INTRODUCTION

Food is one of the three essentials for maintenance of life. The first and foremost goal of any country is to increase the food supply in sufficient amounts of the right nutrient content to meet the needs of an ever increasing population. Such food has to be safe, which implies that its consumption should not give rise to any Food Borne Disease whether from infection, intoxication, contamination, adulteration or other sources.

Food is the most complex part of the environment through which human population is exposed to a variety of contaminants. Further, the WHO conference on nutrition has declared that “Access to safe and nutritionally adequate food is the right of every individual”. But it seems that the right of an individual is misused due to improper safety of foods. This can be said so because millions of people worldwide suffer from diseases of food borne origin.

A majority of Food borne illnesses result from microbial hazards. According to WHO microbes are responsible for approximately 85% of reported food borne disease outbreaks throughout the world. WHO estimated that the diseases caused due to food borne pathogens leads to 76 million human illness each year, 3,25,000 hospitalizations and an unknown number of chronic conditions. This is an overview of the status of the food borne diseases in the world but in many developing countries, including India, food borne illnesses are not that well reported. WHO in 2001 has reported 34,111 cases of diarrhea including 33 deaths in 24 districts of Orissa state; these figures mention the situation of only one state but from this one can imagine the seriousness of the problem at the country and the world level.

The centre for Disease control and prevention reported that there are 250 food borne pathogens such as Clostridium botulinum, E.coli 0157:H7, Listeria monocytogenes, Salmonella spp., Staphylococcus spp., Shigella, Bacillus cereus, Vibrio spp., Clostridium perfringens etc while some viruses are also responsible for food borne illnesses.
Microorganisms have been used in the production of palatable foods, while the actions of other microorganisms have been noted in the form of foodborne diseases or spoilage of foods. The symptoms in foodborne diseases are often mild including nausea, diarrhea, vomiting, though some severe diseases exist. The major sources of microorganisms in food products include the environment, raw materials, and processing steps. Food processing techniques reduce the number of microorganisms present or inhibit their growth in food (Gould et al. 1995, Gould 1996), but the processing steps may also contaminate the final product (Legan and Voysey, 1996). The intrinsic and extrinsic conditions of food products often do not totally exclude the possibility of the growth of microorganisms or the transfer of microorganisms via food into humans. The spoilage of food may be delayed or the spoilage will be due to the growth of different microorganisms if the extrinsic conditions of food are changed (van der Zee and Huis in’t Veld, 1997). In milk and dairy products most of the natural flora are destroyed in pasteurization process and therefore, pathogenic bacteria if present are able to multiply and subsequently cause foodborne diseases (Wong et al., 1988; Schmitt et al. 1990). Some microorganisms such as yeasts and lactic acid bacteria have a dual role in the food industry, while dairies, breweries and bakeries use them in their production, these microorganisms may cause spoilage of other products.

In food industry, the rapid detection (24 h or less) and identification of microorganisms or contamination sources help in making decisions concerning production lots, minimizing the expenses due to unnecessary storage and raw material losses (van der Vossen and Hofstra, 1996). There is increasing demand by the consumers for ready-to-serve, natural and functional food products (Gould et al., 1995; Gould, 1996; van der Vossen and Hofstra, 1996). Changed consumer demands, and corporate concentration together with trade agreements have restructured food production (Wallace, 1992). The problems in larger production units have more detrimental effects when spoilage or food poisoning microorganisms have entered the manufacturing lines (van der Vossen and Hofstra, 1996).
The centralizations of production in Finland have forced food companies to use long distance transportations. Furthermore, international transportation of commodities due to trade agreements has increased (Rees, 1989), the emergence of new pathogens (Notermans and Hoogenboom-Verdegaal, 1992) and the increasing amount of sensitive populations, including the very young, the elderly, pregnant women, and the immunocompromised, set additional demands for microbiological quality control (Gerba *et al*., 1996).

In less developed countries up to 30% of the food produced cannot be used due to the spoilage by the actions of microorganisms, rodents, or insects (Waites and Arbuthnott, 1990) and the number of acute enteritis cases is estimated to be around 300 cases per 1000 people per year, though only about 5% of the foodborne disease cases are reported (Notermans and Hoogenboom-Verdegaal, 1992). Food poisoning diseases have been increasing, and food with high protein content such as fish, meat, milk and poultry are the main incriminating food products (Notermans and Hoogenboom-Verdegaal, 1992; Kukkula, 1998). In Finland, between 1995-1997 the numbers of outbreaks caused by unknown agent have been between 32-34% (Rahkio *et al*., 1997; Kukkula, 1998) and in 1993 as high as 64% (Hirn and Myllyniemi, 1994). The numbers of food- and water-borne outbreaks annually in Finland have been less than 90 and the number of cases involved in the outbreaks less than 6000 persons. However, despite the small number of outbreaks, there is still reason to be cautious. There are new emerging pathogens (*enteropathogenic Escherichia coli*) which have caused severe outbreaks among humans. Traditionally, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella* spp. and *Staphylococcus aureus* have caused few outbreaks annually. Occasional outbreaks caused by *Campylobacter* spp., *Yersinia* spp. or other pathogens have also been reported (Kukkula, 1998). In the beginning of 1999 the most alarming outbreak was due to the consumption of dairy product which contained *Listeria monocytogenes* and was reported in media.

The introduction of the Control at source, Good Manufacturing Practices (GMP), Hazard Analysis of Critical Control Points (HACCP) and Quantitative Risk
Analysis (QRA) have made food production more controlled in a cost-efficient way (Notermans and Mead, 1996). GMP and HACCP protocols should verify for instance that only raw materials without pathogens are used in production, and that processing conditions are well defined to prevent the growth of harmful microorganisms present in the manufacturing lines (Notermans and Mead, 1996). QRA is a stepwise analysis of health risks associated with a particular type of food product. In QRA, estimation is made of the probability of negative health effects after consumption of products (Notermans and Teunis, 1996).

Food microbiological analysis (Table 1) is still needed to verify the performance of the production control systems. The essence of food microbiology has been the enumeration of microorganisms using plate counting methods, though the goal is to verify that a product is safe for human consumption (Sharpe, 1979). It has been estimated that less than 1 % of the microorganisms are culturable (Amann et al., 1995), and not all microorganisms, for instance, different yeasts species are detected by the same media (Beuchat, 1993). There are microorganisms associated with food (Escherichia coli, Salmonella enteritidis, Shigella spp., Vibrio vulnificus, Campylobacter jejuni, Pseudomonas spp.) which are known to have viable but non-culturable forms (VBNC), and they remain potentially virulent during the dormant phase (Roszak and Colwell, 1987; Chmielewski and Frank, 1995; Rahman et al., 1996). Some microorganisms can form spores or other dormant stages when environmental conditions are unfavourable, these and VBNC cells can start to grow when environmental conditions changes to allow their growth (Collins-Thompson et al., 1973; Smith et al., 1984; Scwabe et al., 1990; Dukan et al., 1997; Whitesides and Oliver, 1997; Watson et al., 1998). Furthermore, in food samples about 90 % of the microorganisms can be injured (Hurst, 1977). The nonculturable and injured microorganisms can be detected e.g. by microscopic counting (Wang and Doyle, 1998) or by using polymerase chain reaction (PCR) to amplify their DNA or nucleic acid probes (Brauns et al., 1991; Jaulhac et al., 1992; Josephson et al., 1993; Rahman et al., 1996; Weichart et al., 1997).
Table 1. The food microbiological analysis listed in Food legislation (Anon 1998).

<table>
<thead>
<tr>
<th>Products</th>
<th>Types of analyses*</th>
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</thead>
<tbody>
<tr>
<td>Milk and milk products</td>
<td>Mesophilic aerobic bacteria</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td></td>
<td>Salmonella spp.</td>
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<td></td>
<td>Listeria monocytogenes</td>
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<td></td>
<td>Coliforms</td>
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<td></td>
<td>Escherichia coli</td>
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<tr>
<td>Meat and meat products</td>
<td>Mesophilic aerobic bacteria</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
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<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>Egg and egg products</td>
<td>Mesophilic aerobic bacteria</td>
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<tr>
<td></td>
<td>Salmonella spp.</td>
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<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Fish and fish products</td>
<td>Mesophilic aerobic bacteria</td>
</tr>
<tr>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td></td>
<td>Fecal coliforms</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
</tr>
</tbody>
</table>

*The analyses are performed by using internationally standardized methods (for instance ISO) which currently are conventional methods.

There is a need for new, more rapid and specific detection, quantification and characterization methods for food related microorganisms. The traditional testing has been found unsatisfactory. For instance, biochemical tests used for identification of Shigella spp. do not distinguish it from Escherichia coli and therefore a PCR based identification method was developed (Lampel et al.}
The physiological and immunological methods used to distinguish the yeast species *Candida milleri, Saccharomyces exigus,* and *Saccharomyces cerevisiae* have been found to be unsatisfactory (Middelhoven and Notermans, 1993) and the identification of microorganisms may change when new methods are applied (Oda *et al.*, 1997). Furthermore, there is a lack of a rapid detection method for the causative agent for a diarrheal form of food poisoning caused by *Bacillus cereus.* In order, that PCR methods would be useful in food laboratories there is a need for PCR quantification methods. Rapid and reliable fingerprinting methods should be developed for finding contamination sources. For detection, quantification, and specific identification of food related microorganisms, DNA based methods have and can be developed. Different separate studies were made in this thesis for evaluating all these aspects.

**Microorganisms in food**

The microorganisms present in products are dependent on the microbiological quality of raw materials, hygienic conditions in the production, and how the product has been stored after the production (Table 2).

<table>
<thead>
<tr>
<th>Milk and dairy products</th>
<th>Pseudomonas, Flavobacterium, Alcaligenes, Aeromonas, Serratia, Bacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat and fish products</td>
<td>Pseudomonas, lactic acid bacteria</td>
</tr>
<tr>
<td>Fruits and vegetables</td>
<td>Yeasts</td>
</tr>
</tbody>
</table>

Jay (1992) have listed 36 bacteria genera, 16 mould genera, 14 yeast genera, and five protozoa species associated with food. Several important microorganisms implicated with foodborne diseases include (Gould, 1995; Granum *et al.*, 1995; Notermans and Mead, 1996): *Acetobacter melanogenus,*
Aeromonas hydrophila, Bacillus brevis, Bacillus cereus, Bacillus licheniformis, Bacillus subtilis, Brucella spp., Campylobacter coli, Campylobacter jejuni, Citrobacter spp, Clostridium botulinum, Clostridium perfringens, Enterobacter cloacae, enteropathogenic Escherichia coli, Franciella tularensis, Klebsiella spp., Listeria monocytogenes, Proteus penneri, Salmonella spp., Shigella spp., Staphylococcus aureus, Streptococcus spp., Vibrio parahaemolyticus, Vibrio cholerae and Yersinia enterocolitica.

The prevalence of microorganisms varies in different products depending on the intrinsic and extrinsic conditions (Wong et al., 1988; Van Netten et al., 1990; Te Giffel et al., 1996; Halpin-Dohnalek and Marth, 1989; Parrish and Higgins, 1990; Fleet, 1992; Deák and Beuchat, 1993; Legan and Voysey, 1996). The spoilage microorganisms have been divided into five broad categories (Van der Zee and Huis in’t Veld, 1997):

**Gram-negative rod shaped bacteria**

The most common spoilage microorganisms are *Pseudomonas* spp., particularly in aerobically stored foods with a high aw and relatively neutral pH conditions (meat, fish, poultry, milk and dairy products). Other gram-negative rod shaped bacteria include *Aeromonas* spp., *Photobacterium* spp., and *Vibrio* spp.

Biotechnological processes have been and will continue to be important technological processes in the food industry. For instance yeasts are used to produce 60 million ton of beer, 30 million ton of wine and 600000 ton (dry matter) of baker’s yeast annually (Demain et al., 1998). Some microorganisms, especially, some LAB have beneficial effects on human health (Daly et al., 1998), and one yeast species, namely *Saccharomyces boulardii*, has also even been found to possess therapeutic properties for treating diarrhoea (McFarland and Bernasconi, 1993). The quality control of production strains may be done using DNA based fingerprinting methods to verify the quality of the production strain or the purity of the production lots (Tsuchiya et al., 1992; Roy et al., 1996).
Staphylococcus aureus

Currently, the genus *Staphylococcus* consists of 33 species (Kloos *et al*., 1992, Tanasupawat *et al*., 1992, Chesneau *et al*., 1993, Webster *et al*., 1994, Zakrzewska Czerwinska *et al*., 1995; Lambert *et al*., 1998). The members of the genus *Staphylococcus* have been found to be common to all animals (in skin and in mucosal membranes) (Kloos, 1980; Bergdoll, 1989). Therefore, their presence in food manufacturing lines has been considered as an indicator of poor hygienic conditions (Notermans and Wernars 1991). For instance in one food factory, 44% of the workers were found to harbour enterotoxic staphylococci in their noses (Polledo *et al*. 1985). *S. aureus* and certain other *Staphylococcus* species including *S. capitis, S. cohnii, S. equorum, S. haemolyticus, S. hyicus, S. intermedius, S. lentus, S. simulans*, and *S. xylosus* have been implicated with the production of enterotoxin. Though, only *S. aureus* has been considered to be one of the most important bacteria causing food poisoning (Genigeorgis, 1989; Bergdoll, 1990; Notermans and Hoogenboom-Verdegaal, 1992). About half of the *S. aureus* strains have been found to be enterotoxigenic (Troller, 1976; Ahmed *et al*., 1978, Ewald, 1987; Ewald and Christensen, 1987; Halpin-Dohnalek and Marth, 1989). In microbiological analysis the coagulase positive *S. aureus* has been used as an indicator organism, though enterotoxin production by coagulase negative staphylococcal species have been reported (Breckinbridge and Bergdoll, 1971; Adesiyun *et al*., 1984; Hirooka *et al*., 1988; Bautista *et al*., 1988; Gunn, 1989; Vernozy-Rozand *et al*., 1996).

There are at least ten immunologically different enterotoxins produced by staphylococci and furthermore some immunologically unrecognized toxins (Bergdoll, 1989; Su and Wong, 1995), which are produced during all phases of growth (Czop and Bergdoll, 1974). These enterotoxins are water-soluble proteins (Swaminathan *et al*. 1992), which contain many lysine, aspartate, glutamate and tyrosine residues in their protein primary structure (Bergdoll, 1990) and in their native biologically active form they are resistant to heat and proteolytic enzymes (Bergdoll, 1989). Staphylococcal enterotoxins act as ‘superantigens’ and activate
human defense systems when causing foodborne and other diseases (Marrack and Kappler, 1990).

**Factors influencing microorganisms in food production**

Food manufacturing practises, such as heating, modified atmoshaere packaging, cold storage, and others, have been designed to reduce the numbers of microorganisms present in the raw materials (Gould *et al*. 1995; Gould 1996). Inadequate treatments only injure microorganisms making them unable to form colonies in the selective plates used for detection and they therefore go undetected through quality control (Baird-Parker and Davenport 1965; Hurst 1977). The ability of microorganisms to adapt to changes in growth conditions in food is poorly known (Huis in't Veld, 1996). The range of temperature and pH conditions in which microorganisms are able to grow have often been estimated from laboratory experiments which do not correspond to the conditions found in food. The proteins and fats in food are known to protect staphylococcal enterotoxins and the spores of *Bacillus cereus* (Smith *et al*., 1983; Kramer and Gilbert 1989). The existence of certain indicator organisms in the products, has been considered as a risk factor. Especially, foods which are manually handled after precooking or pasteurisation may be considered to be at high risk (Schmitt *et al*., 1990).

Microorganisms are affected by temperature, pH, chemicals, moisture content, water activity (aw), oxidation-reduction potential (Eh), nutrient content of the raw materials, antimicrobials, and biological structures of food products. Generally, moulds and yeasts are more resistant to different external factors than bacteria e.g. many pathogenic bacteria do not grow below pH 4 (Thomas *et al*., 1993). Low temperatures will slow the metabolic activity of microorganisms, though psychrophilic microorganisms can grow or prefer growing at low temperatures (Legan and Voysey, 1996). Spores of thermophilic bacteria can survive heat treatments commonly used in food production and create a risk for food manufacturing processes (Andersson *et al*., 1995). The strict aerobic microorganisms are not able to grow in modified atmosphere packagings which do not contain oxygen (Gould, 1996), though some microorganisms (yeasts and
moulds) are not totally inhibited in flexible bakery packages in which the concentration of CO2 is lower (Legan and Voysey, 1996). Preservatives such as potassium sorbate (Troller, 1986; Thomas et al., 1993) and nisin produced by some LAB (Blom and Mørtvedt, 1991; Beuchat et al., 1997) may inhibit the growth of other microorganisms. However, the actions of preservation compounds like salt and sugar together with other preservatives are dependent on the concentration of the compounds in the products (Halpin-Dohnalek and Marth, 1989). Starters used in food production and the growth of microorganisms in products affect the growth of pathogenic microorganisms, either promoting or inhibiting their growth rates (Smith et al., 1983; Otero et al., 1993; Cavadini et al., 1998). The natural compounds (oils, lactoferrin, lysozyme, lactate) found in some products are also known to possess antimicrobial activity (Jay, 1992) together with the natural biological structures of food. The outer coverings of fruits, seeds, shells of eggs, and hides of animals provide a barrier against microorganisms. The best preservation action will be achieved by combining many influencing factors (Untermann and Müller, 1992; Thomas et al., 1993; Legan and Voysey, 1996). The processing techniques and subsequent food inspection analysis should ensure that the products do not contain enterotoxins which remain active after processing or harmful microorganisms which may survive and start to grow in the final product when conditions become favourable.

**Current methods in food microbiology**

The safety of food is often determined by detecting the colonies of the 'indicator microorganisms' in suitable media (Table 4). Making the change to more modern techniques is often considered to be difficult (Andrews, 1997) as plate counting methods are standard methods required by legislation to be done by the producer or food inspection offices. High amounts of coliforms in a sample indicate a fecal contamination. Gram-negative bacteria can be found in products with insufficient pasteurization, and staphylococci are present in products which are produced in poor hygienic conditions (Notermans and Wernars, 1991). The coliforms are often the only microorganisms which can be determined using plate counting after overnight incubation. Most of pathogenic microorganisms
used as targets in hygienic quality control, psychrophiles and other slow growing microorganisms require longer incubation times to be detected. This type of post-testing serves only as a trend analysis (van der Zee and Huis in’t Veld, 1997). One single microorganism or small amounts of microbial toxins can be considered potentially harmful in food microbiological analysis and therefore low detection limits are required of the methods used for analysis (Evenson et al., 1988; Wolcott, 1991). The culture based methods are slow for industrial quality control where the contamination source and contaminating strains should be identified preferably before the products reaches the consumers (Cox et al., 1987; Beuchat, 1993; van der Vossen and Hofstra, 1996). Though, there are reports that a certain media performs significantly better than others in detection of specific microorganisms (Niskanen and Aalto, 1978; Rayman et al., 1988), often time-consuming confirmation tests must be applied for reliable results (Niskanen and Aalto, 1978; Noleto and Bergdoll, 1980). For identification of suspected colonies API system have been used successfully (Jasper et al., 1985; Griffiths, 1990; Török and King, 1991; Te Giffel et al., 1996). The plate counting methods have other disadvantages in addition to their inherent slowness and unspecificity. The enumeration of some microorganisms may require additional cultural conditions. For microaerophilic Campylobacter jejuni a suitable oxygen content is needed (Skirrow, 1990), for anaerobes the exclusion of oxygen and for thermophiles high temperatures should be applied (Brock and Freeze, 1969). The common errors associated with microbiological plating are media deficiencies, erroneous incubation temperatures, and mistakes in calculations or writing up of the results (Peterz et al., 1989; Peterz and Steneryd, 1993).

**Milk Microbiology**

Milk is an important food of diet of vast population on earth, due to its high nutritional value for human beings. Milk is an excellent growth medium of microorganism when suitable temperature exists. If it is produced unhygienically and handled carelessly, it gets contaminated very easily leading to its early
spoilage (Oliver et al., 2005). Many milk-borne epidemics of human diseases have been spread by contamination of milk by spoiled hands of dairy workers, unsanitary utensils, flies and polluted water supplies. The same thing can be said for improper handling of foods in the home, restaurants, hospitals, and other institutions.

The quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms.

The contamination of milk and milk products is largely due to human factor and unhygienic conditions. Usually milk gets contaminated with different kinds of microorganisms at milk collecting places. Milk is a major part of human food and plays a prominent role in the diet. Approximately 50 percent of the milk produced is consumed as fresh or boiled, one sixth as yoghurt or curd and remaining is utilized for manufacturing of indigenous varieties of milk products such as Ice cream, Butter, Khoa, Paneer, Rabri, Kheer, Burfi and Gulabjaman. The manufacture of these products is based on traditional method without any regard to the quality of raw material used and/or the hygienic quality of the products. Under such conditions many microorganisms can find access to the milk products.

Coliforms are considered as normal flora of intestinal tract of human and animals. They have been used as indicator organisms for bacteriological quality of milk and its products (Chatterjee et al., 2006). Coliform count is always being taken as a definite index of fecal contamination of milk and its products, that besides the possible presence of enteric pathogens which may constitute health hazards to the consumers. The most important index of microbiological quality is total bacterial count, coliforms, yeast and moulds count and detection of specific pathogens and their toxins (Szita et al., 2008).

Among all micro-organisms *Escherichia coli* is frequently contaminating organism in food and is reliable indicator of fecal contamination and generally present due to insanitary conditions of water, food, milk and other dairy products.
(Jayarao and Henning, 2001). Recovery of *E. coli* from food is an indicative of possible presence of enteropathogenic or toxigenic micro-organism which could constitute a public health hazard. Enteropathogenic *E. coli* (EEC) can cause severe diarrhea and vomiting in infants and young children. Coliforms particularly *Escherichia coli* are frequently used in the microbiological analysis of food as an indicator of poor hygienic condition.

Microbiological examination of milk is essential to find the degree of contamination and enumeration of indicator organisms. The coliform bacteria are able to grow well in a variety of substrates and to utilize a number of carbohydrates and some other organic compounds as food for energy and a number of fairly simple nitrogenous compounds as a source of nitrogen. The coliform group of bacteria is defined as the indicator (faecal coliform) of suitability of milk for drinking (Wells *et al.*, 1991).

**Lactic acid Bacteria in Foods**

Lactic acid bacteria (LAB) are a group of beneficial bacteria which ferment sugars as an energy source to produce large quantities of lactic acid. They are used in the preparation of fermented dairy products and also for pickling of vegetables, baking and wine making, curing fish, meats and sausages (Tamime and Robinson, 1999). Lactobacilli are used for the manufacture of fermented milks like dahi, yoghurt, acidophilus milk, kumiss, bioghurt, cheese and shrikhand (Sellars, 1989). Yoghurt is a semi-solid fermented milk product which originated centuries ago in Bulgaria. Its popularity has increased and it is now consumed in various parts of the world; although the consistency, flavour and aroma may vary from one region to another (Wood 1985). Yoghurt is defined as a coagulated milk product obtained by lactic acid fermentation of milk brought about by *S. thermophilus* and *L. delbruckii* subsp. *Bulgaricus*. A proportion of 1:1 of *L. bulgaricus* and *S. thermophilus* is considered to be optimum for flavour and texture production, (Nakazawa and Hosono, 1992). The optimal growth of LAB in milk depends on their proteolytic system, which hydrolysis milk caseins into peptides and aminoacids. *L. bulgaricus* demonstrates a much stronger proteolytic
activity than does \textit{S. thermophilus}. Stimulation of the growth of \textit{S. thermophilus} is based on the liberation of free amino acids from milk (Wood, 1985).

In recent years, the consumer has been increasingly confronted with so-called functional food products, which are claimed to promote health and well being. Probiotic products are at the centre of such foods. Probiotics are defined as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal balance (Fuller, 1991). Developing probiotic food is a key research and development area for future functional food markets. The most common probiotic bacteria presently used in food products belong to the genera \textit{Bifidobacterium} and \textit{Lactobacillus} (Fuller, 1991). The major conventional criteria that can be used for the selection of microbial strain to be used as probiotics include the following properties (Fuller, 1992; Reuter, 2001):

- Biosafety
- Choice of the origin of the strain
- Resistance to in vivo/vitro conditions
- Antimicrobial activity/antagonisms to pathogens
- Viability/survival and resistance during processing (e.g. heat tolerance or storage)

Based on these criteria and specific properties that are desired, it should be possible to select the probiotic strain that can be helpful therapeutically and nutritionally.

\textit{LAB} are fastidious micro-organisms and their growth is often restricted in milk because of its paucity in essential nutrients. Thus the success of milk fermentation relies most often upon the synergy between \textit{S. thermophilus} and \textit{Lb. bulgaricus}. Because both bacteria are able to grow alone in milk, this indirect positive interaction is called proto-cooperation (Fredrickson, 1977). This positive relationship often has a beneficial effect on bacterial growth and on the
production of lactic acid and aroma compounds. Some of the effectors of this association have been identified and result from the metabolic activities of the two bacterial partners. Indeed, *S. thermophilus* produces pyruvic acid, formic acid and CO$_2$ (Tamime and Robinson, 1999; Zourari *et al.*, 1992) which stimulate the growth of *Lb. bulgaricus*. In turn, *Lb. bulgaricus* produces peptides and amino acids that stimulate the growth of *S. thermophilus* (Bautista *et al.* 1966; Radke-Mitchell and Sandine, 1984), because *S. thermophilus* is only weakly proteolytic when compared with *Lactobacillus*. On the other hand, although the effect of associating these LAB species is often positive, it can also be neutral or detrimental depending on the bacterial strains employed, the type of milk, the method used to heat the milk and the temperature of milk fermentation.

*L. acidophilus* is a member of lactic acid bacteria (LAB) that are used in the manufacture of fermented milk products. *L. acidophilus* is widely used as a probiotic organism in yoghurt, infant formula, dried dietary supplements and many dairy products. It constitutes an important part of the human intestinal bacteria (Gill and Guaner, 2004). Health benefits associated with fermented milk products can be provided by the bacterial starter culture or by dietary adjuncts added after the product is fermented. Improved digestion of lactose, aid in control of serum cholesterol levels, antagonistic action toward pathogens and control of certain types of intestinal cancer are the primary health benefits associated with fermented products containing *Lactobacillus acidophilus*. *Lactobacillus acidophilus* can be added easily to fermented products such as buttermilk of yoghurt. However, for most of the potential health benefits to the consumer, it is necessary that the cell be viable when consumed. Typically, these products are consumed within 2 to 3 weeks after production. Therefore, the cells should survive for at least 3 weeks to provide maximum health benefits to the consumers. The success of the addition of probiotic bacteria in milk products is very dependent on the species and strains used, metabolic interactions with lactic acid starters, fermentation conditions, pH of the product, presence of oxygen, and storage temperature (Ghodussi and Robinson, 1996; Nighswonger *et al.*, 1996; Shah *et al.*, 1995).
**Lactic acid Bacteria and Bacteriocin**

One of the concerns in food industry is the contamination by pathogens, which is a frequent cause of food borne diseases. Over the past decade, recurrent outbreaks of diarrhoea, combined with natural resistance of causative agents, contributed to it’s status as hazard.

The problem of selection of resistant bacteria to antibiotics (Parada, 1980; Chopra et al., 1997; Rao 1998 and Kapil, 2005) and the increasing demand for safe foods, with less chemical additives, has increased the interest in replacing these compounds by natural products, which do not injure the host or the environment.

Biotechnology in the food processing sector targets the selection, production and improvement of useful micro-organisms and their products, as well as their technical application in food quality (Parada et al., 2007).

Antagonistic properties of Lactic acid bacteria (LAB) allied to their safe history of use in traditional food fermented products make them very attractive to be used as biopreservatives (Parada, 1984; Caplica & Fitzgerald, 1999). LAB are usually known as Generally Recognised As Safe (GRAS), used as natural competitive microbiota or as specific starter cultures under controlled conditions (Cintas et al., 2001). Some of these bacteria produce antagonistic substances called bacteriocins, which in small amounts are very active against pathogens (Klaenhammer et al., 1994 & Moreno et al., 2000).

A great number of lactic acid bacteria and other grampositive bacteria produce peptides that display antimicrobial activity (Jack et al., 1995). Because of their wide spectrum of activity, one class of these antimicrobial peptides, the lantibiotics, has received considerable attention in the last few years (De vos et al., 1995). Lantibiotics are posttranslationally modified peptides, containing dehydrated serine and threonine residues and thioether bridges. The most prominent lantibiotic is nisin, which is produced by several strains of *Lactococcus lactis* and is widely used as a food preservative (Delves-Broughton, 1990).
Bacteriocins are an interesting group of biomolecules with antimicrobial properties that may represent a good alternative than antibiotics (Jack et al., 1995). Bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides (De Vuyst & Vandamme, 1994). In general these substances are cationic peptides that display hydrophobic or amphiphillic properties & the bacterial membrane is in most cases the target for their activity (Ennahar et al., 2000). A large number of bacteriocins have been isolated and characterized from LAB & some have acquired a status of potential antimicrobial agents because of their potential as food preservatives and antagonistic effect against important pathogens. The important ones are Nisin, Diplococcin, Acidophilin, Bulgaricin, Helveticin, Lactacin and Plantaricins (Nettles and Barefoot, 1993).

Nisin is a naturally occurring antimicrobial peptide and was discovered in 1928 (Hurst, 1967; Montville & Chen, 1998). The bacteriocin nisin has antimicrobial activity against a wide variety of pathogens and undesirable food borne bacteria such as Listeria, Clostridium, Bacillus and Staphylococcus species (Hurst, 1981). Nisin inhibits spore germination and the growth of a variety of Gram positive bacteria and Gram negative bacteria (with outer membrane first destabilized by EDTA) (Gill & Holley, 2000) and for this reason it is widely used as a natural preservative (Penna et al., 2002).

Nisin was first commercially introduced as a food preservative in UK approximately 30 years ago. It’s first established use was as preservative in processed cheese products and since then numerous other applications in foods and beverages have been identified. It is currently recognized as a safe food preservative in approximately 50 countries. Nisin is used as a preservative in processed cheese, various pasteurized dairy products and canned vegetables. Most recent applications of nisin include its use as a preservative in high moisture, hot baked flour products and pasteurized liquid egg. Uses of nisin to control spoilage lactic acid bacteria have been identified in beer, wine, alcohol production and low pH foods such as salad dressings (Delves et al., 1996).
Random amplification of polymorphic DNA (RAPD) is a PCR method that incorporates a single arbitrarily designed oligonucleotide primer in the amplification reaction to generate DNA fragment polymorphisms (Welsh and McClelland, 1990; Williams et al., 1990). The DNA fragment polymorphisms, or RAPD profiles, have been used to distinguish between closely related bacterial species (Cocconcelli et al., 1995; Permaul et al., 1996). The RAPD technique is simple and quick, and thus it would serve as a convenient alternative protocol to complement the methods currently used to distinguish Lactobacilli strains.

Overall, the broad objectives of the present study were:

- Detection of food borne pathogens by PCR from different food samples available in the market and processed in the laboratory
- Comparison of efficacy of PCR methods with conventional plating methods for efficient detection of food pathogens
- Molecular detection of nisin (a bacteriocin) producing gene in probiotic bacteria
- Use of probiotic lactic acid bacteria in foods and their molecular examination

The specific objectives of the study were:

1. Comparison of different Phenol-chloroform based methods for DNA isolation from E. coli, S. aureus and S. typhi
2. Molecular and bacteriological examination of milk from different milch animals with special reference to coliforms
3. Molecular and bacteriological detection of E. coli, S. aureus and S. typhi from milk samples
4. A comparison of methods for the detection of E. coli O157:H7 from artificially contaminated dairy products by PCR
5. Assessment of viability of probiotic bacteria and competitive growth of L. acidophilus in yoghurt during refrigerated storage using microbiological and molecular methods
6. Effect of pure nisin – a bacteriocin on the growth and survival of selected food pathogens
7. Bacteriocin nisin production by free and immobilized Lactococcus lactis
8. Screening for NisA promoter gene and RAPD profiling of potentially probiotic lactic acid bacteria
9. Molecular detection of food pathogens from retailed fast food samples by PCR
10. Molecular Detection of E. coli, S. aureus and S. typhi from local fast food and juice samples
11. Effect of enrichment media for PCR detection of Listeria monocytogenes and S. aureus from vegetable gravy
REVIEW OF LITERATURE

With the objective of “Detection of foodborne pathogens and use of molecular methods in foods” the review of literature is expressed below, discussed under following sub-heads:

I. Food pathogens *E. coli*, *S. aureus*, *S. typhi* and *L. monocytogenes*

II. Probiotics and Lactic acid bacteria as probiotics

III. Bacteriocins

IV. Nisin (Structure, Organization of gene clusters, Mode of action & applications)

V. Immobilization technique

VI. RAPD-PCR

I. **Food pathogens *E. coli*, *S. aureus*, *S. typhi* and *L. monocytogenes***

Foodborne diseases remain a persistent challenge to the public health. Foodborne disease outbreaks, which cause approximately 76 million illness and 5,000 deaths every year (Mead *et al.*, 1999), have more than doubled in the United States since 1987 (Tauxe *et al.*, 1997). These outbreaks also cause a serious economic loss for both the consumer and the industry. Most reported foodborne diseases of contaminated produce were caused by pathogens such as *E. coli*, *Salmonella* & *Shigella*. Among these outbreaks, *E. coli* caused about every year, resulting in syndromes like hemorrhagic colitis and non-bloody diarrhea (Buchnan *et al.*, 1998). Members of genus *salmonella* are responsible for a large number of food poisoning approximately 1.4 million according to estimates (Mead *et al.*, 1999). Virulent *shigella* species, including *Shigella dysenteriae*, *Shigella boydii*, *Shigella sonnei*, can cause severe illness like hemolytic uremic syndrome with a very low intention dose (10 CFU) (Lindqvist, 1999).
(A) ESCHERICHIA COLI O157:H7

*E. coli* O157:H7 was first recognized as a pathogen in 1982 following two outbreaks in Oregon and Michigan (Jay, 2000). Not all *Escherichia coli* are pathogenic and these organisms are commonly found in the intestinal tracts as part of the normal facultative anaerobic microflora of humans and warm-blooded animals. *Escherichia coli* strains that can cause diarrheal illness are assigned to six specific virulence groups based on virulence properties, mechanism of pathogenicity, clinical syndromes, and distinct O:H serogroups.

These categories include: enteropathogenic *E. coli* strains (EPEC), enterotoxigenic *E. coli* strains (ETEC), enteroinvasive *E. coli* strains (EIEC), diffuse adhering *E. coli* strains (DAEC), enteroaggregative *E. coli* strains (EAggEC), and enterohemorrhagic *E. coli* strains (EHEC) (Doyle *et al*., 1997). In the present study, detection of *E. coli* (EHEC) was mainly carried out.

**CHARACTERISTICS OF EHEC**

*E. coli* O157:H7 was first recognized as a pathogen in 1982 following two outbreaks in Oregon and Michigan. The isolates were clinically different from EPEC and thus a new group of *E. coli*, EHEC, was formed. Distinguishing characteristics of *E. coli* O157:H7 from other groups of *E. coli* include its inability to ferment sorbitol, produce β-glucuronidase, and grow at or above 44.5°C. Most *E. coli* (93 %) of human origin can ferment sorbitol within 24 h, but *E. coli* O157:H7 does not. Most strains (93 %) also have the enzyme β-glucuronidase, which is the basis for detection of *E. coli* in a rapid fluorogenic assay. The assay utilizes 4-methylumbelliferyl β-D-glucuronide (MUG) as an indicator which is hydrolyzed by the enzyme β-glucuronidase to form a fluorogenic product (Padhye and Doyle, 1992). While these traits do hold true for most 0157 strains, sorbitol-fermenting, β-glucuronidase producing *E. coli* O157 have been isolated and are estimated to account for 50 % of *E. coli* O157 infections in Germany. These unusual variants are thought to be a distinct clone of *E. coli* O157 (Law, 2000). Gunzer *et al.* (1992) isolated sorbitol fermenting, β-glucuronidase positive O157 strains in 17 of 44 patients with diarrhea (18) and HUS (26) (Gunzer *et al*., 1992).
Non O157 EHEC is also sorbitol positive and β-glucuronidase positive; making specific identification and differentiation of these EHEC strains more difficult.

E. coli O157:H7 growth studies in trypticase soy broth indicate that the organism grows well between 30-42°C, having generation times between 0.49 h and 0.64 h at 37°C and 42°C, respectively (Padhye and Doyle, 1992). Poor growth of the organism has been observed at 44-45°C, and no growth occurred within 48 h at 10 or 45.5°C. Procedures used to detect fecal coliforms in food use incubation temperatures of 44-45°C; thus E. coli O157:H7 would probably not be detected during normal screening for fecal coliforms using such procedures (Padhye and Doyle, 1992).

Outbreaks
A series of outbreaks involving E. coli O157:H7 have occurred since its identification as a pathogen in 1982. Between 1982 and 2002, a total of 350 outbreaks have been reported in 49 states resulting in 1,493 (17.4 %) hospitalizations, 354 (4.1 %) cases of HUS, and 40 (0.5%) deaths (Rangel et al., 2005). These outbreaks have increased from about 2 per year from 1982 to 1992 to about 17 in 1993 and 30 in 1994. From 1994 to 1997 the average number of outbreaks per year was 31 and in 1998 there were 42 confirmed outbreaks (CDC, 2000). The numbers seem to level off in 1999 with 38 reported outbreaks, but five (13 %) of these outbreaks involved two or more states compared to only two (4 %) of those reported in 1998. Then at the turn of the century, in 2000, the number peaked at 46 outbreaks (Rangel et al., 2005). This increase in the number of reported outbreaks and multistate outbreaks may be due to public health departments’ increased use of pulsed-field gel electrophoresis (PFGE) for subtyping strains. Widely dispersed cases and small clusters within states may be linked by using PFGE. These are cases that in the past may have not been known to be related, perhaps providing further explanation as to the increased reporting of outbreaks (CDC, 2000). Unfortunately some cases go unreported since infected persons having mild or no symptoms and those with non-bloody
diarrhea are not likely to seek medical attention (Doyle et al., 1997). According to the Center for Disease Control and Prevention; *E. coli* O157:H7 causes an estimated 73,500 cases of infection, 2000 hospitalizations, and 60 deaths in the U.S. each year (CDC, 2001).

The majority of outbreaks occur during the warmer summer months. Within the United States, from 1982 to 2002, about 89 % of outbreaks occurred from May to November (Rangel et al., 2005). In 1997 almost half (45.7 %) of the 1,167 recorded *E. coli* O157:H7 cases occurred in the months of July, August, and September (Jay, 2000). Possible reasons for increased summer illness include: (i) increased incidence of the pathogen in cattle or livestock during the summer; (ii) increased human exposure to ground beef or other contaminated foods during the “cook-out” months; and/or (iii) increased improper handling or undercooking of products like ground beef during warm summer months than other months (Doyle et al., 2001). Of the 350 reported outbreaks (1982 - 2002), the majority of transmission routes were as follows: 183 (52 %) foodborne, 74 (21 %) unknown, 50 (14 %) person-to-person, 21 (6 %) recreational water, 11 (3 %) animal contact, 10 (3 %) drinking water, and 1 (0.3 %) laboratory related transmission route. Of the foodborne outbreaks, ground beef was the most common vehicle at 41 %, followed by 23 % unknown, 21 % produce, 6 % other beef, 5 % other foods, and 4 % in dairy products (Rangel et al., 2005). Most foods implicated have been associated with raw or undercooked foods of bovine origin such as raw milk or ground beef. Other foods associated with *E. coli* O157:H7 infections are vegetables, apple cider, cantaloupe, mayonnaise-containing salad dressing, and salami. Outbreaks involving alfalfa sprouts, fruit salads, and coleslaw have also occurred over the years. In recent years contamination of raw fruits and vegetables has become an important issue due to the lack of further processing of these foods.

The first outbreak of *E. coli* O157:H7 infection occurred in Oregon in 1982 rendering 26 individuals ill and 19 hospitalized. Illness for all patients was accompanied with bloody diarrhea and severe abdominal pain. The outbreak was associated with eating undercooked hamburgers at a fast-food restaurant chain.
Another outbreak ensued three months later with the same fast-food restaurant chain. *E. coli* O157:H7 was again implicated as the causative agent and was isolated from patients and a frozen ground beef patty (Doyle *et al.*, 2001). Contamination of municipal water by *E. coli* O157:H7 occurred in December and January of 1990 in Cabool, of the 243 people infected, 86 had bloody diarrhea, 32 were hospitalized, and 4 died (Swerdlow *et al.*, 1992). Another *E. coli* O157:H7 outbreak was associated with a lakeside park near Portland, Ore in the summer of 1991. Twenty-one cases were identified from 19 households. All patients were children and seven were hospitalized. Swallowing lake water was associated with illness and those ill tended to spend more time in the water. Many of the bathers were toddlers and not yet toilet trained (Doyle *et al.*, 2001).

In southeastern Massachusetts in the fall of 1991, an outbreak involving consumption of un-pasteurized apple cider occurred. Twenty-three cases of *E. coli* O157:H7 infection from a total of 13 families was reported and six patients were hospitalized. Investigation at the site where the apple cider was processed indicated that the apples were pressed without washing, the cider was not pasteurized, and no preservatives were used. Before this outbreak, apple cider was considered safe for consumption due to its high acidity and low pH (< 4.0) (Doyle *et al.*, 2001).

The largest multistate outbreak of *E. coli* O157:H7 occurred in four western U.S. states in early 1993. The age of patients ranged from 4 months to 88 years and 90% of patients had eaten at a single fast-food restaurant chain. One hundred seventy-eight were hospitalized, 56 developed HUS, and 4 children died. The cause of the outbreak was due to consumption of hamburgers that were insufficiently cooked by the fast-food restaurant chain.

Investigations at the restaurant revealed that 10 of 16 hamburgers cooked had internal temperatures of below 60°C, which was much lower than the minimum temperature of 68.3°C allowed by the state of Washington (Doyle *et al.*, 2001). The consumption of dry fermented salami was implicated in a multistate outbreak in 1994. Studies later confirmed that *E. coli* O157:H7 can survive the fermentation, drying, and storage of fermented sausage (Doyle *et al.*, 2001).
In Japan, a large outbreak involving 10,000 cases of *E. coli* O157:H7 was associated with the consumption of radish sprouts from May to August 1996. Four separate clusters were identified with the largest involving 6,259 primary school children in Sakai City, Osaka Prefecture, in which 92 patients developed HUS (WHO, 1996; Watanabe *et al*., 1999).

In 1997, the consumption of alfalfa sprouts was directly related to the illnesses of 60 persons caused by *E. coli* O157:H7. Of those that were ill, 44 had bloody diarrhea and 25 were hospitalized. Two had hemolytic uremic syndrome (HUS) and one developed thrombotic thrombocytopenic purpura. In a case-control study, eighteen of 30 case-patients had eaten alfalfa sprouts within 7 days of the onset of illness (CDC, 1997).

In the fall of 2000, 51 people became ill from *E. coli* O157:H7 after visiting a petting farm in Pennsylvania. Patients ranged in age from 1 to 54 (median 4) and symptoms included bloody diarrhea, fever, and vomiting. Sixteen patients, all under the age of ten, were hospitalized. Eight patients developed HUS and one patient experienced renal failure and required renal transplantation. Increased risk of illness was associated with contact with calves and their environment, while hand-washing was protective (Crump *et al*., 2002).

In the summer months of 2002, a multistate outbreak linked by PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance, involved 28 cases in Colorado and six other states. Seven of the patients were hospitalized and five developed HUS. Ground beef was implicated in the outbreak and a nationwide recall of 18.6 million pounds of ground beef was issued (CDC, 2002).

**Growth and Survival of *E. coli* O157:H7 in Foods**

Food model systems using nutrient rich broth have been used to characterize the growth and survival of *E. coli* O157:H7 in foods. Kauppi *et al*. (1996) tested STEC for their ability to grow in different media at low temperatures. The average generation time was higher in brain heart infusion broth (BHI) (13 h) compared to trypticase soy broth (TSB) (10 h) and milk (11 h) at 9.5°C. At temperatures near
rerefrigeration (6.5˚C), the STEC strains did not grow in BHI or TSB, but were able to
grow in milk with a generation time of about 83 h (Kauppi et al., 1996). Most
studies have found that EHEC remain viable in foods at 5°C and can grow at
temperatures of 8˚C or above, verifying the danger associated with temperature
abused foods (Buchanan and Bagi, 1994; Rajkowski and Marmer, 1995; Palumbo et al., 1995; Palumbo et al., 1997). Palumbo et al. (1997) investigated
the effects of low temperature and background microflora on the growth and
shiga toxin production of EHEC in beef and milk. Growth occurred in strains held
at 8° C when no or low background microflora were present. Strains remained
viable at 5° C and in the presence of high background microflora. Shiga toxin
production was detected in low amounts that did not exceed the levels obtained
from broth cultures at 37° C or 5˚C (Palumbo et al., 1997).
Decreased toxin production has been observed in some food substrates. *E. coli*
O157:H7 grown at 37˚C in milk did not produce as much toxin as the same strain
grown in BHI broth under the same conditions (Palumbo et al., 1997).
*E. coli* O157:H7 has the ability to grow over a broad range of pH (4.5 to 9) values
(Zilberstein et al., 1984; Hersh et al., 1996). Since many food environments are
either acidified or have an inherently low pH, the ability of EHEC to survive at low
pH presents a significant food safety threat. Lin et al. (1995) reported that *E. coli*
had a minimum growth pH of 4.4, which was slightly higher than that of
*Salmonella typhimium* (pH 4.0). Previous research has indicated that bacterial
exposure to a sublethal stress can result in subsequent increased resistance to
that particular stress or increased resistance to other stresses (Jenkins et al.,
1988, 1990; Farber and Pagotto, 1992; Buncic and Avery, 1998; Leenanon and
Drake, 2001). Acid adaptation can increase the heat and freeze-thaw resistance
of *E. coli* O157:H7 and nonpathogenic *E. coli*. Cold stress decreased heat
resistance of *E. coli* O157:H7 and nonpathogenic *E. coli*, but allowed for greater
survival during freezing and thawing (Leenanon and Drake, 2001).
Several studies have investigated the growth of *E. coli* O157:H7 in ground beef
(Palumbo et al., 1997; Ajjarapu and Shelef, 1999; Ansay et al., 1999;
Uyttendaele et al., 2001; Tamplin, 2002). These studies demonstrated that *E. coli*
O157:H7 can survive freezing and refrigeration temperatures (5°C) and is able to grow when placed under abusive temperatures (≥ 8°C). In ground beef, the exponential growth rate (EGR) and maximum population density (MPD) of *E. coli* O157:H7 increased with decreasing fat content (Tamplin, 2002). Two native ground beef isolates (unspeciated) were added to sterilized ground beef along with *E. coli* O157:H7; there was a decrease in the EGR and MPD of *E. coli* O157:H7 as the competitive population increased (Tamplin, 2002).

The ability of *E. coli* O157:H7 to grow in fermented products such as sausage (Pond *et al.*, 2001; Uyttendaele *et al.*, 2001), Lebanon bologna (Chikthimmah *et al.*, 2001), cheese (Saad *et al.*, 2001), and buttermilk (McIngvale *et al.*, 2000) has been studied. The sausage fermentation process was able to produce a mean log reduction within a range of 0.3 to 1.33, while the drying stage reduced the population by 1.37 to 2.70 log10. Since Lebanon bologna is not processed above 48.8°C, Chikthimmah *et al.* (2001) investigated effects of salts (NaCl and NaNO₂) on the destruction of *E. coli* O157:H7 during processing. High levels of NaCl (5%) inhibited lactic acid bacteria, resulting in a higher pH (5.0) and better survival of *E. coli* O157:H7. The NaCl concentration had no direct effect on *E. coli* O157:H7, but inactivation of the organism increased significantly at all NaCl concentrations when the pH was lowered from 4.7 to 4.3 and the product heated from 37.7°C to 46.1°C in 5.5 h. (Chikthimmah *et al.*, 2001).

Minas cheese, a fresh Brazilian cheese, provides a suitable environment for pathogens, such as *E. coli* O157:H7. Saad *et al.* (2001) found that cheeses without inoculated lactic acid cultures allowed for a 2 log10 increase in *E. coli* O157:H7 population which was maintained over the storage period. Minas cheese inoculated with lactic acid bacteria controlled the growth of *E. coli* O157:H7 to only 0.5 log10 over a 24 hour period, and this level remained unchanged thereafter (Saad *et al.*, 2001). Acidic condiments have also been investigated (Tsai and Ingham, 1997). *E. coli* O157:H7 did not survive in mustard or sweet pickle relish for more than one hour when stored at 5 or 23°C. However,
\textit{E. coli} O157:H7 was recovered for up to 7 days in ketchup stored at 5°C (Tsai and Ingham, 1997).

**Detection**

Traditional cultural methods for the detection of EHEC in foods require selective enrichment, selective and differential plating, followed by phenotypic identification using biochemical tests and subsequent serotype identification. The fundamental tests used for isolation and detection are found in the \textit{Bacteriological Analytical Manual} (BAM). Within BAM, three selective agars are identified: sorbitol MacConkey agar (SMAC), Hemorrhagic colitis agar (HC), and tellurite-cefixime-sorbitol MacConkey agar (TC SMAC) (Bacteriological Analytical Manual, 1998).

The use of SMAC involves incubation at 35°C for 18 h. Most \textit{E. coli} O157:H7 strains are sorbitol-negative and will form pale colonies as opposed to most other \textit{E. coli} strains and enterics, which are sorbitol-positive and appear bright pink. Confirmation of sorbitol-negative colonies can be tested for \textit{-glucuronidase} (GUD) activity (GUD cleaves the substrate 4- methylumbelliferyl -D-glucuronide (MUG)) by spotting cultures on HC agar with MUG, and then select MUG-negative colonies. For identification of \textit{E. coli} O157:H7, for sorbitol-negative, MUG-negative colonies are tested for agglutination with O157 and H7 antisera. It would be prudent to mention that the presence of high levels of coliforms may mask the presence of O157:H7 when using SMAC. (Bacteriological Analytical Manual, 1998). False positives on SMAC may result when \textit{Escherichia hermanii} and some other enterics are present, or in the presence of \textit{Citrobacter freundii}, which may agglutinate O157 antiserum. Another method, selective plating for the detection for EHEC uses HC supplemented with MUG (note: MUG can be substituted with the colorimetric substrate BCIG). When low numbers of \textit{E. coli} O157:H7 are suspected, the food may be enriched in modified trypticase soy broth containing novobiocin before being plated on selective media. Another procedure uses Tellurite-Cefixime-SMAC as the selective plating media. Recovery of \textit{E. coli} O157:H7 from foods was improved using TCSMAC compared to using HC agar (Hitchins \textit{et al.}, 1995). There exists a small population of non O157 isolates that do not ferment sorbitol, and therefore the incorporation of the
MUG assay should be included in the analysis to distinguish *E. coli* O157:H7 from these atypical *E. coli* isolates. Selective enrichment followed by immunomagnetic separation may also be performed using anti-O157 beads. This method may result in improved assay sensitivity due to pathogen preconcentration after enrichment and prior to selective plating on agar such as TC SMAC (Hitchins *et al.*, 1995).

When presumptive colonies are isolated after enrichment and selective plating, biochemical confirmation is warranted. In particular, EHEC strains will be indole-positive by the spot test. The API or VITEK assays are also useful tests to identify isolates as *E. coli* from TSA-YE slants. Subsequent to biochemical confirmation, latex agglutination using the Prolex *E. coli* O157 Latex Test Reagent kit or RIM *E. coli* O157:H7 Latex Test, an approach for serological identification. A rapid alternative to serological typing involves use of the serotype-specific DNA probe, PF-27. This 18-base oligonucleotide probe targets a unique region of the *uidA* gene in *E. coli* O157:H7. A study of several enteric species on and other STEC (in total 280 bacterial isolates), demonstrated that PF-27 was highly specific only for the O157:H7 serotype (Hitchins *et al.*, 1995).

Only if both the latex and indole test are positive, should the isolates be tested for the presence of *stx1* and *stx2* genes by colony hybridization or polymerase chain reaction. Detection of toxins produced by *E. coli* O157:H7 can be performed using tissue culture assays. (Hitchins *et al.*, 1995).

The method used for isolation of *E. coli* O157:H7 from raw and ready-to-eat meat products is described in the USDA/FSIS Microbiology Laboratory Guidebook. The format involves enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium. Raw ground beef is randomly collected from the package in 65 g sub-samples for a composite sample consisting of 325 g. Enrichment is done in modified enrichment broth with novobiocin for 20 to 24 h at 35°C. Positive, negative, and uninoculated controls are prepared for each group of samples. An ELISA-based *E. coli* O157:H7/NM
screening test can be done after enrichment, in which case positive samples are considered presumptive and are subject to further confirmation. Negative samples, however, are confirmed after such screening (Cray et al., 1998).

There has been much work in recent years to identify optimal selective approach for presumptive identification of *E. coli* O157:H7, particularly with respect to the recovery of sublethally injured cells. Szabo et al. (1986) evaluated the use of hemorrhagic colitis (HC) medium for direct overnight isolation of EHEC strains. Recovery of these strains from artificially contaminated ground beef was greater than 90%. *E. coli* O157:H7 colonies grown on HC medium are blue, do not fluoresce under UV light, and are red following exposure to indole reagent (Szabo et al., 1986). Silk and Donnelly (1997) reported better recovery of injured *E. coli* O157:H7 from apple cider by surface plating on trypticase soy agar (TSA) incubated 2 h at 25° C followed by overlaying with violet red bile agar (VRBA) or sorbitol MacConkey agar (SMAC), compared to the use of other selective or differential media. A modified resuscitation method was also employed using the above method preceded by preincubation of 250 μl trypticase soy broth (TSB) with 1 μl sample for 2 h at 25° C prior to SMAC plating. Despite better recovery by incorporating a resuscitation step, detection was still less efficient than when compared to that obtained using nonselective TSA (Silk and Donnelly, 1997).

Haro-Kudo et al. (1999) determined the best media for use in enrichment procedures for the isolation of *E. coli* O157:H7. The most effective conditions were incubation at 42° C for 18 h in modified TSB (TSB supplemented with bile salts 3, dipotassium phosphate and novobiocin) or modified EC broth with novobiocin for ground beef and radish sprouts (Haro-Kudo et al., 1999). The use of EHEC agar, a variation of sheep blood agar (washed sheep red blood cells added at 5% (v/v) to trypticase soy agar (TSA) supplemented with 10 mmol/L (final concentration) calcium chloride), for isolation of STEC from foods was assessed by Hudson et al., 2000.

Many of the rapid kits available for the detection of *E. coli* O157:H7 require an enrichment step to boost the target cells to detectable levels. Blais et al. (1997a) studied enrichment of *Escherichia coli* O157:H7 in meat using modified *E. coli*
(EC) broth with novobiocin (mEC + n) and the combined effects of agitation and incubation temperature (37°C or 42° C). Results indicated that 42° C without shaking was most effective in suppressing the growth of competitive ground beef microflora while allowing ample growth of E. coli O157:H7 (Blais et al., 1997).

**Staphylococcus aureus**

*S. aureus* is a bacterium belonging to the genus *Staphylococcus*. Staphylococci are toxin producing, Gram positive, catalase positive cocci which grow aerobically but which are capable of facultative anaerobic metabolism. Staphylococcal food borne intoxication is a common cause of bacterial food poisoning.

Staphylococcal food poisoning occurs after the ingestion of food contaminated with the staphylococcal enterotoxin(s). Staphylococcal food poisoning is mainly caused by human strains of *S. aureus* producing SE(A) and/or SE(D), with the majority of strains producing SE(A) alone (Bacteriological Analytical Manual, 1998).

The amount of toxin necessary to cause illness depends on the susceptibility of the person; however, epidemiological studies have shown that only 1μg of SE can cause food poisoning. It should be noted that to produce this amount of SE, an enteroxigenic strain needs to grow to levels of 105 to 106 cells per gram or ml.

Onset of food poisoning symptoms usually occurs between 1 and 7 hours after the ingestion of food containing the SE. Symptoms include nausea, vomiting, abdominal cramps and diarrhoea. In severe cases, collapse may occur. Recovery is usually rapid, i.e. within 2 days.

Food-borne poisoning by staphylococcal enterotoxins (SEs) is an important hazard. SEs, the emetic toxins produced by Staphylococcus aureus, have been divided into five serological types (SEA, SEB, SEC, SED and SEE) (Bergdoll, 1983). In recent years, new types of SEs (from SEG to SE1U) have been reported (Jarraud et al., 2001; Munson et al., 1998; Orwin et al., 2001; Ren et al.,
1994; Su & Wong, 1995; Zhang et al., 1998; Sergeev et al., 2004; Omoe et al., 2003; Lina et al., 2004), but the emetic activity of several of these toxins has not been confirmed. However, various studies have shown that the newly described seg and sei genes are commonly found in food-borne and clinical isolates of S. aureus (Jarraud et al., 2001; McLauchlin et al., 2000; Omoe et al., 2002; Rosec & Giraud, 2002; Becker et al., 2003), suggesting that they have roles as pathogenic factors in staphylococcal food poisoning. Thus, the establishment of a method to detect eight enterotoxin genes (sea, seb, sec, sed, see, seg, seh and sei) is vital for the analysis of staphylococcal food poisoning. Recently, Letertre et al. (2003a) reported the detection of SE-encoding genes based on 59 nuclease multiplex PCR. However, this method cannot detect SE-encoding genes directly from samples because it requires a culturing process. Direct detection of pathogenic genes by PCR is hampered by inhibitors contained in the samples (Rossen et al., 1992; Wilson, 1997).

Among the food samples, milk contains high concentrations of proteins and fats, which are major inhibitors of the PCR assay. In 2000, a large-scale and widespread outbreak of staphylococcal food poisoning caused by the ingestion of dairy products, including lowfat milk and yogurt beverages, occurred in Japan (Asao et al., 2003) affecting a total of 13,420 persons. This incident eloquently indicated the need for a rapid and direct detection method for routine laboratory analysis.

The presence of *staphylococcus aureus* in milk is of major concern in dairy industry. This pathogenic micro organisms is a leading cause of inflammatory bacterial infections and is associated with major economic loss – heat stable enterotoxins produced by *s.aureus* can cause food poisoning. Austrian National Regulations derived from European Regulations apply three levels of classifications for the presence of *S. aureus* in milk of bovine, caprine and bovine milk intended for processing. Without prior heat treatment, the m-vaule is 500 colony – forming units / ml value is 2000. Traditional microbiological method such as plate count or most probable number (MPN) for quantification of *S. aureus* in
milk are time-consuming and require from two days up to six days for quantification & detection.

As a faster alternative, PCR has been used for DNA based direct detection of *S. aureus* in milk. Real-time PCR is a new PCR technique and enables accurate genome based detection and quantification of microorganism.

**Salmonella**

*Salmonella* is one of the leading causes of foodborne illness throughout the world. Estimates indicate that *Salmonella* accounts for approximately 1.4 million illnesses, 17,000 hospitalizations and 600 deaths per year in the U.S (Mead *et al.*, 1999), resulting in a cost of around 4.0 billion dollars annually in medical expenses and productivity losses (Todd, 1989).

A few large-scale salmonellosis outbreaks were recorded in the U.S. during the last century. One occurred in 1974 on the Navajo Indian reservation when approximately 11,000 individuals attended a barbecue. The infections, caused by *S. Newport*-contaminated and improperly handled potato salad, resulted in 3,400 persons becoming ill (Horwittz *et al.*, 1977). In 1985, a massive outbreak of salmonellosis occurred in six states, resulting in 16,284 confirmed cases and subsequently seven deaths. So far, the largest outbreak of foodborne salmonellosis in the U.S. happened in 1994. This outbreak involved at least 41 states and more than 224,000 persons. Zhao *et al.* (2001) investigated the presence of multiplex foodborne pathogens in retail chicken, turkey, pork and beef from the greater Washington D.C. area between June 1999 and July 2000 (Zhao *et al.*, 2001). In a total of 825 samples, *Salmonella* was isolated from 1.9% of 210 beef samples. A more comprehensive study, carried out by the USDA Food Safety and Inspection Service (FSIS) showed that *Salmonella* outbreaks involved 98,204 raw meat and poultry products in the U.S for the period 1998 to 2000 (Rose *et al.*, 2002). This study detected *Salmonella* on 29.2% of 3,481 samples, 15.7% of 735 ground chicken samples, and 10.7% of 24,452 broiler samples.
Due to the contamination of *Salmonella* in foods, a number of recalls have been issued by the food industry. In March 2002, Double D Meat Company Inc. in Louisiana recalled approximately 14,100 pounds of fresh, ready-to-eat pork sausage that may have been contaminated with *Salmonella* (USDA/FSIS, 2002). More recently, a notable recall occurred in October, 2003. M. D. Chavez/Old Santa Fe Trail in New Mexico firm voluntarily recalled approximately 22,000 pounds of beef jerky because of *Salmonella* contamination (USDA FSIS, 2003).

**Characteristics of *Salmonella***

*Salmonella* is a rod-shaped, facultative anaerobic, non-spore forming, Gram-negative bacterium. As a member of the family Enterobacteriaceae, it was named after D.E. Salmon, a bacteriologist who had identified *Salmonella choleraesuis* in 1885 (Tauxe, 1991). The genus includes 2449 serovars under two species, *Salmonella enterica* and *Salmonella bongori*. Based on DNA-DNA hybridization and multilocus enzyme electrophoretic characterization, *S. enterica* is further divided into the six subspecies, *enterica, salamae, arizonae, diarizonae* and *indica*. The serotypes commonly isolated from humans, agricultural products and foods, belong to the subspecies *enterica* (D’ Aoust, 2001). *Salmonella* can grow in temperatures from 2°C to 47°C (with the optimum being from 25°C to 43°C). Most of them can ferment glucose and certain other monosaccharides but generally cannot ferment lactose, sucrose or salicin. It can grow in the low pH environment of the human stomach which may explain the high infectious dose of this pathogen. However, *Salmonella* can be killed under high salt concentrations (Jay, 2000).

The incidence of antibiotic resistance among *Salmonella* strains continues to increase, leading to the emergence of highly virulent serotypes, such as *S. Typhimurium DT104* in Europe and North America. *S. Typhimurium DT 104* is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, and can be tolerant against other medically important antibiotics. This has raised a great public health concern as these antibiotics are usually
utilized as treatments for suspected extraintestinal *Salmonella* infections and serious gastroenteritis in humans (IFT, 2000).

**Disease and virulence**
The clinical syndromes linked to human salmonellosis include enteric fever, enterocolitis, and invasive systematic disease. Enteric fever is caused by *Salmonella* Typhi and *Salmonella* Paratyphi A, B or C. The main symptoms consist of watery diarrhea, prolonged and spiking fever, nausea and abdominal cramps. The serious typhoidal disease was predominant in the U.S. from the later 1800s to 1949 (Tietjen *et al*., 1995). However, it currently seldom occurs, with approximately 824 illnesses, 618 hospitalizations, and 3 deaths per year, mainly as a result of international travel (Mead *et al*., 1999). In comparison, the incidence of outbreaks due to nontyphoidal *Salmonella* (S. Enteritidis, S. Typhimurium and others) continues to increase since the middle of the last century. Estimates indicate that nontyphoidal *Salmonella* accounts for 1.4 million illnesses, 16,430 hospitalizations, and 582 deaths annually in the U.S. (Mead *et al*., 1999). The symptoms of enterocolitis include severe abdominal pain, diarrhea, vomiting and fever. All serotypes of *Salmonella* are potentially pathogenic to humans. The mortality rate of human salmonellosis depends on the age of the subject, with an average of 4.1%. Special populations like newborns, the elderly, and patients with immune deficiencies are particularly prone to the infection (D’Aoust, 2001). In general, the infectious dose of *Salmonella* is approximately $10^5$ CFUs. However, outbreaks may occur from consumption of relatively low numbers of *Salmonella* cells. 14

It has been estimated that more than 200 virulence factors contribute to the pathogenic properties of *Salmonella*. The virulence determinants are encoded by *Salmonella* pathogenicity islands or plasmid (IFT, 2001). During the initial stage of salmonellosis, at least six different adhesions are involved in the intestinal colonization of the pathogen (IFT, 2001). SPI-1 is responsible for the invasion of *Salmonella* into the gastrointestinal epithelium. The proteins secreted by the bacteria are delivered to the host cells and lead to cytoskeletal rearrangement, membrane ruffling, and bacterial uptake macropinocytosis. Once inside, the
engulfed bacteria remain to inhabit and proliferate in the membrane-bound vacuoles. An SPI-2-encoded type III secretion system might account for the survival of *Salmonella* in the intracellular compartment by avoiding lysosomal fusion, a host defense mechanism that usually targets invasive microbes (Hansen-Wester *et al*., 2001; IFT, 2001). In addition, toxins play a notable role in the illness. Most serotypes of *S. enterica* can produce a thermolabile polypeptide enterotoxin that causes diarrhea. Cytotoxins located in the bacterial outer membrane might be involved in spreading the *Salmonella* into deeper host tissues via inhibition of protein synthesis and lysis of host cells. An endotoxin, also located in the bacterial outer membrane, is associated with leukocyte-dependent inflammatory response (D’Aoust, 2001).

*Salmonella* spp. are facultative, intracellular parasites that invade the mucous membrane and are transmitted to humans mainly through meat, eggs, and poultry products (D’Aoust, 1991). The conventional method used for *Salmonella* detection in food relies on pre-enrichment in buffered peptone water (BPW) and enrichment in selective media, followed by isolation on differential media and serological confirmation (International Organization for Standardization, 1991). The main limitation of this method is that it is time-consuming and requires 5 or 6 days during which the food stocks being analyzed are forbidden to be sold. Thus, several research groups have been working to optimize innovative and more rapid methods for the detection of *Salmonella* spp. (Bailey, J. L. 1998; Bennett *et al*., 1998; Fach *et al*., 1999).

Poultry products are frequently contaminated with *salmonella* spp and are consequently thought to be a major source of pathogen for humans. In recent years concern about poultry, meat & other foods contaminated with foodborne pathogens has grown because of the increased incidence of antimicrobial resistant bacteria associated with human illness and the changes observed in the levels and types of resistance found in these organisms (Davis *et al*., 1999; Glynn *et al*., 1998; Logul *et al*., 2003; Seyfarth *et al*., 1997). Conjugative transfer of plasmid often causes acquired antimicrobial resistance phenotypes (Gebreyes *et al*., 2002). The presence of salmonella resistant to multiples antimicrobials in
food chain has been reported by a number of investigators (Chen et al., 2002) raising concern for the medical consequences associated with foodborne illness. 

*Salmonella* and other microorganisms underscore the importance of milk and milk products as vehicles of human infection. For these reasons, the presence of *salmonella* and other human pathogens in unpasteurized milk remains a public health hazard. It is known that high percentage of clinically normal animals on dairy farms can be infected with *salmonella* sp and other pathogen. *Salmonella* continues to be a major food borne pathogen for animals & humans are infected through eating raw or under cooked foods, including meat, poultry, egg & dairy product. Standard methods for detecting *salmonella* spp. are time consuming and involving a pre-enrichment in buffered peptone water, followed by plating on selective agar. Nucleic acid amplification technology has offered the potential for improved detection of *salmonella* in the environment, providing greater sensitivity and dramatically speeding up detection, thereby improving management of outbreaks.

Food and Drug administration gives a high priority to protecting the public from microbial contamination. Foodborne bacteria are listed in bacteriological analytical manual FDA these includes *E.Coli, Salmonella, Shigella, Campylobacter, Yersinia, Vibrio.cholera, I.pseudotuber culsis, V.parahaemolyticus, Staphylococcus aureus, Bacillus cereus, Clostridium perfiringenes & Cl. Botulinum*.

**Listeria Monocytogenes**

In 1966, Gray and Killinger published their classic review of *Listeria monocytogenes* and listeric infections in humans and other animals. Since then, the organism has been implicated as the causative agent in several outbreaks of food-borne listeriosis in North America and in Europe and an understanding of its mechanisms of pathogenicity is growing rapidly (Cossart and Mengaud, 1989). It seemed an appropriate time to review the many aspects of *L. monocytogenes* as
a food-borne pathogen, including its epidemiology, its incidence and growth in foods, and the virulence factors involved in the human disease.

*L. monocytogenes* was first described by Murray *et al.* (1926), who named it Bacterium monocytogenes because of a characteristic monocytosis found in infected laboratory rabbits and guinea pigs. It was renamed *Listerella hepatolytica* by Pirie in 1927 and given its present name by him in 1940 (Gray and Killinger, 1966). The first confirmed isolations of the bacterium from infected individuals, following its initial description, were made in 1929 by Gill from sheep (Gray and Killinger, 1966). Since then, sporadic cases of listeriosis have been reported, often in workers in contact with diseased animals (Cain and McCann, 1986). As a result of food-borne outbreaks, interest in the organism grew rapidly in the 1980s among food manufacturers and government bodies, with a concomitant increase in the published literature (Cossart and Mengaud, 1989; Gellin and Broome, 1989; Lamont *et al.*, 1988; WHO, 1988).

**Microbiology**

*L. monocytogenes* is a gram-positive, nonsporeforming, facultatively anaerobic rod which grows between -0.4°C and 50°C (Junttila *et al.*, 1988, Walker *et al.*, 1987). It is catalase positive and oxidase negative and expresses a P-hemolysin which produces zones of clearing on blood agar. The hemolysin acts synergistically with the 3-hemolysin of *Staphylococcus aureus* on sheep erythrocytes; the substance mediating this effect is known as the CAMP factor after Christie, Atkins, and Munch-Petersen (Christie *et al.*, 1944), the workers who first described the phenomenon in group B streptococci. The organism possesses peritrichous flagella, which give it a characteristic tumbling, motility, occurring only in a narrow temperature range. When the organism is grown between 20 and 25°C, flagellin is both produced and assembled at the cell surface, but at 37°C flagellin production is markedly reduced (Peel *et al.*, 1988). The colonies demonstrate a characteristic blue-green sheen by obliquely transmitted light. *L. monocytogenes* is widely present in plant, soil, and surface water samples (Weis and Seeliger, 1975), and has also been found in silage,
sewage, slaughterhouse waste, milk of normal and mastitic cows, and human and animal feces (McCarthy, 1990).

Rapid Methods to Separate Bacteria from Foods

Immunomagnetic Separation (IMS)

Immunomagnetic separation (IMS) allows for separation of a target microorganism from a population of other competing bacteria providing for easier identification on selective media (Wright et al., 1994). Magnetic beads (2.8 μm diameter) are covalently coated with affinity purified antibodies against specific surface markers on the microorganism. The beads are stored in phosphate buffered saline (pH 7.4 with 0.1% (human or bovine) serum albumin (HSA/BSA) and 0.02% sodium azide) (Dynal Biotech). Dynabeads®, a commercial IMS bead product, are cited in the 8th edition of the Bacteriological Analytical Manual (Feng and Weagant, 2002).

The use of IMS-PCR has proven to be effective in facilitating the detection of *E. coli* O157:H7 in contaminated ground beef and dairy products, for which detection limits of 1 CFU/g or /ml have been reported (Ogunjimi and Choudary, 1999; McIngvale et al., 2002). Tortorello et al. (1998) compared six methods for the detection of *E. coli* O157:H7 in apple juice, including direct fluorescent antibody (DFA), antibody-direct epifluorescent filter technique (Ab-DEFT), direct selective plating on sorbitol MacConkey agar (SMAC), immunomagnetic separation coupled to either selective plating (IMS-SMAC) or the polymerase chain reaction (IMS-PCR), and flow cytometry (FC). The most sensitive (0.1 CFU/ml) and consistent detection of the slowest growing O157 strain was obtained by IMSSMAC and IMS-PCR following 8 hr of nonselective cultural enrichment. Without enrichment, the level of detection was not as sensitive (IMS SMAC, 10 CFU/ml and IMSPCR 103 CFU/ml) (Tortorello et al., 1998).

Karch et al. (1996) compared immunomagnetic separation (IMS) to DNA-based methods and direct culture methods for isolation of *E. coli* O157:H7 strains from patients with HUS. They concluded that IMS was more sensitive (102 CFU/g with
107 coliform background microflora) than direct culture and had an advantage of being less time-consuming and less labor-intensive compared to molecular methods (Karch et al., 1996).

Pyle et al. (1999) explored the use of IMS combined with the cyanoditolytetrazolium chloride (CTC) incubation, used to determine respiratory activity, and its ability to rapidly detect viable *E. coli* O157:H7 compared to plating on SMAC. Following IMS, the bacteria were stained with CTC, exposed to an anti-O157 fluorescein isothiocyanate conjugated antibody (FAB) and filtered for microscopic enumeration using solid-phase laser cytometry. This method detected higher numbers (mean recovery ratios) of target bacteria when compared to plating on SMAC preceded by IMS (improvement of mean recovery ratio—beef, 6.0 times; peptone, 3.0 times; water, 2.4 times); detection limits for ground beef were around 10 CFU/g and for liquid samples were <10 CFU/ml (Pyle et al., 1999).

Bolton et al. (1996) studied detection of *E. coli* O157:H7 in raw meat products using enrichment and four subculture procedures. Enrichment in modified tryptone broth at 42° C for 6 h, IMS, and subculture on to cefixime, tellurite SMAC (ctSMAC) was the most sensitive and selective protocol. This procedure was used in combination with a most probable number technique to assess the number of *E. coli* O157 in naturally contaminated raw meat samples and was effective in the range of < 0.3 to 2300 CFU/g (Bolton et al., 1996). Barkocy-Gallagher et al. (2002) utilized nonselective enrichment prior to IMS to selectively concentrate *E. coli* O157:H7 prior to plating onto ctSMAC (cefixime, tellurite SMAC) and Rainbow agar supplemented with novobiocin and potassium tellurite. Both selective media performed comparably well to traditional methods for recovery of *E. coli* O157:H7 from hide, carcass, and fecal material (Barkocy-Gallagher et al., 2002). Immunomagnetic flow cytometry (IMFC), a combination of flow cytometry and IMS, was shown to be an effective method for detecting low numbers of *E. coli* O157:H7 in artificially contaminated ground beef, apple juice, and raw milk. After a 6 h enrichment and IMFC, total analysis time was only
7 h and the method was able to detect 4 \textit{E. coli} O157:H7 cells/g of ground beef (Seo \textit{et al.}, 1998).

**Metal Hydroxide Immobilization**

Zirconium chloride in water at pH 7.2 results in formation of multiple tetrameric complexes in which zirconium ions are connected by hydroxide bridges. The gelatinous precipitate (zirconium hydroxide) is effective in non-specifically immobilizing bacteria due to the formation of covalent bonds between the hydroxyl groups of the metal hydroxide and the amino acid side chains that are abundant on the surface of bacterial cells (Kennedy \textit{et al.}, 1976). Lucore \textit{et al.} (2000) studied the effectiveness of metal hydroxides (zirconium hydroxide, hafnium hydroxide, and titanous hydroxide) to concentrate pathogens in reconstituted nonfat dry milk prior to detection by cultural and molecular methods.

Following concentration and immobilization steps using zirconium hydroxide and low-speed centrifugation, the sample volume was reduced by 50-fold. The two pathogens tested, \textit{S. enterica} serovar Enteritidis and \textit{L. monocytogenes}, were recovered at a range of 78 to 96 \% of input to 65 to 96 \% of input, respectively. The bacterial-immobilization step was reported to be relatively nonspecific (Lucore \textit{et al.}, 2000) and may provide a less expensive alternative to IMS. The technique has been applied to the concentration of \textit{E. coli} O157:H7 from dairy foods (McKillip \textit{et al.}, 2000b). Recently, Cullison and Jaykus (2002) reported successful magnetization of metal hydroxides. The researchers inoculated nonfat dry milk with 103 to 106 CFU/25 ml of \textit{S. enterica} serovar Enteritidis, \textit{L. monocytogenes}, or \textit{Bacillus cereus} spores. The magnetized carbonyl iron-zirconium hydroxide mixture allowed bacterial recovery to exceed 75\% to >90\%, when assessed on the basis of loss to discarded supernatants, and this metal hydroxide was relatively nontoxic to \textit{S. Enteritidis} and \textit{L. monocytogenes} upon further investigation (Cullison and Jaykus, 2002).

The technique has also been successfully used to prepare food samples for pathogen detection using PCR and RT-PCR (McKillip \textit{et al.}, 2000b; Lucore \textit{et al.}, 2000b).
While bacterial concentration using titanous hydroxide did not improve total DNA yield of *E. coli* O157:H7 inoculated into skim milk, nonfat dry milk, reconstituted whey powder, and cheese, the purity of the DNA obtained following bacterial concentration was improved. *E. coli* O157:H7 inoculated into reconstituted whey powder at 106 CFU/ml could not be detected after simple nucleic acid extraction, but when a bacterial concentration step was incorporated, a band was visualized from an inoculum as low as 104 CFU/ml (McKillip *et al.*, 2000b).

Lucore *et al.* (2000) reported detection limits of 101 to 102 CFU/25 ml for reconstituted nonfat dry milk (11% w/v), ≥102 CFU/ml whole milk, and ≥101 CFU/ml for ice cream for *S. enterica* serovar Enteritidis and *L. monocytogenes* using a metal hydroxide immobilization method and RT-PCR targeting rRNA genes (Lucore *et al.*, 2000).

**Polymerase Chain Reaction**

PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection and identification of specific pathogenic bacteria from different food materials.

Standard conventional detection methods for *E. Coli, salmonella & shigella* are described in FDA bacteriological analytical manual, such as culturing the organisms in selective agar and identifying isolates according to their biochemical or immunological characteristics, these protocols are time consuming & labour intensive.

The polymerase chain reaction is a rapid technique with high specificity and sensitivity for the identification of target organisms. Multiplied PCR is a technology that can amplifying more than two gene sequences in the same reaction. As *E-coli, Salmonella & shigella* have the potential to contaminate same products; it is useful to detect them simultaneously. The applications of multiplex PCR for the detection of *E.Coli, Salmonella & Shigella* have been done with a detection limit of 1.25 CFU/g after a 24 h enrichment (Li *et al.*, 2002). However quantitative detection of target genes is not feasible in multiplex PCR assays
because amplified products can only be visualized in agarose gels after the completion of PCR, and the strength of target band cannot be measured correctly (Lee and Drake, 2001).

Recently, real-time PCR assays were developed for the detection and quantification of pathogen-specific gene product with the use of Taqman TM probes and molecular becons (Lyons et al., 2000; Nogya et al., 2000). Taqman TM probes and molecular becons are oligonucleotides labeled with a reporter and quencher dye at the 5’ and 3’ end respectively. With the amplification of target sequences, probes that attach to the sequences will break, and reporter dye that is suppressed by the quencher dye, will give out fluorescence. The intensity of fluorescence will increase with the process of amplification. The measurement of an incremental fluorescence increases for each PCR cycle. This will provide an accurate estimate of the number of specific pathogenic cells in contaminated food samples. Multiplex real time PCR has been used for detection of different bacterial species from meat samples, realizing the quantiation and identification of target strains at the same time (Li et al., 2005).

Even though concentration of most foodborne pathogens in foods are low, the infection doses of some of these pathogens are quite low. Minimum infection dose of *E. coli* & *Shigella* are approximately 10 CFU, while that of *salmonella* is around 10^6 CFU (Kothary et al., 2001). Enrichment is usually done to increase the concentration of target pathogens in order to increase the detection sensitivity. However, it becomes impossible, after enrichment, to determine the actual initial concentration of pathogen present in food samples (Knutsson et al., 2002).

The PCR protocols for the detection of food borne pathogens are very different; they include use of different PCR cycle machines, annealing temperature and buffer system. For routine laboratory analysis, it could be ideal if PCR methods using same condition could be developed. Not all PCR methods are very sensitive or specific. Some PCR primers have very high free energy of overlapping pentamers at 3’ end that will cause non-specific reactions, some primers have a stable 3’-terminal dimer and some have a stable hairpin primer.
Modification of some existing PCR method and development of new PCR method are necessary for development of a routine PCR protocol. Complex composition of food material can inhibit PCR, PCR cannot distinguish the DNA from live cells or dead cells, food particles interfere with the PCR assay (Porteen et al., 2007).

2. Probiotics and Lactic acid bacteria as probiotics

Probiotic microorganisms (Lactobacillus sp., bifidobacterium sp. & others) have been defined as live microbial food supplements that affect the host animal beneficially by improving its intestinal microbial balance, (Fuller 1989). Goldin and Gorbach (1992) reported that probiotics may be of therapeutic or preventative benefit for a number of pathological states including gastroenteritis, diarrhea, constipation & hypercholesterolemia. Gilliland and Walker (1990) reported that microorganisms should survive passage through the gastric juice after administration to give the probiotic effect.

The beneficial effect of probiotics can be explained by the provision of:

i. Digestible nutrients or digestible enzymes.

ii. Antibacterial substances antagonistic to harmful bacteria.

iii. Live bacteria that metabolize in vivo and provides nutrients, enzymes or antibacterial substances.

iv. Aids absorption of calcium (Fuller 1986).

Lactic acid bacteria as Probiotics

Lactic acid bacteria have a long history of use by man for food production and food preservation. LAB are characterized as Gram positive, non-spore forming bacteria and are naturally present in raw food material and in the human gastro-intestinal tract. The heterogenous group of LAB include various major genera:
**Introduction**

*Lactobacillus, Lactococcus, Carnobacterium, Enterococcus, Lactospheara, Leuconostoc, Streptococcus, Teragenococcus, Vagococcus & Weisella* (Carr et al., 2002).

LAB are widely used as starter cultures for fermentation in the dairy, meat and other food industries. Their properties have been used to manufacture products like cheese, yoghurts, fermented milk products, beverages, etc. These food grade bacteria can also improve the safety, shelf life, nutritional value, flavor and quality of the product (Wood et al., 1995; Leroy & Vuyst, 2004). Moreover, LAB can be used as cell factories for the production of food additives and aroma compounds (Wilbert & Hugenholtz, 2000)

LAB have been cited to be part of human (Fuller, 1991; Goldin, 1990 & Holzapfel et al., 2001) and animal microbiota (Batt et al., 1991; Benno et al. & Perdigon et al., 2001). LAB are regarded as a major group of probiotic bacteria (Collins et al., 1998; Metchnikoff, 1908). The probiotic concept has been defined by Fuller (1989) to mean “a live microbial balance”. Salimen et al. (1999) proposed that probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host. Several Lactobacilli, Lactococci and Bifidobacteria are held to be health benefiting bacteria (Rolfe, 2000; Tuohy et al., 2003).

LAB constitute an integral part of the healthy gastrointestinal microecology and are involved in the host metabolism (Fernandes et al., 1987). Fermentation has been specified as a mechanism of probiotics (Gibson and Fuller, 2000 & Metchnikoff, 1908). LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids & gases (Gibson & Fuller, 2000). LAB synthesize enzymes, vitamins, antioxidants and bacteriocins (Fernandes et al., 1987 & Knorr, 1998). With these properties, intestinal LAB constitute an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990).
Antimicrobial compounds produced by LAB have provided these organisms with competitive advantage over other micro-organisms. Exploitation of antibiosis of LAB is the best choice for not only improving the microbial safety of the food products but as a probiotic preparation because of their natural adaptation to the gut environment. Lactics need to be acid tolerant bacteria and exhibit resistance to lysozyme present in the saliva and other enzymes, gastric mucosa. Probiotics act through suppression of viable count by production of antibacterial compounds, competition for nutrients and adhesion sites, alteration of microbial metabolites and stimulation of immunity. (Mishra & Lambert, 1996). Sellar (1989) reported that the production of lactic acid during yoghurt preparation is important not only to meet commercial requirement but also to provide the following advantages:

1) It acts as a preservative.
2) It contributes a mild sour but refreshing taste.
3) It also enhances the digestibility of casein.
4) It also improves utilization and absorption of minerals.
5) It can stimulate gastric juice secretion.

Micro-organisms used as probiotics:
Lactobacillus acidophilus, L. plantarum, L. casei, L. delbreuckii subsp bulgaricus, L. fermentum, Lactococcus lactis, Bifidobacterium bifidum, B. infantis, Streptococcus salivarius subsp. thermophilus, Enterococcus faecalis
Source: Conway (1996)

Traditional probotic dairy strains of LAB have a long and safe use. There is a considerable interest in extending the range of foods incorporating probiotic organism from dairy foods to infant formulae, baby foods, fruit juice based products, cereal based products and pharmaceuticals (Lee and Salminen, 1995). Nowroozi et al (2004) studied biochemical tests, sugar fermentation pattern and antibacterial activity of Lactobacillus strains isolated from sausage. Antibacterial activities was assessed by agar spot, well diffusion and blank disc method.
Lactobacillus acidophilus

Lactobacillus acidophilus fermented food products have been recommended as a dietary adjunct because of their beneficial effects on health by giving the following characteristics (Gilliland 1979, Klaenhammer 1982 & Kim 1988):

1) Survive in large numbers in the carrier food to which they are added.
2) Normal inhabitants of the intestinal tract.
3) Tolerate the extreme low pH of the human stomach.
4) Survive in bile and phenolic substance concentration that are present in the small intestine of humans.
5) Produce beneficial effects like
   i. Inactivation of carcinogens in the digestive tract and inhibition of their production.
   ii. Enhancement of host immunoactivation.
   iii. Improving the intestinal microflora.
   iv. Cholesterol-lowering action.
6) Take up resistance and multiply in the human large intestine (Gilliland 1979, Klaenhammer 1982 & Kim 1988).

L. acidophilus produces lactic acid, H₂O₂ and antibiotics to suppress the multiplication pathogenic or putrefactive bacteria which cause intestinal problems, diarrhea and digestive upset etc. and contribute to maintenance of health, (Gilliland 1977).

The Physiological action of L. acidophilus

1. Improving the intestinal microflora

Tomic- Karovic & Krivec (1970) demonstrated the therapeutic value of “Acidophilus Zyma“ in the treatment of different intestinal disorders. Freeze dried cultures of L. acidophilus proved successful in controlling the gastrointestinal complaints.
Dave & Prajapati (1994) reported that during fermentation of milk, lactobacilli are known to produce several antimicrobial compounds like lactic acid, acetic acid, \( \text{H}_2\text{O}_2 \) and bacteriocins which are effective against several intestinal pathogens and therefore consumption of these bacteria has been found to be useful in controlling a variety of intestinal disorders.

Gupta et al (1996) found that L.acidophilus strain 301 produced sufficient amount of acid and antibiotic like compound which also showed inhibitory activity towards enteric pathogens like *Salmonella typhi*, *E. coli*, *Proteus vulgaris* & *staphylococcus aureus* when grown in milk as a mixed culture and thus improved the intestinal microflora.

2. **Growth promoting efforts**

Vakil (1965) isolated Acidophilin, a low molecular weight peptide extracted in methanol & acetone from acidophilus milk which showed an antibacterial action against pathogenic bacteria in *vitro*.

*L. acidophilus* produces lactic acid, \( \text{H}_2\text{O}_2 \) & antibiotics and creates an environment for the efficient utilization of nutrients (Nakazawa & Hosono, 1992). Grunewala (1983) reported a significant increase in body weight after 4 weeks in mice given acidophilus fermented product because of the efficiency of utilization of nutrients which was promoted by lactic acid fermentation of *L.acidophilus*.

3. **Cholesterol lowering action**

Gilliland *et al* (1985) noted that when they gave pigs a high – cholesterol feed together with *L. acidophilus*, an increase in blood cholesterol was suppressed due to the consumption of cholesterol by *L. acidophilus* directly in the intestinal tract.

Akalin *et al* (1997) demonstrated that yoghurt fermented with *L. acidophilus* significantly reduced the serum cholesterol concentration in mice, whereas yoghurt fermented with *L. bulgaricus* & *S. thermophilus* did not.
4. Cancer controlling action

Ayebo et al (1981) and Ayebo et al (1982) separated the anti-tumor components from yoghurt by dialysis. When this dialysate was fed to mice for 7 days, Ehrlich tumor cells showed 33% reduction in growth. The anti-tumor effect could not be demonstrated in vitro therefore it was concluded that it might elicit the activation of immunological response against the host without participating directly.


Goldin & Gorbach (1984) reported that acidophilus milk has been found to reduce the activity of fecal $\beta$ – glucosidase, $\beta$ – glucuronidase, nitroreductase and azoreductase which are the enzymes that convert pre-carcinogens into carcinogens.

5. Immunostimulating effect

Patidar (1995) fed five groups of chicks with milk fermented by different strains of L.acidophilus for 8 days and then measured the antibody titre by haemagglutination inhibition test in sera collected upto 5 weeks after feeding. He observed that high titre increased upto the third week and then gradually decline in all treated groups as compared to the control group fed only milk. Significant variations in the titre where observed among the strains of lactobacilli used however, among different strains, L.acidophilus C2 showed the highest antibody titre.

Tomioka & Saito (1992) gave a dose of $2.4 - 4.5 \times 10^6$ cells of different Lactobacillus species to mice & after 24 hours were the mice intraperitoneally infected with E. coli. The surviving mice score after 7 days were 0 in the untreated group but it was highly variable with different Lactobacilli. The highest 80% survival was reported in the group fed L.acidophilus. The action was mainly based on the activation of host macrophage by Lactobacilli in either a T-cell dependent or a T-cell independent manner.
Line- Amster et al (1994) gave cultured milk containing L. acidophilus La1 & Bifidobacteria to human volunteers over a period of 3 weeks. The volunteers were also given an attenuated S. typhi Ty2a, to mimic an enteropathogenic infection. They found that there was significant increase in total serum IgA & almost a 4 fold increase in specific serum IgA titre against S. typhi Ty21a in the test group as compared to the control, which didn’t receive fermented milk.

6. Alleviation of Lactose intolerance

Lactose intolerance is a problem for a large population of the world population for whom lactose acts like a non-digestible carbohydrate, because they have a low amount of intestinal lactose. During fermentation of yoghurt milk containing L. acidophilus produces lactase that hydrolyses lactose to glucose & galactose. This predigestion of lactose reduces the symptoms associated with lactose intolerance in susceptible individuals. Probiotics have been shown to improve lactose digestion & intolerance in some studies, (Pamela Masun, 2001).

Kim (1983) gave yoghurt inoculated with L. acidophilus to men & noted an increase in their intestinal lactase activity which is due to β-galactocidase activity.

Many reports concerning the acid tolerance, bile tolerance & viability of L. acidophilus in milk have attempted to select L. acidophilus for yoghurt & cheese making which have greater possibility of reaching the intestine as viable cells (Prasad et al., 1998; Masuda et al., 2005).

Vogel et al (1998) reported that the lipolytic activity of LAB is important for aroma formation in fermented products. Lipase activities are known to be generally low or negligible in LAB.

Masuda et al (2005) reported that L.acidophilus showed higher lipase activities than L.gasseri & intracellular protease activities were shown to be higher in L.gasseri than L.acidophilus.
Marshall & Cole (1983) reported that L. acidophilus elaborated the enzyme alcohol dehydrogenase, which converted most of the acetaldehyde to alcohol. As a result, lesser quantities of acetaldehyde were present in acidophilus products. Drisko et al. (2005) gave a systemic approach using the polymerase chain reaction (PCR), followed by DNA sequence analysis of the PCR products, which was undertaken to gather direct evidence for the presence of bacteria stated to be present in the probiotic products. They concluded that lack of Bifidobacterium bifidum in 2 probiotic products may be attributed to different preparation standards among probiotic manufacture & identification of additional Lactobacillus species may represent contamination of the samples due to manufacture.

Muller et al (1999) reported that the sensitivity & reproducibility of the detection methods based on PCR are largely dependent on the isolation of sufficient DNA suitable for PCR & in complex food matrices an abundance of potential PCR inhibitors have to be eliminated for successful amplification of target DNA. Herman et al. (1997) & Hahn (1996) reported that within the dairy industry PCR has received much attention mainly to detect bacterial contaminants.

Roland & Joseph (2001) reported that free DNA is, however, distributed mainly in the whey & cream fractions of cow's milk, in fact, the highest concentration of free DNA can be found in the cream layers by the CTAB method.

Temmerman et al. (2003) reported that in the near future, the linkage of real-time PCR to the DGGE (denaturing gradient gel electrophoresis) method may result in a very powerful tool for both qualitative & quantitative analysis of all kinds of fermentation products.

Giraffa & Rossetti (2004) reported that the randomly amplified polymorphic DNA PCR technique (RAPD-PCR) is applied at both species & strain level differentiation of dairy isolates of LAB.

Kandler & Weiss (1986) reported that the species Lactobacillus acidophilus consists of a genetically heterogeneous collection of strains that are difficult to differentiate by simple physiological & biochemical tests.

Stevens & Jaykus (2004) developed a simple, rapid method to concentrate & purify bacteria & their nucleic acids from complex dairy food matrices in preparation for direct pathogen detection using Polymerase Chain Reaction (PCR).

**BACTERIOCINS CLASSIFICATION**

There are a wide number of bacteriocins produced by different LAB & they can be classified according to their biochemical & genetic characteristics (Kleanhammer *et al.*, 1994 & Gonzalez-Martinez *et al.*, 2003).

Bacteriocins produced by LAB are classified into three main groups(Klaenhammer, 1993).

**Class 1**

LANTIBIOTICS

**Class 2**

NON-LANTIBIOTICS

Small heat stable peptides

**Class 3**

Large heat labile protein

(Non-bacteriocin lytic proteins)

**Class 1 Lantibiotics**

Lantibiotics, small (< 5 KDa) heat stable peptides act on membrane structures. They are extensively modified after translation resulting in the formation of characteristic thioether aminoacids lanthionine and methyl lathionine. They arise via a two step process, originated from post translational modifications: firstly, gene encoded serine and threonine are subjected to enzymatic dehydration to
give rise to dehydroalanine and dehydrobutyrine, respectively (Sahl & Blerbaum, 1998). A very well known example of this group is Nisin (Broadbent et al, 1989).

The lantibiotic bacteriocins were initially divided into two subclasses based on structural similarities.

**Subclass 1a** included relatively elongated, flexible and positively charged peptides, they generally act by forming pores in the cytoplasmic membranes of sensitive target species. The prototypic lantibiotic nisin is a member of this group. **Subclass 1b** peptides are characteristically globular, more rigid in structure & are either negatively charged or have no net charge. They exert their action by interfering with essential enzymatic reactions of sensitive bacteria (Deegan et al, 2006).

**Class 2 Non-Lantibiotics**

These Bacteriocins are of variable molecular weight, but are usually small (<10 KDa) heat stable, containing regular amino-acids. This group is divided into 3 sub-groups:

**Class 2a:** Peptides active against Listeria, the characteristic represents are pediocin PA-1 (Venema et al, 1997) and Sakacin P.

**Class 2b:** Formed by a complex of two distance peptides, these peptides have little or no activity and there appears to be no sequence similarities between complementary peptides. In this group are lactococcin G and Plantaricins EF e JK.

**Class 2c:** Small peptides, heat stable which are transported by leader peptides. In this subclass are found only the bacteriocins Divergin A and Acidocin B.
Fig. 1. Overview of the application potential of bacteriocin production by LAB in food quality and safety and in medicine, emphasizing their role as a foods ingredient and in the human gastrointestinal tract, respectively.
Class 3: Big peptides with molecular weight over 30 KDa. In this class are Helveticins J (Joerger & Klaenhammer, 1986) and V (Vaugham et al, 1992), Acidofilicin A and Lactacins A & B.
Most of the low molecular weight bacteriocins are highly cationic at pH 7 and this seems to be a unifying feature of both lantibiotics and non-lantibiotics (Cintas et al., 2001).

Nisin
Nisin, a lantibiotic usually is used as an additive in foods. Of the known lantibiotics, nisin still is the only one with substantial industrial use. In fact to date nisin is the only lantibiotic allowed as a food supplement. In 1969 the FAO/WHO Expert Committee on Food additives stated Nisin to be a safe & natural food additive (FAO/WHO, 1969). Some 15 yrs later nisin was commercially used in at least 39 countries (Hurst, 1983). In 1983 Nisin was incorporated to the EEC food additive list & given the designation E 234 (EEC, 1983). In US, the Food & Drug Agency gave nisin a GRAS status (Generally Recognised As Safe) in 1988 (Federal Register, 1988). By the year 1996 nisin was allowed as a food additive in more than 50 countries, including EU, China & the US (Delves-Broughton et al., 1996).

Structure and Chemical Characteristics of Nisin
Nisin is a peptide formed by 34 amino-acids with a small molecular weight, approximately 3350 Daltons, depending on variant. It’s synthesis is complex involving processing of transcription, transduction, post-transductional modifications, secretion, processing & signs of transduction (Guder et al., 2000). Nisin is soluble and highly stable at acidic solution, at aqueous solution of pH 2, the solubility of nisin is 57 mg/ml & nisin retains it’s biological activity even if it is autoclaved (Hurst, 1981). However, at alkaline pH the solubility decreases and nisin becomes dramatically inactive, probably due to chemical modifications (Liu & Hansen, 1990).
Primary Structure

To date, four natural Nisin variants have been described:

- Nisin A (Gross & Morell, 1971)
- Nisin Z (Graeffe et al., 1991; Mulders et al., 1991)
- Nisin Q (Zendo et al., 2003)
- Nisin U (Wirawan et al., 2006)

The two variants of this bacteriocin, Nisin A & Z differ from each other only by the amino-acid 27. Histidine in nisin A is replaced by Asparagine in nisin Z.

Nisin A, Z, & Q comprise 34 amino-acids of which 8 are post translationally modified. These mature molecules each contain one lanthionine, four methyllanthionines two didehydrobutyrine & one didehydrobutyrine (Gross & Morell, 1971; Graeffe et al., 1991; Mulders et al., 1991 & Zendo et al., 2003).

The lanthionines form five ring structures (designated as rings A, B, C, D & E) & 2 of these rings D & E are fused together to establish a double ring structure. The structure of nisin U is essentially the same, though it is composed of 31 amino-acids and has two didehydrobutyrines (Wirawan et al., 2006).

Faustina et al. (2004) studied the nisin production related to growth conditions of Lactococcus lactis subsp. Lactis ATCC 11454, the effects of various media components & concomitant release of nisin into the media through 5 time transfers. Nisin production was assayed by agar diffusion using Lactobacillus sake as a sensitive test organism. The expression of nisin was strongly influenced by the addition of skimmed milk to both MRS & M17 broth with highest production obtained after the 2nd and 5th transfer respectively with maximum expression after 36 hours of incubation.

Faustina et al. (2004) used a low cost growth media diluted skimmed milk (2.27g & 4.54g total solids) which lead to 3 fold higher and 85 fold higher nisin production respectively and further mentioned that milk subproduct whey can be exploited for nisin production.
Kim (1997) investigated Nisin production by *lactococcus lactis* subsp.lactis using two phase batch culture. A solvent (phenyl methyl silicon oil) was introduced in addition to the aqueous phase. Two phase batch culture supported 21% increase in growth & 24% increase in the level of nisin produced compared to single phase batch culture.

Shioya *et al.* (1999) used a mixed culture system consisting of *Lactococcus lactis* and *Kluyveromyces marxianus* for nisin production. The interaction between lactate production by *L. lactis* & its assimilation by *K. marxianus* was used to control the pH instead of addition of alkali. The interaction of these microorganisms to maintain high level of nisin production investigated and kinetics of growth production of lactate, acetate & nisin by *L. lactis* were investigated.

Hickmann *et al.* (2003) developed a redefined modified method for the turbidimetric assay of nisin estimation in the total incubation period of 6 hours.

William *et al.* (1992) found out the properties of nisin Z and distribution of its gene in *Lactococcus lactis*. The nisin variants were purified to apparent homogeneity, and their biological activities were compared. Identical MICs of nis A and nis Z were found with all tested indicator strains of six different species of gram positive bacteria. The distribution of nisin variants in various lactococcal strains determined by amplification of nisin structural gene by polymerase chain reaction followed by direct sequencing of amplification product.

Papagianni *et al.* (2006) determined bacteriocin activity with bioassays carried out on solid & liquid substrates with indicator micro-organisms i.e. Plate agar diffusion assay & turbidometry. Dose responses & sensitivities were examined & compared over a range of assay variables in standard bacteriocin solutions fermentation broth filtrates & processed food samples. The data produced were analysed statistically using ANOVA technique & pairwise analysis.

Simard *et al.* (1997) purified and characterized the bacteriocin Nis Z produced by *Lactococcus lactis biovar diacetyllactis* UL 719. Purification of bacteriocin was carried out by ammonium sulphate precipitation followed by dialysis and further
purification by RP-HPLC.SDS-PAGE analysis & amino-acid sequence determination and molecular mass determination by mass spectrometry. Ivanova et al. (2000) carried out detection, purification & partial characterization of a novel bacteriocin substance produced by \textit{L. lactis} isolated from Boza(bulgarian traditional cereal beverage).

**ORGANIZATION OF GENE CLUSTERS**

The genes involved in biosynthesis of the model lantibiotic nisin are located on a 70 kb conjugative transposon which also contain the genetic information for sucrose metabolism. The first gene of the nisin gene cluster, nis A encodes 57 amino-acid nisin precursor, consisting of a N-terminal leader sequence followed by the propeptide, from which nisin A is matured. The structural gene followed by ten other genes i.e. nis B, nis T, nis C,nis I,nis P, nis R, nis K nis F, nis E, nis G encoding regulatory proteins, proteases,transport proteins and immunity proteins (Fig.3 A & B).The proteins are encoded by these genes have been found to be homologous to gene products of the gene clusters of other lantibiotics.

Untill now scientific data show that almost all bacteriocin genetic determinants are clustered in operons or regulons. This is not unexpected because in the simplest case the bacteriocin expression needs at least two genes: one structural gene and another one that encodes an immunity protein specific to the produced bacteriocin. In most cases bacteriocin production needs also of specific export machinery and is subjected to some regulation factors, which make bacteriocin operons much more complex. Based on the data obtained, there is a tendency that in general the lantibiotics operons are more complex than those of non-lantibiotics because they need additional genes encoding enzymes for posttranslational modifications with very few exceptions, all bacteriocins are synthesized as prepeptides containing leader sequences.
Fig. 3A. Organization of nisin biosynthetic, regulatory and immunity genes (Regulated promoters are indicated by P* and constitutive promoters by P. An inverted repeat is indicated by IR)
The nisin precursor is modified by the putative enzymes NisB and NisC and translocated across the membrane by the exporter NisT. The precursor is extracellularly processed by NisP, resulting in the release of mature nisin. NisK senses the presence of nisin in the medium and autophosphorylates. The phosphate-group is transferred to NisR, which activates transcription of the genes.
nisABTCIP and nis FEG.NisI,F,E and G protect the cell from the bacteriocidal activity of nisin. P: Promoter region, P*: nisin-regulated promoters.

Lantibiotics functionally depend on post translational modifications other than cleavage of leader sequences, their operons are some of the most complex among bacteriocins. One of the best characterized bacteriocin operon is that encoding the biosynthesis of nisin. The nisin gene cluster contains eleven genes and is usually designed as nisABTCIPRKEG. Of these genes, nis A encodes the nisin A precursor peptide, nis B and nis C encode putative enzymes involved in the post translational modification reactions, nis T encodes a putative transport protein if the ABC translocator family that is probably involved in the extrusion of the modified nisin precursor, nis P encodes an extracellular protease involved in precursor processing, nis I encodes a lipoprotein involved in the producer self protection against nisin; and nisin FEG a encodes putative transporter proteins that have also been implied in immunity. The proteins encoded by nis R and nis K have shown to be involved in the regulation of nisin biosynthesis. Nis R, a response regulator, and nis K a sensor histidine protein kinase belong to the class of the signal transduction regulatory systems proteins.

The gene cluster contains two promoters. The first one is located upstream in the nis A gene, and the second one between the genes nis P and nis R. It is interesting that an inverted repeat located between the genes nis A and nis B could act as a rho-independent terminator suggesting sophisticated regulation of the expression of the genes within the gene cluster.

The following path for nisin biosynthesis has been proposed:

1) nis A translated to pre-nisin A.

2) Pre-nisin A is translated to precursor nisin A by the products of the genes nis B and nis C, at this stage several disulfide bridges are made as some amino-acids are transformed to unusual ones as well.

3) Finally the precursor nisin A is exported out of the cell by the products of the genes nis T and nis P at the same time while the leader peptide is cleaved and the final product, nisin A obtained.
Although nis A specific transcription is already present in early exponential phase, detectable active nisin starts to appear in mid-exponential phase (when biomass reached to 50 % of maximum biomass) & increased to reach it’s maximum at the beginning of stationary phase (Hirsch,1951 & Buchman et al.,1988). The late appearance of active nisin was suggested to be the result of delayed expression of maturation enzymes (De Vuyst & Vandamme, 1992).

In general, nisin production is growth associated & shows a cell density dependent characteristics (Kuipers et al., 1995). However, maximization of growth doesn’t necessarily result in maximization of nisin production.

**Antimicrobial Activity Of Nisin**

Interestingly nisin effectively kill bacteria is nano molar concentrations while many non-lantibiotic poreformer antimicrobial peptides only work into micromolar range. This remarkable potency of nisin was suggested as a result of its dual killing mechanism, targeted pore formation & inhibition of cell wall synthesis (Brotz & Sahl, 2000; Wiedmann et al., 2001).

A specific target of nisin for pore formation is an is an integral component of the eubacterial cytoplasmic membrane, called Lipid II. According to the model proposed by Hasper et al(2004) Nisin first forms a 1:1 complex with lipid II because one more nisin molecule binds to lipid II , giving a 2:1 nisin:lipid II complex. Finally a staple pore complex is formed by the insertion of nisin molecule into a perpendicular orientation with respect to membrane surface, giving final pore complex of 8 nisin & 4 Lipid II molecules with diameter 2-2.5 nm (Wiedemann et al., 2004). Binding of nisin on lipid II not only causes collapses of proton motive force & membrane integrity via formation, but also interfere with cell wall synthesis by blocking lipid II from incorporation into peptidoglycan (Wiedemann et al., 2001).

In bacterial spore, effect of nisin is known to be different from that against vegetative cell & is mostly sporostatic. Nisin affects the post germination stages of spore development by inhibiting pre-emergent swelling, the outgrowth & formation of vegetative cells (Hitchins et al., 1963 & Gould, 1964).
Model for the Mode of Action of Lantibiotics

Several models have been proposed to explain the membrane activity of nisin. Most of the models are based on the “barrel save” model, initially proposed for nisin by Sahl et al (1991) in the current model (Fig.4) it is assumed that nisin in particular the C-terminal region of the molecule, binds to anionic phospholipids by means of electrostatic interactions. In-vivo, the binding of nisin to bacterial membranes might also involve the interaction with peptidoglycan precursor lipid II. This more specific interaction was suggested to be mediated by the N-terminall part of nisin, as nisin shares a homologous N-terminal ring pattern. The membrane associated peptide or atleast part of them, insert into the membrane, thereby taking an orientation parallel to the membrane surface. The hydrophobic residues of nisin interact with the fatty acyl chains of the lipids, while the polar residues are located at the membrane spanning orientation and form a cluster around a central water filled pore (Step 4a). It is likely that this process is preceeded by aggregation of peptides, although this process remains speculative. In an alternative so called wedge model it was proposed that the bound anionic lipids co-insert with the peptide, resulting in bending of the lipid surface, giving rise to wedge like pores (Step 4b). Both models assume that nisin exposes its charged residues to the lumen of the pore, which is expected to the result in anion selectivity of the nisin pore. It was proposed that the C-terminal region of the peptides switches across the membrane to form a pore. Possibly the electrochemical membrane gradient facilitates this process, by pulling the charged C-terminus across the membrane. The transmembrane orientation of the peptides is presumably only transient. Upon disassembly of the pores, the peptides are assumed to flip back to an orientation parallel to the membrane surface.
Fig. 4 Model for the mechanism of action of nisin. At first nisin binds to the surface of the membrane (1), followed by insertion of the molecule into the membrane (2). The peptides switch to a membrane spanning orientation, involving translocation of the C-terminus of the peptides across the membrane (3). A transmembrane aqueous pore is formed, most likely consisting of several peptides (4). Membrane phospholipids might co-insert, resulting in bending of the lipid surface (4b).

Applications of Nisin
Nisin was confirmed as GRAS by U.S. Food & Drug Administration (FDA, 1988) & is allowed for use as a preservative in about 50 countries around the
The suitability of nisin as a food preservative arises from many good characteristics such as non-toxicity & heat stability at low pH values. Moreover, nisin is not used clinically digested by enzyme α-chymotrypsin and it does not add color or flavor to food.

The principal commercial application of nisin are in food & beverages which are pasteurized but not sterilized such as processed cheese, milk, clotted cream, dairy desserts, ice-creams mixes, liquid egg & hot baked flour products. It is also used in canned products to prevent spoilage by thermophillic, heat resistant spore formers. Another application includes alcoholic beverages such as beer & wine in which it is used to control spoilage due to lactic acid bacteria(Davies & Delves- Broughton,2000).

Apart from important application as a food preservative nisin is now widely used for the production of bovine mastitis(Sears et al.,1991).Moreover, nisin was found to be very effective in preventing oral plaque & gingivitis. Thus nisin shows a potential for many dental applications(Howell et al.,1993),with all these applications demand for nisin keeps increasing & thus these is a need for economical production of nisin.

**Immobilization**

Immobilization has been characterized as a technique that confines a catalytically active enzyme or cell within a reactor system & prevents its entry into mobile phase which carries the substrate & product (Roscwear, 1984).

Physical entrapment in a porous matrix is the most flexible & most popular approach employed for whole cell immobilization (Freeman, 1984;Klein & Varlop, 1983) According to Freeman (1984), a process designed for efficient entrapment of whole cells should allow for the following:

1. High retention of cell viability, the biological activity of entrapped cells should not be impaired by immobilization conditions.
2. The porosity of formed gel should be uniform and controllable free exchange of substrates, products cofactors, gases and so forth is essential for efficient performance of immobilized components.

3. The gel obtained should retain good mechanical, chemical & biological stability. It should not be easily degraded by enzymes, solvent, pressure changes or shearing forces.

4. The gel should be composed of reasonably priced components & the process should be amenable to ready scale up.

Immobilization provides various advantages over traditional batch fermentation processes with freely suspended cells, including maintenance of stable & active biocatalysts over long periods of time accelerated reaction rates, high volumetric productivity & improved product recovery.

**ADVANTAGES**

- Reuse of biocatalysts (due to entrapment)
- Maintenance of stable & active biocatalysts (due to separation of cell growth & metabolite production due to entrapment).
- Accelerated reaction rates due to high cell densities & elimination of non-productive cell growth phases.
- High volumetric productivity (due to continuous process & removal of desired metabolites & metabolic inhibitors etc.
- Improved process control (due to continuous process, produced separation & removal of metabolic inhibitors).
- Improved product recovery (due to physical separation of biocatalyst & desired products within reactor).
- Increased possibilities for secondary metabolite formation (due to cell aggregation).
- Improved efficiency of production (due to reuse of biocatalyst recycling of cofactors and co-enzymes, high reaction rate, removal of inhibitors)
- Biochemical processing using immobilized cells is a relatively new approach to biocatalysts (Venkatsubramanian, 1979)
The following categorization of methods for immobilization of microbial cells has been provided by Klein & Wagner (1983):

1. Physical crosslinking (flocculation)
2. Covalent crosslinking
3. Adsorption on insoluble matrices
4. Covalent binding to insoluble matrices
5. Physical entrapment in porous materials
6. Encapsulation

Entrapment methods are based on inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium, while still allowing mass transfer of nutrients & metabolites (Durand & Novarro, 1978).

Ivanova et al (2000-2002) used calcium alginate beads encapsulated lactic acid bacteria for bacteriocin production. As a result of immobilization, the bacteriocin production was increased approximately 50% compared to batch fermentation with free cells. The immobilized cells could be reused up to three times after filtration and resuspension in new medium.

Wan et al. (1995) studied nisin production with suspended cells and Ca-alginate immobilized cells. They found that in repeated batch fermentation the immobilized cells produced less nisin than the suspended cells, perhaps because of diffusional limitation in alginate gels. However, the same study also showed that, in continuous fermentation at a high dilution rate, the nisin titer from immobilized cells was equal to that obtained from batch fermentation.

**RAPD-PCR**

PCR based protocols that use short oligonucleotides of random sequence as primers to amplify polymorphic DNA from anonymous genomes hold promise for population and ecological genetic studies. Williams et al (1990) who first
developed the technique proposed the term “Random Amplified Polymorphic DNA (RAPD) for this class of genetic markers which is widely used.

The main PCR-based technique that has been applied to the study of population is RAPD analysis (Williams et al., 1990). Randomly amplified polymorphic DNA (RAPDs) are one of a family of techniques that produce arbitrary fragment length polymorphism & are collectively described as multiple arbitrary amplicon profiling (MAAP) (Caeano-Anolies, 1994). Other methodologies in this family include those using an arbitrary primed polymorphic chain reaction (AP-PCR) (Welsch & McClelland, 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolies et al., 1991) anchored inter-simple sequence repeat (ISSR) & PCR (Zietkiewicz et al., 1994).

The RAPD technique utilizes single, arbitrary, decamer DNA oligonucleotide primers to amplify regions of the genome using PCR (Welsch & McClelland, 1990; Williams et al., 1990; Hadrys et al., 1992 & Williams et al., 1993). Priming sites are though to be randomly distributed throughout a genome & polymorphism in these region results in differing amplification products. The methodology is simple & has been widely used for the assessment of ‘genetic diversity’ investigation of hybridization and introgression events, genetic variation within species, to determine relationships between closely related species & genotypes within a species to identify particular genotypes & processes, etc. (Kresovich et al., 1992; Vierling & Nugyen 1992; Hsiao & Riesoberg, 1994).

RAPD technique can be used to determine taxonomic identity access kinship relationship, detect interspecific gene flow, analyze hybrid speciation & create specific probes. Advantages of RAPDs include stability for work on anonymous genomes applicability to work where limited DNA is available, efficiency & low expense (Hadrys et al., 1992). It is also useful in distinguishing individuals or accessions (Karp et al., 1996).

By using different primers, molecular characters can be generated that are diagnostic at different taxonomic levels. RAPD amplification products can be either variable (polymorphic) or constant (non-polymorphic). In RAPD analysis of
several individuals within species, constant fragments diagnostic for genus may be identified, as well as fragments which are polymorphic among species of the genus (Hadrys et al., 1992).

In the last few years various molecular typing methods such as RFLP, Pulsed field gel electrophoresis, ribotyping and PCR-derived techniques, randomly amplified polymorphic DNA (RAPD), repetitive extragenic, palindromic and enterobacterial repetitive intergenic consensus PCR have been used to distinguish between lactic acid bacteria strains. Recently RAPD analysis has been used to estimate the diversity among several genera of bacteria such as Lactobacillus, Enterococcus, Lactococcus and Staphylococcus isolated from many sources (Cocconcelli et al., 1995; Corroler et al., 1998; Morea et al., 1998; 1999; Rebecchi et al., 1998) and 16s rDNA sequence analysis for the taxonomic identification of biotypes.

It is also possible to use a combination of two or more 10 mer oligonucleotides (multiplex RAPD) in a single PCR to generate RAPD profiles making it possible to discriminate between Lactobacillus strains.

RAPD techniques have been extensively used in the typing of lactic acid bacteria (Taillez et al., 1996). Randomly amplified polymorphic DNA analysis has been used to monitor population dynamics in food fermentation and to estimate the diversity of Lactobacillus strains in cheeses (Baruzzi et al., 2001; Berthier et al., 2001; Bouton et al., 2002) whey culture (Cocconcelli et al., 1997), sausage fermentation (Morea et al., 1998; Rebecchi et al., 1998) and maize dough (Hayford et al., 1999). Du Plessis and Dicks (1995) used RAPD to separate species of the Lactobacillus group (Lb. acidophilus, Lb. crispatus, Lb. amylovorus, Lb. gallinarum) which are difficult to distinguish on the basis of simple physiological and biochemical tests. Cocconcelli et al. (1997) demonstrated that the community of thermophilic lactobacilli that dominates in cheese is composed of a limited number of bacterial strains belonging to the Lb. helveticus and Lb. delbrueckii ssp. lactis species.