pure milk or in milk supplemented with casein hydrolysate. Survival in fermented milk products during storage at low temperature (5-7°C) was also studied. Fourteen of 17 strains completely lost their viability in the 1st week of storage. The loss of viability was lower in less acidic products. Good survival in fermented milk products is an exceptional property among bifidobacteria and should be a criterion for selecting strains for manufacturing products with probiotic effects. Misra and Kulla (1994)
stated that 'Biogarde' yoghurt with a 'walnutty' aroma, pleasant flavour and firm consistency could be obtained by incubating fortified cow milk (16% TS) at 45°C/4h with 2% of 1:1 *Lactobacillus delbrueckii* var. *bulgaricus*: *Bifidobacterium bifidum* starter.

Salama and Hassan (1994) reported the use of starter cultures in preparation of cultured milk products and the observations were as follows: (i) yoghurt, *Streptococcus salivarius* var. *thermophilus* (SST) + *Lactobacillus delbrueckii* var. *bulgaricus* (LbB); (ii) acidophilus yoghurt, *L. acidophilus* (LbA) + LbB + SST; (iii) acidophilus-bifidus yoghurt, SST + LbB + LbA + *Bifidobacterium bifidum* (BB); (iv) bioghurt, LbA + SST; and (v) Biogarde, Biogarde ABT culture (LbA + BB + SST). During storage of the products for 15 days at 6°C, titratable acidity increased whilst pH decreased. Titratable acidity was higher in (i), (ii) and (iii) than (iv) and (v) throughout storage. Total organoleptic scores for fresh products of (i)-(v) were 84.34, 89.67, 93.67, 81.50 and 94.00, respectively. It was concluded that LbA and BB could be safely incorporated into starters to improve the therapeutic properties of yoghurt.

Kumar *et al.* (1995) studied on the addition of 5 strains of bifidobacteria isolated from infant faecal samples together with *Lactobacillus delbrueckii* var. *bulgaricus* LBR3 and *Streptococcus salivarius* var. *thermophilus*, STW, and incubated at 42°C/4 h for short-set- (SS) and 30°C/20 h for long-set (LS) yoghurt. Control SS and LS yoghurt had titratable acidity of 0.68 and 0.99% lactic acid, yoghurt starter had a log count of 7.90 and 8.68/ml, and overall acceptability (10-point scale) of 7.5 and 8.5, respectively. When bifidobacteria were included in SS and LS yoghurt, the
acidity ranged from 0.72 to 0.82% and from 1.02 to 1.35%, yoghurt starter log count ranged from 8.25 to 8.50/ml and from 8.78 to 9.08/ml, bifidobacterial log count ranged from 7.90 to 8.32/ml and from 8.68 to 8.92/ml and overall acceptability ranged from 8.6 to 9.5 and from 8.5 to 9.1, respectively.

Huang and Huang (1995) studied the manufacture of yoghurt using the following combinations: (i) *B. longum* CCRC 14605, (ii) *S. thermophilus*, (iii) *Lactobacillus delbrueckii* ssp. *bulgaricus* and (iv) *L. acidophilus* for making A and B yoghurts with (i):(ii):(iii) cultures in 1:1:1 and 2:2:1 ratios respectively, and C and D yoghurts with (i):(ii):(iv) cultures in 1:1:1 and 2:2:1 ratios respectively. Optimum growth temperature of all starter combinations was 40°C, and incubation time (to pH 4.6) and acidity were 6-7.5 h and 0.68-0.75%, respectively. Viable counts of (i) were >10⁷ cfu/ml after 8 days of storage was noticed. Acidity was similar in B and C yoghurts.

Rybka and Kailasapathy (1995) observed that yoghurt bacteria viability is important in providing a number of therapeutic benefits to consumers. In yoghurts with ABS culture, *L. acidophilus*, *Bifidobacterium* spp. and *S. thermophilus* the counts after culture were 4.0 x 10⁷, 9.0 x 10⁶ and 2.8 x 10⁹ cfu/ml and after 36 days of refrigerated storage the corresponding counts were 10⁷, 4.9 x 10⁵ and 4.5 x 10⁸ cfu/ml. In traditional yoghurt with AB culture, the viable counts of *L. acidophilus*, *Bifidobacterium* spp., *L. bulgaricus* and *S. thermophilus* after culture were 4.0 x 10⁶, 8.6 x 10⁶, 1.2 x 10⁸ and 1.6 x 10⁹ cfu/ml while after 62 days of refrigerated storage the corresponding counts were 1.2 x 10⁶, 1.5 x 10⁶, <10², 10⁸ cfu/ml.
Martin (1996) examined the survivability of two strains of *Bifidobacterium longum*, two of *B. adolescentis*, two of *B. infantis* and one each of *B. breve* and *B. bifidum* in yoghurt. Approximately $10^7$ viable cells/g were added to pH 4.2 yoghurt, and survival over 56 days at 4-6°C was determined. Some strains of *B. adolescentis* and *B. longum* survived well in yoghurt while selected strains of *B. bifidum* and *B. breve* were not as acid-tolerant.

Sanders *et al.* (1996) reported that the probiotic milk product manufactured contained $10^7$ cfu/ml of *L. acidophilus* LH1, $10^7$ cfu/ml of *Bifidobacterium* BG9 and $5 \times 10^7$ cfu/ml of *S. thermophilus* SG9. Lactic, but not psychrotrophic, populations remained stable during storage.

Sezgin *et al.* (1996) conducted studies on three different cultured milk products produced using *L. acidophilus*, *B. bifidum*, *L. bulgaricus* [*L. delbrueckii* ssp. *bulgaricus*] and *Streptococcus thermophilus* cultures. Titratable acidity, total volatile fatty acids and tyrosine levels were highest and pH lowest in the AB milk, whereas these properties were similar in the AB yoghurt and ABT milk. However, the difference in acetaldehyde contents between AB milk and ABT milk was not significant. It was concluded that the ABT milk was the product most preferred by the respondents, followed by AB yoghurt and then AB milk.

Rybka and Fleet (1997) determined the viable populations of the probiotic bacteria in 50 commercial yoghurts. *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were $>10^7$ cfu/g in 54 and 68% of the samples, respectively. The viable populations of *L. acidophilus* and *Bifidobacterium* species were $>10^6$ cfu/g only in 24 and 14% of the samples, respectively.
Yoghurts with high counts of *L. delbrueckii* ssp. *bulgaricus* tended to be more acidic (pH <4) and contained *L. acidophilus* and/or *Bifidobacterium* species at populations <10⁶ cfu/g.

Xie-JiZhi *et al.* (1997) studied the effect of three manufacturing methods on growth of bifidobacteria after 1-15 days of cold storage. Methods I and II, in which 3 starter strains were used, resulted in counts of 7.0 x 10⁸-7.7 x 10⁸ cfu/ml and 6.6 x 10⁸-7.3 x 10⁸ cfu/ml, respectively. Method III, in which yoghurt culture:bifidobacteria cultured milk were used at a ratio of 1:2, resulted in counts of 6.8 x 10⁸-9.4 x 10⁸ cfu/ml.

Dechter and Hoover (1998) evaluated the ability of strains representing six species of *Bifidobacterium* with probiotic potential to survive low-temperature storage period. Cultures were also evaluated for their ability to ferment skim milk and retain viability during storage at 4°C. In cultured skim milk, *B. breve* 15700, *B. bifidum* 29521 and *B. animalis* 25527 tolerated a final product pH of 4.75 with >1 x 10⁸ cfu/ml remaining after 14 days of storage at 4°C.

Payne *et al.* (1998) inoculated 70-ml aliquots of reconstituted skim milk containing a standard yoghurt culture with *Bifidobacterium bifidum*, *B. longum* and *B. adolescentis* (concentration in 10% inoculum of 6.6 x 10¹⁰, 9.3 x 10⁸ and 1.78 x 10⁸ cfu/ml, respectively). Provided that the initial concentration of *Bifidobacterium* was about 10⁸-10⁹ cells/ml milk, therapeutic doses survived in yoghurt for up to 21 days.

Karagul *et al.* (1999) reported about the manufacture of sweetened low fat (1%) plain yoghurt and low fat Swiss-style strawberry and lemon yoghurts. After addition of the yoghurt cultures and *Lactobacillus acidophilus*
and *Bifidobacterium longum*, yoghurt samples were incubated at 43°C until desirable pH values of 5.0 or 4.2 were reached. The yoghurt was stored at 4°C for sensory evaluation by an expert panel on days 7, 21 and 45. A consumer panel evaluated carbonated and non-carbonated yogurts on day 21. The results of the study showed that the carbonation had no significant effect on the acceptability of yoghurt during shelf-life. Also, the carbon dioxide treatment did not alter the sensory characteristics of yoghurt as noted either by expert panelists or by consumers.

Gregurek (1999) studied the effects of level of inoculum on viability of probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* spp.) during yoghurt manufacture and subsequent storage at 4°C. Starter was added at 0.5, 1.0, 1.5 or 2.0 g/10 L. Samples were taken immediately after starter culture addition and at intervals up to 35 days of storage. For all three starters, bifidobacteria were more stable than *L. acidophilus*. Post-acidification was slightly higher in yoghurts prepared with lower levels of starter inoculum. It was concluded that viability of probiotic bacteria in yoghurt was affected by associative organisms and pH.

Shah (2000) discussed the viability and survival of *L. acidophilus* and *Bifidobacterium* spp. in products in terms of the effects of antagonism between various groups of organisms, growth requirements of bifidobacteria and oxygen toxicity in bifidobacteria. Approaches to improve viability of probiotic bacteria were also considered which include selection of acid and bile resistant strains, use of glass vs. plastic containers, level of inoculum, 2-step fermentation, microencapsulation of bacteria, stress adaptation, addition of micronutrients, use of ascorbic acid as an oxygen scavenger,
addition of cysteine, and use of sonication to release β-galactosidase from yoghurt bacteria.

Vinderola et al. (2000) studied effects of pH (3.5, 4.5 and 5.5, adjusted with lactic acid) of acidified milk on viability of L. acidophilus and B. bifidum. Plate counts were conducted weekly on inoculated 1-ml yoghurt and acidified milk samples stored at 5°C for up to 4 weeks. L. acidophilus generally exhibited higher sensitivity to environmental conditions in yoghurt than B. bifidum. Full-fat yoghurt was a more inhibitory medium than reduced-fat yoghurt, especially for B. bifidum. At pH 4.5, reduction in viable cell counts observed between acidified milk and yoghurt samples generally differed while loss of cell viability in yoghurt samples showed wider fluctuations. A pH of less than or equal to 4.5 in yoghurt stored at 5°C was generally detrimental to the viability of probiotic bacteria.

Hughes and Hoover (1995) evaluated commercial milk and two brands of yoghurt containing bifidobacteria for viability of bifidobacteria and lactic acid bacteria during refrigerated storage at 4°C. The yoghurts were evaluated at 3, 2, and 1 week prior and past their expiration. Viability of bifidobacteria and lactic acid bacteria in milk and yoghurt remained above 10^6 cfu/ml or until the expiration date of the respective products. This microbial concentration is the recommendation minimum dose to receive the health benefits of these organisms.

Lamoureux et al. (2002) in their investigation found that the survival of bifidobacteria was drastically affected during storage of yoghurts, except for products containing B. animalis, in which viable counts remained at >10^6 cfu/g after 28 days of storage at 4°C oligosaccharides with a degree of
polymerization of three were produced during the pre incubation step (0.31 to 0.68%), and the amount in the final products varied according to the species of bifidobacteria inoculated during the pre-incubation step or the concentration of bifidobacteria used as second inoculum during the fermentation process. In fact, the higher concentration of oligosaccharides measured at the end of the fermentation process (0.72%) and after 28 days storage period (0.67%) was obtained for yoghurts containing B. infantis. However, yoghurts containing B. breve showed higher β-galactocidase activities and had lower lactose concentrations after the fermentation process and the storage period than the other yoghurts. The use of a mixed cultures of bifidobacteria (B. animalis, B. infantis, or B. breve) thus altered the production of yoghurts in which bifidobacteria can survive in relatively high cell numbers and contain appreciable amount of oligosaccharides.

2.5.2. Ingredient supplementation on the quality of probiotic yoghurt

Klaver et al. (1993) tested several Bifidobacterium strains belonging to different spp. for their growth potential in milk alone or in milk supplemented with casein hydrolysate. Of 17 strains, 15 did not grow in milk. However, growth required the presence of peptides or amino acids derived from casein degradation. Since these strains lack proteolytic activity, they could be grown by adding casein hydrolysates or by co-culturing with proteolytic species.

Proulx et al. (1994) determined the growth rates and acid production of the most common dairy-related Bifidobacterium spp. in the presence of casein hydrolysates prepared by hydrolysis of sodium caseinate with three proteolytic enzymes. It was concluded that tryptic digests of casein could be used to supplement milk media for growth of bifidobacteria. The importance
of amino acid composition for growth-promoting activity of casein hydrolysates was stressed. Baig et al. (1996) observed that incorporation of whey solids stimulated the growth of *S. thermophilus* and *B. bifidum* in yoghurt, but the count of *L. delbrueckii* ssp. *bulgaricus* was reduced when *B. bifidum* was incorporated. Examination of the organoleptic properties of the yoghurts showed that both forms of whey solids were satisfactory replacements for dried skim milk. Fortification by whey protein concentrate improved the textural properties. Supplementation by *B. bifidum* had only a marginal effect on the flavour of the product.

Ruperez (1998) discussed the role of oligosaccharides as functional food ingredients and highlighted their beneficial effects for consumer health due to their non-carcinogenicity, low energy content and promotion of the growth of beneficial bacteria in the colon. Ravula and Shah (1998) studied the effect of acid casein hydrolysate on the viability of yoghurt bacteria and probiotic bacteria during a storage period of 12 weeks. The counts of *L. acidophilus* and bifidobacteria decreased to \(<10^2\) cfu/g in the control samples, whereas the counts were \(>10^5\) cfu/g in the samples supplemented with acid casein hydrolysate or cysteine.

Dave and Shah (1998) investigated the effects of cysteine, whey powder, whey protein concentrate, acid casein hydrolysates, or tryptone on the viability of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and bifidobacteria. Changes in pH, titratable acidity, redox potential, and viability of bacteria were monitored during 24 h of fermentation and refrigerated storage (4°C) of yoghurt for 35 days. The drop in pH or the increase in acidity and redox potential was dependent on the added ingredients. The
addition of cysteine, whey protein concentrate, acid casein hydrolysates, or tryptone improved the viability of bifidobacteria to a variable extent, but whey powder failed to improve their viability.

Bruno et al. (2002) observed that the levels of growth of bifidobacteria was affected by the type of prebiotic added and the initial viable cell counts prior to refrigerated storage. They also stated that the viability of all 5 strains of bifidobacteria tested was reduced. However, the retention of viability during the 4-week storage was significantly higher ($P<0.05$) when they were grown in the presence of a probiotic.

### 2.5.3. Nutritional and health benefits of probiotic cultures

Zbikowski (1981) observed an increase in *Bifidobacterium bifidum* contents in rat faeces and decrease in *Escherichia coli* contents in a study on three strains of *Bifidobacterium bifidum*. McNamara et al. (1989) found that the plasma total LDL and HDL were not affected by the yoghurt or the low fat milk concentrate. Suzuki et al. (1991) studied on mice by giving a diet containing yoghurt until the age of 121 weeks and given a diet containing one of three treated milk diets viz., skim milk, yoghurt, cultured milk of bifidobacteria until 97 weeks old. The levels of some serum constituents in the mice from groups 2 and 3 were significantly different (uric acid, higher in 2 ($P < 0.01$); blood urea nitrogen, lower in 2 ($P < 0.05$); creatinine, lower in 2 and 3 ($P < 0.05$) from those in mice from one group I.

Goh et al. (1994) reported that feeding of control diet containing 20% casein, modified control diet with 10% yoghurt (CY) or 10% ginseng (CG) or ginseng-supplemented yoghurt (CGY) had no significant effect on glucose levels in mice. Mean serum cholesterol level (mg/dl) at 3 weeks in mice fed
control, CY, CG and CGY diets, respectively, were as follows: total cholesterol - 158.6, 145.0, 136.3 and 141.5 mg/dL, high density lipoprotein (HDL) cholesterol - 65.2, 71.2, 77.5 and 79.2 mg/dL, low density lipoprotein (LDL) cholesterol – 93.4, 73.8, 58.8 and 62.3 mg/dL, free cholesterol - 47.8, 43.2, 40.7 and 41.1 mg/dL, and cholesterol ester - 110.8, 101.8, 95.6 and 100.4 mg/dL.

Moussa et al. (1995) used the diets viz., dry diet alone (control I) or together with (2); fresh cow milk (control II), (3) fresh yoghurt, (4) bio grade for the feeding of 30 mature albino rats, divided into 6 groups for 30 days and reported that with exception of HDL cholesterol, serum of rat receiving (2) diet, had higher level of all measured parameters than that of rat fed (1) diet, whereas feeding of any of cultured milks reduced the levels. The reduction in serum cholesterol, triglycerides, total lipids and LDL cholesterol varied between type of cultured milk and their overall benefits was placed in the order (5) > (6) > (4) > (3).

Athrayilkkalathil et al. (1997) studied the possible hypocholesterolaemic properties of milk and yoghurt. Fifty four albino rats were divided into 9 groups and were given (i) a basal diet (control), (ii) basal diet + cholesterol, (iii) as (ii) + whole milk, (iv), (v) and (vi) as (ii) + standard yoghurt fortified with dried skim milk, condensed whey or lactose-hydrolysed condensed whey respectively, and (vii), (viii) and (ix) as (ii) + bifidus yoghurt containing *Bifidobacterium bifidum* + the three additives respectively. Cholesterol was given at 5 g/kg body weight. After 30 days, triacylglycerides, total cholesterol, HDL-and LDL-cholesterols were measured in serum. Groups (iii) and (iv) had no hypocholesterolaemic effect, but (vi), (vii), (viii)
and (ix) had lowered serum cholesterol. In general, yoghurts changed HDL-cholesterol a little, but tended to increase the concentration of triacylglycerides. There was a marked decrease in the concentration of LDL-cholesterol in rats given either type of yoghurt fortified with whey proteins.

Beena and Prasad (1997) concluded studies on the rats which were fed with experimental diets consisting of yoghurt and bifidus yoghurts containing whey proteins and confirmed the presence of viable lactic acid bacteria in the intestine. Coliform count reduced considerably in the product-fed group than the controls. They also evaluated the effect of yoghurt and bifidus yoghurt fortified with skim milk powder, condensed whey and lactose hydrolyzed, condensed whey on serum cholesterol and triacyl glycerol levels in rats. The rats were fed with the following diets (1) basal diet (control), (2) basal diet + cholesterol, (3) as (2) + whole milk, (4)(5) and (6) as (2) + standard yoghurt fortified with dried skim milk powder, condensed whey or lactose-hydrolyzed condensed whey, respectively and (7),(8)and(9) as (2) + bifidus yoghurt containing Bifidobacterium bifidum + the 3 additives, respectively. Cholesterol was added at 5 g/kg body weight. After 30 days of feeding trials, triacyl glycerols, total cholesterol, HDL- and LDL-cholesterol levels were measured in blood serum. Group (2) and (4) had no cholesterolaemic effect but (6), (7), (8) and (9) showed lowered serum cholesterol. Yoghurt increased the concentration of triacyl glycerol in rats. This study showed that bifidus yoghurt and yoghurt fortified with whey protein can reduce total and LDL-cholesterol in rats.

Yaeshima et al. (1997) administered yoghurt containing B. longum BB536 (Bifidus yoghurt) to adult volunteers and found that the administration
of Bifidus yoghurt significantly increased the number and relative percentage of fecal bifidobacteria. It decreased the fecal ammonia concentration and increased the organic acid content. The defecation frequency was significantly increased by Bifidus yoghurt. The colour was changed to yellow and the consistency to soft. It was concluded that the administration of Bifidus yoghurt improved the intestinal environment, faecal characteristics and defecation frequency. Agerholm et al. (2000) also made some feeding trials by feeding 450 ml yoghurt daily to four groups of men and women during the study of effect of probiotic milk products on risk factors for cardiovascular disease. The CAUSIDO culture reduced LDL-cholesterol and increased fibrinogen in the over weight subjects at a 450 ml consumption per day.

Kedar et al. (2000) studied the effect of maize oil or butter without bacterial strains and butter diets supplemented with yoghurt (*Bifidobacterium bifidum* and *Lactobacillus acidophilus*) strains and their mixture on the total serum cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride levels, as well as, saturated and unsaturated fatty acids and the total cholesterol of liver tissue in rats. They reported that supplementation of butter diets with yoghurt or probiotics strain significantly reduced the serum cholesterol, HDL- and LDL-cholesterol and triglyceride levels while their mixture had the lowest amount of total cholesterol, LDL and triglyceride levels. Supplementation of butter diets with either yoghurt or probiotics strains reduced the number of both fecal enterococci and coli forms, but increased the number of lactobacilli. *L. acidophilus* showed the highest capability to reduce the growth of both enterococci and coli forms followed by *B. bifidum* and yoghurt.
strains. Rats fed with *B. bifidum* diets exhibited higher number of lactobacilli in their feces than rats fed with butter diets without these bacterial strains.