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2.0 REVIEW OF LITERATURE

2.1 MICROORGANISMS AND HUMAN HEALTH

Human health is one of the main reasons behind food choices and this has led to a diverse range of food formulations with specific functionalities that provide better health and wellness. One of the common health disorders associated with the diet pattern is that of gastrointestinal (GI) disorders. Such GI disorders can be prevented to a certain extent through routine consumption of foods with specific functionality (Sandholm et al. 2006). Hence, the concept of functional foods evolved as the role of food in the maintenance of health and well-being received increased scientific and commercial interest (Swinbanks and O’Brien, 1993). Lactic acid bacteria (LAB) and bifidobacteria are well known for their extensive use in the preparation of functional food products (Sandholm et al. 2006). These organisms have been termed as ‘probiotic bacteria’, which does impart certain specific health promoting attributes through oral feeding (Marks, 2004). Simultaneous with probiotics, the other term ‘prebiotics’ are known to be non-digestible food ingredients (higher polysaccharides) that beneficially affect the host by selectively stimulating growth and/or activity of selected group of bacterial genera and species that are normal inhabitants of colon (Gibson and Roberfroid, 1995).

In order to exert health benefits on the host, probiotics must be able to grow in the human intestine and therefore, should possess the ability to survive passage through gastrointestinal tract (GIT), which involves exposure to acidic environment in the stomach and bile in the small intestine (Kearney et al. 2008). Most of the probiotic strains are natural inhabitants of the human intestine and are generally regarded as safe (GRAS) along with acid and bile tolerance and ability to adhere to gut epithelial cells (Lankaputhra and Shah, 1998; Dunne et al. 2001). Hence, the best designed route for the entry of these probiotic bacteria is the diet, both for animals and human beings.
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(Farnworth, 2008). Fermented foods based on milk, cereals and legumes are among the most accepted food carriers for delivery of viable probiotic cultures (Kearney et al. 2008). Probiotic bacteria and their fermentation products appear to influence the human health, wherein they provide a colonization resistance against potential pathogenic microorganisms (Gmeiner et al. 2000).

In several studies, researchers have demonstrated the ability of probiotic bacteria to inhibit pathogenic bacteria by the production of organic acids like lactic and acetic acids during fermentation process, which lowers the pH of the intestine and consequently inhibits growth of the undesirable bacteria (Goktepe, 2006). In addition to these beneficial health effects, researchers have demonstrated that the major end products of fermentation in humans are the short chain fatty acids (SCFA) like those of acetate, propionate, and butyrate (Cummings, 1981). Besides, few other antimicrobial substances produced widely by lactic acid bacteria include hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins (Ouwehand and Vesterlund, 2004). Probiotic bacteria like LAB and bifidobacteria are also known to synthesize folate, vitamin B\textsubscript{12} and vitamin K, which are vital components of the human diet and involved in the biosynthesis of nucleotides and cofactors in many metabolic reactions (Barrett et al. 2004).

There has been substantial evidence for the benefits of probiotics and prebiotics in the lowering of (i) lactose intolerance through the activity of β-galactosidase; (ii) antibiotic associated diarrhoea; (iii) colon carcinogenesis; (iv) hypcholesterolemic effect and (v) gut mucosal dysfunction (Gallaher and Khil, 1999; Isolauri et al. 2001; Salminen et al. 2004; Schmid et al. 2006, Casci et al. 2007).
2.2 FERMENTED TRADITIONAL FOODS OF INDIA

2.2.1 Background scenario

Fermentation is one of the oldest food preservation methods, which became popular with the dawn of civilization, as it led to the development of a variety of tastes, forms and other sensory attributes. It appears that fermentation originated in the settlements of Indian subcontinent that predate the great Indus Valley civilization. As a process, fermentation consists of the transformation of simple raw materials into a range of value added products by utilizing the phenomena of growth of microorganisms and their activities on various substrates, wherein the knowledge of microorganisms is essential to understand the process of fermentation. At present, a number of fermented foods and beverages have evolved over the years (Farnworth, 2008).

2.2.2 Milk based foods

Fermentation of milk, either knowingly or unknowingly has occurred since early times, resulting in various fermented milk products. Fermented milk products are known for their taste, nutritive value and therapeutic properties. The nature of these products has differed from region to region depending on the indigenous microflora, which in turn depends upon the surrounding environmental factors (Tamime and Robinson, 1988). The most popular traditional fermented products of Indian subcontinent are dahi, mishti doi, lassi, shrikhand and others. The use of desired microorganisms as in the case of controlled fermentation would greatly enhance the chances of obtaining products with uniform and consistent quality products of acceptable attributes (Rati Rao et al. 2006).

2.2.2.1 Dahi

Dahi is one of the most popular fermented milk products of the Indian subcontinent liked for its mild acidic taste and pleasant flavour. A good quality dahi is of firm and uniform
consistency with a sweet aroma and a clean acid taste. The surface is smooth and glossy and usually a cut surface is trim, free from cracks and gas bubbles. The nutritional and therapeutic values of dahi are rated as high when compared to that of the milk used for its preparation. Dahi is easy to digest and it has been found to have certain health attributes (Sinha and Sinha, 2000). One of the earliest average composition of dahi prepared from whole milk is as follows (on percentage basis): water 85-88%, fat 5-8%, protein 3.2-3.4%, lactose 4.6-5.2%, ash 0.7-0.75%, lactic acid 0.5-1.1%, calcium 0.12-0.14% and phosphorus 0.09-0.11% (Laxminarayana et al. 1952).

The commonly associated lactic acid bacterial cultures in the inoculum are strains of Lactococcus lactis ssp. lactis, Lc. lactis ssp. cremoris, Lc. lactis ssp. diacetyllactis, Leuconostoc cremoris, Lactobacillus delbrueckii ssp. bulgaricus, Lb. acidophilus, and Lb. helveticus. Selection of a good starter culture is important to obtain a good flavour with desirable characteristics. Use of both mesophilic and thermophilic starters in various combinations has been reported (Baisya and Bose, 1975; Sharma and Jain, 1975). Addition of probiotic cultures such as Lb. acidophilus and Bifidobacterium bifidum along with the regular lactic cultures for dahi preparation help to increase the therapeutic and nutritional value of dahi (Vijayendra, 1994). Dahi was found to be a potential source of lactic acid bacteria, mainly species of Lactobacillus active against foodborne pathogenic and spoilage bacteria such as Staphylococcus aureus, Bacillus cereus, Bac. licheniformis, Bac. subtilis, Escherichia coli and Klebsiella sp. and Pseudomonas sp. (Balasubramanyam and Varadaraj, 1994). Further, a strain of Lb. delbrueckii ssp. bulgaricus producing a heat stable bacteriocin with broad spectrum antibacterial activity and a potential for use as a food biopreservative was isolated from dahi (Balasubramanyam and Varadaraj, 1998).
2.2.2.2 Mishti doi

*Mishti doi* is a popular variety of sweetened dahi available in the eastern region of India. It is also known as *lal dahi* or *payodhi*. It is generally characterized by light brown colour and firm body with cooked or caramelized flavour. Two formulations for the preparation of *mishti doi* are identified (Gupta et al. 2000). One combination involves use of *Streptococcus salivarius* ssp. *thermophilus*, *Lb. acidophilus* and *Lb. delbrueckii* ssp. *bulgaricus* and the other formula has *Lb. acidophilus*, *Lc. lactis* ssp. *lactis* and *Saccharomyces cerevisiae*. Microbiological examination of market samples of *mishit doi* revealed the presence of yeasts such as *Saccharomyces*, *Candida* and *Rhodotorula*, besides the LAB like *Lactobacillus*, *Lactococcus* and *Streptococcus*. Proteose peptone, a minor protein of milk does play a very important role in imparting the brown colour to this milk product (Mann and Joshi, 1997).

2.2.2.3 Lassi

*Lassi* also known as buttermilk in Indian context is another popular lactic fermented milk-based beverage of the Indian subcontinent, which is consumed mainly during hot and warm seasons. It is a by-product obtained during the preparation of *desi* butter from *dahi* by churning. It is also made by breaking the set *dahi* with the agitator and the addition of a required amount of water, sugar or salt and flavour compounds. The composition of *lassi* varies based on the type of milk used, the extent of dilution during churning and the efficiency of fat removal. The average composition (on percentage basis) of *lassi* is: water 96.2%, fat 0.8%, protein 1.29%, lactose 1.2%, lactic acid 0.44%, ash 0.4%, calcium 0.6% and phosphorus 0.04% (Rangappa and Achayya, 1974). The shelf life of *lassi* has been extended by more than 6 d at 37°C by the addition of 0.03-0.35% sodium metabisulphite and the sulphur flavour imparted by this preservative could be masked by
the addition of 0.07-0.09% crushed green ginger and 0.5-0.7% salt (Bhanumurthy and Trehan, 1970).

The preservative effect of nisin in enhancing the shelf life of lassi has been studied, wherein lassi could be stored 32-48 h at 30°C with the addition of Nisaplin at a concentration of 200-500 IU/ml, whereas, the stability was 8-10 d at refrigerated storage (Naresh and Prasad, 1996). To enhance the therapeutic value of lassi, a method has been standardized to prepare lassi using probiotic culture of Lb. acidophilus along with Str. thermophilus to get a desirable flavour in the final product (Patidar and Prajapathi, 1998).

2.2.2.4 Shrikhand

Shrikhand is a sweetened lactic fermented product widely consumed in western and northern parts of India. It is a product having a refreshing taste with pleasant aroma, smooth and homogenous texture and firm consistency. Shrikhand preparation involves products of curd (dahi) by lactic fermentation of whole milk, either cow’s or buffalo’s milk, followed by the draining of whey from the curd through a suspended muslin cloth bag for 6-8 h. The resulting solid mass (known as chakka) is uniformly mixed with ground sugar (44-45%) and made into a semisolid mass to which flavouring substances such as cardamom and saffron are added. The lactic cultures used are of mesophilic lactics, the same as those involved in the preparation of dahi (Patel and Abdel-Salam, 1986).

The technological and microbiological aspects of shrikhand preparation are reported in an earlier study (Reddy et al. 1984). Preparation of dietetic shrikhand using buffalo skim milk fermented with a 2% combined culture of Lb. acidophilus (NDRI-AH1) and Str. salivarius ssp. thermophilus (NDRI-YHS) was reported to reduce the high fat content in the final product (Subramanian et al. 1997). A change in the profile of mineral content from milk to the final product of shrikhand was studied (Boghra and Mathur,
Post-production heat treatment (PPHT) of *shrikhand* at 70°C for 5 min enhanced the shelf life up to 15 d at 35°C and above 70 d at 8-10°C (Prajapati et al. 1993a, b). *Shrikhand* powder was prepared by subjecting the product to spray drying at 160-170°C of inlet temperature and 100°C of outlet temperature. Dehydrated *shrikhand* had a shelf life of 90 d when stored at 30°C in gas packed containers (Mahajan et al. 1979; De and Patel, 1990).

There are also several other traditional milk-based fermented foods, which are unique to certain regions of India and are not covered in the present review.

### 2.2.3 Cereal and legume-based foods

Cereal and legume-based foods are a major source of economical dietary energy and nutrients, worldwide. Often, the regional specific cereals or legumes are subjected to natural or controlled fermentation to obtain desirable final products which are nothing but fermented foods. The involvement of desirable microorganisms, particularly those of lactic acid bacteria, yeasts, and fungi have been well documented (Wang and Hesseltine, 1981; Campbell-Platt, 1987; Steinkraus, 1995). A major proportion of the cereal and legume based foods are fermented by lactic acid bacteria, wherein these organisms possess the ability to increase palatability, keeping quality, safety, and nutritive value of the raw materials, either cereals or legumes or both. The successive growth phase of microorganisms in fermenting cereals and legumes also favors yeast growth, which often occurs as a component of mixed microflora and imparts specific characteristics for the product (Salovaara, 1993).

#### 2.2.3.1 Idli

Among the closely related types of traditional fermented foods based on cereal and legume combination is that of *idli*. *Idli* is a white, fermented (acid leavened), steamed,
soft and spongy texture product, widely popular and consumed in the entire South India. It has been documented that idli batter fermentation has been in use since 1100 AD (Ramakrishnan, 1979). Idli is the resultant product from the naturally fermented batter made from washed and soaked milled rice (Oryza sativus) and dehulled Black gram dhal (Phaseolus mungo). From a nutritional and health status point of view, idli appears to be an ideal human food for people of all ages and at all times. Investigations into the primary aspects of idli batter fermentation were initiated as early as in 1955 at the Central Food Technological Research Institute, Mysore, India. Several researchers have used different proportions of Black gram cotyledons to rice ranging from 4:1 to 1:4 weight to weight (w/w) for making idli with a preference for 2:1 and 3:1 over 4:1 (Lewis et al. 1955; Desikachar et al. 1960; Radhakrishnamurthy et al. 1961; Steinkraus et al. 1967; Reddy et al. 1981).

Studies have demonstrated the optimum fermentation conditions for obtaining good idlis as well as the physiochemical and microbiological changes taking place over intermittent periods of incubation at varying temperatures (Desikachar et al. 1960; Yajurvedi, 1980; Thyagaraja et al. 1992). Usually, the microorganisms that develop during the initial and later soaking of the ingredients are sufficient to bring about the fermentation. The microbiological changes during the fermentation period have shown the involvement of varied genera and species of lactic acid bacteria and yeasts. The bacterial organisms, identified as a part of the microflora, included Leu. mesenteroides, Lb. delbrueckii, Lb. fermentum, Lb. lactis, Lb. brevis, Str. faecalis and Pediococcus cerevisiae, while the yeast flora comprised Torulopsis candida, Torp. holmii, Candida cacaoi, Can. fragicola, Can. kefyr, Can. tropicalis, Hansenula anomala and Rh. graminis. Further, these studies have shown the predominance of major flora at various stages of fermentation in idli (Mukherjee et al. 1965; Steinkraus et al. 1967; Venkatasubbaiah et al. 1984; Soni, 1987; Soni and Sandhu, 1990; Thyagaraja et al. 1991, 1992).
Two significant changes occurring in idli fermentation are acidification and leavening of the batter. Comprehensive studies on the various changes accompanying idli batter fermentation have shown that besides successive increase in microbial populations, the pH declined to 4.4-4.9 from an initial pH of 6.6 (Soni and Sandhu, 1990; Thyagaraja et al. 1992). Several attempts have been made to improve the idli fermentation by standardization of various physio-chemical factors. An increasing rate of idli batter fermentation was observed to accompany a rise in temperature (Desikachar et al. 1960). Fortification of idli batter with glucose at 1% level showed a beneficial effect on gas production and leavening during fermentation (van Veen et al. 1967). Similarly, the relationship between the type of microflora and the biochemical attributes revealed an increase in the water soluble group B vitamins during idli fermentation (Lakshmi, 1978).

As a step towards convenience in the preparation, the concept of a dry blend mixture was proposed as early as in 1960 (Desikachar et al. 1960). In a similar approach, a process relating to an improved means of providing inocula (lactic acid bacteria and yeast) in ready to use form idli fermentation has been developed (Varadaraj et al. 2001). In a direction towards reducing the fermentation time of idli batter and increasing the shelf life of fermented idli batter, a Indian patent has been filed (Varadaraj et al. 1999). Simultaneously, the flavour profile of such controlled fermented idli batter has shown the presence of desirable flavour compounds such as ketones, diols, and acids for a period of 8 d of storage. This flavour profile can be a reliable qualitative and quantitative parameter for objective assessment (Agrawal et al. 2000).

Plantaricin LP84, a bacteriocin produced by Lb. planatrum NCIM 2084 was able to retard the growth of the foodborne pathogens such as Bac. cereus F 4810, Esch. coli D 21, and Staph. aureus FRI 722 during idli batter fermentation. However, these pathogens
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occurring as contaminants in *idli* batter can survive and grow under conditions of natural fermentation (*Jama and Varadaraj, 1999*).

### 2.2.3.2 Dosa

*Dosa* is a fermented, thin, crisp, fried and pancake like product widely consumed in south India and parts of western India. In recent years, *dosa* is becoming popular in other parts of India as well as in the Indian restaurants elsewhere in the world. The batter preparation for *dosa* has been almost the same as that of *idli* batter, except for the proportion of milled rice and Black gram dhal. The product is prepared by soaking separately equal quantities of milled rice and dehulled Black gram dhal for a period of 6-8 h at ambient temperature (25-30°C) and then grinding them together into a fine paste with requisite quantity of water (2.0-2.5 parts w/w). The batter is then allowed to undergo natural fermentation for a period of 10-14 h at ambient temperature (25-30°C). The fermented batter is then fried using oil into a thin, crisp, pancake like product.

Similar to *idli* batter fermentation, traditional *dosa* batter fermentation has also revealed the occurrence and role of lactic acid bacteria in combination with yeasts in bringing about various biochemical changes (*Soni et al. 1985, 1986*). The predominant species identified were *Leu. mesenteroides*, *Lb. fermentum*, *Lb. delbrueckii*, *Ped. cerevisiae*, *Sac. cerevisiae*, *Han. anomala*, and *Kluyveromyces* sp. Usually, these microorganisms come from the raw materials like rice and Black gram dhal (*Soni and Sandhu, 1990*). *Dosa* batter fermentation also resulted in increased biochemical attributes, including that of water soluble group B vitamins such as thiamine, riboflavin, and cyanocobalamin.

Attempts were made to prepare *dosa* like products by replacing Black gram with other legumes like soy beans. Soy bean-based *dosa* batter was found to be nutritious, but less preferred organoleptically (*Soni and Sandhu, 1989*).
2.2.3.3 **Kadhi**

*Kadhi* is a traditional fermented culinary food item with a mild acidic taste and a typical cooked flavour, widely consumed in most parts of India as a culinary food item. Traditionally, *Kadhi* is prepared by boiling stirred *dahi* (lactic fermented) with 5-8% (w/w) Bengal gram dhal flour (*besan*) as a thickening agent. Considering the potential use of antagonistic LAB, studies have been undertaken to evaluate the effectiveness of antibacterial properties of selected LAB towards foodborne pathogenic and spoilage bacterial species occurring in pre-processing and post-processing contaminants in *kadhi*. The study emphasized the benefits of using pure cultures of antagonistic LAB such as *Lb. delbrueckii* ssp. *lactis* CFR 2023 and *Lb. delbrueckii* ssp. *bulgaricus* CFR 2028 in the preparation of *kadhi* with advantages desirable quality attributes and preservation against foodborne pathogens (*Balasubramanyam and Varadaraj, 1995; Varadaraj et al. 2000*).

The efficacy of the antagonistic cultures of *Lactobacillus* species was evaluated in terms of microbiological and sensory parameters in *kadhi* during storage at ambient and refrigeration temperatures (*Balasubramanyam, 1996*). Research studies have been attempted to modify the *kadhi* preparation to provide better nutritional status through the use of lesser quantities of Bengal gram flour obtained from pre-soaked seeds. On the basis of sensory attributes and the characteristic consistency of *kadhi*, the product prepared using Bengal gram flour (from pre-soaked seeds at 40 mg/g) was found to have better acceptance as against the product prepared by conventional method using 100 mg/g of *besan* (*Rati Rao et al. 2006*).

2.2.3.4 **Dhokla**

*Dhokla*, a steamed fermented product, is a traditional food popular in western India, particularly in the States of Gujarat and Maharashtra. The product has a soft and spongy
texture which is prepared from a mixture of Bengal gram dhal (*Cicer arietinum*), dehulled Black gram dhal (*Phaseolus mungo*) and milled rice (*Oryza sativa*) in the ratio of 2:1:1. The mixture of legumes and cereal after 6-8 h of soaking in water is then ground into a grainy consistency. The resulting batter is mixed with curds in 1:1.5 (w/w) and allowed to ferment for 16-18 h. The fermented batter is prepared into suitable shapes and steamed. The steamed product is then seasoned with oil, spices and greens (coriander leaves) prior to consumption (*Ramakrishnan, 1979; Steinkraus, 1995*).

In the background of limited studies, in one of the earlier studies on the microbial and biochemical changes occurring during naturally fermented *dhokla* batter, it was established that the population levels of LAB and yeast increased during 0-18 h of fermentation period. The microflora usually comprised *Lb. fermentum*, *Leu. mesenteroides* and *Han. silvicola*. The increase in microbial counts was accompanied with the changes in pH, titratable acidity and volatile fatty acids (*Neeta Joshi et al. 1989*).

Studies with the use of antagonistic isolates of *Lactobacillus* spp. in the preparation of *dhokla* batter revealed that spoilage bacterial species like *Bac. laterosporus*, *Bac. licheniformis*, and *Bac. subtilis* occurring as pre-processing contaminants were unable to increase in their population levels, while the same spoilage organisms were able to survive and increase in their population levels in *dhokla* batters prepared using a non-antagonistic isolate of *Lactococcus* sp. (*Balasubramanyam, 1996; Varadaraj et al. 2000*).

2.2.3.5 Punjabi warri

*Punjabi warri*, a legume-based fermented and dried product is very popular in north India. *Warries* are spicy, hollow, brittle, fried balls of about 5-8 cm diameter each and are generally used as a condiment in cooking with vegetables, soup, or Indian sambhar
(dhal-based spicy liquid). Dehulled Black gram dhal (*Phaseolus mungo*) after being washed and soaked overnight in water are ground to a batter of pasty consistency, which is then supplemented with a variety of spices, molded into small balls and subjected to fermentation and drying in open atmosphere for 4-8 d (Sandhu and Soni, 1989).

Microbiological and biochemical aspects associated with *punjabi warri* have been the subject of study in a few research investigations (Sandhu and Soni, 1989; Soni and Sandhu, 1990). These studies have established that the development and prevalence of microorganisms were affected by the seasons; summers being more favourable for bacteria and winters for yeasts. These microbial types tend to increase significantly with the progress in fermentation. The microorganisms which bring about acidification and leavening in *warries* are those of *Leu. mesenteroides*, *Lb. fermentum*, and *Str. faecalis*. In addition, the yeast flora encountered were those of *Sac. cerevisiae*, *Pichia membranaefaciens* and *Trichosporon beigeli*. The microbiological changes have been associated with biochemical and nutritional changes. An increase in the amylase and proteinase activities was observed during *warri* fermentation, during which period there was also an appreciable increase in levels of water soluble B-vitamins such as thiamine, riboflavin and cyanocobalamin.

The use of LAB as biopreservative in *punjabi warri* has been the subject of few studies. In order to enable the benefits of biopreservatives in *warries*, the product was prepared using antagonistic isolates of *Lb. delbrueckii* ssp. *bulgaricus* CFR 2028 and *Lb. delbrueckii* ssp. *lactis* CFR 2023. In these *warries*, the spoilage bacterial species like *Bac. laterosporus*, *Bac. licheniformis* and *Bac. subtilis* were retarded in their growth during the storage period of 10 d at 25-30°C, which happens to be the traditional method of simultaneous fermentation and drying (Balasubramanyam, 1996; Varadaraj et al. 2000).
In addition to the above, there are several other documented cereal and legume-based traditional foods popular in specific regions of India. However, detailed scientific and technological studies have not been much documented about these foods.

2.2.4 Milk and cereal / legume-based foods

2.2.4.1 Rabadi

*Rabadi* is a fermented beverage popular in north western parts of India, particularly the State of Rajasthan. The product is prepared from a mixture of pearl millet flour (*Pennisetum typhoideum*) or wheat flour and buttermilk, which is then placed in an earthen container and allowed to ferment for 4-6 h at ambient temperature. The fermented product is diluted with water, boiled and salted to taste. Due to the process of natural fermentation and lactic flora coming from buttermilk, lactobacilli count appears to increase (*Dankher and Chauhan, 1987*). *Rabadi* fermentation of freshly ground wheat millet flour brought about significant increase in HCl-extractability of calcium, iron, copper, zinc, manganese and phosphorus (*Gupta et al. 1991*). Consumption of such fermented foods may help to ameliorate the prevalent mineral deficiencies due to their limited bioavailability from such coarse grains (*Mahajan and Chauhan, 1987*; *Khetarpaul and Chauhan, 1989, 1991*). The effect of processing parameters such as dehulling, cooking and fermentation on antioxidants present in pearl millets during *rabadi* preparation revealed that cooking and fermentation results in enhanced flavonoids (*Gupta and Nagar, 2010*). Further, using germinated pearl millet grains, optimization of *rabadi* preparation was undertaken by response surface methodology. The most acceptable product was prepared using 5.3% flour and 72% water based on the type of curd (*Modha and Pal, 2011*).
2.2.4.2 Kulcha

*Kulcha* is a popular product consumed in north India, which of late, is gaining popularity in other regions of India. The product is prepared from white wheat flour mixed with milk, sugar, salt, curds, dry yeast, baking powder and water. The ingredients are well mixed and kneaded into soft stiff dough. The dough is subjected to fermentation for a period of 6-8 h at ambient temperature. The fermented dough is divided into equal size balls which are then rolled or flattened by hand to thick round/disc shaped *kulchas* of about 6" in diameter. The smoothly flattened dough is baked in a tandoor (metallic baking tray or special oven made of clay) to obtain a golden brown colour. *Kulchas* are served hot (Rati Rao et al. 2006).

2.2.4.3 Naan

*Naan* is leavened flat bread baked in a clay oven called the tandoor and is widely consumed by the people in north India. In recent times, *naan* is becoming more popular in other regions of this country. The product is prepared from a mixture of white wheat flour, milk, egg, baking powder, salt, sugar, and curds. The dough is allowed to ferment at ambient temperature for 1-2 h. Then the dough is divided into equal sized medium balls, which are then rolled out into oblong shapes on a lightly flattened surface. The flattened dough is put on a wet cloth and then transferred onto the inner wall of pre-heated tandoor (clay oven) wall. The final product known as *naan*, when fully baked is crisp and brown on both sides, which is removed using skewers and served hot, topped with butter (Rati Rao et al. 2006).

2.2.5 Vegetable-based fermented foods

The ancient civilization knew well about the existence of natural microflora and its role in fermentation of vegetables, which could result in palatable foods for human
consumption. Vegetables do contain low sugar content, have low sugar content, neutral pH and their composition is not favourable for spontaneous growth of LAB. However, over centuries, people have traditionally developed lactic fermentation methods that could stabilize and improve nutritional quality of vegetables. Fermented vegetables represent an essential element of human diet. Lactic acid fermentation that enhances the organoleptic and nutritional quality of the vegetables has remained more as a household or cottage level process.

Spices and herbs do impart a fine flavour and plays a determining role in fermented vegetables. Aromatic compounds in spices (including terpenes and polyphenols) often have antimicrobial effect, which selectively promote LAB. Certain spices such as garlic, cloves, chillies (unripe and ripe) do inhibit undesirable microflora. Many sulphur compounds (as in garlic) have antibacterial properties. Mustard seed contains allyl isothiocyanate, a volatile aromatic compound with antibacterial and fungal properties, which are known to inhibit yeasts. In India, ground mustard seed or oil is widely used in traditional lactic fermentation for prolongation of shelf life of the product. Chemical preservatives such as sorbic and benzoic acids and their salts have been in use in the development of fermented products based on vegetables (Montet et al. 1999). Salt concentration induces plasmolysis in vegetables, thus promoting anaerobiosis for proliferation of lactic acid bacteria. A few of the well known vegetable-based lactic fermented products are presented in the following paragraphs.

The popular types of pickles consumed by the human population of India have been those based on unripe mangoes, goose berries, lemons, swallow root (Decalepis hamitonii) and a variety of mixed vegetables. In general, most of these pickles are prepared at the house-hold level by subjecting the vegetables to natural fermentation. In specific type of pickles, preservation is achieved through lactic acid fermentation and in the presence of high concentrations of salt. The process involves washing of raw
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materials, followed by cutting into suitably sized shapes and mixed with salt at a level of two times more than that of weight of the raw materials. Requisite spice powders are also used in the preparation. The spice powder mix mainly consists of chillies, mustard, and coriander seeds. The mouth of the containers are closed properly and kept for 7–8 days at ambient temperature (20–30°C) for the natural fermentation to take place in situ. During the storage period, acidity develops and a certain amount of aging provides the acceptable organoleptic attributes to the pickles. Although not much research has been done into the nature of microflora and other attributes, it is thought that the microflora mainly comprises lactic acid bacteria and to a certain extent acetic acid bacteria.

In another specific type of pickle which is devoid of any liquid, the raw materials for pickle preparation are the same as described previously and preservation is through high concentration of salt, spice mix and edible oil. This mixture is subjected to intermittent mild frying with oil. After 3 to 4 similar treatments, the whole mixture is placed in a clean container and the mouth of container is closed properly. It is very essential to see that the pickle mixture is layered with sufficient quantity of edible oil, wherein the oil used depends upon the specific regions of this country. In this specific type of preparation, fermentation occurs naturally with the predominance of LAB, which can survive and grow in presence of high salt concentrations. Irrespective of the type of pickle preparation, the shelf life is quite reasonable extending even up to periods of 6 months and beyond, if proper practices of hygiene and sanitation are in-place during preparation and subsequent storage. In the absence of any microbiological studies of the nature of pickle fermentation, from the product profile, it would appear that species of *Pediococcus* tend to predominate over other LAB.

There are several other traditional fermented vegetable-based foods, which are more popular in eastern, northern and north-eastern regions of India. A few of them known by traditional names are *gundruk, sinki, khalpi, mesu, kachampulli* and others.
2.3 NUTRITIONAL STATUS OF INDIAN TRADITIONAL FOODS

In the background of a diverse range of traditional foods of India, the most popular and widely consumed ones are those based on either milk only or cereals and legumes with milk. The scientific knowledge base of nutritional benefits derived by milk and milk-based products are well documented. On the other hand, the same is not true with those of cereals and legumes-based foods, as the wide ranging region specific available raw materials are a store-house of complex nutrients. This complexity linked with the type of fermentation process, product preparation parameters and final profile gives ample opportunities to bring into focus the importance of nutritional constituents in cereals and legumes-based Indian traditional foods.

Cereals and legumes are considered to be one of the most important sources of dietary proteins, carbohydrates, vitamins, minerals and fibre for human nutrition. Often, the nutritional quality of cereals and legumes are not on par with those of milk and milk products. This is more attributed to the complex nature of macronutrients as well as the prevalence of antinutritional factors, which makes it difficult in terms of their bioavailability (Chavan and Kadam, 1989). Besides, processing unit operations like soaking, sprouting, milling, fermentation and cooking/heating have enabled the improvement of nutritional attributes of cereals and legumes (Mattila-Sandholm, 1998).

In general, natural fermentation of cereals and legumes leads to a decrease in the level of complex carbohydrates such as non-digestible poly- and oligosaccharides. Further, certain amino acids and vitamins, especially B group vitamins may be synthesized and becomes available. Increased amounts of riboflavin, thiamine, niacin, and lysine due to the action of LAB in fermented blends of cereals were reported in few of the studies (Hamad and Fields, 1979; Sanni et al. 1999). Fermentation also provides optimum pH conditions for enzymatic degradation of phytate which is present in cereals in the form of complexes with iron, zinc, calcium, magnesium and proteins. Such a
reduction in phytate may increase the bioavailability of iron, zinc and calcium by several folds (Stewart and Getachew, 1962; Gillooly et al. 1984; Chavan and Kadam, 1989; Khetarpaul and Chauhan, 1990; Nout and Motarjemi, 1997; Haard et al. 1999).

Fermentation is known to extend the shelf life and impart improved and acceptable texture, taste and aroma of the final product. During cereal fermentations several volatile compounds are formed, which contribute to a complex blend of flavours in the products (Chavan and Kadam, 1989). The presence of aromas representative of diacetyl, acetic acid and butyric acid make fermented cereal and legume based products more appetizing. Traditional fermented foods prepared from most common types of cereals and legumes (such as rice, wheat, corn or sorghum and soy bean) are well known in many parts of the world. Some are utilized as colourants, spices, beverages and breakfast or light meal foods, while a few of them are used as main foods in the diet. In most of these products the fermentation is natural and involves mixed cultures of yeasts, bacteria and fungi. Often, the predominant microflora may be functional parallelly, while few others may become functional in a sequential manner with a changed environment due to fermentation process.

The common fermenting bacteria are species of Lactobacillus, Leuconostoc, Streptococcus, Pediococcus, Micrococcus and Bacillus. The fungal genera known to be commonly present are Aspergillus, Cladosporium, Fusarium, Penicillium and Trichothecium. The common fermenting yeasts are species of Saccharomyces, which usually predominates in an alcoholic fermentation (Steinkraus, 1998). The type of bacterial flora developed in each fermented food depends on the water activity, pH, salt concentration, temperature and the composition of the food matrix.

Lactic acid fermentation contributes towards the safety, nutritional value, shelf life and acceptability of a wide range of cereal based foods (Oyewole, 1997). In many of those processes, cereal grains, after cleaning are soaked in water for a few days during
which a succession of naturally occurring microorganisms will result in a population dominated by LAB. In such fermentations, endogenous grain amylases generate fermentable sugars that serve as a source of energy for the lactic acid bacteria. Fermentation is often just one step in the process of fermented food preparation. Other operations such as size reduction, salting or heating also affect the final product properties (Nout and Motarjemi, 1997).

A range of indigenous fermented foods prepared from cereals in India utilizes cereals in combination with legumes, thus improving the overall protein quality of the fermented product. Cereals are deficient in lysine, but are rich in cysteine and methionine, legumes are rich in lysine, but deficient in sulphur containing amino acids. Thus, by combining cereal with legumes, the overall protein quality is improved (Campbell-Platt, 1994). In the fermented foods like idli, dosa and dhokla, the fermenting desirable microflora are considered essential for leavening of the batter and for acid production in idli (Purushothaman et al. 1993; Ramakrishnan, 1993). Fermentation of idli batter appears to significantly increase the essential amino acids and simultaneously reduce the antinutrients (phytic acid), enzyme inhibitors and flatus causing sugars (Steinkraus et al. 1993).

2.3.1 Bioavailability of minerals, vitamins and proteins as affected by phytate

For several decades, concerns have been raised about the role of phytic acid in reducing mineral bioavailability. Phytic acid is a ubiquitous plant constituent present in nuts, cereals, legumes and oilseeds, it has a potential for binding positively charged proteins, amino acids and minerals in foods. The resulting complexes are insoluble and makes it difficult for humans to hydrolyze during digestion, which implies that they are less available for absorption. Phytate forms chelating conjugates with nutritionally important minerals such as calcium, magnesium, copper, iron (Fe$^{2+}$ and Fe$^{3+}$), zinc,
cobalt, and manganese. Solubility is a pre-requisite for absorption of most minerals, although solubility at neutral pH has been shown to be less important for calcium absorption (Heaney et al. 1990). The chemical structure of phytic acid is indicative of strong chelating potential. Phytic acid has six strongly dissociated protons (pKs 1.1 to 2.1) and six weakly dissociated protons (pKs 4.6 to 10.0). The effect on minerals is observed through the formation of phytate-mineral (M) or peptide-mineral-phytate complexes. When the complex includes peptides, bioavailability of proteins and enzymatic activity may be reduced (Deshpande and Cheryan, 1984). Humans lack sufficient intestinal phytase to degrade the complexes. There are several factors which determine the effect of phytate on mineral bioavailability.

2.3.1.1 pH

The effect of pH on mineral-phytate and protein-mineral-phytate interactions has been reviewed by Champagne and Phillippy (1989). As the pH increases and under sufficient phytate concentrations, phytic acid becomes more ionized and begins binding cations. pH also affects the charge of peptides. On the acidic side of the isoelectric pH of the peptide, the negatively charged carboxyl groups can react directly with the positively charged amino group. On the alkaline side of the isoelectric pH of the peptide, wherein carboxy groups are negatively charged, binding occurs through positively charged mineral ions. This can occur in processing or during digestion. For example, at higher pH, zinc was more associated with phytate in soy protein isolates than at 4.5 pH (Khan et al. 1990). A higher pH environment would typically occur in the intestine. However, Champagne and Phillippy (1989) reported that high intra-luminal gastric pH leads to the formation of calcium-zinc-phytate complexes as early as in the stomach following the ingestion of soy protein isolate.
2.3.1.2 Mineral and phytate interactions

The relative binding strengths of different minerals to phytic acid vary greatly. Chelation strength increases with increasing atomic number of the mineral moving from the alkaline earth metals through transition metals in the periodic table. Vohra et al. (1965), using titration curves of phytate as free acid in the presence of single cations, reported that phytate forms complexes with cations in the following descending order of strength:

\[ \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+} > \text{Fe}^{3+} > \text{Ca}^{2+} \]

The decreasing order of stability of phytate-mineral complexes is as follows:

\[ \text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+} \]

Consequently, zinc is the essential mineral most affected by phytate. In one of the research studies, the presence of unhydrolyzed phytate in unleavened bread was considered to be responsible for zinc deficiency in the Middle East. Zinc deficiency was corrected by leavening or by zinc supplementation to the phytate-rich cereal legume diet of Egyptians and Iranians (Prasad et al. 1963; Reinhold et al. 1973; Navert et al. 1985).

Phytate concentrations have to be sufficiently high to exert a substantial effect on mineral bioavailability in the diet. In soy, low levels of phytate can inhibit iron absorption. It is not clear whether phytate concentration per se or the ratio of phytate to minerals in the foods is dominant in influencing mineral bioavailability. It is possible that these factors have different impacts for different nutrients. The molar ratio of phytate to zinc has been reported to be a major factor in influencing bioavailability of zinc in breakfast cereals fed to rats. Molar ratios of phytate:zinc of >10 were associated with zinc deficiency symptoms (Morris and Ellis, 1980, 1981). The phytate to calcium/zinc molar ratio was even a good indicator of zinc bioavailability from seeds than was the phytate-zinc molar ratio, especially at lower protein intakes (Davies et al. 1986; Fordyce et al. 1987). The phytate:mineral molar ratios have been used to predict bioavailability of
several minerals. There are several reasons that could explain why phytate:mineral molar ratios in a food lacks predicted mineral bioavailability. There is no fixed stoichiometric relationship between phytate and any particular mineral, because it depends on the presence of multiple ions, pH, temperature and ionic strength. Further, the intestinal milieu alters these factors. The pH varies along the digestive tract and endogenous secretions can dilute the phytate:mineral ratios. The specific environment at the site determines absorption, but has problems to be readily measured.

2.4 PREBIOTICS

2.4.1 Definition and / Nomenclature

The human gut microflora is affected by many factors such as age, drug therapy, diet, host physiology, peristalsis, local immunity and in situ bacterial metabolism (Berg, 1996). However, diet is probably the most significant factor determining the type of gut flora that develops based on the type of foods, which provide the main nutrient source for colonic bacteria. This interactive development has led to the concept of prebiotics. The term prebiotics was proposed by (Delzenne and Roberfroid, 1994) for the non-digestible oligosaccharides (NDOs) used as food ingredients to modify the composition of endogenous gut microflora. The definition was developed by Gibson and Roberfroid (1995) who named a prebiotic as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health”. This definition only considers microbial changes in the human colonic ecosystem. Later, it was considered timely to extrapolate this into other areas that may benefit from a selective targeting of specific microorganisms and to propose a revised definition of a prebiotic (Gibson et al. 2004) as “a selectively fermented ingredient that allows specific changes, both in the
composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health”. Prebiotics are considered to stimulate selective bacterial groups such as bifidobacteria, lactobacilli and eubacteria, which resides in the colon and benefits human health (Roberfroid, 2000).

The major prebiotics are non-digestible oligosaccharides (NDO’s, recently referred to as resistant oligosaccharides), which includes fructo-oligosaccharides, gluco-oligosaccharides, galacto-oligosaccharides, transgalacto-oligosaccharides, isomalto-oligosaccharides, xylo-oligosaccharides, soy bean oligosaccharides and also resistant starches, sugar alcohols and difructose anhydride. Inulin-type fructo-oligosaccharides have been the ones most investigated as prebiotics and are considered as typical 'bifidogenic factors' (Crittenden et al. 2001). The non-digestible oligosaccharides are complex carbohydrates which are resistant to hydrolysis by acid and enzymes in the human digestive tract due to the chemical configuration. Therefore, prebiotics have also gained importance as functional food ingredients/products (Lee and Prosky, 1995).

2.4.2 Non-digestible higher polysaccharides

Oligosaccharides, such as lactulose, fructo-oligosaccharides, transgalacto-oligosaccharides have received increased attention, especially because they have been shown to be effective in stimulating the growth of bifidobacteria and lactobacilli in human large intestine (Gibson et al. 1995; Bouhnik et al. 1997). In the food industry, simple oligosaccharides are used as bifidogenic substances and some infant products contain them as it may provide few of the benefits attributed to oligosaccharides in human milk (Rivero-Urgell and Santamaria-Orleans, 2001). At least two types of oligosaccharides exist in cereal grains namely (i) galactosyl derivatives of sucrose, stachyose and raffinose and (ii) fructosyl derivatives of sucrose, fructo-oligosaccharides (Henry and Saini, 1989). Cereal bio-processing through enzymatic reactions or fermentation can
also produce a large range of oligosaccharides with potential prebiotic properties. The α-amylase present in the cereal grain can hydrolyze the gelatinized starch granules, and the extent of the hydrolysis could be regulated through temperature control.

2.4.3 Significance of prebiotics

The concept of prebiotics is only 14 years old and has stimulated research in the areas of both nutrition and medical sciences. The significance of prebiotics in human health could be attributed to the following factors (Macfarlane et al. 2006):

(i) the growing belief towards the existence of a healthy or balanced gut microbiota,
(ii) the ability of prebiotics to alter the composition of microbiota towards deriving a more healthy profile,
(iii) as an alternative to probiotics, which can be difficult to handle in some foods and
(iv) prebiotics currently in use, especially inulin and its derivatives and galacto-oligosaccharides (GOS) are relatively easier to prepare or extract from plant sources.

Inulin-type fructo-oligosaccharides have been the ones most investigated as prebiotics. Much of the focus has been on their ability to enhance growth of species of *Lactobacillus* and *Bifidobacterium* in the intestinal tract, primarily in the large intestine. Even, galacto-oligosaccharides have a similar effect (Sako et al. 1999). These bacteria can hydrolyze such oligosaccharides and use them as an energy source to support their growth. In the large intestine, prebiotics, in addition to their selective effects on bifidobacteria and lactobacilli, influence many aspects of bowel function through fermentation products. Prebiotics can be metabolized in the large gut by bacterially produced enzymes (Cummings and Englyst, 1995). The metabolism of these carbohydrates produces a variety of products, such as short-chain fatty acids (SCFA) like acetate, propionate and butyrate as well as organic acids (lactate, succinate and
pyruvate). These products may have various effects on host health (Delzenne and Roberfroid, 1994; Gmeiner et al. 2000).

Many studies have shown that SCFA, being the main acidic products of bacterial fermentation, contribute towards a low colonic pH resulting in the direct inhibitory activity towards important gastro-intestinal pathogens and provide metabolic energy for the host (Gibson, 2004). Short chain fatty acids are not only a source of energy for tissues, but can also have important effects on host physiology. Acetate is metabolized in systemic areas like muscle, while propionate is transported to the liver and used to generate ATP. Butyrate is an important source of energy for the colonocytes and is thought to have anti-tumour properties. It also improves the bioavailability of minerals such as calcium and magnesium (Cummings et al. 1989).

One advantage of the prebiotic over the probiotic approach is that the former does not rely on culture viability. Prebiotics are ingredients in the normal human diet and as such they do not pose as great a challenge from the aspects of safety and consumer acceptability as do probiotics (Wells et al. 2008). The currently recognized prebiotics in Europe are fructo-oligosaccharides, galacto-oligosaccharides and lactulose. Prebiotics are added to many foods, including yoghurts, cereals, breads, biscuits, milk desserts, ice creams and so forth (Wells et al. 2008). In vitro studies have shown an increased population of bifidobacteria and lactobacilli by prebiotics (Wang and Gibson, 1993; Gibson and Wang, 1994; Probert and Gibson, 2002). The majority of clinical trials in humans have focused on demonstrating their efficacy in increasing intestinal levels of bifidobacteria and sometimes lactobacilli in fecal samples of healthy subjects (Kolida et al. 2002; Macfarlane et al. 2006).

Prebiotics are known to exhibit, both important technological characteristics and interesting nutritional properties. In food formulations, they can significantly improve organoleptic characteristics (Franck, 1999). Most of the prebiotics in commercial
applications are NDOs (Delzenne and Roberfroid, 1994). They are obtained either by extraction from plants like oligofructose by enzymatic hydrolysis of chicory inulin or by synthesis (trans-glycosylation reactions) from mono- or disaccharides such as fructo-oligosaccharides from sucrose and galacto-oligosaccharides from lactose (Crittenden and Playne, 1996).

2.4.4 Categories of prebiotics
The prebiotics are categorized based on certain established criteria (Gibson et al. 2004). These criteria are:

i) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastro-intestinal absorption,

ii) fermentation by intestinal microflora and

iii) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being.

Resistance, in the first criterion, does not necessarily mean that the prebiotic is completely indigestible, but it should guarantee that a significant amount of the compound is available in the intestine (especially the large intestine) to serve as a fermentation substrate.

2.4.4.1 Established prebiotic oligosaccharides
Most widely researched prebiotics fall in the group of oligosaccharides. Oligosaccharides are sugars consisting of 2 to 20 sugar units. Some occur naturally as in asparagus, chicory, garlic, onion and soy bean. There are several properties of oligosaccharides, which serve as the desired attributes of prebiotics. High selectivity and efficiency in metabolism by bifidobacteria and lactobacilli have enabled the supplementation of prebiotic at low dosage. Oligosaccharides are reported to possess receptor sequences
that inhibit adhesion of pathogens to mucosal cells. Binding of the pathogens to specific receptor sites in oligosaccharides is postulated to increase host resistance to infection, reducing the likelihood of pathogen establishment and subsequent elaboration of virulence (Gibson et al. 2000).

2.4.4.1.1 Fructans

The only prebiotic for which sufficient data has been generated to allow an evaluation of their possible classification as functional food ingredient are the inulin-type fructans, which include native inulin, enzymatically hydrolyzed inulin or oligofructose and synthetic fructooligosaccharides (Roberfroid, 1998; Roberfroid et al. 1998). Inulin and oligofructose are the natural constituents of many common vegetables, fruits and cereals. There has been widespread and common knowledge on their natural occurrence and consumption as human food and animal feed for years (Van Loo, 1995; Mosfegh, 1999). Inulin and oligo-fructose are recognized as dietary fibres in most countries (Hoebregs, 1997).

The commercial source of inulin for functional food application is chicory root. The production process involves extraction of naturally occurring inulin by diffusion in hot water, followed by refining, evaporation, and spray drying. Chicory inulin is a linear chain of fructo-furanose residues with β 2, 1 linkages (Gibson, 2004; Franck and De Leenheer, 2005). A specific combination of long-chain inulin and oligofructose (1:1), known as Synergy1 has been developed to offer enhanced nutritional benefits. Its unique chain length distribution makes it active throughout the whole length of the colon, with the shorter chains being fermented more rapidly in the proximal colon and the longer chains reaching more distal parts of the gut (Van Loo, 2004).

The resistance of inulin type fructans to digestive processes has been extensively studied and demonstrated by in vitro and in vivo methods (Roberfroid,
1993). Several in vitro studies support the selective stimulation of bacterial growth by inulin. This has been carried out in defined pure culture fermentation and using a mixed fecal inoculums, both in batch and continuous culture (Wang and Gibson, 1993; Gibson and Wang, 1994a; Roberfroid et al., 1998). Similarly, in vivo studies demonstrated that feeding of oligo-fructose or inulin, or a mixture of both in rats, selectively stimulated the growth of bifidobacteria as well as lactobacilli, while reducing the number of clostridia. Such treatments also increased the relative proportion of butyrate indicating a change in bacterial activity (Levrat et al., 1991; Campbell et al., 1997; Kleessen et al., 2001; Poulsen et al., 2002).

The efficacy of inulin has also been evaluated with a view to its administration to formula-fed infants observed an increase in bifidobacteria and lactobacilli in infants who received formula milk supplemented with a mixture of long chain inulin and galactooligosaccharides, indicating its prospects in infant nutrition (Coppa et al., 2002; Moro et al., 2002).

2.4.4.1.2 Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) also called as trans-galactosylated oligosaccharides are synthesized by galactosyl transfer reactions catalyzed by β-galactosidase utilizing lactose as a substrate. Lactose acts as both glycosyl donor and acceptor in such reactions and a complex series of oligosaccharides are formed (Albayrak and Yang, 2002). The commercial products are not pure and they typically contain around 50% GOS with 38% glucose and 12% lactose on a weight basis. The principal components are trisaccharides and tetrasaccharides and GOS contain mainly β 1-4 and β 1-6 linkages with lesser amounts of β 1-2 and β 1-3 (Rabiu et al., 2001).

The data on non-digestibility do not fully match the requisite criteria of prebiotics. However, there are suggestions that GOS do reach the colon intact and generally
display a consistent prebiotic effect in mixed fecal cultures. In comparative studies with other prebiotics using batch fecal cultures and molecular microbiological analyses, GOS displayed good selectivity for bifidobacteria and decreased clostridial populations (Tomomatsu, 1994; Rycroft et al. 2001; Palframan et al. 2003; Tzortzis et al. 2005). Galacto-oligosaccharides has also shown good selectivity in three-stage models of the human colon, particularly for lactobacilli as well as reduced the β-glucosidase, β-glucuronidase and arylsulphatase activities, considered to be undesirable in the colon (McBain and Macfarlane, 2001). Adding a mixture of oligosaccharides (90% GOS and 10% long chain inulin) to infant formula milk has been shown to increase fecal bifidobacteria in both pre-term and term infants (Dubey and Mistry, 1996; Knol, 2001; Rivero-Urgell and Santamaria-Orleans, 2001; Boehm et al. 2002; Moro et al. 2002; Vandenplas, 2002).

2.4.4.1.3 Lactulose

Lactulose is produced by alkaline isomerization of lactose to produce 4-O-β-galactopyranosyl-D-fructose, (Harju, 1986). The commercial lactulose product is very pure and is available in crystalline and syrup formulations. These are currently targeted at medical applications and no food grade form of lactulose currently exists in the market. Although lactulose has a long history of use as a laxative and for treatment of hepatic encephalopathy, it can act as a prebiotic at sub-laxative doses. It is used in Japan in foods and has FOSHU status in several food vehicles (Rastall, 2007). Investigations of the enzymatic degradation of lactulose have found that human and calf intestinal β-galactosidases did not degrade lactulose (Gibson and Angus, 2000). In one of the earliest studies on lactulose fermentation using 37 species of bacteria in pure culture, it was found that Bacteroides oralis and vulgatus, Bifidobacterium bifidum, Clostridium perfringens, Lb. casei ssp. casei and four other strains of Lactobacillus spp.
fermented lactulose (Sahota et al. 1982). However, the in vitro data presently available do not demonstrate a selective stimulation of bacterial growth in mixed populations of microorganisms.

Tomoda (1991) fed yoghurt supplemented with lactulose to healthy volunteers and reported a significant increase in fecal bifidobacteria, but no total anaerobic count was performed and no other bacteria were enumerated, providing no evidence of selective stimulation of growth. In a well planned microbiological study, it was established that there was a selective and significant increase in fecal bifidobacteria and decreases in Cl. perfringens, streptococci, bacteroides and lactobacilli (Terada et al. 1993). In another study using randomized, double blind, placebo-controlled trials, there was more evidence of lactulose enabling significant increase in Bifidobacterium, Lactobacillus and Streptococcus, concomitant with significant decrease in Bacteroides, Clostridium, coliforms and Eubacterium (Ballongue et al. 1997). Through the use of fluorescent in situ hybridization, studies have also demonstrated a statistically significant and selective increase in bifidobacteria, following the feeding of lactulose (Tuohy et al. 2002).

2.4.4.2 Emerging prebiotic oligosaccharides

Gluco-oligosaccharides, isomalto-oligosaccharides, lacto-sucrose, polydextrose, soy bean oligosaccharides and xylo-oligosaccharides are oligosaccharides for which preliminary or even promising data already exist. However, the evidence for prebiotic status is still not sufficient and at present they are not classified as prebiotics. The prebiotic potential of several other compounds has also been investigated. However, evidence pointing towards any prebiotic effect is too sparse to justify a detailed review and a classification as prebiotic at the present time. These compounds include germinated barley, oligodextrans, gluconic acid, gentio-oligosaccharides, pectic
oligosaccharides, mannan oligosaccharides, lactose, glutamine, hemicellulose-rich substrate, resistant starch and its derivatives, oligosaccharides from melibiose, lactoferrin-derived peptide and N-acetylchito-oligosaccharides (Gibson et al. 2004).

2.4.4.2.1 Isomalto-oligosaccharides

The preparation of isomalto-oligosaccharides (IMO) includes hydrolysis of starch by the combined action of α-amylase and pullulanase, followed by isomerization of the resultant malto-oligosaccharides by α-glucosidase (Kohmoto et al. 1998, 1991) that catalyzes a transfer reaction converting the α 1-4 linked malto-oligosaccharides into α 1-6 linked IMO with different molecular weights. They do not strictly qualify as prebiotics as they are partially metabolized by the human small intestine (Oku and Nakamura, 2003). It is desired that precise human trials supported by molecular biology techniques could lead to a prebiotic claim for IMO.

2.4.4.2.2 Soy bean oligosaccharides

Soy bean oligosaccharides (SOS) are α-galactosyl sucrose derivatives (raffinose, stachylose). They are isolated from soy beans and concentrated to form the commercial product (Crittenden, 1996). Raffinose and stachyose have been suggested, but not really demonstrated to reach the colon after feeding to humans (Oku, 1994). The fermentation properties of these oligosaccharides have been studied either as mixtures of oligosaccharides or as individual components. In an early study, Minami (1983) studied the fermentation of raffinose in pure cultures and found it to be metabolized by bifidobacteria and a range of enteric organisms. Hayakawa et al. (1990) compared pure raffinose and stachyose with refined SOS. In a pure culture study, bifidobacteria (with the exception of Bif. bifidum) and lactobacilli (with the exception of Lb. casei)
metabolized the test sugars. The in vitro data presently available do not demonstrate a selective stimulation of bacterial growth.

2.4.4.2.3 Gentio-oligosaccharides

Gentio-oligosaccharides (GeOS) are β 1-6 linked gluco-oligosaccharides with a dp ranging from 2 to 7. They are commercially available as Gentose by Nippon Shokuhin Kako in Japan. As they are β-linked they have a bitter taste, which limits their applications in food products. Gentio-oligosaccharides are claimed to be prebiotic, but there is very little published data to support this claim. The prebiotic potential has been shown in one of the in vitro studies (Rycroft et al. 2001a). To date, there have been no peer reviewed articles on the prebiotic activity of GeOS in humans and consequently there is no information available on health attributes.

2.4.4.2.4 Xylo-oligosaccharides

Xylo-oligosaccharides (XOS) are prepared by enzymatic hydrolysis of xylan from corn cobs. The commercial products are predominantly composed of the disaccharide xylobiose with small amounts of higher oligosaccharides (Yamada, 1993). The parent molecule, xylan, is recognized as a dietary fiber indicating that it may reach the colon intact. The most informative studies on XOS were those carried out by Okazaki et al. (1990). These researchers carried out an initial pure culture study involving a wide range of bacteria, wherein it was shown that XOS was metabolized by a majority of bifidobacteria and lactobacilli. A recent pure culture study by Jaskari (1998) has shown that XOS from oat spelt xylan was metabolized by bifidobacteria as well as by bacteroides, Clostridium difficile and E. coli. However, lactobacilli did not metabolize the XOS. In general, pure culture study would not reflect the true situation in the colon. Crittenden and Playne (2002) suggested that bifidobacteria were able to utilize XOS,
but not xylan. The \textit{in vitro} data presently available do not demonstrate a selective stimulation of bacterial growth. A study in rats was carried out by Campbell et al. (1997), wherein a significant increase in the population bifidobacteria was evidenced. As it stands, the evidence prebiotic status of XOS is still not sufficient, hence is not yet classified as a prebiotic (Roberfroid, 2008).

2.4.4.3 Potential prebiotics

2.4.4.3.1 Pectic oligosaccharides

Pectins represent an interesting and abundant resource of complex oligosaccharides. Pectic oligosaccharides (POS) prepared by enzymatic hydrolysis of citrus and apple pectins have been evaluated as prebiotics by \textit{in vitro} studies (Olano-Martin et al. 2001, 2002; Hotchkiss et al. 2003). Studies with pure cultures of \textit{Bif. angulatum}, \textit{Bif. infantis} and \textit{Bif. adolescentis} revealed better growth on POS than on the highly methylated citrus pectin. The prebiotic activity of POS prepared from orange peel by acid extraction has also been evaluated in the studies of Manderson et al. (2005). Further, POS produced higher levels of butyrate than did FOS.

2.4.4.3.2 Gluco-oligosaccharides

Gluco-oligosaccharides (GOS) are synthesized by the action of the enzyme dextran sucrase (EC 2.4.1.5) on sucrose in the presence of maltose. Gluco-oligosaccharides can also be produced via fermentation in the presence of \textit{Leu. mesenteroides}. Branched chain oligomers produced using \textit{Leu. mesenteroides} B-742 have been shown to be readily utilized by bifidobacteria and lactobacilli in a pure culture study by Chung and Day, (2002), but not by \textit{Salmonella} spp. or \textit{Esch. coli}. Djouzi et al. (1995) found that GOS were utilized by only a few species of \textit{Bifidobacterium} like \textit{Bif. breve}, \textit{Bif. pseudocatenulatum} and \textit{Bif. Longum}. 
2.4.4.3.3 Lactosucrose

Lactosucrose is produced from a mixture of lactose and sucrose using the enzyme β-fructofuranosidase (Playne and Crittenden, 1996). The fructosyl residue is transferred from sucrose to the C1 position of the glucose moiety in the lactose, producing a non-reducing oligosaccharide (Hara et al. 1994). In chronically constipated patients receiving lactosucrose, Kumemura (1992) found a significant increase in bifidobacteria and a significant decrease in clostridia. Ohkusa et al. (1995) carried out a volunteer study involving feeding a normal diet supplemented with lactosucrose. A significant increase in bifidobacteria compared to pre-trial values was seen, together with a significant decrease in bacteroides compared to samples one week after termination.

2.4.5 Interactions between prebiotics and microbiota

Prebiotics (as well as probiotics) have been regarded as functional food ingredients because of their putative beneficial effects. A functional food is a food that contains one or a combination of components that interact with physiological functions in the body to improve them or to reduce the risk of associated diseases. Much work has been done to identify the possible mechanism of prebiotics to positively influence different metabolic functions in the body that are useful for host physiology as well as for the reduction of risk or even the treatment of some pathologies in its early stages (Milner, 1994). Presently, there are only 2 food ingredients that fulfill the prebiotics criteria i.e., inulin-type fructans and trans-galacto-oligosaccharides (Roberfroid, 2007). The efficacy of prebiotics in promoting human health is strongly related to their chemical structure. In general, feeding of FOS leads to (i) an increase in the population of *Bifidobacterium* spp. and *Lactobacillus* spp., (ii) increase in SCFA levels, (iii) decreases the numbers of *Clostridium* spp., *Fusobacterium* spp., *Bacteroides* and (iv) lowers pH (O’ Sullivan, 1996; Fuller and Gibson, 1997; Gibson et al. 1995; 1996). As a consequence of the
metabolism of the FOS by fermentative bacteria, SCFA and lactic acid are produced. Both lead to a drop in the pH of the large intestine. This is beneficial for the organism as it constitutes an ideal medium for the development of the bifidogenic flora and, at the same time, limits the development of bacteria which are considered pathogens (Rosenfeldt et al. 2002).

The underlying mechanisms of prebiotic induced alterations are not yet known. Substantial experimental data (Seifert and Watzl, 2008) suggest that prebiotics induce their immunological effects by the following means:

- Selective increase/decrease in specific bacteria that modulate cytokine and antibody production
- Increase in intestinal SCFA production and enhanced binding of SCFA to G-coupled protein receptors on leukocytes
- Partial absorption of prebiotics resulting in local and systemic contact with the immune system
- Interaction of prebiotics with carbohydrate receptors on leukocytes

2.5 PROBIOTICS

2.5.1 Definition and nomenclature

The concept of probiotics was introduced long before that of prebiotics (Gibson and Roberfroid, 1995; Hamilton-Miller et al. 2004). The term probiotic ("for life") was originally proposed by Lilley and Stillwell, (1965) as an antonym to the term antibiotic and was thought to be used for microbial substances, which promote the growth of other microorganisms. A few years later, the term probiotic was used in the context of animal feeds by Parker, (1974) and Fuller, (1989). The word probiotic is derived from the Greek meaning “for life” (Bengmark, 2000). Research on probiotic has exploded at nearly an
exponential rate over the last 15 years, since the first workable definition for probiotics was proposed. This definition, proposed by Fuller, (1989) was “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. Later on, probiotic has been defined as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Reid et al. 2003). Like prebiotics, probiotics do modify the composition of the gut microflora and, as a consequence, they have been shown to influence both intestinal and body functions.

The concept of probiotics, though not known in its present definition has been around for almost 100 years, as could be seen from the documented studies of lactobacilli in soured milks and the treatment of infant diarrhoea with bifidobacteria by (Tissier, 1906; Elie Metchnikoff, 1908). At the beginning of the 20th century, the Russian Nobel laureate Elie Metchnikoff associated the observed longevity of Bulgarian peasants with their high consumption of live microbes in fermented milk products (Metchnikoff, 1908). In 1930, the Japanese scientist Minoru Shirota isolated a lactic acid bacterium from the feces of a healthy infant. Five years later, one of the first fermented milk drinks thought to support intestinal health was produced with the strain he developed and named ‘Yakult’. Today, probiotic food products containing bifidobacteria and/or lactobacilli are consumed by millions of people worldwide.

Probiotics are mainly consumed by eating foods or supplements that contain a mono or mixed culture of live bacteria. The microorganisms exist in a viable state in foods like dairy products or as lyophilized forms in supplements. The beneficial effects attributed to the consumption of probiotic bacteria include improving intestinal tract health, enhancing the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, decreasing the prevalence of allergy in susceptible individuals, and reducing the risk of chronic disorders like
ulcerative colitis and colorectal cancers (Bengmark, 2000; Cunningham-Rundles et al. 2000; Pochapin, 2000; Saavedra, 2000; Schultz and Sartor, 2000; Sherwood and Gorbach, 2000; Vanderhoof, 2000; Kopp-Hoolihan, 2001; Levy, 2002). The probiotic bacteria that can be attributed to improving health are mainly from two genera: *Lactobacillus* - e.g., *Lb. acidophilus*, *Lb. casei*, *Lb. bulgaricus*, *Lb. plantarum*, *Lb. salivarius*, *Lb. rhamnosus* and *Lb. reuteri* and *Bifidobacterium* - e.g., *Bif. bifidum*, *Bif. longum* and *Bif. infantis* (Tannock et al. 1999).

Maintaining or improving human health is not the only target market for probiotics. Farm animals are also a big market as studies have implicated probiotics in many positive attributes such as reduced scouring in calves (Beeman, 1985), improved weight gain and feed efficiency for beef cattle (Parra and DeCostanzo, 1992), improved milk yield in dairy cattle (Huber, 1997), increased weight gain in chickens (Lan et al. 2003), control of post-weaning diarrhoea in pigs (Kyriakis et al. 1999) and reduced *Salmonella* in chickens (Fritts et al. 2000).

Intestinal probiotics are dominated by members of *Bifidobacterium* and *Lactobacillus*, as these two genera have a long history of safe use and have GRAS (generally regarded as safe) status. The origin of the strains used in probiotics can be either freshly isolated from a human or animal host or from a culture collection. However, as stated by Havenaar et al. (1992), the choice of where to get a probiotic strain depends on the specific purpose of the probiotic. For example, if only transient activity of the probiotic is needed, such as for lactose digestion, then it is not necessary for the probiotic to have characteristics that would enable it to colonize the host. Most probiotic effects in the gastro-intestinal system would be enhanced if the probiotic would be able to compete with the indigenous flora. This requires a more careful selection of strains. Although all the criteria for this purpose are not currently known for any intestinal organism, there is one general consensus, wherein the probiotic should originate from
the same animal species that it is intended to target (Mattila-Sandholm, 1997; Dunne et al. 1999)

### 2.5.2 Selection criteria and attributes

Probiotics are associated with beneficial health effects, and may be selected for prevention and treatment of diseases (Alvarez-Olmos and Oberhelman, 2001; Shanahan, 2002; Guarner and Malagelada, 2003). They are being used extensively as food supplements that beneficially affect the host by improving its microbial balance (Fuller, 1989; Forestier et al. 2001), are quickly gaining interest as functional foods. An increasing number of food supplements as well as pharmaceutical preparations are being promoted with health claims based on several characteristics of certain strains of lactic acid bacteria (LAB), particularly from the genera *Lactobacillus*, *Enterococcus* and also the genus *Bifidobacterium* (McFarland and Elmer, 1997; Kaur et al. 2002). Five major aspects may be taken into account as key criteria for the selection of an appropriate functional strain (Holzapfel et al. 1998; Salminen et al. 1998b; Hammes and Hertel, 2002; Holzapfel and Schillinger, 2002; Mare and du Toit, 2002; Reuter et al. 2002), which are as follows:

1. General aspects, e.g., origin, identity, and resistance to mutations
2. Technical aspects (growth properties in *vitro* and during processing, survival and viability during transport and storage)
3. General physiological aspects (resistance against environmental stress and to the antimicrobial factors prevailing in the upper GIT as encountered during the stomach-duodenum passage [pH 2.5, gastric juice, bile acid, pancreatic juice], adhesion potential to intestinal epithelium)
4. Functional aspects and beneficial features (adhesion, colonization potential of the mucosa, competitiveness, specific antimicrobial antagonism against pathogens,
stimulation of immune response, selective stimulation of beneficial autochthonous
bacteria, restoration of the “normal” population)

5. Safety aspects (no invasive potential, no transferable resistance against
therapeutic antibiotics, no virulence factors)

The above-mentioned requisites were further expanded (Salminen et al. 1996,
1998a; Berg, 1998) to include:

- Each potential probiotic strain should be documented
- Extrapolation of data even from closely related strains is not acceptable
- Only well-defined strains, products, and study populations should be used in trials
- Where possible, all human studies should be randomized, double blinded, and
  placebo controlled
- Results should be confirmed by independent research groups
- Preferentially, the study should be published in a peer-reviewed journal

There are a number of features that are important considerations for strain selection.
The usefulness as a probiotic is its ability to survive the transit to its intestinal target via
the stomach and duodenum (O’Sullivan, 2001).

2.5.2.1 Source of strains

Even though essentially all animals contain an abundance of species/strains of both
Lactobacillus and Bifidobacterium, it is now widely accepted that an effective human
probiotic should be of human origin. The underlying reason for this is that human
intestines are sufficiently different from those of animals such that the isolates suited to
those environments would not necessarily be suited to the human intestine. Currently, an
animal isolate of bifidobacteria (Bif. animalis) is widely used commercially in human
probiotics, although it is often declared as B. longum by the manufacturer (Klein et al.
This strain may not be a very effective probiotic, but it may have some health benefits during its transient passage through the intestine at high numbers. Although not presently researched sufficiently, it is likely there is a large diversity in human intestines depending on age, sex, race, and diet. With a better understanding of these diversities, future probiotic cultures may consist of multiple strains of the same species to account for this diversity and enable beneficial effects in a wider group of people (O’Sullivan, 2001).

2.5.2.2 Tolerance to acid and bile

The high acid conditions of the stomach require that the organism should have a high tolerance to acid. This is frequently measured by evaluating its ability to survive pH 3 or lower for 3 hours, an average passage time through the stomach (Charteris et al. 1998). Many probiotics do not have their stress defenses constitutively expressed and thus would be killed with this treatment. They can often be primed by exposing them to mild stresses that can induce stress defenses to enable them to withstand greater stresses. Therefore, potential isolates for probiotic cultures need not necessarily be directly resistant to pH 3, as long as they tolerate it after prior priming at a higher pH. This may easily be accomplished when yogurt is used as the delivery vehicle for the probiotic, as the mild acid conditions may be sufficient to effectively prime many isolates. Similarly, isolates need sufficient tolerance to bile to enable safe passage through the duodenum to their site of action. This is generally measured by simply plating out isolates on media containing bile salts. This process, however, largely measures direct resistance to bile rather than just tolerance. In vivo, a probiotic culture’s stress response will already be strongly induced following passage through the stomach. It is known that exposure to one stress can induce a response that protects cells against multiple stresses (Duwat et
al. 2000). As the stress response is already induced at that stage, it may be capable of surviving the bile in the duodenum.

### 2.5.2.3 Adherence to intestinal cells

The survival and establishment in a natural ecosystem such as the human intestine, the bacterium needs to be able to attach to the available attachment sites in the intestine. In the absence of ability to attach, an organism can only be transient, thus limiting its potential effectiveness. Adhesion of the microorganisms to the intestinal mucosa is thought to be a precondition for colonization (O’Sullivan, 2001). Under these conditions probiotic bacteria can produce antagonist effects on pathogens by different mechanisms such as competition for nutrients, release of antimicrobial substances, and prevention of the subsequent attachment of pathogens by competitive exclusion. Different studies have suggested that adhesive probiotic bacteria can prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract (Benno and Mitsuoka, 1992).

Currently, adherence is measured primarily using two *in vitro* cell lines Caco-2 and HT-29, as these essentially represent a single cell from the intestines of two individuals. Positive attachment to these cell lines can be viewed as a good indicator of their potential to attach (O’Sullivan, 2001). Some adhesive strains of *Lactobacillus* showed a strong inhibition of the adhesion of foodborne pathogens by a combined effect of both bactericidal activity and competition for attachment sites (Todoriki et al. 2001). Results obtained with *Lactobacillus* and *Bifidobacterium* indicate that surface-mediated properties, such as aggregation, can have a role in adhesion and colonization (Perez et al. 1998, Cesena et al. 2001). Commercial probiotic strains such as *Lb. rhamnosus* GG are able to slightly reduce S-fimbria-mediated adhesion of *Esch. coli* to glycoproteins
extracted from feces. In addition, adhesion of *Salm. typhimurium* was significantly inhibited by probiotic *Lb. johnsonii* LJ1 and *Lb. casei* Shirota (*Tuomola et al. 1999*).

**2.5.2.4 β-Galactosidase activity**

A significant proportion of the population has some degree of intolerance to lactose. This is especially true of certain racial groups, such as African, American and Hispanic. People with northern European heritage have the lowest instance of lactose intolerance. The lactic acid bacteria are excellent digesters of lactose due to production of β-galactosidase or phospho-β-galactosidase. This feature of a probiotic organism is pertinent when the culture is intended to reduce the symptoms of lactose malabsorption. It should also be noted that most wild-type isolates have very low constitutive levels of this enzyme but have much higher levels following growth in lactose as the sole carbon source. The induced β-galactosidase level is, therefore, the pertinent feature for a probiotic organism intended to aid subjects with lactose digestion (*O’Sullivan, 2006*).

**2.5.2.5 Stability and viability**

This is a measure of the suitability of an organism for survival and competition in an ecosystem. The human intestine is a complex and enclosed ecosystem and is arguably one of the most difficult to study. Consequently, all of the features a probiotic organism should have to compete effectively in this environment are not yet understood. However, from general principles of bacterial competition, some criteria are presently important. Essentially these principles are competition for nutrients and production of antimicrobial compounds (*O’Sullivan, 2001*). Carbon sources available in the intestine can be quite variable. Therefore, strains with a varied metabolic capability would have an advantage over strains with a limited metabolic capability. The ability to metabolize nutrients that are not metabolized by the host would be very advantageous for a species. One
example is oligosaccharides, which are not metabolized by humans and, therefore, are available for microbial growth. Very few bacteria can metabolize oligosaccharides, of which few selected species of bifidobacteria and lactobacilli can do so with a significant advantage when these substrates are present.

The subject of prebiotics arose from a derivation of this concept in providing oligosaccharides such as inulin or FOS to give the resident bifidobacteria and lactobacilli a competitive advantage in their respective environments. Screening for strains that can efficiently utilize certain prebiotics will therefore enable the probiotic to be ingested along with the prebiotic, thus providing a competitive advantage for the organism when both arrive in the nutrient limiting large intestine. This concept of consuming a probiotic with a prebiotic is referred to as synbiotics (Gibson and Roberfroid, 1995).

2.5.2.6 Beneficial attributes

The production of antimicrobial compounds gives an organism the additional advantage to inhibit their competitors. Examples of antimicrobial compounds are organic acids, \( \text{H}_2\text{O}_2 \) and bacteriocins. All lactic acid bacterial cultures produce organic acids, while only a few of them produce bacteriocins that are proteinacious in nature. Therefore, it is considered to be more favourable to select an organism for probiotic purposes, if it can also produce bacteriocins. Several bacteriocins of lactic acid bacteria have been described and they differ in the spectrum of target organisms that they can inhibit (O’Sullivan, 2001). Many probiotic lactobacilli have been shown to be bacteriocinogenic such as \( \text{Lb. acidophilus} \) (Yamato et al. 2003), \( \text{Lb. casei} \) (Palacios et al. 1999), \( \text{Lb. reuteri} \) (Kabuki et al. 1997), \( \text{Lb. rhamnosus} \) (Bernardeau et al. 2001), \( \text{Lb. gasseri} \) (Toba et al. 1991), \( \text{Lb. salivarius} \) (Arihara et al. 1996) and \( \text{Lb. johnsonii} \) (Abee et al. 1994). It is considered more desirable to have a broader spectrum of activity to provide the host better protection against GI infections. Only one species of \( \text{Bifidobacterium} \)
namely *Bif. bifidum* has been shown to produce a bacteriocin (Yildirim et al. 1999), suggesting that members of this genera may depend on other competitive features for aggressively colonizing the large intestine. Another competitive feature for bifidobacteria may be the ability to out-compete other large intestine flora for iron (O’Sullivan, 2001).

Iron is an essential element for all living cells, with exception of *Lb. plantarum* and *Borrelia burgdorferi* are notable exceptions (Archibald, 1983; Posey and Gherardini, 2000). This metal is a required cofactor for a wide range of basic biochemical mechanisms, although it can be toxic at elevated amounts, because of its ability to generate free radicals. Certain microorganisms respond to iron limitation by secreting siderophores, low molecular weight compounds produced to solubilize, bind and transport enviromental iron to their cells (Neilands et al. 1987). In general, dominant colonizers of an environment have better scavenging systems and can inhibit the growth of other competing organisms by depriving them of iron (O’Sullivan, 1990; O’Sullivan and O’Gara, 1992).

As the large intestine is of neutral pH, competition for iron has to be an important feature for survival in this environment. Bifidobacteria are superior competitors in the large intestine, wherein studies have indicated that bifidobacteria compete successfully in their environment against other bacteria such as *Esch. coli* by depriving them of iron for growth. However, these studies were performed in batch cultures, wherein the production of organic acids would reduce the pH and make iron more bioavailable. Research studies with feeding of infants with cow milk fortified with iron had higher counts of *Esch. coli* and less bifidobacteria in their feces than infants fed unfortified milk (Mevissen-Verhage et al. 1985). Besides, investigations have also suggested that bifidobacteria use iron scavenging to compete against *Esch. coli* in the large intestine (Kullen et al. 1997; O’Sullivan, 2001). Such probiotic microorganisms appear to be promising candidates for the treatment of intestinal disorders produced by abnormal gut...
microflora and altered gut mucosal barrier functions (Salminen et al. 1996a, 1996b; Alvarez-Olmos and Oberhelman, 2001).

2.5.3 Protocols for commercial probiotic preparations

The pathway to development involves the initial isolation and characterization of probiotic cultures, testing using in vitro assays, experiments with animal models, dose and safety evaluation and finally determination of the efficacy in patients with the disease indication (McFarland, 1999). Probiotics do share similar requirements with other commercial market products. Therefore, the process of producing probiotics should have standardized protocols for manufacture and quality control procedures. The production of beneficial microorganisms (now known as probiotics) is based on a long history of fermentation processes and does not rely upon advanced technology (McFarland and Elmer, 2006).

Besides the conventional morphological, cultural and biochemical characteristics, the method of identification of specific probiotic culture is based on molecular techniques (polymerase chain reaction or other genotyping methods), carbohydrate fermentation patterns, biotyping and DNA and RNA homology grouping (Heller, 2001). Strain identification is important for both quality control and differences in clinical efficacy for the differing strains of probiotics (Clements et al. 1983).

At the same time, the type of food product that will deliver the probiotic should allow the probiotic to survive in high numbers and have its characteristics compatible with the probiotic preparation. The various manufacturing steps for various probiotic foods including milk, cheese, yoghurts, kefir and cottage cheese have allowed for the survival of Lactobacillus strains (Reid et al. 2003). The final probiotic preparation needs to be evaluated for the specific attributes and viable populations of the culture used to prepare the product. Probiotics, whether sold as dried cultures or added to foods need to
have a sufficiently long shelf life to reach the consumer at high concentrations and in a viable state. Lyophilized or freeze-dried preparations of probiotics have the advantage of longer shelf life and have no need for refrigeration. The evidence from clinical trials suggests that doses over $10^8$ to $10^{10}$ CFU/d are necessary to obtain a therapeutic effect. Most oral doses to deliver this intake of viable microbes have ranged from 1 to 3 g/d for infants and 1 to 15 g/d in adults (Silva et al. 1987).

If the probiotic has a distinct or unique property, such as the production of a substance that targets a specific pathogen or is formulated in a manner that allows more probiotic to reach its target organ, then this would give the probiotic a competitive edge when it comes to marketing. A few of the specific attributes include the production of hydrogen peroxide by Lactobacillus species (Sanders and Klaenhammer, 2001; Mastromarino et al. 2002), production of bacteriocins (Silva et al. 1987), other inhibitory substances by Lb. acidophilus LA1 (Bernet-Camard et al. 1997) and the antimicrobial agent reuterin by Lb. reuteri (Casas and Dobrogosz, 2000). As probiotics are often given to patients with acute disease, another advantage for a potential probiotic is the ability to survive in the presence of antibiotics or concurrent medications. Such probiotics as Saccharomyces species have the advantage of not being susceptible to antibiotics (other than antifungal agents).

As with other commercial products on the market, manufacturers need to have established quality control procedures and methods to test them. Several studies have found discrepancies between probiotic product labeling and independent laboratory analysis of the probiotic cultures contained in the product (Yeung et al. 2002). Further, of the 16 probiotic products tested, only 4 contained the Lactobacillus species listed on the label and 69% had contaminants (Hughes and Hillier, 1990). Similarly, 64 Lactobacillus strains from various sources were tested and found that 6 contained Lb. plantarum, which was not indicated on the product label (Zhong et al. 1998).
The probiotics to have their desired effect need to be delivered safely to their targeted site of action. The traditional target sites for probiotics are the upper and lower gut. Probiotics targeting the intestine clearly encounter the greatest hurdles in order to be delivered to their targeted site. The biggest hurdles are the acid conditions of the stomach and the bile in the duodenum. Studies have been undertaken using microencapsulation technologies to protect probiotic bacteria during processing, storage and transit through simulated gastric conditions. Some of these studies were involved with bifidobacteria, which are the primary probiotic bacteria targeting the large intestine. Encapsulation using calcium alginate or kappa-carrageenan has shown good promise at protecting cells during processing and storage (Adhikari et al. 2003; Talwalkar and Kailasapathy, 2003). However, microencapsulation using cellulose acetate phthalate protected bifidobacteria quite impressively during spray drying and during prolonged exposure to gastric simulated conditions (Favaro-Trindale and Grosso, 2002).

Probably the longest history of proven health benefits and safety of probiotic bacteria in foods is documented for *Lb. casei* strain Shirota and some strains of the *Lb. acidophilus* group (Shirota et al. 1966). Over the past 40 years in Japan and more than 30 years in Germany, LAB cultures of human origin are applied in the manufacture of fermented milk products. Viable strains of *Lb. acidophilus* and *Bif. bifidum* were introduced in Germany during the late 1960s into dairy products, because of their expected adaptation to the intestine and the sensory benefits for producing mildly acidified yoghurts (Schuler-Malyoth et al. 1968). In Germany, such products first became known as mild yoghurts or bio-yoghurts, whereas in USA, acidophilus milk was developed. The functional properties and safety of particular strains of *Lb. casei/paracasei*, *Lb. rhamnosus*, *Lb. acidophilus* and *Lb. johnsonii* have extensively been studied and are well documented (Salminen and Tuomola, 1998; Salminen et al. 1998; Salminen et al. 2000; Fooks and Gibson, 2002).
Viable probiotic strains with beneficial functional properties are at present found among a wide and diverse number of microbial species and genera. They are supplied in the market either as fermented (mainly yoghurt type) food commodities or in lyophilized forms, both as food supplements and as pharmaceutical preparations. Most strains currently in use as probiotics in food, nutrition and in pharmaceutical preparations are members of the LAB. A number of ‘non-lactic’ strains like *Propionibacterium freudenreichii*, *Prop. jensenii*, *Prop. acidopropionici* and *Prop. thoenii* as well as *Sac. cerevisiae (boulardii)* are also available in the market mainly as pharmaceutic preparations and some also as animal feed supplements (*Holzapfel, et al. 1998; Fooks and Gibson, 2002; Huang and Adams, 2004*).

### 2.5.4 Health and therapeutic attributes

The interest in probiotics has increased due to a higher flexibility in medical care choices and alternatives to the dominance on antibiotic therapy (*McFarland and Elmer, 2006*). The use of probiotic strains (particularly lactobacilli and bifidobacteria) has been promoted as a means to balance the gut microbiota. In fact, their potential preventive and therapeutical effects have received renewed research and industrial interest (*Salminen et al. 1998; Ouwehand et al. 1999; Guandalini et al. 2000; Felley et al. 2001; Saavedra, 2001; Kalliomaki et al. 2003*). The characteristics of a successful probiotic are acid and bile tolerance, antimicrobial activity against intestinal pathogens and ability to adhere and colonize the intestinal tract. Probiotic organisms include lactobacilli, lactococci, bifidobacteria and *Saccharomyces* with several species of Lactobacillus being most studied. Beneficial effects include control of diarrhoea, alleviation of lactose intolerance and inhibition of intestinal pathogens (*Bhatia et al. 1989; Reddy et al. 1998; Fonden et al. 2000*). Other effects studied include reduction in
cholesterol level, enhanced immune response and antimutagenic and anticarcinogenic activities (Agarbaek et al. 1995; Fuller and Gibson, 1997; Kimura, 1997).

2.5.4.1 Treatment of diarrhoea

It is widely accepted that the normal gastro-intestinal microflora exert a protective role against attack by enteric pathogens (Savage, 1977; Simon and Gorbach, 1984; Tannock, 1995). Tissier (1906) first advocated the ingestion of live bifidobacteria as a remedy against diarrhea in children. Bifidobacteria have been used to successfully treat intestinal disorders (Salminen et al. 1996; Phuapradit et al. 1999). A few studies have supported the use of a Lactobacillus strain, specifically Lb. rhamnosus GG for the prevention and treatment of diarrhoea in children (Saavedra et al. 1994; Oberhelman et al. 1999; Guandalini et al. 2000). Antibiotic associated gastro-intestinal disturbances are a well recognized problem. It has been observed that Bif. longum delivered with Lb. acidophilus decreased the incidence of ampicillin-associated diarrhoea and the time required for recolonization (Black et al. 1991). Various lactobacilli (Lb. acidophilus, Lb. bulgaricus and Lb. rhamnosus), Bif. longum, Enterococcus faecium and the yeast Sac.boulardii have been tested in randomized, placebo-controlled clinical trials (Surawicz et al. 1989; McFarland et al. 1995; Arvola et al. 1999; Vanderhoof et al. 1999).

Diarrhoea is a common symptom of any kind of gastro-intestinal disorders. One of them is known as traveller’s diarrhoea. In this case, persons on travel to different places are usually given a prescription of antibiotics for use if diarrhoea develops. Probiotics have the potential to decrease such antibiotic use (and hence antibiotic resistance) when taken prophylactically to prevent the occurrence of traveller’s diarrhoea. Both Lb. rhamnosus and Sac. boulardii have been shown to have modest efficacy (Oksanen et al. 1990; Kollaritsch et al. 1993; Hilton et al. 1997).
Yet, another type of diarrhoea is that of pediatric diarrhea, which in developing countries cause childhood morbidity and mortality. The etiology involves bacteria and parasites. Improved sanitation and nutrition may lead to decreasing the extent of this problem, but routine use of an inexpensive probiotic preparation could have protective benefits. Culture of *Lb. rhamnossis* has been studied for the prevention of diarrhoea in undernourished Peruvian children ([Oberhelman et al. 1999](#)).

In the case of acute diarrhoea, oral rehydration is the prime answer for treatment, but several studies show that added probiotics can help speed recovery. Probiotic preparations containing any one or more of the cultures like *Lb. rhamnosus / Lb. reuteri / Lb. acidophilus / Lb. bulgaricus, Str. thermophilus, Bif. infantis* and *Sac. boulardii* have been shown to reduce the period of diarrhoea in several studies conducted in different countries ([Elmer et al. 1996; Boudraa et al. 2001; Elmer, 2001; Lee et al. 2001; Rosenfeldt et al. 2002a,b](#)) and in a multicentered European trial ([Guandalini et al. 2000](#)).

### 2.5.4.2 Clostridium difficile disease

A rare, but more serious and even life-threatening adverse event of antimicrobial therapy is overgrowth of *Clostridium difficile*. The resulting elaboration of *Cl. difficile* toxins A and B is followed by an intense and characteristic ileal and colonic inflammation termed pseudo-membranous colitis. Standard treatment is a course of metronidazole. More difficult cases require oral vancomycin therapy. The approach would be to administer the probiotic during and for some time after *Cl. difficile* treatment with antibiotics, as a means to normalize the gut microbial flora and to establish colonization resistance against *Cl. difficile* regrowth. Few probiotics have been tested to prevent relapse of *Cl. difficile* associated colitis in controlled clinical trials. There is limited evidence for success with *Lb. rhamnosus* ([Gorbach et al. 1987; Biller et al. 1995](#)), but only *Sac. boulardii* has
been tested in placebo-controlled trials. Encouragingly, this yeast treatment reduces relapse by more than 50% (McFarland et al. 1994; Surawicz et al. 2000).

2.5.4.3 Alleviation of constipation

Constipated bowel movement is a significant problem for many people, especially with the elders. The low population of intestinal bifidobacteria in the elders may be a contributing factor to constipation. Kleessen et al. (1997) recently showed that feeding inulin (a prebiotic that enhances bifidobacteria in the large intestine) to constipated elderly individuals with constipation significantly increased bifidobacteria population in their intestines and also had a laxative effect. A study in Japan also reported that feeding a *Bifidobacterium* cultured milk to elderly people significantly improved stool frequency (Yaeshima, 1996). It is possible that a laxative effect of bifidobacteria will only require high numbers of transient bifidobacteria through the intestine.

2.5.4.4 Alleviation of the symptoms of lactose maldigestion

Dairy foods are a very important part of a healthy diet, but several individuals suffer from symptoms of lactose maldigestion. Fermented dairy products appear to be more tolerable to lactose mal digesters than non-fermented foods, because of a decrease in lactose concentration of the food, as a result of microbial metabolism and perhaps the delivery of β-galactosidase (Kolars et al. 1984; Savaiano and Levitt, 1987). Several studies have investigated the contribution of both lactobacilli and bifidobacteria for the improvement of lactose tolerance. A *Lb. acidophilus* strain with good bile and acid tolerance was found to reduce symptoms associated with ingesting lactose (Mustapha et al. 1997).
2.5.4.5 Enhancement of immune function

In recent years, studies have also shown the ability of probiotics to modulate the immune system in human beings, including activation of natural killer cells (Gill et al. 2001a,b). Probiotics have been shown to boost host immune status via stimulation of specific and non-specific immune pathways. This can involve modification and regulation of humoral, cellular and non-specific immunity (Matsuzaki et al. 1998; Matsuzaki and Chin, 2000; Isolauri et al. 2001; Madsen et al. 2001; Cross et al. 2002). Some reported positive *in vivo* effects of probiotics include amplified mucus production, macrophage activation by lactobacilli signaling, stimulation of secretory IgA (therefore increased production), decreased proinflammatory cytokine production and increased peripheral immunoglobulin production (Kaila et al. 1992; Fukushima et al. 1998; Perdigon et al. 1999; Miettinen et al. 2000; Mack and Lebel, 2004).

2.5.4.6 Suppression of tumorigenesis

A few species of *Bifidobacterium* have been suggested to be associated with anticarcinogenic, antimutagenic and antitumorigenic activities. The suggested mechanism for these health claims is plausible, but there is no real direct human evidence to support the claims. However, some animal studies are supportive of a role for bifidobacteria and some lactobacilli in the suppression of tumorigenesis. In a rat feeding study, cultures of *Bif. longum* reduced carcinogenesis by a food mutagen, 2-amino-3-methylimidazo [4,5-f] quinoline (Reddy and Rivenson, 1993). In another study, a strain of *Bif. longum* was found to suppress azomethane-induced colon carcinogenesis in rats (Singh et al. 1997). Certain strains of lactobacilli have also been shown to significantly suppress intestinal tumors in rats by chemical mutagens (McIntosh et al. 1999). Further research is essential to comprehend these effects in humans and elucidate roles for specific strains of probiotic bacteria and their metabolites.
2.5.4.7 Cholesterol reduction

There are claims that consumption of fermented milk significantly reduces serum cholesterol (Mann and Spoerry, 1974; Gilliland et al. 1985; Gilliland, 1989). In case of hyper-cholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks. The principal site of cholesterol metabolism is the liver, although appreciable amounts are formed in the intestines. Claims are strong that certain \textit{Lb. acidophilus} strains and few \textit{Bifidobacterium} species are able to lower cholesterol levels in the intestine. Cholesterol coprecipitates with deconjugated bile salts as the pH declines as a consequence of lactic acid production by the LAB (Marshall, 1996). The role that bifidobacteria cultures may play in lowering serum cholesterol is not well understood. In rat models, serum cholesterol was lowered by feeding of bifidobacteria in a mechanism that may involve HMG-CoA reductase (Homma, 1988).

In this direction, findings of research investigations have indicated that a factor is produced in the fermented milk, which inhibits cholesterol synthesis in the body (Gilliland, 1989). Another theory is that \textit{Lb. acidophilus} deconjugates bile acids into free acids, which are excreted more rapidly from the intestinal tract than are conjugated bile acids. As free bile salts are excreted from the body, the synthesis of new bile acids from cholesterol can reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977b). A third hypothesis is that reduction of cholesterol may also be due to a co-precipitation of cholesterol with deconjugated bile salts at lower pH values as a result of lactic acid production by the bacteria (Kailasapathy and Rybka, 1997).

2.5.5 Safety of probiotics and food applications

Bacterial species that have traditionally been regarded as safe are used in probiotics, the prominent cultures used include lactic acid bacteria and bifidobacteria that inhabit
the intestinal tracts of humans and animals. Most probiotics are marketed as foods or pharmaceuticals. Consideration of the safety of probiotics is therefore of utmost importance. The safety of the microbes that have been used traditionally in probiotics has been confirmed through a long period of existence in human and animal systems (Kurmann et al. 1992; Mayra-Makien and Bigret, 1993). Ecologically, bifidobacteria are present as the predominant bacteria in the intestinal tract of breast-fed infants and are considered to contribute to the health of infants (Drasar and Hill, 1974; Mitsuoka, 1978).

The factors that must be addressed in the evaluation of safety of probiotics include pathogenicity, infectivity, virulence factors (toxicity), metabolic activity and the intrinsic properties of the microbes. A few methods for assessing the safety of LAB through in vitro animal and human clinical studies indicated that few of the current probiotic strains do not meet the required safety standards (Donohue and Salminen, 1996). Salminen and Marteau, (1997) also proposed studies on intrinsic properties, pharmacokinetics and interactions between the host and probiotics as a means to assess the safety of probiotics. The absence of pathogenicity and infectivity is a requisite of probiotic safety. The safety of a bacterial strain may be evaluated by considering questions such as whether invasion of the host by the bacteria leads to infection, whether infection results in severe outcome, and the effect of association of the bacteria on the host. Assessment of the ability to cause opportunistic infection is difficult. The acute and chronic toxicity tests probably provide circumstantial evidence. However, observations of the passage of bacteria across the intestinal barrier and invasion of the host body by translocation provide more direct data for determining infectivity.

Another requisite of probiotics is that the probiotic bacteria should not produce harmful substances by metabolic activities. One test is to determine whether the bacteria convert food components or biological secretions into secondary substances harmful to
the host. For example, some intestinal bacteria act on proteins and their digested products to produce ammonia, indole, phenols and amines (Drasar and Hill, 1974). Although Lactobacillus and Bifidobacterium species have not been reported to produce any harmful compounds, the data on the production and consumption of ammonia are interesting. The enzyme activities related to the consumption and generation of ammonia in Bifidobacterium sp. of human origin were measured and compared with other bacteria of the intestinal flora. Bifidobacterium spp. have a lower deaminase activity involved in the production of ammonium from amino acids but a higher ammonia assimilation activity (Araya-Kojima et al. 1995, 1996). Secondary bile acids are important harmful substances that are produced by intestinal bacterial actions on body secretions. They may exhibit carcinogenicity by acting on the mucous-secreting cells and promoting their proliferation, or they may act as promoters of carcinogenesis (Cheah, 1990). Many intestinal bacteria, including Bifidobacterium and Lactobacillus species can deconjugate conjugated bile acids (Midtvedt and Norman, 1967).

The issue of the isolation of antibiotic resistant bacteria has also been a point of debate (Chomarat and Espinouse, 1991; Maskell, 1992). Many strains of Enterococcus, including those isolated from infection sites have been shown to be resistant to many antibiotics (Gray et al. 1991). These resistant bacteria may have acquired antibiotic resistance independently by contact with the antibiotics or they may have acquired it by transformation. Natural antibiotic resistance of bifidobacteria and lactobacilli has been reported (Matteuzzi et al. 1983; Gupta et al. 1995). This undesirable transfer of resistance or conferment of resistance to endogenous bacteria should not be inherent property of probiotics. Although special purpose probiotics for use in combination with antibiotics have been developed through the introduction of multiple resistance to the bacteria, probiotics generally should not be designed to carry more resistance than is required for a specific purpose (Miyazaki et al. 1991).
Probiotic bacteria are applied in many different products worldwide. In addition to food products, probiotic cultures are also used in pharmaceuticals and animal feeds. Most definitions of probiotics are based on live bacteria that confer a health benefit for the consumer. It is important that probiotic products contain an effective dose of living cells during their market shelf life. A few of the applications of probiotic bacteria are presented briefly in the following paragraphs.

Probiotic bacteria have been applied in fermented dairy products for many years. In some cases, fermented milk products are mono-cultures of probiotic bacteria, but also include multiple cultures as a means to promote and enhance acidification process and provide the desired texture and flavour. Many lactobacilli and bifidobacteria survive in fermented milk products for a period of 4 to 8 weeks. There are several parameters that may influence the growth and survival of the probiotics like thyhe type of starter culture, fermentation temperature, pH, sugar content, presence of oxygen, packaging material, fruit preparations and other ingredients. Probiotics may also be applied to unfermented milk products such as milk-based sweet or acidified drinks and ice cream.

Similar to dairy products, fruit juices have been shown to be suitable carriers for probiotics. There is a growing interest in applying probiotics to fermented meat products. Lactic acid bacteria have been used for the fermentation of meat products for many years and at present, some strains are also utilized as protective cultures. Probiotics might be an instrument to change the perception of meat products towards a healthier image. Freeze-dried probiotic bacteria are applied to infant nutrition powders and powdered milk drinks. In these products, the water activity is very low, which is essential for the stability of freeze-dried bacteria.

Most probiotic food supplements and over-the-counter products are available as powders, tablets and capsules. As these products also contain dried bacteria, the water activity in the final product must be very low. Another critical parameter for tablets is the
pressure applied in tableting and the heat that is produced. An enteric coating can be applied on tablets and capsules in order to protect the bacteria from the acidic environment in the stomach and improve their survival rate.

Thus, an ideal probiotic would be one that can survive passage through the gastrointestinal tract, establish itself permanently in the small intestine and colon and provide specific health benefits for the host by eliciting (i) an immune response, secretion and production and (ii) synthesis of compounds such as short chain fatty acids, lactic acid and bacteriocins. As a source of energy, this probiotic would selectively utilize a prebiotic to achieve enhanced population (Bezkorovainy, 2001).

2.6. FOOD ASSOCIATED YEASTS

At present, approximately 750 yeast species are recognized, but only a few are frequently isolated. Relatively few natural habitats have been thoroughly investigated for yeast species (Boekhout and Phaff, 2003). Yeasts are traditionally characterized by morphological, physiological and biochemical criteria (Kreger-van Rij, 1987; Kurtzman and Fell, 1998). Since the 1970s, a large amount of data on the chemical composition of cell walls, capsular polysaccharides, whole-cell hydrolysates, antigenic determinants and enzyme patterns have provided valuable information for yeast taxonomy (Phaff, 1984). Traditional identification procedures are based mostly on morphological criteria of vegetative and sexual reproduction including ultrastructural studies of cell walls, septae, and spores. Identification of vegetative reproduction includes modes of budding (multilateral, bipolar), splitting cells, formation of arthroconidia and ballistoconidia and some other microscopic features of morphology (e.g., shape and size of cells, development of true hyphae or pseudohyphae), as well as characteristics of macroscopic growth (colony type, colour, formation of pellicle on liquid medium, etc.).
The progress in molecular biology has led to the development of new techniques for the detection, identification and typing of microorganisms. In comparison with phenotypic properties, the genotype represented in nucleic acid sequences is more stable, because it is not changed by environmental influences during growth. Nevertheless, DNA molecules are subject to mutations and sequence rearrangements that result in differences (polymorphisms) between closely related species and even strains belonging to the same species (Deak, 2007). Various DNA-based methods have updated the taxonomy and classification of yeasts including the recognition and identification of species as well as their infraspecific relationships (Kurtzman, 2006). A few commercially available systems provide accurate and reliable results, giving 90% or more agreement with data obtained by traditional methods (Deak, 2007).

2.6.1 Yeasts of common occurrence in foods

Yeasts within the genus Saccharomyces are best known for their positive contribution to food and beverage production, especially through their involvement in the fermentation process (Rose and Harrison, 1970, 1993). A few others involved in food fermentations are presented briefly as below:

2.6.1.1 Saccharomyces cerevisiae

The cells are globose, ovoidal or elongate (3.0-8.0) x (5.0-10.0) µm and are usually isolated or in small groups after 3 days growth in 5% malt extract at 25°C. After one month at 20°C, sediment is present in the liquid medium. Colonies on 5% malt agar at 20°C are butyrous, light cream-coloured and surface is smooth, usually flat, occasionally raised or folded and opaque. Pellicles are not formed in liquid medium. Pseudohyphae are either not formed or are rudimentary. Vegetative cells are transformed directly into persistent asci containing one to four globose to short ellipsoidal ascospores. Ascospore
formation, observed almost exclusively on acetate agar was usually below 10%, except in highly fertile homothallic strains, wherein sporulation ranged from 40-95% in 6-10 days at 20°C (Kurtzman and Fell, 1998).

2.6.1.2 *Saccharomyces kluyveri*

The cells are globose, ellipsoidal or cylindroidal (3.5-7.0) x (4.0-11.0) µm, single, in pairs or in clusters after 3 days growth in 5% malt extract at 25°C. After one month at 20°C, sediment and a ring are present. Colonies on 5% malt agar at 20°C are cream-coloured to tan, fairly flat and smooth to raised and wrinkled or folded, often sectored, glossy or dull, margin undulating or lobate and sometimes fringed with pseudohyphae. Pellicles are not formed in liquid medium. Pseudohyphae are usually formed. Vegetative cells are transformed directly into asci containing one to four globose to subglobose ascospores. Sporulation was observed on acetate agar after 4 days at 25°C (Kurtzman and Fell, 1998).

2.6.1.3 *Schizosaccharomyces pombe*

*Schizosaccharomyces pombe* cells are globose, ellipsoidal or cylindroidal (3.0-5.0) x (5.0 15.0-24.0) µm, single, in pairs or small groups. After one month at 20°C, sediment is present and sometimes a thin ring. Colonies on 5% malt agar at 20°C are brownish, dull or glistening, slightly raised, striated, and the margin is entire or sinuous. Pellicles are not formed. Conjugation of vegetative cells precedes the formation of evanescent asci containing two to four globose to ellipsoidal ascospores. The ascospores may cohere in small groups upon release. Haploid heterothallic strains may be encountered. When observed under Scanning Electron Microscope, the ascospore surface is warty (Mikata and Banno, 1987). Sporulation was observed on malt-, potato- and corn meal agars.
after 5-6 days at 25°C (Martini and Martini, 1998). It is involved in the fermentation of wine from grapes and cider from apples (Fleet, 2006).

2.6.1.4 Candida spp.

*Candida* is the nomenclatural type genus of the family Candidaceae. The genus *Candida* can be defined only as a broad group of asexual ascomycetous yeasts, which is characterized by a typical two-layered cell wall not containing xylose, rhamnose or fucose and not showing positive Diazonium blue B or urease reactions. The cells of *Candida* may be of spherical, ellipsoidal or cylindrical shape, budding multilaterally and may form true or pseudohyphae. It comprises 163 species as listed in the 4th edition of *The Yeasts* (Kurtzman and Fell, 1998). A few of the species known to predominant in foods are *Can. famata* in meat products, *Can. pelliculosa* and *Can. krusei* in acidic foods, *Can. kefyr* in dairy products, *Can. colliculosa* in low aw foods and *Can. valida* in beverages. Further, *Can. versatilis*, *Can. etchellsii*, and *Can. holmii* can be frequently isolated from foods of low water activity (high-sugar products, concentrates, dried foods, pasta, bakery products, etc.). Besides, *Can. versatilis* and *Can. etchellsii* are important osmotolerant species that play a key role in soy sauce fermentation (Hanya and Nakadai, 2003).

2.6.1.5 Kluyveromyces spp.

*Kluyveromyces* cells are ovoidal, ellipsoidal, cylindrical to elongate. Pseudomycelium may be formed. True hyphae are not produced. Glucose is fermented vigorously and nitrate is not assimilated. Diazonium blue B reaction is negative. *Kluyveromyces* species produce diffusible non-carotenoid Bordeaux-red pigments imparting a red colour (Stratford, 2006). The important species are *Kluy. lactis* and *Kluy. marxianus* (Lachance, 1998). Both species synthesize β-galactosidase enzyme and ferment the
lactose. Strains of *Kluy. marxianus* shows a high inulinase activity and this species is also known for the production of extracellular polygalacturonase.

### 2.6.2 Role of yeasts in fermented foods

Fermentation has been in use from ancient civilization as an effective and low-cost method to preserve the quality and safety of foods. Apart from this primary role, fermentation adds value and enhances nutritional quality and digestibility through biological enrichment and provides dietary enrichment through aroma and flavour production as well as improves textural attributes of food substrates. All these changes are effected by microorganisms, which are naturally present (spontaneous fermentation) or added (controlled fermentation) in raw materials, break down complex carbohydrates and proteins into more easily digestible forms. Among the fermentation microorganisms, yeasts constitute one of the most important groups of microorganisms that are exploited for commercial purposes (*Romano et al. 2006*). The role of yeasts in different types of foods is highlighted below:

#### 2.6.2.1 Dairy products

Dairy products provide a unique ecological niche for the selection and growth of specific yeast species. Relatively few yeast species occur in dairy products such as milk, cream, yoghurt, butter, cheese, kefir and koumiss. Dairy yeasts share a number of physiological and biochemical characteristics such as the fermentation or assimilation of lactose, a high proteolytic or lipolytic activity, utilization of lactic or citric acids, growth at low temperatures and tolerance to elevated salt concentrations (*Fleet, 1990*). Yeasts are in most cases used as secondary starter cultures in order to enhance the aroma production or to facilitate the growth of other microorganisms (*Romano et al. 2006*).
The production of cheeses involves a maturation stage characterized by a complex ecology of yeasts, bacteria and filamentous fungi (Devoyod and Desmazeaud, 1971; Fleet, 1990; Jakobsen and Narvhus, 1996; Viljoen, 2001). The microbial interaction between this microbiota determines the quality, safety and acceptability of the final product. Several yeasts assist the starter cultures in cheeses by expression of their proteolytic and lipolytic activities as well as the formation of aroma components during ripening (Siewert, 1986; Besançon et al. 1992; Welthagen and Viljoen, 1999).

In case of fermented milks, yeasts belonging to the genera Candida, Galactomyces, Kluyveromyces, Saccharomyces and Torulaspora are involved (Oberman and Libudzisz, 1998). Galactomyces geotrichum is used as commercial starter culture in the production of Villi, a Scandinavian fermented milk product. The cultures of Sac. unisporus and Kluy. marxianus are used as commercial starter cultures in the production of milky kefir. Strains of Sac. cerevisiae has also been reported to be involved in the fermentation of a number of indigenous African fermented milk products known under names such as amasi, nono and rob (Okagbue and Bankole, 1992; Abdelgadir et al. 2001; Gadaga et al. 2001).

2.6.2.2 Sourdough breads

Bread-making is one of the oldest biotechnology processes. Spontaneous souring with natural microflora and the use of special starter cultures are both used in sourdough bread baking. Sourdough is an important part of cereal fermentation and its preparation is based on traditional customs of each country. Sourdough could be considered a complex biological system which includes a mixed microbial population mainly represented by yeasts and lactic acid bacteria (Romano et al. 2006). The most frequent yeast species detected in sourdoughs are Sac. cerevisiae, Sac. exigus, Can. krusei, Can. milleri, Can. humilis, Pichia anomala, Pic. subpellicosa and Torp. holmii (Rossi,
1996; Gullo et al. 2002). In bread-making many different functional properties have been defined for yeasts. The most important function of baker’s yeasts is leavening by producing CO$_2$ via the alcoholic fermentation of the sugars (Paramithiotis et al. 2000). The production of CO$_2$ increases the dough volume resulting in bread with characteristic a light and spongy texture.

Baker’s yeast also influences the development of the dough gluten structure, which results expansion of the dough owing to CO$_2$ production. Furthermore, yeasts produce primary and secondary metabolites, such as alcohols, esters and carbonyl compounds which contribute to the development of the characteristic bread flavour (Hansen and Hansen, 1994; Damiani et al. 1996; Martinez-Anaya, 1996). Some of these compounds are volatile and are baked out of the bread. In addition, through their enzymatic activities, such as proteases, lecithinase, lipases, α-glucosidase, β-fructosidase and invertase, yeasts can affect not only the organoleptic characteristics, but also the overall appearance of the final product. In fact, these enzymatic activities have an influence on the dough stickiness and rheology, as well as on the crust colour, crumb texture and firmness of the bread (Antuna and Martinez-Anaya, 1993; Collar et al. 1998).

### 2.6.2.3 Fermented foods

Several traditional cereal fermented foods of India and Africa depend on spontaneous fermentation as could be seen in products such as kenkey, koko, banku, punjabi warries, papadams, jellabis, pozol, etc. Kenkey fermentation is dominated by *Aspergillus*, *Rhizopus* and *Penicillium* in the initial fermenting stages, succeeded by *Lb. brevis* and *Acetobacter* spp. in the fermenting dough. Wild types of yeasts including *Sac. cerevisiae* are present at all stages of the fermentation, contributing to the flavour by producing esters and ethanol (Muller and Nyarko-Mensah, 1972). *Koko* fermentation comprises
the LAB (Ped. cerevisiae, Leu. mesenteroides and Leu. fermenti) and yeasts. Punjabi warries and papadams include the yeasts Sac. cerevisiae and Candida spp., while jellabis are prepared with Sac. bayanus (Batra and Millner, 1974).

The production of soy sauce represents a typical sequential inoculation method making use of a two-stage process. The first stage is an aerobic process growing Asp. oryzae or Asp. sojae on soy beans and wheat which amylopectin hydrolyses the starch and liberates sugars (Hesseltine and Wang, 1967; Yokotsuka, 1985; Verachtert and Dawoud, 1990). In second stage, an anaerobic fermentation with Lb. delbrueckii, Ped. halophilus and Zygosaccharomyces rouxii takes place. The LAB proceed to grow and produce lactic acid, which decreases the pH, encouraging the growth of Zyg. rouxii, which results in vigorous alcoholic fermentation (Yong and Wood, 1976). Other osmophilic yeasts such as Can. etchelsii and Can. versatilis present produce phenolic compounds and furfural, which are desirable flavour enhancers (Morimoto and Matsutani, 1969; Noda et al. 1980; Wood and Hodge, 1985; Yokotsuka, 1985).

Coffee beans and cocoa beans (chocolate) undergo natural, indigenous fermentations in the primary stages of their processing, where the growth and activities of a diverse range of yeasts like Hanseniaspora, Candida, Pichia, Issatchenkia, Kluyveromyces and Saccharomyces species have been documented. Essentially, these yeasts assist in degradation of bean pulp and contribute to the production of chocolate flavour precursors (Schwan and Wheals, 2003, 2004).

2.6.2.4 Probiotic yeasts

Lactic acid bacteria are widely recognized as the main probiotic cultures, but there is increasing interest in adding other organisms to the probiotic list, which includes yeasts (Klaenhammer, 2001). Viable preparations of Sac. cerevisiae have been used as supplements to animal and poultry feeds resulting in improved growth and health of
these hosts (Lyons et al. 1993). Further, there is an expanding interest in using yeasts as probiotics in the aquaculture industry (Gatesoupe, 1995). With respect to humans, *Sac. cerevisiae var. boulardii* has been successfully used over the last 20 years as an oral, biotherapeutic agent to treat patients with severe cases of diarrhoea and other gastrointestinal disorders (McFarland and Bernasconi, 1993; Czerucka and Rampal, 2002). The yeast colonizes the intestinal tract and acts as a probiotic with almost all the attributes.

The role of yeasts in all types of foods is not covered in this review, as they do not directly relate to the subject of this thesis. It is well known that in a given spontaneous fermentation, a wide range of yeasts are involved at some stage of the fermentation process as in case of grape wines, brewing, alcoholic beverages and fermented sausages. Yeasts are also significant in that they act as a source of ingredients and additives in food processing. Besides, the predominance of baker’s and brewer’s yeasts, they are a rich source of nutrient supplements, because of their high levels of vitamin B group, proteins, peptides, amino acids and trace minerals. Yeasts are used for the production of antioxidants, aromas, flavours, colours and vitamins (Halasz and Laszity, 1991 and Reed and Nagodawithana, 1991). Flavour ingredients based on yeast extracts, yeast autolysates and dried yeast preparations represent the most commercially significant products extracted from yeasts and are used extensively in the food industry as a source of savoury, roasted, nutty, cheesy, meaty and chicken flavours. In addition, some extracts are specifically enriched in their contents of glutamic acid and nucleotides that function as strong flavour enhancers (Dziezak, 1987; Kollar et al. 1992; Nagodawithana, 1992; Stam et al. 1998).
2.6.3 Phytase from yeasts

2.6.3.1 Significance of phytate

The interest in phytate hydrolysing enzymes and their application in the animal industry have advanced significantly over the past decades (Mullaney et al. 2000). This is because about two-thirds of the phosphorus of feedstuffs of plant origin is present in the form of phytate (Nelson, 1967). Phytic acid is an abundant plant constituent comprising 1 to 5% by weight of edible legumes, cereals, oilseeds, pollens and nuts. It is an organic form of phosphorus, which is chemically a myo-inositol hexakis-dihydrogenphosphate (IP6). The molecular formula of phytic acid is C$_6$H$_{18}$O$_{24}$P$_6$ and its molecular weight is 659.86. Salts of phytic acid are called phytates. It is the primary source of inositol and storage form of phosphorus in plant seeds that are used as animal feed ingredients. Most foods of plant origin contain 50 to 80% of their total phosphorus as phytate (Harland and Morris, 1995). This molecule is highly charged with six phosphate groups extending from the central myo-inositol ring. For this property, phytic acid is considered to be an antinutritional factor for humans and monogastric animals as it acts as an excellent chelator of cations such as Ca$^{2+}$, Mg$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$ and as it complexes the basic amino acid group of proteins, thus decreasing the bioavailability of protein and nutritionally important minerals and inhibits number of digestive enzymes such as α-amylase, trypsin, acid phosphatase and tyrosinase (Maga, 1982; Harland and Morris, 1995; Wodzinski and Ullah, 1996; Dvorakova, 1998).

Phytate phosphorus is largely unavailable to monogastric animals due to the absence or insufficient amount of phytate degrading enzymes in their gastrointestinal tract (Maenz and Classen, 1998; Boling et al. 2000). Since phytic acid cannot be reabsorbed, feeds for pigs and poultry are commonly supplemented with inorganic phosphate in order to meet the phosphorus requirement (Reddy et al. 1982). Meanwhile, the unutilized phytate phosphorus from plant feeds is excreted, becoming an
environmental pollutant in areas of intensive animal agriculture, which is degraded by soil microorganisms releasing phosphorus in the soil. Excessive phosphorus in soil runs off to lakes and rivers causing eutrophication and stimulating growth of aquatic organisms that may produce neurotoxins injurious to marine animals and humans (Cheryan, 1980; Lei and Porres, 2003). In this background, there is a considerable interest in phytate degrading enzymes, i.e., phytases which hydrolyze the phosphate moieties from phytate, thereby resulting in the formation of free orthophosphate (Pi) and lower inositol phosphates, with reduced negative effect on the availability and uptake of nutritionally important minerals (Sandberg, 1999; Yano et al. 1999).

2.6.3.2 Phytate in cereals and legumes

Phytate (myo-inositol hexakisphosphate, InsP6) widely occurs in plant seeds and/or grains, roots and tubers, fruits and vegetables, nuts, pollen of various plant species and organic soils (Caldwell and Black, 1958; Baldi et al. 1987; Harland and Oberleas, 1987; Reddy et al. 1989; Wolters et al. 1993; Ravindran et al. 1994). Phytates are the primary storage form of both inositol and phosphate in all seeds and grains and occurs primarily as a salt of mono- and di-valent cations in discrete regions of grains and seeds (Cosgrove, 1966; Tanaka and Kasai, 1981; Lott, 1984; Lott and Ockenden, 1986). It rapidly accumulates in grains and seeds during their ripening period and maturation, accompanied by other substances such as starch, proteins, and lipids (Mebrahtu et al. 1997; Honke et al. 1998). Phytate concentration may vary in different tissues of a seed/grain/fruit (Wada and Lott, 1997). Phytate is used by all seeds/grains/fruits as a mineral nutrient store for the growing seedlings. It provides substantial myo-inositol, P, K, and Mg to the growing seedling, and perhaps Ca, Fe, Zn, and Mn (Lott, 1984; Batten and Lott, 1986; Lott et al. 1995).
In monocotyledonous seeds such as cereals, phytate is associated with specific components or parts within the grain and can be preferentially separated with those components. The starchy endosperm of wheat and rice grains is almost devoid of phytate, as it is concentrated in the germ and aleurone layers (pericarp) of the cells of the grain. Corn differs from most other cereal grains, as 88% of phytate is concentrated in the germ portion of the grain (O’Dell et al. 1972). Corn endosperm has small amounts of phytate (3.2% of total phytate). Rice and wheat germ portions contain appreciable amounts of phytate, but a major portion of phytate is found in the aleurone layers or pericarp. Of the total phytate, 84–88% has been reported to be present in the bran part of the rice (Resurreccion et al. 1979). In pearl millets, the majority of the phytate appears to be present in germ and bran fractions (Simwemba et al. 1984). In dicotyledonous seeds, such as beans and other seeds, phytate is distributed throughout the cotyledon and located within the subcellular inclusions of protein bodies (Lott and Ockenden, 1986; Lott, 1984).

The phytate content varies from whole cereal grains to milled fractions and to products prepared, especially the fermentative process in bread making. During fermentation of bread dough, some of the phytate in the dough is hydrolyzed by wheat and bacterial and yeast phytases, which may result in reduced phytate content, especially in breads made with white flours. As most of the phytate in cereals is located in the aleurone layers (bran), milling of cereals and subsequent separation of bran result in a significant reduction of phytate in flours (Singh and Reddy, 1977).

Phytate phosphorus accounts for the major portion (>80%) of total phosphorus in cereals and cereal products. Of the total phosphorus, phytate phosphorus represents 73.7–81% in brown rice, 51–61% in polished rice, 60–80% in wheat, 55–70% in barley, 48.7–70.9% in oats, 38–66% in rye, 18–73% in triticale, 71–88% in corn, 63.9–90.5% in sorghum, 58.3–78% in finger millet (ragi), 70.4% in foxtail millets, 64–85.7% in rice bran,
49.6–93% in wheat bran and 54% in oat bran (Frolich and Nyman, 1988; Eechkhout and Depaepe, 1994; Ravindran et al. 1994).

Phytate content ranges from 0.17 to 9.15% in whole beans, 0.58 to 4.20% in bean flours and bean protein products and 0.05 to 5.20% in bean-based foods. Among the legumes, crude soy bean oil and soy milk appear to contain the lowest amounts (<0.12%) of phytate. The wide variations reported for phytate content within the same type of bean may be due to differences in cultivars, growing conditions and locations. Because most of the phytate in beans is distributed in the cotyledons, removal of the hull or seed coat typically leads to a higher phytate content of beans (Beal and Mehta, 1985; Davies and Warrington, 1986). In beans and bean products, of the total phosphorus, phytate phosphorus accounts for 50–70% in soy beans, 27–87% in lentils, 40–95% in chickpeas, 39.5–95% in broad beans, 36–53% in peas, 75–76% in pigeon peas, 70–87% in linseed, 31–60% in lima beans, 63.2–69% in Green gram, 29.8–71.8% in cow peas, 74.4–79% in Black gram, 68–72% in red kidney beans, 73.2–93.3% in dolichos beans, 57–81.6% in peanuts, 44–73% in winged beans, 77% in tempe, 94.5% in tofu, 60–60.9% in soybean meals, 87% in defatted soy flour and 62% in soy protein isolate (Olghobo and Fetuga, 1982; Thompson and Erdman, 1982; Ferrando, 1983; Lott, 1984; Kotaru et al. 1987; Reddy and Pierson, 1987; Deka and Sarkar, 1990; Eechkhout and Depaepe, 1994; Ravindran et al. 1994).

2.6.3.3 Phytase

Phytases (myoinositol hexakisphosphate phosphohydrolase EC 3.1.3.8 and 3.1.3.26) belongs to the family of histidine acid phosphatases, a subclass of phosphatases, which catalyze the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, myo-inositol and inorganic phosphate, all utilizing a phosphohistidine intermediate in their phosphoryl transfer reaction (Mitchell et al. 1997). Initially, two classes of phytases
were classified by IUPAC-IUB in 1975, 3-phytases (EC 3.1.3.8) and 6-phytases (EC3.1.3.26) based on the position specificity of initial hydrolysis of phytates. Thus, in recent years, the number of enzymes described as phytases has increased (Konietzny and Greiner, 2002).

On the basis of pH of activity, phytases can be broadly categorized into two major classes namely the histidine acid phosphatases and alkaline phytases. However, the focus has been on acidic phytases due to their applicability in animal feeds and broader substrate specificity than those of alkaline phytases. The phytases exhibit variations in structure and catalytic mechanism and consequently, they have been categorized into histidine acid phosphatases (HAPs), β-propeller phytases (BPP) and purple acid phosphatases (PAP) based on their active site motifs (Mullaney and Ullah, 2003). Among these, phytases belonging to HAPs are the most widely studied and have been isolated from bacteria, yeast, filamentous fungi and plants (Van Etten et al. 1991; Mullaney et al. 2000). Recently, a fourth class of phytase has been added to this classification, i.e. a novel phytase from *Selenomonas ruminantium* as it shares no sequence homology with other microbial phytases (Chu et al. 2004).

Research studies have shown that microbial sources are more promising for the production of phytases on a commercial level and in cereal based foods. Microbial phytases are easily produced and extracted when synthesized extracellularly in a culture medium. In general, they may be synthesized by the same microbial starter used for food processing, natural or genetically modified strains of bacteria such as *Esch. coli*, *Bac. subtilis*, *Bac. amyloliquefaciens*, *Pseudomonas sp.*, *Aerobacter aerogenes*, *Enterobacter sp.* and *Klebsiella sp.*; yeasts such as *Schwanniomyces castellii*, *Schw. occidentalis*, *Han. polymorph* and *Rh. gracilis*; and fungi such as *Asp. niger* and *Asp. ficuum* are some of the most important species used for the production of microbial phytases (Pandey et al. 2001).
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There are reports on the supplementation of animal feed with yeast phytase to improve the nutritional status of feed (Stahl et al. 2000). An enzyme to be used in supplementing animal feeds must retain activity when exposed to 80–85°C for a few seconds during pelleting. The important characteristics of phytase to be used in practical applications are high specific activity, pH optima corresponding to various regions of the animal digestive tract, resistance to stomach proteases, drying and high temperature. This creates a need for phytases with an optimal combination of various properties (Zinin et al. 2004).

2.6.3.4 Phytate hydrolysis

The degradation of phytate (myo-inositol hexakisphosphate, InsP6) is of nutritional importance, because the mineral binding strength of phytate decreases and the solubility increases when phosphate groups are removed from the inositol ring resulting in an increased bioavailability of essential dietary minerals. Major efforts have been made to reduce the amount of phytate in foods by following different food processing techniques such as soaking, malting, hydrothermal treatment and fermentation. These unit operations of processing will increase the naturally occurring plant phytase activity. In addition, phytate can be hydrolyzed by the addition of microbial phytase enzyme, which is mainly derived from fungi, yeasts and bacteria (Nayini and Markakis, 1983; Lonnerdal et al. 1989; Sandberg and Svanberg, 1991; Brune et al. 1992; Larsson and Sandberg, 1992; Sandstrom and Sandberg, 1992; Svanberg et al. 1993; Sandberg et al. 1999; Pandey et al. 2001). Phytase catalyzes the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, myo-inositol and inorganic phosphate. An enzymatic synthesis has the advantage of high stereospecificity and mild reaction conditions. The use of phytase has been shown to be very effective in producing different inositol phosphate species.
Review of Literature

D-myo-inositol 1,2,6-trisphosphate, D-myo-inositol 1,2,5-trisphosphate, L-myo-inositol 1,3,4-trisphosphate and myo-inositol 1,2,3-trisphosphate were successfully prepared using phytase derived from Sac. cerevisiae (Siren, 1986a, b). Lambrechts et al. (1992) screened 21 yeast strains for their ability to hydrolyze phytates and among all these, Schw. castellii CBS 2863 exhibited the highest phytase activity. In their studies, Sandberg and Andlid, (2002) tested various strains of Sac. cerevisiae, Sac. boulardii, Debaryomyces hansenii, Rh. Rubra and Rh. glutinis as well as several tropical species such as Metschnikowia lochheadii, Can. drosophilae and Can. tolerans by growing them in a medium containing IP6 as the sole phosphorus source followed by HPLC analysis of the degradation products.

The properties and applications of immobilized phytases from microorganisms have been studied by many researchers (Ullah and Cummins, 1987; Ullah and Phillippy, 1988; Dischinger and Ullah, 1992; Greiner and Konietzny, 1996; Liu et al. 1999). Quan et al. (2003b) reported the immobilization of whole cells of Can. krusei having high phytase activity in alginate gel beads as an application of the preparation of myo-inositol phosphates. It has also been investigated that during dough fermentation different strains were able to hydrolyze IP6 and generate myo-inositol with lower degree of phosphorylation. Many bakery products such as bread have a significant content of IP6, which can be hydrolyzed by microorganisms (Garcia-Estepa et al. 1999). Specifically, during the bread production, endogenous (from cereals) and microbial (yeasts or/and lactic acid bacteria naturally present in flour or added as starter) phytase enzymes are both active.

2.6.3.5 Phytase production and its assay

Phytase activity is usually measured by the amount of inorganic phosphate released per min from a selected substrate under certain pH and temperature. There has been
intensive research on the substrate specificity and affinity of different phytases. Microbial phytases (Asp. niger, Esch. Coli and Bacillus sp.) seem to have a high affinity to phytic acid, whereas plant phytases and some fungal enzymes such as the one from Asp. fumigatus have a broader substrate specificity and are capable of degrading the lower inositol phosphates. Phytases are able to degrade phytic acid to the monophosphate ester of inositol as the final product (Ullah and Phillippy, 1994; Wyss et al. 1999a; Greiner et al. 2002).

Most isolated phytases have their pH optima in the range of 4.5–6 and the temperature optima of most plant and microbial phytases range from 45 to 60°C. As the commercial feeds are often pelleted using high temperature (60–80°C) and steam, all feed enzymes need to be heat stable to avoid substantial activity loss during this process. Chemical coating of phytases has been used to improve their heat stability. An effective phytase needs to have a strong resistance to hydrolytic breakdown by digestive proteinases in the digestive tract. Fungal and bacterial phytases show different sensitivities to pepsin and trypsin (Kerovuo et al. 1998; Rodriguez et al. 1999).

Research studies have shown that available inorganic phosphorus content of the medium influences the synthesis of the phytase enzyme (Shieh and Ware, 1968). The most commonly used phosphorus sources for phytase production are KH$_2$PO$_4$ and K$_2$HPO$_4$. The regulatory effect of high P on phytase synthesis was confirmed in few of the earlier studies (Howson and Davis, 1983; Han and Gallagher, 1987; Ullah and Gibson, 1987). Phytase production by Can. krusei was controlled by phosphate concentration in the medium (Quan et al. 2001). Maximum production occurred in the medium containing 0.5 mg phosphorus per 100 ml. An increase in the concentration to more than 5 mg per 100 ml caused inhibition of phytase synthesis.

Several investigators have optimized the nutritional and physical parameters for maximizing the production of yeast phytases. Phytase production using yeast cultures
such as *Schw. castellii, Sac. occidentalis, Han. polymorph, Arxula adeninivorans* and *Rh. gracilis* have generally been carried out in submerged fermentation (SmF) systems (Pandey et al. 2001). In *Schw. castellii*, phytase production was carried out continuously in a fermenter aerated at 1 vvm and agitated at 600 rpm. Galactose (1%) was found to be the preferred carbon source and sodium phytate (0.06%) was required for phytase secretion (Segueilha et al. 1992). *Rhodotorula gracilis* produced phytase constitutively, and a high enzyme titre was attained in a medium lacking phytate in 18 h (Bindu et al. 1998). Mayer et al. (1999) developed an efficient process for the low cost production of phytases using *Han. polymorpha*. Glucose or glucose syrups were used as main carbon sources during fermentation.

*Arxula adeninivorans* secreted high levels of phytase during its active growth phase at 44°C. Galactose was found to be a better carbon source than glucose, while yeast extract (1%) and peptone (1%) served as good nitrogen sources. Phytate did not show an inducible effect on phytase production (Sano et al. 1999). The highest phytase yield by *Can. krusei* WZ-001 was attained in 48 h (late exponential phase) at 30°C and pH 7.0. Glucose (5%) and polypeptone (0.7%) were the appropriate carbon and nitrogen sources, respectively and phytase synthesis was repressed by the presence of phosphate in the medium (Quan et al. 2001). Phytase production from *Pic. anomala* has been extensively studied. A high phytase titre was attained in a synthetic medium that contained glucose (4%) and beef extract (1%), supplemented with Fe²⁺ (0.15 mM), at 20°C in 24 h (Vohra and Satyanarayana, 2001).

An enhancement in the titres was achieved using the statistical approach namely response surface methodology (RSM), wherein the interactions among 3 variables (glucose, beef extract and inoculum density) were studied. The use of RSM in biotechnological processes is gaining immense importance for the optimization of enzyme production (Vohra and Satyanarayana, 2002; Rao and Satyanarayana, 2003;
Chadha et al. 2004). The final concentrations of glucose (2%) and beef extract (0.5%) were half of those used initially (Vohra and Satyanarayana, 2002b). A medium consisting of cane molasses, a by-product of the sugarcane industry, was formulated that supported enhanced phytase yield with reduced production cost as compared to a synthetic glucose-beef extract medium (Vohra and Satyanarayana, 2004). There was a significant increase in the production of cell-bound phytase in cane molasses medium (176 U/g, dry biomass) as compared to synthetic medium (100 U/g, dry biomass). An overall 86.6% enhancement in phytase yield was attained in optimized cane molasses medium using fed-batch fermentation. The economical cane-molasses medium was further optimized using statistical approaches such as Plackett-Burman design and RSM to attain a higher biomass yield and cell bound phytase in Pic. anomala (Kaur and Satyanarayana, 2005).

The marine yeast strain Kodamae ohmeri BG3 isolated from the gut of a marine fish (Hexagrammes otakii) was found to secrete a large amount of phytase into the medium. The crude phytase produced by this marine yeast showed the highest activity at pH 5.0 and 65 °C. The optimal medium for phytase production contained oat 10.0 g/l, ammonium sulfate 15.0 g/l, glucose 30 g/l, and NaCl 20.0 g/l, while the optimal cultivation conditions for phytase production were pH 5.0, a temperature of 28 °C, and a shaking speed of 170 rpm. Under the optimal conditions, over 557.9 mU/ml of phytase activity was produced within 72 h of fermentation at the shake flask level. This is a very high level of phytase activity produced by yeasts (Li et al. 2008).

2.6.3.6 Purification and characteristics of phytase

Purification studies on phytases were usually performed with an aim to study phytase characteristics (Pandey et al. 2001). Properties of enzymes are important in determining their potential in varied applications. Phytase from Sac. cerevisiae, Sac. occidentalis,
Schw. castellii, Arxula adeninivorans, Pic. anomalala, Pic. rhodanensis, Pic. spartinae, Pic. pastoris, Kodamaea ohmeri and Debaryomyces castellii have been purified to homogeneity and their biochemical and catalytic properties have been reported (Vohra and Satyanarayana, 2003). Extracellular phytase has been reported in Sac. cerevisiae, Sac. occidentalis, Schw. castellii, Arxula adeninivorans, Pic. anomalala, Pic. spartinae, Pic. rhodanensis, Pic. pastoris, and Kodamaea ohmeri (Segueilha et al. 1992; Han and Lei, 1999; Han et al. 1999; Sano et al. 1999; Nakamura et al. 2000; Lee et al. 2007; Li et al. 2008; In et al. 2009; Nuobariene et al. 2012) An intracellular phytase occurs in Sac. cerevisiae and Pic. anomalala (Nakamura et al. 2000; Vohra and Satyanarayana, 2001). Various methods have been used for purifying enzymes, including ammonium sulphate / acetone precipitation, ultrafiltration, ion exchange, hydrophobic interaction and affinity / gel filtration chromatography.

Phytase from Schw. castellii, which was extracellular, purified by anion exchange and gel filtration chromatography resulted in 18.4 fold purification and had a high molecular weight of 490 kDa with a glycosylation rate of 31%. The structure of the glycosylated protein was tetrameric, with one large subunit (MW 125,000) and three identical small subunits (MW 70,000). The enzyme was optimally active at pH 4.4 and 77°C and stable for 1 h at 74°C. The enzyme had broad substrate specificity with phytate as its preferential substrate. Phytase of Schw. castellii was slightly inhibited in the presence of 5 mM Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\) and Fe\(^{2+}\). The cations Zn\(^{2+}\) and Cu\(^{2+}\) (0.5 mM) caused around 50% inhibition of activity; while 5 mM Zn\(^{2+}\) and Cu\(^{2+}\) strongly inhibited the reaction. There was no significant inhibition of phytase of Schw. castellii by the presence of inorganic phosphate in the reaction mixture upto 40 mM. Phytate was completely dephosphorylated by the phytase and the Km value was 38 μM (Segueilha et al. 1992).
Partial purification of phytase from *Arxula adeninivorans* was achieved by Sephadex G-50 filtration and DEAE chromatography. The purified phytase showed the maximum activity at pH 4.5 and 75°C. It was generally observed that the presence of substrate along with the enzyme prevents thermal denaturation above optimal temperatures. The yeast phytases were not heat resistant in the absence of substrate phytate. Pre-incubation of the enzyme at 75°C for 20 min in the absence of substrate led to 90% inactivation. The enzyme was less sensitive to low concentrations of various ions (Mg$$^{++}$$, Ca$$^{++}$$, Zn$$^{++}$$) and also relatively resistant to high concentrations of NaCl like 60% of full activity at 1.0 M (Sano et al. 1999).

A phytase from *Can. krusei* WZ-001 was purified to homogeneity by ion-exchange chromatography, hydrophobic interaction chromatography and gel filtration that resulted in ~34-fold purification and ~6% yield. The phytase of *Can. krusei* WZ-001 was optimally active at pH 4.5 and 40°C. This phytase was easily inactivated at temperatures above 50°C and was strongly inhibited by Zn$$^{2+}$$ and Mg$$^{2+}$$ (1 and 5 mM), but not inhibited to any significant extent in the presence of Ba$$^{2+}$$ and Pb$$^{2+}$$. Further, this phytase of *Can. krusei* also exhibited a broad substrate specificity, but with maximum activity on phytate (Quan et al. 2002).

Phytase from *Pic. anomala* was purified to homogeneity by a two-step process of acetone precipitation, followed by anion exchange chromatography using DEAE-Sephadex that resulted in ~22% fold purification and ~20% yield. The enzyme had a molecular weight of 64 kDa. It was optimally active at 60°C and pH 4.0. This enzyme was found to be highly thermostable and acid-stable, with a half life of 7 and 8 d at 60°C and pH 4.0, respectively. At 80°C, the half life of phytase could be increased from 5 to 30 min by the addition of 10% w/v of sucrose, lactose and arabinose. The enzyme exhibited broad substrate specificity, since it acted on p-nitrophenyl phosphate, ATP, ADP, glucose-6-phosphate besides phytic acid. There was no requirement of metal ions.
for activity. SDS was observed to be highly inhibitory to phytase activity. Sodium azide, DTT, β-mercaptoethanol, EDTA, toluene, glycerol, PMSF, iodoacetate and N-bromosuccinimide did not show inhibitory activity. The enzyme was inhibited by 2, 3-butanedione, indicating the involvement of arginine residues in catalysis. Phytase activity was not inhibited in the presence of inorganic phosphate up to 10 mM. The shelf life of the enzyme was 6 months at 4°C and there was no loss in the activity on lyophilization (Vohra and Satyanarayana, 2002).

Among the several yeast species screened for extracellular phytase activity, it was found that these phytases had an optimum pH of 4 to 5 when measured at optimal temperature of 50–60°C. However, at 37°C, many strains produced another phytase with the optimum pH of 3 to 4. This means either there is more than one phytase or the same protein changes its optimal pH range depending on temperature. *Pichia spartinae* and *Pic. rhodanensis* showed the highest levels of phytase activity. The enzyme of *Pic. spartinae* exhibited the highest optimal reaction temperature at 75 to 80°C, whereas for *Pic. rhodanensis*, it was 70 to 75°C with optimum pH being 3.6 to 5.5 and 4.5 to 5, respectively (Nakamura et al. 2000).

The extracellular phytase from *Kodamaea ohmeri* BG3 (marine yeast) was purified to homogeneity with a 7.2 fold increase in specific activity as compared to that in the supernatant by ammonium sulphate fraction, gel filtration chromatography (Sephadex G-75) and anion-exchange chromatography (DEAE Sepharose Fast Flow Anion-Exchange). According to the data obtained from SDS-PAGE, the molecular mass of the purified enzyme was estimated to be 98.2 kDa and the enzyme was shown to be a monomer based on the results of gel filtration chromatography. The optimal pH and temperature of the purified enzyme were 5.0 and 65°C, respectively. The enzyme was stimulated by Mn²⁺, Ca²⁺, K⁺, Li⁺, Na⁺, Ba²⁺, Mg²⁺ and Co²⁺ (at a concentration of 5.0 mM), but it was inhibited by Cu²⁺, Hg²⁺, Fe²⁺, Fe³⁺, Ag⁺, and Zn²⁺ (at a concentration of
5.0 mM). The enzyme was also inhibited by phenylmethylsulfonyl fluoride (PMSF), iodoacetic acid (at a concentration of 1.0 mM) and phenylgloxal hydrate (at a concentration of 5.0 mM), but not so by 1.0 mM of EDTA and 5.0 mM of 1,10-phenanthroline (Li et al. 2008).

An extracellular acid phytase from Sac. cerevisiae CY strain was isolated from the mash of traditional Korean Yakju was characterized (In et al. 2009). The purified enzyme had a molecular weight of 55 kDa with higher activity at pH 3.6 and temperature of 40°C. The phytase exhibited broad substrate specificity and was completely inhibited by Fe³⁺ and Hg²⁺ and strongly inhibited to an extent of 91% by Ba²⁺,Co²⁺,Cu⁺,Cu²⁺,Fe²⁺,Mg²⁺ and Sn²⁺ at 5 mM concentration (In et al. 2009). Isolates of Sac. cerevisiae with specific activities of 10.6 and 8.2 U/10 CFU were isolated from sourdoughs (Nuobariene et al. 2012).

The growing interest in microbial phytases has given opportunities for research studies based on molecular biology techniques such as recombinant DNA technology to understand aspects relating to the active site, amino acid sequence, heterolgous gene expression for enhanced phytase production, site directed mutagenesis for improving desired characteristics and potential application in foods and feeds. Phytase encoding gene was cloned and sequenced, besides elucidating the 3D structure in Pic. anomala. Further, enzyme could effectively dephosphorylate phytate present in wheat flour and soy milk (Vohra et al. 2011).

2.7 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are Gram-positive, catalase negative, devoid of cytochromes, non-spore forming and non-flagellated rods or cocci, which produce lactic acid as the major end product during the fermentation of carbohydrates. The rods / cocci are present in varied forms like single, pairs, chains and tetrads. They are generally associated with
habitats rich in nutrients, such as various food products (milk, meat, beverages, vegetables), but some are also members of the normal flora of the mouth, intestine and vagina of mammals (Axelsson, 2004).

The concept of the LAB as a group of organisms developed at the beginning of the 1900s, preceded by pioneering scientific and technical developments during the later part of the 19th century. The interactions of LAB in foods enjoyed early attention of scientists and resulted in the significant contribution by Pasteur on lactic acid fermentation in 1857, followed by the first isolation of a pure bacterial culture, Bacterium lactis by Lister in 1873. Use of starter cultures for cheese and sour milk production was introduced almost simultaneously in 1890 by Weignann in Kiel and Starch in Copenhagen. This opened the way for industrialisation of food fermentations (Stiles and Holzapfel, 1997).

Important progress in the systematic of LAB was made when the similarity between milk souring bacteria and other LAB from other sources was recognized. Orla-Jensen (1919) used the following characteristics as a basis for classification of LAB into different genera, which was largely based on morphology (cocci / rods / tetrad formation), mode of glucose fermentation (homo- or heterofermentation), growth at different temperatures (10°C and 45°C), configuration of the lactic acid produced (D / L), ability to grow at high salt concentrations, and acid or alkaline tolerance. Chemotaxonomic markers such as fatty acid composition and constituents of the cell wall are also used in classification. In addition, the present taxonomy relies partly on true phylogenetic relationships, which have been revealed by extensive work on determining rRNA sequences. The most promising for routine use are 16S rRNA gene sequencing, PCR-based fingerprinting techniques and soluble protein patterns.

The taxonomic status of LAB is well established and there has been a continuous update on the genera and species, which are documented in Bergey's Manual of

2.7.1 Probiotic LAB

Considering the diverse nature of LAB, it would be more appropriate in this review to present only those genera / species of LAB, which are more commonly used as probiotics.

2.7.1.1 Lactobacillus acidophilus

The most well known species of probiotic bacteria is Lb. acidophilus. It is widely used as probiotic culture in dairy and pharmaceutical products, because this species is one of the most dominant lactobacilli in the human intestine (Sandine, 1979). This may be contributed by their non-iron requirement for growth, extremely high hydrogen peroxide tolerance, ability to operate using homofermentative metabolism and are aerotolerant despite the complete absence of a respiratory chain. The best researched single strain of acidophilus is probably the DDS-1 strain. Research studies have also shown that Lb. acidophilus DDS-1 strain also alleviates lactose intolerance by producing significant amounts of the lactose digesting enzyme lactase, inhibits gastro-intestinal pathogens by producing antimicrobial substances such as acidophilin and helps alleviate dermatitis and other skin conditions by altering gut flora amongst other things (Environmental Illness Resource – Blog in the Net). Some Lb. acidophilus strains like LB, LA1, BG2FO4 and LCFM express adhesive factors that foster adhesion to human intestinal cells in vitro (Coconnier et al. 1992, 1993; Bernet et al. 1994; Sanders and Klænhammer, 2001). While adherence in vivo may occur, this has not been well demonstrated and
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Exogenously administered lactobacilli eventually drop below detectable concentrations in stool samples. It was concluded that daily consumption would be needed to maintain high intestinal levels of this probiotic strain. Several *Lb. acidophilus* strains have been shown to produce antimicrobial substances *in vitro*, but production *in vivo* of concentrations high enough for a direct inhibition of pathogen growth has not been demonstrated (Coconnier et al. 1993; Aroutcheva et al. 2001; Sanders and Klaenhammer, 2001).

2.7.1.2 *Lactobacillus rhamnosus*

*Lactobacillus rhamnosus* GG is the most studied lactobacilli-based probiotic. This strain of *Lactobacillus* was isolated from the feces of a healthy human by Gorbach and Goldin (hence the GG) as reported by Silva et al. (1987). It has been variously designated as *Lb. casei* ssp. *rhamnosus* strain GG or *Lb. rhamnosus* GG. The culture of *Lb. rhamnosus* GG is stable to bile and acidic pH and described to adhere to intestinal mucosal cells *in vitro* (Goldin et al. 1992). Silva et al. (1987) described a microsin from this strain that had relatively broad spectrum antibacterial activity as measured by an agar diffusion assay. In one of the studies, it was demonstrated that *Lb. rhamnosus* GG survived in gastric juice for 4 h at pH levels of 7, 5 and 3, but not at pH 1. Fecal samples were analyzed from healthy adult volunteers receiving *Lb. rhamnosus* GG in the form of fermented milk or whey for 28 d and the same strain was recovered in all the samples tested. Further, the observation of *Lb. rhamnosus* GG being isolated 7 d after stopping ingestion of the fermented whey product indicated that this probiotic had the capacity to persist and colonize (Goldin et al. 1992).

Furthermore, a human study quantitating *Lb. rhamnosus* GG in fecal and colon biopsy samples revealed that the probiotic was found in all biopsy and fecal samples 14 d after cessation of treatment, but had mostly disappeared at 28 d (Alander et al. 1999).
Feeding *Lb. rhamnosus* GG to healthy volunteers for 4 weeks decreased fecal β-glucuronidase specific activity, whereas feeding *Str. thermophilus* or *Lb. bulgaricus* did not reveal any decreasing trend (Goldin et al. 1992). A study in rats showed that daily feeding of *Lb. rhamnosus* GG before and during dimethylhydrazine injections decreased the incidence and number of colon tumors, compared to no probiotic feeding (Goldin et al. 1996). Another interesting finding was that *Lb. rhamnosus* GG enhanced the mucus-binding ability of *Bif. lactis* (Ouwehand et al. 2000) indicating that one probiotic might influence the adhesion of another.

2.7.1.3 *Lactobacillus bulgaricus*

This organism is slightly different from most of the probiotic bacteria in that it is a ‘transient bacteria’. It is referred to in this way, because unlike most probiotic bacteria, it does not adhere to the intestinal wall and colonize, rather it simply passes through the digestive system. However, *Lb. bulgaricus* has many beneficial effects like enhancing the digestibility of milk products and other proteins and producing natural antibiotic substances that specifically target pathogenic bacteria. In this sense, *Lb. bulgaricus* can be more so potential as a positive supporter to those bacterial species that have the ability to colonize. It could also suppress inflammatory immune reactions in the intestinal wall thus preventing tissue damage (Bai et al. 2004). In another study, a substance produced by *Lb. bulgaricus* was shown to stimulate activity in part of the gut immune system called the Peyer’s patches which provide defense against pathogenic organisms within the gut (Kitazawa et al. 2003).

2.7.1.4 *Lactobacillus plantarum*

This bacterium is the most prevalent species in most of the naturally fermented foods based on cereals, legumes, vegetables and meat. It has the ability to block receptor sites
for Gram negative bacteria and so is effective as an antibiotic. It is an important player in antimicrobial defense and is effective against both extra- and intra-cellular pathogens. The culture of *Lb. plantarum* is also capable of digesting semi-digestible fibres such as those found in onions, garlic, wheat, oats, rye and yeast and may therefore help with digestive problems like flatulence. Recent research has shown that *Lb. plantarum* has the ability to break down bile acids and lower cholesterol (*Jones et al. 2004*) and is extremely resistant to stress conditions including high temperature and concentrations of ethanol, extremes of pH and the freeze drying process that would normally kill lactic acid bacteria (*Alegria et al. 2004*).

### 2.7.1.5 *Lactobacillus casei*

This species is commonly found in probiotic dairy foods, hence the name 'casei', which relates to the milk protein casein. It was reported that this culture is supposed to have the most potent protective activity against *Listeria* spp. The culture of *Lb. casei*, in the form of the shirota strain found in yakult probiotic yoghurt drinks has been shown to significantly inhibit the growth of the peptic ulcer causing bacteria *Helicobacter pylori* (*Sgouras et al. 2004*). A probiotic drink containing the shirota strain has also been shown to reduce the severity of constipation as evidenced by both patient response to questionnaires and physical examinations (*Koebnick et al. 2003*). Finally, a study with malnourished mice showed that *Lb. casei* (combined with FOS), when given along with a re-nutrition diet enhanced the immune response and increased resistance to certain pathogenic bacteria in the digestive tract (*Cano and Perdigon, 2003*).

### 2.7.1.6 *Lactobacillus reuteri*

Another probiotic LAB is *Lb. reuteri*. Strains of this microbe are widespread in nature and can be isolated from a variety of food products, animal products and from the human
gastro-intestinal tract. Under in vitro conditions, *Lb. reuteri* produces 3-hydroxypropionaldehyde from glycerol, wherein 3-hydroxypropionaldehyde has a relatively broad spectrum antimicrobial activity (Talarico et al. 1988). Reutericin 6, a bacteriocin produced by *Lb. reuteri* strain LA 6 has also been identified (Toba et al. 1991). It is not clear as to whether these antimicrobial substances are produced in the human intestine in concentrations high enough to directly inhibit pathogens. However, *Lb. reuteri* strains have received considerable commercial attention as a probiotic for both human and animal uses (Casas and Dobrogosz, 2000, Wadstrom et al. 1987).

Jacobsen et al. (1999) did an interesting evaluation of 47 *Lactobacillus* strains with respect to properties that might be important for probiotic activity. Among the tested strains, only *Lb. rhamnosus* GG, *Lb. rhamnosus* 19070-2 and *Lb. reuteri* DSM 12246 were relatively resistant to acid and bile, highly adherent to Caco-2 cells in culture, showed antimicrobial activity against enteric pathogens and could be isolated from most stool samples from 12 volunteers after ingestion.

### 2.7.1.7 *Lactobacillus sporogenes*

Several studies reported that *Lb. sporogenes* was able to lower cholesterol levels by 104 points. It produced a highly significant reduction in LDL cholesterol (undesirable) levels and a small, but significant increase in HDL cholesterol (desirable). This study offers the prospect of using *Lb. sporogenes* as a side-effect free alternative to drug therapy in the treatment of high cholesterol and heart disease. In a multi-centre double-blind placebo controlled trial, *Lb sporogenes* was found to be nearly twice as effective as placebo in reducing the number of episodes and duration of diarrhoea following antibiotic treatment in children (La Rosa et al. 2003).
2.7.1.8 *Streptococcus thermophilus*

Similar to *Lb. casei*, this bacterium has been shown to aid recovery from malnutrition due to short-term fasting and reduce the associated intestinal atrophy in animal studies (Dock et al. 2004). The culture of *Str. thermophilus* is also known to have powerful antioxidant activity, protecting the body from dangerous free radicals, which increase in the body due to aging, stress, sugar, antibiotics, chemicals and toxins. It has also been shown to have anti-tumor activity that is more effective against colon cancer cells.

2.7.2 Alpha-D-galactosidase

2.7.2.1 Potentiality

Alpha-Galactosidase (α-D-galactoside galactohydrolase E.C.3.2.1.22) is an exo-glycosidase enzyme widely distributed in nature and is responsible for in vivo cleavage of terminal α-1-6-linked D-galactosyl residues from a wide range of simple and complex substrates including oligosaccharides of raffinose family sugars (raffinose, stachyose, melibiose, verbascose) and polysaccharides of galactomannans, locust bean gum and guar gum. It also acts on glycoconjugates, glycoproteins and glycosphingolipids. Some of α-galactosidases are also known to catalyze transgalactosylation reactions especially at high concentrations of substrate (Dey and Pridham, 1972; Gote et al. 2006).

Interest in this enzyme arises from their potential technological and health applications. The most important industrial application of α-galactosidase is presently in beet sugar industry, pulp and paper industry, soy food processing and in animal feed processing (Linden, 1982; Prashanth and Mulimani, 2005). In beet sugar industry, it is extensively used to remove raffinose from beet molasses, a major impurity in sugar production and thus improve the yield of crystallized sugar (Liang et al. 1989). In soy food and animal feed processing industry, α-galactosidase is used to degrade the raffinose family of sugars such as raffinose, stachyose and verbascose in food and feed.
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materials such as soy meal or soy milk (Mulimani and Ramalingam, 1995; Kawanura et al. 2006).

Human beings and monogastric animals are deficient in pancreatic α-galactosidase (α-Gal), hence α-galactosides are not digested in the duodenum. As a result, they pass into the large intestine, wherein they are degraded by gas-producing intestinal bacteria such as Clostridium spp. and Bacteroides spp., yielding considerable amounts of CH₄, CO₂ and H₂. The abnormal accumulation of flatulent rectal gas thus provokes gastro-intestinal disorders such as abdominal pain, nausea, diarrhoea and increased peristalsis (Rackis, 1981). As a means to overcome these drawbacks, many attempts have been made to eliminate the α-galactosides from some of the foods, especially soy beans. They consist of tedious physical methods, which include soaking, germination, water extraction, and ultrafiltration (Kim et al. 1973; Ku et al. 1976; Ibrahim et al. 2002).

Alternatively, the use of α-Gal as a biotechnological approach for removal of α-galactosides from leguminous seeds has been proposed (Cruz et al. 1981; Ganiats et al. 1994; Slominski, 1994). In this approach, the use of microbial α-Gal offers a promising solution for the degradation of α-galactosides, especially during soy milk fermentation by LAB. Among the LAB, Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. casei, Lb. lactis, Lb. salivarius, Lb. reuteri, Lb. cellubiosis and Lb. buchneri can hydrolyze α-galactosides to digestible carbohydrates (Silvestroni et al. 2002).

2.7.2.2 Cultural conditions and assays

Alpha-D-galactosidase enzyme activity from microbial sources has been characterized by a large number of researchers. The presence of α-galactosidase enzyme in lactobacilli was reported as early as in 1973. The cells were grown in a medium containing glucose as the only energy source. The culture conditions under which the α-
galactosidase activity was detected suggest that it is a constitutive enzyme present in the soluble fraction of the cells of *Lb. fermentum*, *Lb. brevis*, *Lb. buchneri*, *Lb. cellobiosis* and *Lb. salivarius* subsp. *salivarius* (Mital et al. 1973).

The α-galactosidases from different lactobacilli hydrolyze naturally occurring α 1-6 linked galactosides. The cultures of *Lb. fermentum* and *Lb. brevis* hydrolyze the stachyose more completely than raffinose. For complete hydrolysis of stachyose and raffinose, both β-fructofuranosidase and α 1-6 galactosidase activities are needed. As the former was absent in both organisms, only α 1-6 galactosidase acted on the galactose component of raffinose and stachyose. In general, under optimum conditions, about 50% of the stachyose and 33% of the raffinose could be hydrolyzed, which is about the maximum hydrolysis to be expected by α-galactosidase activity of LAB. Among the substrates tested, the order of hydrolysis was melibiose > stachyose > raffinose for β-fructofuranosidase negative cell-free extracts; for β-fructofuranosidase positive cell-free extracts, the order of hydrolysis was melibiose > raffinose > stachyose in decreasing rates of activity. The α-galactosidases from different lactobacilli showed optimum activity in pH range 5.2 to 5.9.

Studies on α-galactosidase activity of *Lb. plantarum* ATCC 8014 grown in various sugars at 30 and 37°C revealed that raffinose exhibited the strongest induction, followed by galactose, melibiose and lactose (Silvestroni et al. 2002). The enzyme α-galactosidase was characterized in the hetero-fermentative strains of *Lb. fermentum* CRL 236 and *Lb. fermentum* CRL 251 that have been used in the preparation of fermented soy bean products. The optimum temperature was found to be 45°C. The enzyme was inactivated at temperatures higher than 55°C, but remained active during storage at low temperatures (0, -30 and -70°C) for 5 months. Enzyme activity was observed within a 5.0-6.5 pH range, while optimum pH was dependent on the specific strain assayed. The addition of Zn$^{2+}$ to the reaction buffer exerted a slight negative effect
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upon the activity, while Hg$^{2+}$ and p-chloromercuribenzoate produced a strong inhibition. These results would indicate the presence of SH groups in the catalytic site of the enzyme. In the assay conditions used in this study, the cell-free extracts of *Lb. fermentum* CRL 236 and *Lb. fermentum* CRL 251 showed an activity of 78 UE/mg, protein and of 47 UE/mg, protein, respectively (*Garro et al. 1993*).

Generally, oligosaccharides have been used in the food industry as a source of energy or as sweeteners. In the present scenario of extensive knowledge base, oligosaccharides find new applications as immune-stimulating agents or prebiotic compounds able to modulate the colonic microflora towards a healthy balance (*Gibson and Roberfroid, 1995*). This usually involves selectively increasing the levels of bifidobacteria and lactobacilli at the expense of less desirable bacteria (*Fuller and Gibson, 1998*). Because of its beneficial attributes, recently there is an increase in the utilization of α-D-galactosidase enzyme from the *Lactobacillus* and *Bifidobacterium* spp. for synthesis of novel oligosaccharide structures to be used as prebiotics. *Tzortzis et al. 2003* studied on the glycosidic activities of *Lb. reuteri*. It has been grown in the medium containing raffinose as a carbon source, wherein there was an increase in the α-galactosidase enzyme production at the beginning of the stationary growth phase. A 64 kDa enzyme was purified by ultra- and gel-filtration and characterized for its hydrolytic and synthetic activity. Highest hydrolytic activity was found at pH 5.0 at 50°C. The crude cell-free extract was further used in glycosyl transfer reactions to synthesize oligosaccharides from melibiose and raffinose. The α-galactosidase from *Lb. reuteri* was found to synthetically act at the C-6 hydroxyl group of the galactosyl residue in, both raffinose and melibiose transgalactosylation reactions without any other linkages formed (*van Laere et al. 1999*).

The behaviour of *Lb. fermentum* CRL 722 and CRL 251 under different pH conditions (pH 6.0, 5.5, 5.0, 4.5) was evaluated and it was observed that at pH 5.5, α-
galactosidase activity of these isolates were high (5.0 U/ml). This study was undertaken to optimize the growth condition of *Lb. fermentum* with elevated levels of α-galactosidase activity to be used in the reduction of non-digestible oligosaccharides (NDOs) in soy products when used as starter cultures (LeBlanc et al. 2004).

Among the cultures of LAB, α-galactosidase activity is estimated by the rate of hydrolysis of p-nitrophenyl-α-D-galactopyranoside substrate. The reaction mixture consists of 100 μl of 0.1 M sodium acetate buffer of pH 5.0; 100 μl of enzyme preparation and 100 μl of 2 mM p-nitrophenyl-α-D-galactopyranoside (pNPGal) or other synthetic substrates. The reaction is run for 15 min at 45°C and stopped by the addition of 1 ml of 0.5 M sodium carbonate. One enzyme unit (U) is defined as the amount of protein required to produce one μmol of p-nitrophenol per min (De Fatima Viana et al. 2005).

Optimization of microbial α-D-galactosidase production through the use of Response surface methodology (RSM) has become increasingly favourable, because this method is robust and effective in analyzing responses that are affected by many factors and their interactions. In optimization processes, RSM is less time-consuming and tedious compared to the conventional one factor at a time method (Liong and Shah, 2005).

Alpha-D-galactosidase activity of strains of probiotic bacteria like *Lb. acidophilus* FTCC 0291, *Lb. casei* FTCC 0442, *Lb. fermentum* FTD 13, *Lb. acidophilus* ATCC 4962 and *Bif. bifidum* BB12 was evaluated. The strain of *Lb. acidophilus* FTCC 0291 displayed the highest specific α-D-galactosidase activity and was thus selected to be optimized for the growth in soy whey medium supplemented with 7 nitrogen sources (including peptone, tryptone, meat extract, vegetable extract, yeast extract, urea and ammonia) using RSM. In addition, the growth properties of the probiotic in soy whey medium such as pH, production of acids and utilization of sugars within the optimized range for
maximum growth were also evaluated. The results revealed that optimum growth of \textit{Lb. acidophilus} FTCC 0291 was achieved in soy waste medium, peptone, meat extract and vegetable extract. The validation experiment showed that the prediction generated from the model using RSM was reliable. Analyses of growth, pH and titratable acidity showed that increased growth in the medium was accompanied by an increase in levels of lactic and acetic acids. The decreased concentration of reducing sugars and oligosaccharides at regions of high growth indicated increased utilization of the sugars (Fung \textit{et al.} 2008).

The application of statistical methods for the optimization of \(\alpha\)-D-galactosidase from \textit{Streptomyces griseoloalbus} in submerged fermentation was also investigated. Screening of variables to find their relative effect on \(\alpha\)-D-galactosidase production was done using Plackett-Burman design. Out of the eleven factors screened, salinity, magnesium sulphate and temperature were found to influence the enzyme production significantly. The optimal levels of these variables and the effect of their mutual interactions on enzyme production were determined using Box-Behnken design. The interaction between salinity and magnesium sulphate concentration was found to enhance \(\alpha\)-D-galactosidase production, whereas temperature exhibited an influence independent of the other two factors. Using this statistical optimization method, the \(\alpha\)-D-galactosidase production was increased from 17 to 50 U/ml (Anisha \textit{et al.} 2008).

\textbf{2.7.2.3 Purification and characterization}

Alpha-D-galactosidase has been purified and characterized from a variety of sources such as plants, animals and microorganisms (fungi, yeasts and bacteria). The enzyme was purified by ammonium sulphate precipitation, gel filtration and ion exchange chromatography and was demonstrated to be homogeneous by slab gel electrophoresis and molecular weight of this enzyme was estimated by gel filtration. Generally \(\alpha\)-galactosidases found in yeast, mold and plant seeds have an optimum pH over a wide
range of 3 to 6 and are also stable in acidic pH levels. However, bacterial α-galactosidases showed narrow pH optima and are stable over an alkaline pH range (Ulezlo and Zaprometova, 1982).

The purification and properties of α-D-galactosidase from *Lb. fermentum* was the focus of a study in one of the earlier research investigations. This enzyme had different characteristics from those isolated from fungi, yeasts and plant seeds. Its molecular mass was 194.5 kDa and composed of four subunits of 45 kDa each. The maximum activity occurred at 45°C and in a pH range of 5.0-6.5. A strong inhibition of α-galactosidase activity was found in the presence of 0.1 mM HgCl and 0.01 mM p-chloromercuribenzoate. However, the enzyme was not affected by CaCl$_2$, CuSO$_4$, MnCl$_2$, FeCl$_3$, CdCl$_2$, NaCN, NiSO$_4$, KCN, FeSO$_4$, or other chemical products such as EDTA, dithiothreitol and β-mercaptoethanol (Garro et al. 1996).

The glycosidic activity of *Lb. reuteri* was also studied. It has been grown in the medium containing raffinose as a carbon source, wherein there was an increase in the α-galactosidase enzyme production at the beginning of the stationary growth phase. A 64 kDa enzyme was purified by ultra- and gel-filtration and characterized for its hydrolytic and synthetic activity (Tzortzis et al. 2003).

### 2.7.2.4 Molecular characterization

Genes encoding α-D-galactosidase have been cloned from various sources such as humans, plants, yeasts, filamentous fungi and bacteria (Sumner-Smith et al. 1985; Bishop et al. 1986; Liljestrom and Liljestrom, 1987; Aslanidis et al. 1989; Overbeeke et al. 1989; Aduse-Opoku et al. 1991; den Herder et al. 1992; Zhu and Goldstein, 1994; Margolles-Clark et al. 1996; Shibuya et al. 1997). On the basis of primary structure similarities and hydrophobic cluster analysis, α-galactosidase encoding genes have been classified into three different glycosyl hydrolase families (Henrissat
and Bairoch, 1993). The structural differences among these enzymes also may contribute to differences in activity (Luonteri et al. 1998).

A few α-galactosidase genes have been cloned and sequenced in LAB to characterize the genes involved in the metabolism of α-galactosides. In Str. mutans, the α-galactosidase gene is associated with the multiple-sugar metabolism operon (Russell et al. 1992). This is essential for growth in the presence of melibiose and raffinose. In Str. pneumoniae, this gene is essential for raffinose utilization and its activity is stimulated by raffinose and catabolic repression is by sucrose, but not glucose (Rosenow et al. 1999). In Carnobacterium piscicola, the α-galactosidase determinant is grouped with two β-galactosidase genes and both enzymatic activities are repressed in the presence of glucose or lactose during growth (Coombs and Brenchley, 2001).

The first genetic characterization of α-galactosidase from Lb. plantarum ATCC 8014 was undertaken, wherein the melA gene involved in α-galactoside catabolism in lactobacilli was studied. The study described cloning, sequencing and characterization of α-Gal gene (melA) from Lb. plantarum ATCC 8014 and its expression in Esch. coli. The result shows that melA gene encodes a 738-amino acid protein (MelA) with a deduced molecular mass of 84 kDa. Molecular weight assessment of active MelA showed that it occurs as oligomers and that the monomers are inactive. Northern hybridization revealed that melA is expressed in Lb. plantarum ATCC8014 and that it is transcribed from its own promoter. Regulation occurs at the transcriptional level, i.e., melA is induced by the α-galactoside melibiose and partially repressed by glucose (Silvestroni et al. 2002).

LeBlanc et al. (2004) have cloned and expressed the α-Gal structural genes from Lb. plantarum ATCC 8014 and from guar in Lc. lactis. The gene products were directed to different bacterial compartments to optimize their possible applications, in terms of the removal of α-galactosides from soy-derived products. The ability of Lc. lactis
to survive the hostile conditions of the human gastrointestinal tract has made it the model bacterium for the delivery of gene products in the gut (Klijn et al. 1995).

2.7.3 Bile salt hydrolase

2.7.3.1 Significance

Bile salt hydrolase (BSH) is an enzyme (cholylglycine hydrolase; EC 3.5.1.24) that catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and deconjugated bile salts i.e. free bile acids (Tanaka et al. 2000). The major bile salt modifications in the human large intestine include deconjugation, oxidation of hydroxyl groups at C-3, C-7 and C-12 and 7α/β–dehydroxylation (Ridlon et al. 2006). It is generally considered that bile salts are first deconjugated by intestinal bacteria before they are further metabolized (Batta et al. 1990; Hill, 1995). Conjugated bile salts are absorbed from the small intestine (ca. 97%) and are returned to the liver by the hepatic portal circulation (Gorbach, 1969). Many reports have been suggested that conjugated bile salts are deconjugated, dehydroxylated, dehydrogenated, and desulfated in the intestines by microbial enzymes (Hylemon and Glass, 1983). Bile salt hydrolase has been isolated and characterized from several species of intestinal bacteria such as Lactobacillus sp., Bifidobacterium sp., Cl. Perfringens, Bacteroides fragilis ssp. Fragilis, and Listeria monocytogenes (Stellwag and Hylemon, 1976; Gopal-Srivastava and Hylemon, 1988; Lundeen and Savage, 1990; Grill et al. 1995).

The BSH enzyme responsible for bile salt deconjugation during enterohepatic circulation has been detected in several LAB species inherent to the GI tract (Chikai et al. 1987; Walker and Gilliland, 1993; De Smet et al. 1994, 1998; du Toit et al. 1998). It has also been suggested that BSH activity should be a requirement in the selection of probiotic organisms with cholesterol lowering properties. Interestingly, in a study of the cholesteremia in a tribe of Maasai conducted by Mann and Spoerry, (1974), serum
cholesterol levels of Maasai men decreased after consumption of large amounts of milk fermented with a native Lactobacillus strain. The reduction of cholesterol by LAB has been demonstrated in humans, mice and pigs (Kawase et al. 2000; Haberer et al. 2003).

The ability to tolerate bile is one of the requisite properties of probiotic bacteria and there are many studies on bile tolerance of probiotic LAB (Garriga et al. 1998; Jin et al. 1998). Several reports have indicated that consumption of fermented milk products or cultured dairy products supplemented with Lb. acidophilus led to the reduced concentrations of serum cholesterol (Harrison and Peat, 1975; Tortuero et al. 1975; Grunewald, 1982; Gilliland et al. 1985; Danielson et al. 1989). Midtvedt and Norman, (1967) reported that Lb.arabinosus deconjugated both taurocholic and glycocholic acids, whereas Lb. brevis deconjugated only glycocholic acid. The cultures of Lb. acidophilus, Lb. casei and Lb. delbrueckii did not deconjugate either of the two bile acids. However, the correlation between BSH activity and high tolerance to bile salts is still under debate.

In one of the earlier studies, several strains of Lb. acidophilus of human origin were characterized for their in vitro activity with respect to bile resistance, bile salt hydrolysis and cholesterol assimilation. Some data suggest that both Lb. acidophilus and calcium can enhance the reduction of serum cholesterol in pigs that had been fed a high cholesterol diet, probably through alteration in the enterohepatic circulation of bile acids (Buck and Gilliland, 1994). Vander Meer et al. (1990) postulated that the excretion of fecal bile acids was increased by the binding of these acids to calcium phosphate, thus making them insoluble and suppressing their reabsorption into the enterohepatic circulation.

Research investigations being undertaken over the years have revealed that microbial BSHs do play a role in the detoxification of bile salts and also increase the
intestinal survival and persistence of producing strains. Therefore, BSH activity by a probiotic bacterium may be desirable since it could maximize its prospects of survival in the hostile environment of the GI tract. Increased intestinal survival is likely to increase the overall beneficial effects associated with the strain (Begley et al. 2006).

2.7.3.2 Cultural conditions and assays

In one of the earlier studies, the distribution and extent of BSH activity among LAB was undertaken with 300 strains encompassing the genera of *Bifidobacterium* and *Lactobacillus* as well as *Lc. lactis, Leu. mesenteroides* and *Str. thermophilus* (Tanaka et al. 1999). It was hypothesized that high BSH deconjugation activity associated with the stationary phase of culture was a result of reduced pH levels in the medium (Corzo and Gilliland, 1999). In one of the *in vitro* studies, it was observed that cholesterol was reduced by *Lb. acidophilus* ATCC 43121 and L1 when the pH was maintained at 6.0 (Noh et al. 1997). Brashears et al. (1998) hypothesized that cholesterol removal was not solely contributed to bile salt deconjugation and co-precipitation. The regulation of BSH activity by pH is still not clear, although BSH activity was shown to be higher at lower pH values. Furthermore, different strains of the same bacterial species exhibited different BSH activity under similar pH levels (Lunden and Savage, 1990; Corzo and Gilliland, 1999).

A correlation between growth and BSH activity has been reported by for a strain of *Lb. plantarum* (De Smet et al. 1994). Some studies have shown that the optimal pH for bile salt deconjugation by lactobacilli is approximately 6.0 (Walker and Gilliland, 1993). In their studies, Gilliland and Speck (1977) reported that deconjugation of bile acids by *Lb. acidophilus* required a low oxidation reduction potential and that the BSH system was constitutively expressed. Pereira et al. (2003) studied the *in vitro* BSH activity of a number of LAB strains in view of their *in vivo* potential to lower cholesterol
through enhanced BSH activity. The study of BSH activity as a function of growth revealed marked differences in behaviour among *Lb. fermentum* KC5b, *Str. bovis* ATCC 43143 and *Enterococcus faecalis* UK873. The BSH activity of *Str. bovis* was growth related. However, in the other two strains, bile salt deconjugation increased only when cultures reached their maximum cell density and no correlation was detected between growth and deconjugation activities.

Optimization studies using RSM has been applied to study the reduction/removal of cholesterol by in vitro experiments. The study was focused on optimization of cholesterol removal by *Lb. casei* ASCC 292 in the presence of FOS and maltodextrin through response surface approach, which led to better understanding of the interactions involved in cholesterol reduction (*Liong and Shah, 2005a*). Similarly, in the case of *Lb. acidophilus* ATCC 4962, optimization was achieved for cholesterol removal in the presence of mannitol, FOS and inulin (*Liong and Shah, 2005b*).

In another study, the focus was to evaluate the bile salt deconjugation ability, BSH activity and cholesterol removal ability from co-precipitation with deconjugated bile by lactobacilli strains, as a means to select strains for cholesterol lowering properties. The results showed that all lactobacilli strains studied were able to deconjugate, both sodium glycocholate and sodium taurocholate. Substrate preference for BSH was more towards sodium glycocholate than sodium taurocholate, while *Lb. acidophilus* had better deconjugation ability and BSH activity than *Lb. casei*. The strains of *Lb. acidophilus* ATCC 33200, 4356, 4962 and *Lb. casei* ASCC 1521 showed highest bile salt deconjugation and BSH activity compared to other strains studied. These strains also showed highest deconjugation capability and BSH activity in experiments using concentrations of sodium glycocholate and sodium taurocholate that resemble the human bile, and pH levels that are similar to the pH of human intestine. This indicates
that these strains may exert effective deconjugation activity \textit{in vivo} (Liong and Shah, 2005c).

\subsection*{2.7.3.3 Purification and characterization}

Several BSH enzymes from cultures of \textit{Bacteroides vulgates}, \textit{Bacteroides fragilis} ssp. \textit{fragilis}, \textit{Cl. perfringens}, \textit{Lactobacillus} spp. and \textit{Bifidobacterium} spp. have been purified and characterized (Irvin et al. 1944; Nair et al. 1967; Gopal-Srivastava and Hylemon, 1988; Lundeen and Savage, 1990; Grill et al. 1995). In general, BSHs are intracellular, oxygen sensitive and pH optima of 5-6 (Begley et al. 2006). These purified enzymes differ in their physical, kinetic and genetic properties.

The strain of \textit{Lb. johnsonii} 100-100 had four proteins with BSH activity. The four proteins were designated as BSH-A, -B, -C and -D. All four proteins consist of one or two polypeptides. The peptides had molecular weights of 42 kDa and 38 kDa and were designated as \(\alpha\) and \(\beta\), respectively. The approximate native molecular weights of BSH A, B, C, and D were 115 kDa, 105 kDa, 95 kDa, and 80 kDa, respectively. The BSH activity of \textit{Lb. johnsonii} 100-100 differs from that of other reported genera / species. When conjugated bile salts were added to suspensions of stationary phase cells, the activity increases as much as three to five-folds within 20 min (Lundeen and Savage, 1990, 1992a; Savage et al. 1995; Elkins and Savage, 1998).

Three strains of \textit{Lb. acidophilus}, two from human intestinal origin (016 and L1) and one from porcine intestinal origin (ATCC 43121) were tested for their bile salt deconjugation activity. The optimum pH for deconjugation of sodium glycocholate was between 4 and 5.5 for all three strains. For deconjugation of sodium taurocholate, the optimum pH was in the range of 3.5 to 4.5 for strains L1 and ATCC 43121 and pH 5 to 6 for strain O16. The molecular mass of the enzyme in all three strains of \textit{Lb. acidophilus} was estimated to be 126 kDa by Sephadex G-200 gel filtration. All three strains exhibited
more bile salt hydrolase activity towards sodium glycocholate than towards sodium taurocholate (Corzo and Gilliland, 1999).

2.7.3.4 Molecular characterization

Genes encoding BSH have been cloned from *Lb. plantarum*, *Cl. perfringens*, *Lb. johnsonii*, *Bif. Longum*, *Bif. Bifidum*, *Bif. adolescentis* and *Listeria monocytogenes* (Christiaens et al. 1992; Coleman and Hudson, 1995; Elkins and Savage, 1998; Tanaka et al. 2000; Elkins et al. 2001; Glaser et al. 2001; Dussurget et al. 2002; Kim et al. 2004b; Kim et al. 2005). The BSHs have been classified as N-terminal nucleophilic (Ntn) hydrolases with an N-terminal cysteine residue. This Cys-1 becomes a catalytic center after removal of the initiation formyl methionine by an auto-proteolytic process, which is one of the common features of the Ntn hydrolase family. The thiol (SH) group of Cys-1 has been shown to be essential for BSH catalysis (Suresh et al. 1999).

Christiaens et al. (1992) first reported the isolation of a conjugated bile acid hydrolase gene from a *Lb. plantarum* strain by the use of a direct conjugated bile acid hydrolase (CBH) plate assay. The nucleotide sequence of the *cbh* gene and the deduced amino acid sequence were described. Further, the gene was cloned in an *Esch. Coli - Lactobacillus* shuttle vector and reintroduced into the parental *Lactobacillus* strain. By this way, overproduction of the CBH enzyme was obtained. Preliminary characterization of the gene product showed that it is a cholyglycine hydrolase (EC 3.5.1.24) with only slight activity against taurine conjugates. The optimum pH was between 4.7 and 5.5 and optimum temperature ranged from 30 to 45°C. Southern blot analysis indicated that the cloned gene had similarity with genomic DNA of bile acid hydrolase-active *Lactobacillus spp.* of intestinal origin.

The BSH enzymes are considered to be especially relevant for microbes that reside in the mammalian intestinal system, where lactobacilli are considered to be
among the most important participants in bile salt deconjugation *in vivo* (Tannock et al. 1989).

2.8 BIFIDOBACTERIA

2.8.1 Characteristics

It was Tissier (1900), who initially observed and isolated bifidobacteria (earlier known as Bacillus bifidus) from the faeces of breast-fed infants at the Institute Pasteur in Paris. He observed an abundance of an irregular Y shaped bacterium in the faeces of breast-fed infants compared with those of bottle fed infants. This bacterium was anaerobic, Gram-positive and did not develop gas during its growth. The taxonomic status of bifidobacteria was highly debatable for a long period of time. This bacterium was assigned to several genera such as *Bacillus, Bacteroides, Tissieria, Nocardia, Lactobacillus, Actinomyces, Bacterium* and *Corynebacterium* (Mitsuoka, 1984). As the classification and identification of microorganisms at the beginning of the century were based on morphological, cultural and biochemical characteristics, Orla-Jensen (1924) used new criteria such as metabolic and enzymatic characteristics to separate bifidobacteria from *Lactobacillus*. This distinction was later confirmed by De Vries and Stouthamer (1967), who demonstrated the presence in bifidobacteria of a specific enzyme namely the fructose-6-phosphate phosphoketolase (F6PPK) and the absence of aldolase and glucose-6-phosphatase dehydrogenase, two enzymes found in the lactobacilli (Scardovi and Trovatelli, 1965).

In 1965, with progress in molecular genetics, it was shown that the percentage of guanine + cytosine (G + C %) in the DNA of *Bifidobacterium* differed from that of *Lactobacillus*, *Corynebacterium* and *Propionibacterium* (Sebald et al. 1965; Werner et al. 1966). Stackebrand et al. (1997), through 16S rRNA analysis, proposed a novel
Bifidobacteria are non-motile, non-spore forming rods of variable appearance, usually somewhat curved and clubbed and are often branched. Freshly isolated strains may have forms ranging from uniform to branched, bifurcated Y and V forms, spatulate or club shapes. However, in unfavourable media conditions, bifidobacteria show branching and pleomorphism, although they are mostly rod-shaped in their natural habitat (Rasic and Kurmann, 1983). Bifidobacteria are described as strictly anaerobic, although the sensitivity of some species and strains to oxygen is known to vary (Scardovi, 1984; Shimamura et al. 1992; Ahn et al. 2001; Talwalkar and Kailasapathy, 2003; Simpson et al. 2004). Bifidobacteria are acid-tolerant microbes and their optimum pH for growth is between 6.5 and 7.0. Strains of Bif. lactis and Bif. animalis can survive exposure at pH 3.5 (Matsumoto et al. 2004), whereas Bifidobacterium strains kept in an environment above pH 8.5 do not survive (Biavati et al. 2000). The cell walls of bifidobacteria have a typical Gram-positive structure, consisting of a thick peptidoglycan envelope containing polysaccharides, proteins and teichoic acids (Gomes and Malcata, 1999). Bifidobacteria are saccharolytic organisms and all characterized strains have the ability to ferment glucose, galactose and fructose. Differences occur between species in their ability to ferment other carbohydrates and alcohols (Scardovi, 1986).

There are two types of phosphoketolases that have been described in the genus Bifidobacterium, a F6P phosphoketolase specific enzyme (F6PPK) in human species and a dual substrate xylulose 5-phosphate/fructose 6-phosphate (X5P/F6P) phosphoketolase in animal species (Grill et al. 1995). Among the 32 species that have been described, Bif. longum, Bif. bifidum, Bif. animalis, Bif. breve and Bif. infantis have
been extensively studied for their effects on human health and incorporated into dairy
products and therapeutic preparations (Biavati and Mattarelli, 2001).

2.8.2 Potential probiotic bifidobacteria

The genus *Bifidobacterium* consists of 32 species, of which 10 species are dominant in
human feces. Six sub-species have been described belonging to three different species
namely *Bif. animalis, Bif. pseudolongum* and *Bif. thermacidophilum* (Yaeshima et al.
1992; Dong et al. 2000; Zhu et al. 2003; Masco et al. 2004). Further, *Bif. longum*
consists of three biotypes namely infantis, longum and suis types (Sakata et al. 2002).

Bifidobacteria have now been successfully applied to a range of food matrices
beyond dairy yoghurt and fermented milk. To simulate the bifidogenic effect of human
breast milk, bifidobacteria and bifidogenic oligosaccharides are now included in some
dehydrated infant formulas (Mountzouris et al. 2002; Vandenplas, 2002). Bifidobacteria
have been reported to grow and survive in a variety of cheeses and
remain viable in ice cream and in frozen yoghurt (Daigle et al. 1999; Davidson et al.
2000; Vinderola et al. 2000; McBrearty et al. 2001; Ryhanen et al. 2001; Haynes and
Playne, 2002).

2.8.2.1 *Bifidobacterium bifidum*

This bacterium is one of the major constituents of the normal flora in the colon and is
also the most common species of *Bifidobacterium* present in probiotic products. One of
the studies reported that *Bif. bifidum* can significantly reduce the intestinal concentration
of endotoxin, which is made up of the cell walls of dead bacteria and is toxic, if allowed
to build up (Griffiths et al. 2004). In another study, *Bif. bifidum* of human origin was
found to adhere well to the intestinal wall and significantly reduce the ability of
pathogenic *Esch. coli* to do the same (Gagnon et al. 2004). Probiotic drink prepared by
using *Bif. bifidum* had significant antioxidant action and was able to protect the intestinal lining from lipid peroxidation (Ito et al. 2001).

### 2.8.2.2 Bifidobacterium longum

*Bifidobacterium longum* is another species of bifidobacteria commonly found in probiotic products. This species is able to eliminate the nitrates commonly found in foods ingested by humans and also reported to inhibit the action of vero cytotoxin produced by some strains of *Esch. coli* (Kim et al. 2001). In another study, *Bif. longum* has also been shown to have a protective effect against infection with *Salm. typhimurium*, possibly due to an anti-inflammatory action (Silva et al. 2004).

### 2.8.2.3 Bifidobacterium infantis

*Bifidobacterium infantis* is known to have an inhibitory action on invasive pathogenic bacteria such as *Esch. coli*. Research investigation has shown that this inhibition is achieved through more than one mechanism. Inflammatory bowel disease is thought to be caused by *Bacteroides*, which are a normal component of the gut flora. The culture of *Bif. infantis* has the ability to reduce the growth of *Bacteroides* and also significantly inhibit the inflammatory response caused by them in the gut lining (Shiba et al. 2003). In another research study, wherein formulations containing *Bif. Infantis* was used, it has been found to have positive benefits in the treatment of irritable bowel syndrome and diarrhoea. Of all the strains of bacteria in the formulation, *Bif. infantis* was found be one of the species that had colonized the intestines of patients to the highest degree (Brigidi et al. 2001).
2.8.3 Alpha-D- galactosidase

Similar to the cultures of LAB, it has been well established that species of Bifidobacterium do exhibit α-D-galactosidase activity. Several research investigations have focused on aspects relating to the cultural and assay parameters for this enzyme activity. Bifidobacteria inhabit the large intestine of warm-blooded animals and are thought to have an important, but poorly understood eco-physiological role within the gastrointestinal microbiota, which may influence host health (Mitsuoka, 1990; Degnan and Macfarlane, 1994; Marx et al. 2000; Klijn et al. 2005). Physiological and genomic studies have shown that bifidobacteria have the ability to efficiently metabolize certain short-chain oligosaccharides such as fructo-oligosaccharides, galacto-oligosaccharides and gluco-oligosaccharides (Djouzi et al. 1995; Kaplan and Hutkins, 2000; Marx et al. 2000; Palframan et al. 2003; Holt et al. 2005; Klijn et al. 2005). Many of these oligosaccharides have shown potential as prebiotics to selectively promote the growth or activity of Bifidobacterium species in animals and humans (Holt et al. 2005). Several studies were conducted to determine the influence of carbohydrates on α-galactosidase activity in Bifidobacterium spp. The α-galactosidase and α-glucosidase are found in most Bifidobacterium species including Bif. adolescentis and are thought to be involved in α-linked oligosaccharide metabolism (Tochikura et al. 1986; Desjardins et al. 1990).

Species of Bifidobacterium including Bif. adolescentis have been reported to express basal or constitutive level of α-glucosidase and α-galactosidase activity when grown on glucose as the sole carbohydrate source (Tochikura et al. 1986; Roy et al. 1991; Degnan and Macfarlane, 1994; Xiao et al. 2000; Holt et al. 2008). The activity of α-galactosidase in Bif. adolescentis was clearly enhanced, when the organism was cultivated on a variety of α-linked or β-linked disaccharides or raffinose regardless of whether the carbohydrate was a galactoside or glucoside. However, the highest enzyme activity was detected when the organism was cultivated on α-linked carbohydrates,
particularly galactosides such as melibiose and raffinose (Roy et al. 1991; Xiao et al. 2000; Holt et al. 2008).

In their studies, Garro et al. (1994) characterized the α-galactosidase enzyme from *Bif. Longum* and observed that the enzyme activity was maximal in a range of 40-45°C and at pH of 5.8. At temperature above 60°C, the enzyme was completely inactivated, but it maintained 100% activity during storage at low temperatures (4, -30, -70°C) for 3 months. The addition of Hg²⁺ to the reaction buffer produced a strong inhibitory effect, while Mn²⁺ exerted a slight positive effect upon activity. The addition of EDTA, inhibitors and other metal ions had no effect on the enzyme α-galactosidase.

Purification and characterization of α-D-galactosidase from *Bifidobacterium* spp. has been the subject of several research studies. Leder et al. (1999) showed that *Bif. adolescentis* DSM 20083 efficiently hydrolyzed and assimilated α-D-galactose-oligosaccharides during in vitro fermentation. Alpha-galactosidase, the responsible enzyme for in vivo degradation of these sugars was released from the cells by ultrasonic treatment and purified to 36-fold by ultrafiltration, ammonium sulphate precipitation, anion-exchange chromatography and size-exclusion chromatography. Two protein bands were constantly observed in SDS-PAGE. Electrophoretically homogeneous α-galactosidase was only obtained by electroelution. The enzyme had an apparent molecular mass of 344 kDa and 79 kDa as determined by size-exclusion chromatography and SDS-PAGE, respectively. Activity staining after non-denaturing SDS-PAGE indicated an apparent molecular mass of 145 kDa. Thus, a tetrameric structure of the protein is suggested. The α-galactosidase showed optimal activity at pH 5.5 and 55°C. Lower pH values and higher temperatures rapidly inactivated α-galactosidase. The enzyme hydrolyzed specifically α-galactosidic linkages, and α-1,3-linkages were hydrolyzed at a higher rate compared to α-1,6-linkages. It demonstrates
the physiological relevance of α-galactosidase of bifidobacteria to the degradation of endogenous galactosides in the intestine.

In another study on the effect of different sugars on the growth of *Bifidobacterium* spp. Roy *et al.* (1991) reported that high growth of this organism could be obtained by addition of galactose-containing sugars to the medium. Xiao *et al.* (2000) have studied the assimilation of raffinose, a galactose containing sugar as well as production and characterization of α-galactosidase from *Bif. breve*, which was highly induced by raffinose. The findings revealed that *Bif. breve* assimilated raffinose about 4-fold more effectively than other intestinal bacteria. The enzyme activity of *Bif. breve* grown on raffinose was highly and specifically increased. Its activity was 30-fold higher than that of the same culture grown on glucose. Melibiose was also effective for production of the enzyme. The enzyme was purified to homogeneity from *Bif. breve*. It is a homo-dimer with a molecular mass of about 160 kDa and optimum pH of 5.5–6.5. The enzyme exhibited high substrate specificity for α-galactoside although it had slight activity for α-glucoside.

Simultaneous with purification and characterization, advances in molecular biology has enabled in bringing about certain understanding of this enzyme at the molecular level. *Bifidobacterium adolescentis* produces α- and β-galactosidases, β-xylosidase and α-glucosidase (Van Laere *et al.* 1997). Besides hydrolase activity, some of the enzymes from bifidobacteria show transferase activity (Nunoura *et al.* 1996; Hung and Lee, 1998; Van Laere *et al.* 1997, 1999). This transglycosylation activity of glycosidases can be used for the enzymatic synthesis of various oligosaccharides. These oligosaccharides might act as growth promoting factors for bifidobacteria.

Van den Broek *et al.* (1999) have cloned and expressed α-galactosidase gene from *Bif. adolescentis* in *Esch. coli*, as a means to increase the production of α-galactosidase in a heterologous host. The recombinant enzyme was purified,
characterized and subsequently nucleotide sequence determined. The recombinant enzyme when purified by anion exchange chromatography method showed total of 83 U of α-galactosidase activity. The purification factor was 5.2 and the molecular weight was 84 kDa.

Studies have indicated that the native α-galactosidase consist of four subunits in *Bif. adolescentis*. In addition to hydrolytic activity, α-galactosidase showed transglycosylation activity and can be used for the production of α-galacto-oligosaccharides (*Leder et al. 1999; Van den Broek et al. 1999*). *Zhao et al. (2008)* studied a novel α-galactosidase gene (*Aga2*) from *Bif. breve* 203. This gene was cloned and expressed in *Esch. coli*. The recombinant enzyme *Aga2* was purified and characterized. It contained an ORF of 2226 bp nucleotides encoding 741 amino acids with a calculated molecular mass of 81.5 kDa. The Aga2 enzyme was highly active towards *p*-nitrophenyl α-D-galactopyranoside. It was a new oligosaccharide produced by glycosidase and contained galactosidase-α-1,4 linkage, a novel galactosidic link formed by microbial α-galactosidase.

### 2.8.4 Bile salt hydrolase

Although, bile salt hydrolase (BSH) has been widely studied in several genera, the genus *Bifidobacterium* has been reported to possess higher BSH activity than other bacterial groups. Wide distribution and high enzyme activity of BSH in the genus of *Bifidobacterium* has been reported by some research groups (*Grill et al. 1995, 2000; Tanaka et al. 1999*). In this study, BSH from all species of *Bifidobacterium* showed a higher deconjugation rate on glycine conjugated bile salts than on taurine conjugated forms. Considering the fact that the majority of human bile salts are glycine conjugated forms, BSH enzymes from bifidobacteria might be important in the deconjugation of bile salt in the human intestine.
Although the bile tolerance mechanisms of bifidobacteria are poorly understood, strains expressing increased resistance to bile have been obtained by adaptation to gradually increasing concentrations of these compounds. The acquisition of resistance to one bile salt also conferred cross-resistance to others, increased resistance to low pH and induced stable changes in carbohydrate fermentation profiles and some glycosidase activities (Margolles et al. 2003; Noriega et al. 2004). It has been also shown that this acquisition induces changes in membrane protein profiles (Margolles et al. 2003) and in the ability to adhere to human intestinal mucus (Gueimonde et al. 2005). Therefore, it is reasonable to hypothesize that highly adhesive bile-salt resistant derivative strains would have also acquired an enhanced ability to inhibit the adhesion of enteropathogens by competitive exclusion.

Perrin et al. (2000) examined the influence of FOS and their monomeric components on bile salt resistance of Bif. breve ATCC 15700, Bif. longum ATCC 15707 and Bif. animalis ATCC 25527. For the three strains tested the growth was identical and bile salts had the same inhibitory effect on growth, irrespective of the carbohydrate used. The survival of Bif. breve and Bif. longum, in the presence of glycodeoxycholic acid depended on carbohydrates. In the case of Bif. animalis, the presence of any carbohydrate in the incubation medium did not enhance the viability of the strain. However, in the three deconjugating strains of Bifidobacterium studied, the presence of FOS during the growth led to improved resistance to the bactericidal effect of the bile salt as compared with their monomeric components (glucose and fructose).

In recent years, the possibility of using bile salt deconjugation by bifidobacteria to lower serum cholesterol levels in hypercholesterolemic patients or to prevent hypercholesterolemia in normal individuals has received increased attention. In one of the research studies, all bifidobacteria cultures could deconjugate both sodium glycocholate and sodium taurocholate. However, more sodium glycocholate was
deconjugated compared to sodium taurocholate. This was consistent with higher substrate specificity by BSH for glycine conjugated bile compared with taurine-conjugated one. However, the highest level of activity was observed with glycochenodeoxycholic acid and had higher affinities for glycine-conjugated bile acids than for taurine-conjugated bile acids (Tanaka et al. 2000).

Based on genetic data of *B. longum*, Tanaka et al. (2000) found that BSH was an intracellular enzyme. The enzyme was released either by sonication or by other cell disruption methods or by lysis in assays performed with whole cells. Sonication of cells under acidic conditions has been found to result in loss of enzyme activity, while mechanical rupturing minimized loss of activity (Shah and Jelen, 1990; Shah and Lankaputhra, 1997). Total BSH activity did not correlate well with protein concentration in the cell extracts and thus did not produce a linear correlation with specific activity. This may be due to several reasons such as the sensitivity of BSH to oxygen and different homogenization efficiency (Tanaka et al. 1999).

It has been reported that bifidobacteria are one of the most predominate culture existing in the human colon, and possessed higher BSH activity than other probiotics. Screening experiments using 30 strains of bifidobacteria revealed that only two strains did not show BSH activity and all positive strains contained constitutive intracellular BSH enzymes (Benno et al. 1989; Kim and Lee, 2003). Earlier studies revealed that the co-precipitation of cholesterol with deconjugated bile was correlated with the pH of media (Tanaka et al. 2000). The pH optimum for BSH activity at 37°C was between 5.0 and 7.0, with the maximum activity at pH 6.0 and temperature between 40 and 45°C. The enzyme was stable in the range of pH values of 4.0 to 8.0 (Tanaka et al. 2000). The solubility of cholic acid was found to decrease with decreasing pH values, because cholic acid is insoluble in pH less than 5.0 due to its pK of 5.0–6.0 (Brashears et al. 1998). Theoretically, cholesterol co-precipitation would increase with decreasing pH
values. At decreasing pH levels, taurine-conjugated bile salts will remain ionized in solution, glycine-conjugated bile salts will be partially precipitated without hydrolysis and conjugated bile salts will be precipitated (Dashkevics and Feighner, 1989).

Liong and Shah (2005d) have examined the bile salt deconjugation ability, BSH activity and cholesterol removal from co-precipitation with deconjugated bile by five bifidobacteria strains (Bif. longum 536, Bif. infantis 1912, Bif. longum 1941, Bif. breve ATCC 15698 and Bif. infantis ATCC 17930) in order to select strains with cholesterol lowering properties. All bifidobacteria strains studied were able to deconjugate both glycine- and taurine-conjugated bile, although more cholic acid was released from the deconjugation of sodium glycocholate compared to sodium taurocholate. This was consistent with higher substrate preference of BSH for glycine-conjugated bile than taurine-conjugates. Based on these findings, they hypothesized that the in vitro removal of cholesterol upon deconjugation activity on sodium glycocholate and sodium taurocholate was contributed by several factors: (a) co-precipitation with deconjugated bile (cholic acid) at pH levels below 5.0, (b) higher substrate preference of BSH for glycine-conjugates, which led to higher cholesterol co-precipitation with the deconjugated bile upon deconjugation of sodium glycocholate and (c) co-precipitation with sodium glycocholate at pH levels below 4.0.

The purification and characterization of BSH from species of Bifidobacterium has been the subject of several research investigations. Grill et al. (1995) purified the BSH enzyme from Bif. longum BB536. Its apparent molecular mass in denaturing polyacrylamide gel electrophoresis was approx. 40 kDa. The intact enzyme had a relative molecular weight of approx. 250 kDa, as determined by gel filtration chromatography, suggesting that the native BSH of Bif. longum was probably a hexamer and located in the intracellular space. The purified enzyme was active towards both glycine and taurine conjugates of cholate, deoxycholate and chenodeoxycholate. The pH
optimum was in the range of 5.5 to 6.5. A loss in BSH activity was observed after incubation at temperatures higher than 42°C, with 50% of the BSH activity being lost at 60°C. The importance of free sulfhydryl groups at the enzyme active centre was suggested.

In a similar study, BSH from *Bif. longum* SBT2928 was purified and characterized. The enzyme had a native molecular weight of 125 kDa to 130 kDa and a subunit molecular weight of 35 kDa as determined from the deduced amino acid sequence indicating that the enzyme is a tetramer. The pH optimum of *Bif. longum* BSH was between 5 and 7 and the temperature optimum was 40°C. The enzyme was strongly inhibited by thiol enzyme inhibitors, indicating that a Cys residue is likely to be involved in the catalytic reaction. The BSH of *Bif. longum* can hydrolyze all six major human bile salts and at least two animal bile salts. A slight preference for glycine-conjugated bile acids was detected based on both the specificity and the *Km* values. The nucleotide sequence of *bsh* was determined and used for homology studies, transcript analysis and construction and analysis of various mutants (*Tanaka et al. 2000*).

*Kim et al. (2004a)* investigated the biochemical characteristics of BSH enzyme in bifidobacteria. Bile salt hydrolases were purified to electrophoretic homogeneity from strains of *Bif. bifidum* ATCC 11863, *Bif. infantis* KL412, *Bif. longum* ATCC 15708, *Bif. longum* KL507 and *Bif. longum* KL515. Three different types (A, B and C) of BSH were revealed during the purification study, exhibiting the type-specific characteristics in their electrophoretic migration and elution profiles based on anion exchange and hydrophobic interaction chromatographic columns. The subunit molecular mass estimated by SDS–PAGE was around 35 kDa and the native molecular mass in all the studied *Bifidobacterium* strains was estimated to be between 130 and 150 kDa by gel filtration chromatography, indicating that all BSH enzymes have tetrameric structure. All BSH enzymes from five strains hydrolyzed six major human bile salts and they showed a
better deconjugation rate on glycine-conjugated bile salts than on taurine-conjugated forms.

The application of molecular biology has provided significant insights into the gene sequences of BSH. Among the species of *Bifidobacterium*, *Bif. longum* strains have been the most studied (Kaufmann et al. 1997). Genus and species specific primers have been developed for the detection and identification, mostly on the basis of 16S rRNA sequences (Matsuki et al. 2003). Since BSH activity is commonly detected in almost all species of *Bifidobacterium*, research investigations of the conserved and variable regions of the *bsh* genes from various species have been undertaken for the development of alternative phylogenetic markers for the genus *Bifidobacterium* (Tanaka et al. 1999). Among *bifidobacterium* species, description about molecular cloning, sequencing, and characterization of a BSH enzyme has been reported from *Bif. longum*, *Bif. bifidum* and *Bif. adolescentis*.

Kim et al. (2004b) described the molecular cloning, sequencing, and biochemical characterization of a BSH enzyme from *B. bifidum* ATCC 11863. In their earlier study, Kim et al. (2004a) described the purification of three different types of BSH that are found in the genus *Bifidobacterium* and their study reported some minor differences in their biochemical characteristics. In this study, supporting evidence for such differences was provided by molecular characterization. The *bsh* gene was cloned from *Bif. bifidum* and the DNA flanking the *bsh* gene was sequenced. Comparison of the deduced amino acid sequence of the cloned gene with previously known sequences revealed high homology with BSH enzymes from several microorganisms.

In another study, a gene coding for BSH from *Bif. adolescentis* was cloned and expressed in *Esch. coli* and the nucleotide sequence was determined. The BSH of *Esch. coli* transformants was produced intracellularly in the absence of bile salts. A unique *bsh* promoter (Pbsh) sequence was identified by using a Neural Network Promoter
Prediction. In spite of their high-level sequence homology with other \textit{bsh} genes in the \textit{Bifidobacterium} species, their genetic organization surrounding the \textit{bsh} gene and their promoter sequences are different depending on the species (\textit{Kim et al. 2005}).