Chapter I.

General Introduction

Agriculture is the base of Indian economy. Agriculture forms 26% of the national GDP and approximately 75% of India’s population live in villages.
and depend on crop and livestock farming for their livelihood. Livestock production including dairying plays a multipurpose role in the agriculture systems of India. (FAO, 2011; NIC, 2011). Dairying plays a dynamic role in India’s agro-based economy. The dairy sector in India has shown remarkable development in the past decade. Today, India ranks first in the world in terms of milk production with 112.5 million tons per year during 2009-10 (Anon, 2011).

Milk may be defined as the normal secretion of the mammary gland of mammals. Milk contains many essential nutrients, such as carbohydrates, proteins, lipids, minerals and vitamins. According to Hindu mythology as well as the Indian traditional medical practices, cow’s milk has rejuvenator, health protecting and health promoting properties and hence can be referred to as one of the best vitalisers. Most of the changes which take place in the flavour and appearance of milk, after it is drawn from the udder are the results of the activities of microbes. Milk as it is secreted by the gland of the mammals is free of microorganisms. However, microorganisms associated with the teat move up the teat canal and into the interior of the udder. This causes even aseptically drawn milk to contain microorganisms, mostly bacteria. Bacteria in aseptically drawn milk are usually limited in number and include mostly Micrococci, Lactococci, Staphylococci, Streptococci and Bacillus (LCT, 2011).

Milk may become contaminated with bacteria during or after milking. The mammary glands of cows can become inflamed due to a bacterial infection called mastitis. During mastitis, very high numbers of bacteria are present in the udder and are excreted through the milk. Some disease causing
organisms (pathogens) can be shed through cow feces and may contaminate the outside of the udder and teats, the farm environment (bedding, for example) and the milking equipment. Although optimal growth conditions for bacteria/pathogens are varying, milk contains important nutritional components for growth, and, therefore, it is also an ideal medium for the growth of most of the bacteria/pathogens. Temperature plays an important role in bacterial growth. Many bacteria prefer to grow at body temperature (86-98°F, 30-37°C), but may grow at lower temperatures (such as refrigerator temperature) at slower rates.

The area of dairy microbiology is large and diverse. Clean milk is generally defined as “Milk drawn from the udder of healthy animals, which is collected in clean dry milking pails and free from extraneous matter like dust, dirt, flies, hay, manure, etc. Clean milk has a normal composition, possesses a natural milk flavour with low bacterial count and is safe for human consumption”. Some bacteria may be specifically added to milk for fermentation to produce products like yogurt and cheese. The spoilage and pathogenic bacteria present in milk and dairy products may cause spoilage of milk or disease in consumers, respectively. Besides being a health hazard, contamination of milk and milk products can lead to huge economic losses. The employment of hygienic practices at the time of milking is therefore one of the first and most important steps in clean milk production.

Clean milk production results in milk that is safe for human consumption, free from disease producing microorganisms, has a high keeping quality, can be transported over long distances, has a high commercial value and is a high quality base suitable for processing resulting in high quality
finished products. Milk needs to be protected from all possible sources of microbial contamination. Potential sources of contamination of milk are dung, water, utensils, soil, feed, air, milking equipment, animal and the milker. Contamination of milk can occur during storage and transport (FAO, 2011).

Human illness from milkborne pathogens is usually associated with consumption of raw milk or products made from raw milk. In the past 20 years, foodborne illnesses from dairy product consumption have been predominantly associated with Salmonella enterica, Listeria monocytogenes, Campylobacter jejuni, and Escherichia coli O157:H7. These organisms have been isolated from bulk tank samples (Jayarao et al., 2001; 2006; Van Kessel et al., 2004). Because there is a risk of pathogen contamination in milk produced from healthy cows under sanitary milk conditions, pasteurization of milk prior to consumption will destroy pathogens and provide protection for illness associated with milk borne pathogens. Occasionally, human illness has been linked to pasteurized milk products but these cases usually have been a result of contamination of the product after pasteurization or improper pasteurization (Oliver et al., 2005).

Mastitis is recognized as the most costly disease in dairy cattle. Losses to the USA dairy industry is about $2 billion per year. Mastitis is one of the economically most important diseases affecting the Indian dairy industry causing an annual economic loss of Rs.7165.51 crores (US$ 1492.8 millions) (Bansal and Gupta, 2009). Mastitis reduces milk production and alters milk composition. The magnitude of these changes in individual cows varies with the severity and duration of the infection and the causative microorganisms. Mastitis is commonly caused by bacteria like Staphylococcus aureus, Streptococcus
agalactiae, *E. coli*, *Klebsiella*, *Listeria monocytogenes* etc. These microorganisms produce toxins that can directly damage milk producing tissue of the udder and the presence of bacteria initiates inflammation within the udder tissue in an attempt to eliminate the invading microorganisms. The inflammation contributes to decreased milk production and is primarily responsible for the compositional changes observed in milk fat and protein content. Mastitis not only reduces dairy producers’ profits but also results in important and costly losses to processors due to poor quality milk. Awareness of the economic losses associated with mastitis is resulting in a desire for mastitis control programs (Hurley, 2011).

Dairy cattle are considered as the primary reservoir of Shiga toxin-producing *Escherichia coli* (STEC) and the main route of STEC infections in humans is via consumption of contaminated food (Hussein and Sakuma, 2005; Meng *et al.*, 2007). STEC strains have been isolated from a large variety of different foods including raw-milk cheeses (Caro and Garcia-Armesto, 2007; Stephan *et al.*, 2008) and although the first STEC outbreaks were associated with consumption of contaminated and undercooked hamburgers, subsequent outbreaks have incriminated both animal and plant origin foods (Erickson and Doyle, 2007). Clinically, STEC infection is characterized by non-bloody-to-bloody diarrhoea, haemorrhagic colitis, thrombocytopenia and fatal haemolytic uremic syndrome (HUS) (Bertholet-Thomas *et al.*, 2011). Fecal specimens examined from healthy cattle during the investigations of two sporadic cases of hemolytic uremic syndrome (HUS) associated with raw milk consumption and an outbreak of gastroenteritis and HUS caused by *E. coli* 0157:H7 demonstrated that dairy cattle are a reservoir of *E. coli* 0157:H7 and other
STEC (Wells et al., 1991). Enterohemorrhagic *E. coli* (EHEC) infection can occur through ingestion of improperly cooked food and raw milk contaminated with bovine feces containing *E. coli* 0157:H7. There is a growing concern over the emergence of highly virulent non-O157 STEC serotypes that are globally distributed. The recent outbreak of non O157 *E.coli* i.e. O104:H4 in more than 14 European countries has caused 42 deaths and more than 3792 people were ill (WHO, 2011). Studies in India have confirmed that cattle are the principal reservoir of non-O157 serotypes (Khan et al. 2002). In India, limited information is available regarding the STEC in animals including cattle (Pal et al., 1999), sheep (Bhat et al., 2008), fish (Sanath Kumar et al., 2001), beef (Khan et al., 2002) and human faeces (Khan et al., 2002).

*Listeria monocytogenes* is a foodborne pathogen of great concern for the food industry and food producing companies. Due to its physiological characteristics, such as resistance to acidic and sodium chloride stress, ability to grow at low temperature and possibility to form biofilms (Harvey et al., 2007), it can persist and/or re-contaminate food products, thereby representing an important risk for the safety of the consumers (Olesen et al., 2009; Gardan et al., 2003; Liu et al., 2002; Pan et al., 2006). The term “Listeria hysteria” was coined towards the end of 1980s following a series of listeriosis outbreaks due to the consumption of soft-cheese and ready-to-eat (RTE) meats in the UK. Recently, this emerged again in the large outbreaks in Canada caused by deli meats (Warriner and Namvar, 2009) and in USA (Anon, 2011). The increase in the occurrence of listeriosis, reported in the community summary report on foodborne outbreaks in the European Union in 2007 (Anonymous, 2009), warns for the need of special attention to this foodborne pathogen in order to
combat its presence in foodstuffs. *Listeria* spp. are ubiquitous bacteria widely distributed in the environment (Liu *et al.*, 2006). Among the eight species of *Listeria*, only *Listeria monocytogenes* is commonly pathogenic for humans. Although human listeriosis occurs only sporadically (Farber and Peterkin, 1991 and Schuchat *et al.*, 1991) several outbreaks have been observed during the last two decades (McLauchlin *et al.*, 2004). It is established that food-borne transmission constitutes the main route of acquisition of listeriosis (Farber and Peterkin, 1991; Pinner *et al.*, 1992). Although the incidence of the first human case of listeriosis was reported by Nyfeldt (1929), it is only since 1981, after the three well investigated listeriosis epidemics, first caused by coleslaw (Schlech *et al.*, 1983), second caused by whole and 2% fat milk (Fleming *et al.*, 1985) and third caused by consumption of soft Mexican-style cheese (Linnan *et al.*, 1988), that this organism came to be considered as a foodborne pathogen. Multinational outbreak from dairy products was reported recently by Fretz *et al.* (2010). Large majority of patients with listeriosis have an underlying condition which predisposes to infection by interfering with T cell-mediated immunity. It can cause serious infections such as meningitis or septicemia in newborns, immunocompromised patients, and the elderly or lead to abortion (Vazquez-Boland *et al.*, 2001). At great risk are pregnant women and the unborn child, alcoholics, drug abusers, diabetics, patients receiving treatments which alter their natural immunity such as AIDS patients, patients with malignancies and the elderly (WHO, 1988). Infection acquired early in pregnancy may lead to abortion, stillbirths or premature delivery. When the infection is acquired late in pregnancy, it can be transmitted transplacentally and lead to neonatal listeriosis which may be manifested at birth or late in the
neonatal period. Non-perinatal listeriosis is seen mainly in immunocompromised adults and children. Typical overt listeriosis presents as sepsis and meningitis. Although the presence of *L. monocytogenes* has been reported from a wide variety of foods, the incidence in tropical foods is very low (Karunasagar and Karunasagar, 2000). The epidemiology and risk management of listeriosis in India has been reviewed (Barbuddhe *et al.*, 2011).

Hence, in the present study, isolation of *Listeria* from milk samples was undertaken.

The availability of subtyping procedures to track individual strains involved in outbreaks, and to examine the epidemiology and population genetics of bacteria, is integral to control and prevention programs aimed at limiting infections. Application of subtyping methods also provides insight into the population genetics, epidemiology, ecology, and evolution of bacteria. A variety of conventional, phenotypic, and DNA-based subtyping methods have been described for differentiation of *L. monocytogenes* and *E. coli* beyond the species and subspecies levels (Graves *et al.*, 1999). While phenotype-based methods have been used for many years to subtype *L. monocytogenes* and other food-borne pathogens, DNA-based subtyping methods are generally more discriminatory and amenable to inter-laboratory standardization and are thus increasingly replacing phenotype-based subtyping methods (Wiedmann, 2002; Karama and Gyles, 2009; Foley *et al.*, 2009). Commonly used phenotype-based subtyping methods for *L. monocytogenes* and other food-borne pathogens include serotyping, phage typing, and multilocus enzyme electrophoresis (MLEE) (Seeliger and Hohne, 1979; Weintraub, 2007). The genetic subtyping approach encompasses PCR-based approaches (e.g., random
amplified polymorphic DNA and amplified fragment length polymorphism), PCR-restriction fragment length polymorphism (PCR-RFLP), ribotyping, pulsed-field gel electrophoresis, and DNA sequencing-based subtyping techniques (e.g., multilocus sequence typing (MLST)) (Wiedmann, 2002; Karama and Gyles, 2009; Hyytiä-Trees et al., 2007).

Raw (unpasteurized) milk can be a source of food borne pathogens. Raw milk consumption results in sporadic disease outbreaks. Pasteurization is designed to destroy all bacterial pathogens common to raw milk, excluding spore-forming bacteria and possibly Mycobacterium paratuberculosis, but some people continue to drink raw milk, believing it to be safe.

The territory of Goa with the high rainfall (3000 mm), 100 km. long coastal boundary with the available water resources and moderate temperature ranging from 24°C to 32°C, provides ample scope for income generation through agriculture and livestock production. The territory has about 100,000 cattle and 45,000 buffaloes. The human population of the state is around 14 lakhs in addition to the visiting tourists and migrant labourers which accounts for about one third of the local population (Anon, 1997).

Food borne illnesses continue to pose a threat to human health. Foods of animal origin are usually implicated as a vehicle for such illnesses. Production of milk and its products involves a long sequence of operations from harvesting to final consumption during which it is exposed to various microorganisms. The climate in Goa is very congenial with high humidity and relatively constant temperature throughout the year which in turn favours the rapid multiplication of the microbes in foods of animal origin. The microbial growth is undesirable as it may cause spoilage as well as food-borne illnesses.
Microbiological examination of milk is essential to find the degree of contamination. The assessment of microbial load at various stages of manufacture or processing may serve as a useful tool for quality assessment and improvement which will result in longer shelf life which is a desirable market requirement. The detection of coliform bacteria and pathogens in milk indicates a possible contamination of bacteria either from the udder, faecal sample, milk utensils or water supply used (Olson and Mocquot, 1980; Bonfoh et al., 2003). However, keeping milk in clean containers at refrigerated temperatures immediately after milking process may delay the increase of initial microbial load and prevent the multiplication of micro-organisms in milk between milking at the farm and transportation to the processing plant (Bonfoh et al., 2003). Contamination of mastitis milk with fresh clean milk may be one of the reasons for the high microbial load of bulk milk (Jeffery and Wilson, 1987).

Guaranteeing a greater food safety level for consumer products warrants an integrated approach to controlling food safety throughout the entire food chain (Stefan, 1997; Valeeva et al., 2005). A number of regulations have been developed and introduced to assure food safety at different stages of the food production chain. Given the many potential and emerging hazards along the chain, it is of practical importance to prioritize attributes. Spoilage and contamination may occur in the milk chain as a result of poor hygiene, long periods of transportation and lack of proper storage facilities. Deficient hygiene has often been considered to be one of the major causes of spoilage of products resulting in a loss of income, both for farmer and smallholder dairies. Although many studies are reported on analysis of milk collected at different
stages of processing, data is lacking particularly on the analysis of milk in production chain i.e from farm to table. Hence, the proposed study was envisaged with the following objectives.

a) To assess the microflora of milk at different points of collection and processing.

b) To standardize the conventional methods of isolation of microorganisms.

c) To isolate the specific pathogens viz. *Escherichia coli* and *Listeria monocytogenes* from milk and milk products.

d) To analyse the sources of contamination of milk.

e) To characterize the specific pathogens by molecular techniques.