Discussion
Chapter 5

Discussion

Fermented foods are consumed all over the world as these are considered to promote health and prevent diseases. Health promoting and disease preventing features of fermented foods are implicated with the presence of beneficial microorganisms that are derived from fermentation are known as probiotics. India is traditionally rich in fermented foods, but the nature of the products and the base material used for the preparation of fermented foods vary from region to region (Sekar and Mariappan, 2007). Traditional fermented foods are one of the rich sources for isolation of lactic acid bacteria (LAB). In recent years many papers have been published on isolation and characterization of LABs from traditional fermented foods (Oyedeji et al., 2013; Ishola and Tayo, 2012; Osuntoki et al., 2008; Sulieman et al., 2007). In the present investigation, attempt has been made to characterise the probiotic potential of the fermented foods and beverages of Himachal Pradesh. The detailed studies on microbiological, biochemical, technological and probiotic characteristics of fermented foods have been undertaken and the results are discussed in following sections.

Further the efficacy of finally selected LAB isolates to decontaminate the foods borne pathogen i.e. L. monocytogenes in the meat and ready to eat salad was analysed. Finally, the selected LAB isolate was fermented to produce the probiotic soymilk.

5.1 Isolation of lactic acid bacteria

Fermented foods and beverages are being extensively studied all over the world for their nutritional and health benefits. Among the all microflora present in fermented foods, LAB received the tremendous attention due to its various positive health promoting properties and GRAS (generally regarded as safe) status. Exploration and characterization of LAB present in traditional fermented foods is prerequisite to document the health and probiotic attributes of traditional foods. Himachal Pradesh, a northwest Himalayan state comprises of tribal districts and rural areas where traditional fermented foods and beverages are very popular.

In all 221 lactic acid bacteria and yeast isolates were isolated from 60 different fermented food products from various areas of Himachal Pradesh as listed in Table 4.1. A number of fermented foods (e.g. Siddu, Chilra, Marchu, Manna, Dosha, Bhatru, Bedvin roti, Bagpinni and Churpa) and fermented beverages (e.g. Sura, Chhang, Lugri, Daru, Angoori and Behmi) have been earlier documented, which have become an integral part of the food and nutrition of the rural and tribal people of Himachal Pradesh (Thakur et al., 2004; Sourabh et al., 2010).
Fermented foods are known to be the favourable niche for LAB isolation as a number of investigations have been conducted focussing on the isolation of *Lactobacillus* from fermented foods across the world. *Lactobacillus* species have been isolated from traditional Nigerian fermented foods such as *Fufu*, *Garri*, *Nono* and *Ogi* (Adenike et al., 2007) and Senegal fermented foods (Diop et al., 2007). Similarly, *Lactobacillus acidophilus* and *L. casei* have been isolated from *appam* batter and pickles, and characterized (Jamuna and Jeevaratnam, 2004). Several species of LAB were isolated from *borde* an Ethiopian cereal beverage (Abegaz, 2007), Nigerian fermented dairy products *wara*, *nunu* and unpasteurized yogurts (Osuntoki et al., 2008), fermented cassava by Kostinek et al. (2008), fermented dairy products (Ishola and Tayo, 2012) and Nigerian fermented food products *Fufu* and *Ogi* (Oyedeji et al., 2013).

### 5.2 Characterization of lactic acid bacteria

The first discriminatory trait used to identify and characterize any bacterium is based on cell morphology. Gram’s staining; endospore staining and capsule staining are the most widely employed methods for characterization of LAB (Patel et al., 2012). Lactic acid bacteria are generally defined as a group of bacteria, which have characteristics of low G+C, non-spore forming, gram-positive rod and cocci, fermentative, catalase negative, facultative anaerobe, non-motile and acid tolerant (Hutkins, 2006).

All the isolates selected as presumptive LAB were Gram positive and catalase negative rods or cocci, non-sporulating and non-motile. They were fermentative rather than being oxidative in nature. The characteristics exhibited by the presumptive LAB isolates were compared with those of standard strains for their identification (Sneath et al., 1986; Sharpe, 1979). *Lactobacillus* are rod shaped organisms that can be either hetero or homofermentative and are widespread in many plant and animal sources. *Lactobacillus* spp. are more tolerant to acid than the other genera of lactic acid bacteria (cocci) and this property makes them abundant in the final phases of many food fermentations when other organisms are inhibited by the low pH (Devirgiliis et al., 2008). This might be the main reason that around 70 % of the LAB isolates from traditional fermented foods of Himachal Pradesh were *Lactobacillus* spp.

Some of the technological and biochemical characters are very important in classification of LAB. However, the basis of classification includes mode of glucose fermentation, temperature optima, pH optima, tolerance to salt, and hydrolysis of diverse
hexose and pentose sugars. Moreover, some specific tests such as nitrate reduction, arginine hydrolysis, citrate utilization etc. that are based on metabolic activities are also used to characterise LAB (Stiles and Holzapfel, 1997).

LAB isolates were characterized according to the method recommended by Bergey's Manual of Systematic Bacteriology (Brinner et al., 2005). Generally, biochemical properties of the presumptive LAB isolates agreed with the description of Kandler and Weiss (1986) and confirmed with Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986). The species of presumptive Lactobacillus was identified by carbohydrates fermentation pattern, growth at 15 °C, 37 °C, 45 °C and growth at different NaCl concentration, arginine hydrolysis, citrate utilization, carbohydrate fermentations, growth at different pH (both acidic and alkaline values) and at different lactic acid and acetic acid concentrations as previously reported by Cullimore (2008).

The majority of LAB isolates grew at 15 °C and 45 °C which was similar to the findings of Hugas et al. (1993); Papamanoli et al. (2003). Hugas et al. (1993) reported that all Lactobacillus strains could grow well at 6.5-10 % NaCl, whereas the growth of LAB was variable in the present studies which suggest that growth at different temperatures and salt concentrations might be strain dependent. Few of the LAB isolates showed growth at pH 2.5 and 3.5 and majority of them exhibited good growth at pH 8.5 and 9.5 which was in agreement to the result shown by Papamanoli et al. (2003).

5.3 Probiotic characterization of LAB

Significant efforts have been made to identify potential probiotic LAB isolated from humans or from fermented foods because of their benefits. Stringent selection criteria for the identification of probiotic strains are now being considered essential (Wang et al., 2010). For specifically selecting highly potent probiotic strains, the safety and functional properties of LAB, such as survival in the GI tract (acid and bile tolerance), antibiotic resistance, adhesion to intestinal cells, antimicrobial activity, and production of β-galactosidase are highly important and should be studied using reliable in vitro and/or in vivo screening methods (Vinderola and Reinheimer, 2003; Minelli et al., 2004; Ronka et al., 2003). The outcomes of the probiotic characterization of LAB isolates are being discussed in following sections.
5.3.1 Tolerance to acidic pH

Resistance to gastric conditions is one of the in vitro tests frequently suggested for the evaluation of the probiotic potential of an individual LAB strains (Monteagudo-Mera et al., 2012). The time taken by food from entrance to release from stomach was reported to be 90 min (Berrada et al., 1991). Acid tolerance of bacteria is important not only for withstanding gastric stresses, but is also a prerequisite for their use as dietary adjuncts.

The in vitro survival test revealed that several LAB isolates (L1, L19, L21, L26 and L40) were resistant to pH 2 even after 3 h of exposure, while most of the isolates showed reduced viability after being exposed to pH 2. These results were similar with those of the previous studies, where Lactobacillus strains were viable even after being exposed to pH values of 2.5-4.0, but showed reduced viability at lower pH values (Mishra and Prasad, 2005; Angelis et al., 2006). Maragkoudakis et al. (2006) examined 29 Lactobacillus strains of dairy origin in vitro for their probiotic potential, and the survivability of different strains under the simulated gastric conditions varied which was consistent with our results. Wang et al. (2010) has also declared good survival rates for 11 Lactobacillus strains (except one strain) isolated from infant faeces in MRS broth at pH 3.0, which was in agreement with our present results.

The acid tolerance of lactic acid bacteria has been linked to the induction of H*-ATPase activity (Matsumoto et al., 2004; Ventura et al., 2004). Therefore, the variation in the acid tolerance of the selected probiotics might be related to the difference in H*-ATPase activity in the probiotics (Cotter and Hill, 2003; Guo et al., 2009). It was presumed that the F1F0-ATPase proton pump was solely responsible for the survival of LAB in acidic environments. Combination of constitutive and inducible strategies resulting in the removal of protons (H+), alkanization of the external environment, changes in the composition of the cell envelope, production of general shock proteins and chaperones, expression of transcriptional regulators, and responses to changes in cell density can all contribute to survival in acidic conditions (Cotter and Hill, 2003).

5.3.2 Resistance to bile salt

Bile plays a fundamental role in specific and nonspecific defense mechanisms of the gut, the magnitude of its inhibitory effects is determined primarily by the concentration of bile salts (Charteris et al., 1998). Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes which are mainly composed of lipids and fatty acids, affecting the cell permeability, viability and
interactions between the membrane and the environment (Succi et al., 2005). Therefore, bile tolerance is considered as an important characteristic of *Lactobacillus* strains, which enables them to survive, grow, and exert their action in gastrointestinal tract.

According to Sanders et al. (1996), *Lactobacillus* strains which could grow and metabolize in normal physical bile concentration could survive in gastrointestinal transit. The concentration of bile varies from 0.5 to 2 % during the first hour of digestion; the levels may decrease during the subsequent period. Most foods pass through the small intestine by 12 h (Clark and Martin, 1994). Hence tolerance of LAB was evaluated by exposing the cells to 1 and 2 % bile salt solution up to 12 h at 30 °C.

In present study, the tolerance of the LAB isolates was tested in three different concentrations of 0.5, 1.0, and 2.0 % (w/v) of bile salt. Out of total 40 isolates, only 20 isolates survived at 2 % bile salt concentration and these exhibited fairly good bile tolerance with survivability levels greater than 89 % after exposure to 2 % concentration. For the result of more than 15 isolates, there was significantly decrease in viability at higher bile concentration i.e. 2 % and the same pattern was observed in earlier reports by Succi and Coppola (2005); Hamon et al. (2011). These results are in agreement with those acquired from similar previous studies, where *Lactobacilli* strains were viable even after being exposed to bile range of 0.3 % - 0.7 %, but showed diminished viability at higher bile concentration i.e. 2 % (Tulumoglu et al., 2013; Wang et al., 2010).

All the selected probiotic isolates could grow in the medium with bile salts and possessed bile salt tolerance. Thus these could survive in bile salts in concentrations simulating the small intestine environment (0.5 % w/v). Two percent oxgall (bile salt) represents the extreme concentration obtained in animal or human intestines during the first hour of digestion; afterwards, the normal level of bile salt in intestine is around 0.3 % (Kacem and Karam, 2006).

Bile salts are toxic for living cells, since they disorganize the structure of the cell membrane which is composed of lipids and fatty acids (Papamanoli et al., 2003) and bile salt tolerance is considered one of the essential properties required for lactic acid bacteria to survive in the small intestine (Succi et al., 2005). Some *Lactobacilli* are able to hydrolyze these toxic bile salts with bile salt hydrolase enzyme, weakening their detergent effect (Erkkila and Petaja, 2000). The resistance to bile salts varies a lot among the *Lactobacillus* species and even among strains (Xanthopoulos et al., 1997) and the mechanism is still unknown (Erkkila and Petaja, 2000). Bile salt tolerance of some
microorganisms is possibly due to the presence of bile salt hydrolase (BSH) and some transporter proteins, which are functionally related to each other to respond efficiently to the stress from bile salts (Kim and Lee, 2008).

5.3.3 Cholesterol assimilation

The in-vitro study of cholesterol removal of Lactobacilli has been regularly utilized as a screening tool for selection of probiotic strains with diverse health promoting characteristics (Madani et al., 2013). High level of serum cholesterol has been associated with risks of coronary heart disease. The use of probiotic bacteria to reduce serum cholesterol levels has attracted wide attention of the consumers, researchers and the physicians.

LAB showed greater cholesterol assimilation in the media containing deconjugated bile (cholic acid), in contrary to the conjugated bile (taurocholic acid) and oxgall (contain both conjugated and deconjugated bile) which was in agreement with the results shown by Liong and Shah (2005). This may be due to more inhibitory effect of conjugated bile (taurocholic acid) towards LAB isolates as compared with deconjugated bile (cholic acid) and oxgall. The resistance of the LAB toward deconjugated bile in comparison to conjugated bile may be because conjugated bile salts have greater solubility and detergent activity, and may, therefore be more toxic than their deconjugated counterparts i.e. low solubility index (Liong and Shah, 2005).

Some strains of Lactobacillus have been identified to demonstrate cholesterol reducing capability in in-vitro (Kim et al., 2008; Sirilun et al., 2010). Lim et al. (2004) found that many LAB strains were able to reduce cholesterol in MRS broth regardless of the presence of oxgall. The emulsifying feature of bile affected cholesterol removal supplemented with different bile concentration (1-3 mg/ml) which was higher than in the medium without bile (Tok and Aslim, 2010). It was also reported that the inhibitory effect of the bile has considerable effect on the cholesterol removal ability (Lin et al., 1998). It is well known that the uptake of cholesterol by some lactic acid bacteria takes place in the presence of bile salts and it resulted partially from cholesterol co-precipitation together with deconjugated bile salts (Ziarro, 2009).

Cholesterol assimilation by growing cells was significantly higher than resting and dead counterparts; however, there was no significant difference (p<0.05) in the level of cholesterol removal by resting and dead cells. The capability of strains to remove cholesterol in dead and resting stage indicated that cholesterol might also be removed via
binding to cells (Liong and Shah, 2005). Higher cholesterol removal by growing cells indicated that the degree of bound cholesterol might be dependent on the growth of cells (Kimoto et al., 2002). Although cholesterol assimilation occurred mainly with growing cells, results on cholesterol removal from media by heat-killed cells indicated the potential of non-viable cells to reduce cholesterol concentration in the gastrointestinal system.

The mechanisms underlying the lowering of cholesterol have been recommended to involve assimilation of cholesterol, destabilization and co-precipitation of the cholesterol micelles, physiological action of the end products of short chain fatty acids by fermentation, cholesterol adherence to the bacterial cell wall or its incorporation into bacterial cells, bile salt hydrolase (BSH) activity of the Lactobacilli (Liong and Shah, 2005; Sirilun et al., 2010), cholesterol oxidize activity (Ahire et al., 2012), and finally production of some functional peptides (Kim et al., 2008).

All the isolates did not exhibit the BSH activity, despite that it had the ability to reduce cholesterol from cell-free broth by active cells. This suggests that the reason for cholesterol-lowering activity of strains without BSH activity might be due to the acid produced from natural lactic acid fermentation of these lactobacilli strains. The precipitation of cholesterol in supernatant appears to be related to the deconjugation of bile salts and their subsequent precipitation at low pH which was in agreement to the results reported by Klaver et al. (1993); Brashears et al. (1998); Sirilun et al. (2010).

5.3.4 Lysozyme resistance

Lysozyme, a hydrolytic enzyme found in mucous secretions is able to cleave the peptidoglycan layer of the bacterial cell wall. For orally applied probiotics the conditions in the gastrointestinal tract are the major selection criteria for microbial strains, and lysozyme in the oral cavity may lyse gram positive lactic acid bacterial cells (Surono and Nurani, 2001). The lysozyme content in saliva varies from 10-200 mg/L. Therefore their ability to survive at these lysozyme concentrations can be an additional parameter for selecting probiotics (Saran et al., 2012). Lysozyme contained in human saliva is the first step to be passed. Resistance to lysozyme has been attributed to the peptidoglycan structure in the cell wall, physiological state of the cell and lysozyme structure in the medium (Cunningham et al., 1991).
All the selected isolates showed a high resistance to 150 μg/ml of lysozyme which was previously reported by other authors (Turchi et al., 2013; Bosch et al. 2011; Zago et al. 2011). Lysozyme is capable of lysing bacteria, but doesn’t significantly impair activities of lactic acid bacteria (Lodi et al., 1983) which is similar to the present studies. Some of the LAB isolates were sensitive to 150 μg/ml lysozyme, but some showed considerable resistance (Brennan et al., 1986). Neviani and Veaux (1991) reported the acquisition of lysozyme resistance in \textit{L. helveticus} cells grown in milk, MRS agar containing lysozyme due to cell adaptation.

5.3.5 Antibiotic susceptibility

A key requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes. Ingestion of bacteria carrying such genes is undesirable as horizontal gene transfer to recipient bacteria in the gut could lead to the development of new antibiotic-resistant pathogens (Salminen et al., 1998; Saarela et al., 2000). There is also a potential for antibiotic resistance transfer within the gastrointestinal tract from commensal or probiotic bacteria to other bacteria or potential pathogen (Snydman, 2008). \textit{Lactobacilli} display a wide range of antibiotic resistance naturally, but in most cases antibiotic resistance is not of the transmissible type.

Among antibiotic resistances, vancomycin resistance is of major concern because vancomycin is one of the antibiotics broadly efficacious against clinical infections caused by multidrug-resistant pathogens (Woodford et al., 1995). Such resistance is usually intrinsic, chromosomally encoded and non-transmissible (Morrow et al., 2012; Klein et al., 1998). In our study, we found that all the LAB isolates were resistant to vancomycin (Table 4.9). These results are similar to those of Tulini et al. (2013); Tulumoglu et al. (2013); Zhou et al. (2005). Several species of \textit{Lactobacillus} including \textit{L. rhamnosus} and \textit{L. casei} are intrinsically resistant to vancomycin. There is an underlying possibility that vancomycin resistance could be transferred to other bacteria but there are no such reports till date (Ashraf and Shah, 2011). Resistance to vancomycin by \textit{Lactobacillus} strains has been attributed to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal dipeptide D-Ala-D-Ala, which is the target of the antibiotic (Coppola et al., 2005; Danielsen and Wind, 2003).

The profiles of antibiotic susceptibility of LAB have been documented by many researchers (Zoumpopoulou et al., 2008; Ammor et al., 2007; D’Aimmo et al., 2007). Various opinions exist as to whether it might be desirable that some probiotic strains
show resistance to specific antibiotics that are involved in antibiotic-induced diarrhoea (Charteris et al., 1998). On the other hand, the commercial introduction of probiotics containing antibiotic resistant strains may also have negative consequences due to its transferrable resistance to intestinal pathogens (Curragh and Collins, 1992). However, according to previous studies (Charteris et al., 2001; Danielsen and Wind, 2003) the antibiotic resistance observed for *Lactobacillus* strains in this work, are considered to be intrinsic or natural resistance because it is chromosomally encoded and, therefore, non-transmissible. Resistance to aminoglycoside antibiotics, such as gentamicin is considered to be intrinsic in the *Lactobacillus* genus and is attributed to the absence of cytochrome-mediated electron transport, which mediates drug uptake (Mera et al., 2012). Previous studies also confirmed the lower resistance of the lactobacilli species studied here towards chloramphenicol (Temmerman et al., 2002; Choi et al., 2003; Maragkoudakis et al., 2006; Argyri et al., 2013). Most of the isolates belonging to the *Lactobacillus* genus were susceptible to ampicillin, penicillin G, streptomycin and erythromycin and susceptibility of these antibiotics were also reported by Mera et al. (2012). Conversely, few of the *Lactobacillus* isolates show resistance to erythromycin and it is considered as transferable acquired resistances which are frequently found among LAB (Danielsen and Wind, 2003). However, our study indicated that there are lesser chances of occurrence of such transferable resistance gene in the LAB isolates from fermented foods.

The transmission of antibiotic resistance genes to unrelated pathogenic or potentially pathogenic bacteria in gut is a major health concern resulting in a spread of antibiotic resistance strain (Moreno et al., 2006; Patel et al., 2012). On the other hand, intrinsically antibiotic resistance *Lactobacillus* strain may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of the various antimicrobial agents (Lavanya et al., 2011). For this reason, at this stage of the present study, the LAB isolates showing transferable antibiotic resistances, although they display some probiotic properties were excluded as candidate probiotics, they may be reconsidered when the necessary tools for an objective assessment of their safety are developed.

**5.3.6 Antimicrobial activity**

Another essential condition for LAB with probiotic activity is the inhibitory effect on the growth of pathogenic bacteria (Mera et al., 2012). Use of LAB as biopreservatives has been approved in several studies, owing to their antagonistic activities toward various
foodborne pathogens such as *S. aureus*, *E. coli*, *S. dysenteriae* and *Y. enterocolitica*, etc (Pinto et al., 2006). The bacteria used as indicators in this study included Gram-positive bacteria, such as *Staphylococcus aureus*, *Bacillus subtilis*, and Gram-negative bacteria such as *Yersinia enterocolitica*, *Pseudomonas aeruginosas*, *Shigella dysenteriae* and *Escherichia coli*.

Antimicrobial activity by LAB isolates was checked by bit/disc method (Barefoot and Klanhammer, 1983) and well diffusion method (Kimura et al., 1998) against pathogenic microorganisms but at times the results may be misleading because of different factors viz, aggregation, non-diffusible bacteriocin, and protease inactivation and concentration effects. Some bacteriocin producing strains which showed positive activity with agar spot test/bit disk method/cross streak method have shown negative results with well diffusion assay (Stiles and Hasting, 1991).

The LAB isolates in the present investigation showed antagonistic potential toward almost all of the above mentioned pathogenic bacteria; however, they did not significantly inhibit the growth of *Y. enterocolitica* (Table 4.11). In this study, isolates L1, L6, L9, L11, L14, L16, L21, L22, L23, L36 and L40 showed satisfactory abilities to inhibit various pathogenic bacteria in agreement with the research results reported by Yukskekdag et al. (2004) and Tulumoglu et al. (2013). This antagonistic LAB may be effectively used in various foods to protect against pathogenic bacterial contamination that may occur during the manufacturing processes and storage.

The antimicrobial activity of lactic acid bacteria might be due to a number of agents. The possible mechanisms of action for this protection include the production of acid (Makraux and Vuyst, 2006; Shah, 2007) and other by-products of bacterial metabolism (Ghalfi et al., 2006; Kuleasan and Cakmakci, 2002). Among these are diminished pH levels, competition for substrates, the production of substances with a bactericidal or bacteriostatic action, including bacteriocins and bacteriocin-like substances (Pan et al., 2009). The other antimicrobial agents are primary the organic metabolic end-product such as lactic acid and acetic acid, diacetyl, hydrogen peroxide, carbon dioxide, aldehydes (Yukskekdag et al., 2004). The capacity to produce different antimicrobial compounds may be one of the critical characteristics for effective competitive exclusion of pathogen survival in the intestine and expression of a probiotic effect for the host (Ouwehand and Salminen, 1998). The acidic conditions in the stomach may even enhance the activity of these antimicrobial compounds (Ganzle et al., 1999).
Furthermore, these probiotic characteristics may partly be based on the production of relevant concentrations of lactic acid in the microenvironment, which in combination with a detergent such as bile salts, inhibits the growth of Gram-negative pathogenic bacteria (Begley et al., 2005).

5.3.7 Cellular autoaggregation

The autoaggregation ability is one of the key factors that determine the ability of the probiotic strain to adhere to the oral cavity, gastrointestinal tract and urogenital tract. Aggregation properties are important characteristics of bacterial strains that are used as probiotics (Kaushik, 2009). *Lactobacilli* with aggregation ability and hydrophobic cell surface could have more chance for adhesion to intestinal cells (Martin et al., 2005). The cellular aggregation helps not only in the transient colonization but also in providing a protective shield to the host system due to formation of a bacterial biofilm over the host tissue (Richard et al., 2003). *In-vitro* evaluation of autoaggregation and ability to coaggregate with potential enteric pathogens can be used for preliminary screening and selection of the best probiotic strain.

Variations in the percentage autoaggregation were seen in all tested LAB isolates i.e. 1.52-72.94 % which was in similar to that reported earlier by Tulumoglu et al., 2013: 24-80%, Collado et al., 2008: 5.0-73.7% and Collado et al., 2007: 11.3-58.4%. It was reported by Kos et al. (2003) that the cell surface proteins (S-layer proteins) influenced autoaggregation property and adhesiveness of *L. acidophilus* M92. Collado and Salminen (2007) reported that lactic acid bacteria strains presented higher autoaggregation abilities than the pathogens after incubation of 24 h. Goh and Klaenhammer (2010) analyzed that the aggregation promoting factors increases self-aggregation with incubation time. So our results confirmed the results of Tomas et al., (2005); Collado and Salminen (2007) and Goh and Klaenhammer (2010) that the autoaggregation increases with increase in incubation time.

The ability of probiotic bacteria to form cellular aggregates is considered to be a desirable characteristic, as they can potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or coaggregation with commensal organisms on the intestinal mucosa or by direct coaggregation with the pathogens to facilitate clearance (Bujnakova and Kmet, 2002; Schachsteik et al., 2004). In addition, studies have suggested aggregation as an important mechanism for genetic exchange, adhesion, and colonization in the host environments, as well as
immunomodulation of colonic mucosa (Cesena et al., 2001; Voltan et al., 2007). The cellular aggregation helps not only in the transient colonization but also in providing a protective shield to the host system due to formation of a bacterial biofilm (Richard et al., 2003) over the host tissue. It has been suggested that cellular aggregation is important to promote the colonization of beneficial microorganisms in several ecological niches like the GI or urogenital tracts (Tomas et al., 2007; Kmet and Lucchini, 1997).

5.3.8 Cellular coaggregation

The coaggregation assay is a reliable method to evaluate the close interaction between Lactobacilli and pathogenic bacteria (Collado, 2007; Soleimani et al., 2010) in which Lactobacilli could release antimicrobial substances in a very close proximity (Botes et al., 2008). Food-associated Lactobacilli possessing ability to coaggregate with pathogens are of special interest with regard to potential applications. Our results (≥ 60%) are in agreement with previous reports of Kos et al. (2003); Jankovic et al. (2012) that showed a high autoaggregation percentage and microscopic clustering of cells which may increase adhesion to intestinal epithelial cells.

5.3.9 Bacterial adhesion to hydrocarbons (BATH)

The BATH test has been extensively used for measuring cell surface hydrophobicity in lactic acid bacteria (Kos et al., 2003; Vinderola et al., 2004; Canzi et al., 2005). The beneficial effect of probiotic bacteria has been attributed to their ability to colonize human and animal gastrointestinal tracts (Cole et al., 1989). Hydrophobicity of the bacterial cell surface is the one of the most important factor that is involved in the adhesion of probiotics. It has been reported that, compared to hydrophilic strains, hydrophobic lactobacilli adhered better to intestinal epithelial cells (He et al., 2009). As bacterial cells alter their membrane fluidity under various environmental conditions, growth conditions may have a profound effect on the fatty acid composition of their lipids and subsequently on the hydrophobicity and adhesion ability of bacterial strains. As suggested by others, the high cell surface hydrophobicity of the LAB strains could indicate their potential to attach to the epithelial cell lining of the intestine and resist the movement of digesta (Lee and Salminen 1995).

With different hydrocarbons, maximum adhesion was with hexadecane (62.33%), followed by toluene (58.65%), xylene (48.41%) and octane (32.72%) as shown in Table 4.14 which is in agreement to the findings of Shobharani and Agrawal, (2011) in which
maximum adhesion was with toluene (46.11%) followed by xylene (41.23%), hexadecane (24.15%) and octane (20.56%). Mojzani et al. (2007) have found adhesion of *Lactobacillus* strain to toluene (27%) and xylene (12.4%), which are lesser than what was observed in the present studies. Maximum hydrophobicity was found to be present in n-hexadecane as also reported earlier (Dewan and Tamang, 2007; Aswathy et al., 2008; Divya et al., 2012).

The microbial adhesion to hydrocarbons has been widely used to measure the cell surface hydrophobicity of bacteria (Orlowski and Bielecka, 2006). Through this adhesion property, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors (Otero et al., 2004). The large differences in the cell surface hydrophobicity could be due to variation in the level of expression of cell surface proteins among strains of a species as well as due to environmental conditions which could affect the expression of surface proteins (De Vries et al., 2006; Ramiah et al., 2007).

The microbial adhesion to hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent (Martin et al., 2005). A high percentage of isolates adhered to xylene, a polar solvent; suggested the hydrophobic nature of the cell surface of these strains, important for binding of probiotics to gastric mucosa (Kumar et al., 2012). Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of (glyco)proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Rojas and Conway, 1996; Pelletier et al., 1997). It is known that only pronase and pepsin-sensitive surface molecules are responsible for cell surface hydrophobicity in bacteria.

### 5.3.10 Exopolysacharride production

Exopolysaccharides are a major component of the bacterial biofilm with a well-documented impression on adherence of bacteria to host cells (Lenda et al., 2011). Exopolysaccharides (EPS) of microbial origin are gaining importance because of their applications in food and other industries (Vijayendra et al., 2008). Among the wide variety of polysaccharide producing microorganisms, LAB have gained special attention because of the remarkable property of the polymers they synthesize and as they do not bear any health risk which are GRAS (generally regarded as safe). LAB strains often produce polymeric substances such as exopolysaccharides (EPS) which enhance the
colonization of probiotic bacteria by cell to cell interactions in gastrointestinal tract (Kanmani et al., 2013). Besides the use of EPS producing strains as food thickeners, emulsifiers, or gelling agents to modify the rheological properties and texture of products (Madiedo and Reyes-Gavila, 2005), EPS will remain longer in the gastro-intestinal tract, thus enhancing the colonization by probiotic bacteria (German et al., 1999). Unlike the EPS produced by other microorganisms, many health benefits such as antitumor effects (Kitazawa et al., 1998), cholesterol lowering ability (Pigeon et al., 2002) and immune-stimulatory activity (Chabot et al., 2001) have been attributed to EPS produced by LAB. The chemical composition, chain length, and structure of subunits together with the molar mass and radius of gyration of the EPS molecule determine the physical characteristics and thereby their viscosity-intensifying properties (Tuinier et al., 2001; Ruas-Madiedo et al., 2002).

The EPS-producing colonies (EPS*) have a characteristic smooth and shiny appearance which allows them to be easily distinguished from the EPS- variants (Reddy et al., 1996). All the isolates screened for EPS production produce slimy white ropy colonies on skimmed-milk ruthenium agar plates which are in agreement with the earlier reports (Chabot et al., 2001; Dabour et al., 2009). Ruthenium red stains the bacterial cell wall, producing pink colonies from non-ropy strains. In ropy strains, EPS prevents uptake of the stain, and the colonies appear white (Stinegele and Mollet, 1995).

5.3.11 Haemolytic activity

Haemolysis is a known virulence factor among pathogenic microorganisms (Rushdy and Gomaa et al., 2013). Assessment of haemolytic activity has also been used in the in vitro evaluation of probiotic safety. No evidence of haemolytic activity was found in faecal, blood and probiotic Lactobacillus strains; however, some strains of Lactobacilli express α- haemolysin (Baumgartner et al., 1998). The examined isolates were γ-haemolytic (i.e., no haemolysis) on blood agar plates. Santini et al. (2010) and Cosentino et al. (2012) have observed that none of the probiotic strains possess haemolytic activity it has also been reported that haemolysis is rarely present in fermented food LAB (Maragkoudakis et al., 2006).

5.3.12 Proteolytic activity

The proteolytic activity is considered desirable for the growth of lactic acid bacteria (LAB) in milk. Moreover, it is involved in the development of some organoleptic
characteristics in different fermented milk products (Axelsson, 1998). The production of high quality of fermented dairy product such as cheese and fermented milk is dependent on the proteolytic system of the starter bacteria used. Proteolytic enzymes from LAB play an important role in the degradation of casein and peptides leading to production of free amino acids (Neito-Arribas et al., 2010). These amino acids contribute directly or indirectly in dairy product flavor, since they are precursors of other catabolic reactions, which produce volatile aroma compounds (Williams and Banks 1997). However, highly proteolytic strains are not always the most suitable as starter cultures, since excessive proteolysis can cause uncontrolled production of bitter peptides and other undesirable compounds (Buffa et al., 2005).

In the present study, proteolytic activity as tested by agar plate method was recognizable by the presence of a clear halo in the plates. The results for 40 LAB isolates tested for proteolytic activity are shown in Table 4.16. Only 21 strains possessed this feature. Same results were reported by Marroki and Marroki, (2013); Kumar et al. (2013) on skimmed milk agar plates.

The potent proteolytic lactic acid bacteria could potentially generate a large variety of peptides, including bioactive sequences and especially, strain selection, based on the specificity of proteolysis and are probably all factors that remarkably influence the proteolytic activation of encrypted bioactive peptides (Kumar et al., 2013). The proteolytic system of lactic acid bacteria is very complex. It is composed of an extracellularly located serine proteinase, a transport system specific for di-, tri-, and oligopeptides, and a multitude of intracellular peptidases (Kunji et al., 1996).

5.3.13 Bile salt hydrolase activity

One beneficial effect resulting from human consumption of lactic acid bacteria with active bile salt hydrolase, or products containing them, is a reduction in serum cholesterol levels (Pereira and Gibson, 2002). LAB are projected to lower cholesterol levels through an interaction with host bile salt metabolism via the enzymatic deconjugation of bile salts (De Smet et al., 1998; Tahri et al., 1997). It has also been suggested that BSHs are detergent shock proteins that protect the bacteria from toxicity of bile acids in the gastrointestinal tract (Adamowicz et al., 1991; Flahaut et al., 1996).

None of the isolates from fermented foods and beverages in the present investigation displayed bile salt hydrolase activity which was similar to the findings reported by Botes et al. (2008); Begley et al. (2006); Quezada et al. (2013). Tanaka et al.
Chapter 5

Discussion

(1999) studied more than 300 lactic acid bacterial strains and reported that BSH activity was found primarily in organisms isolated from the gastrointestinal tracts of mammals, while organisms isolated from fermented milk preparations and vegetables did not exhibit BSH activity. However, recent microbial genome analyses suggest that the organisation and regulation of genes encoding BSH differ between species and genera (Ridlon et al., 2006) and even among strains (Tanaka et al., 1999).

Bile salt hydrolase (BSH) activity is a controversial subject and still under debate. The phenomenon was initially associated with natural tolerance to bile salts (De Smet et al., 1994; Moser and Savage, 2001). However, recent studies have shown that bile salt resistance of Lactobacilli could not be associated with the presence of BSH (Gilliland and Speck, 1977; Schmidt et al., 2001).

Most often, bile salt hydrolase activity has been detected in strains indigenous to the gastrointestinal tract (Toit et al., 1998); an environment rich in conjugated and unconjugated bile acids (Martoni et al., 2008). To date, BSH activity has not been detected in bacteria isolated from environments from which bile salts are absent e.g. Lactococcus lactis or Streptococcus thermophilus (Ahn et al., 2003).

5.3.14 Phosphoketolase activity

Negative phosphoketolase activity was present in all tested isolates which is in agreement to the result obtained by Martínez et al. (2012).

5.3.15 Hydrogen peroxide tolerance

Stress adaptation of strains can be done by exposing strains to lethal or sub-lethal conditions (Alvarez-Ordonez et al., 2008; Anous et al., 1999). Stresses such as use of ethanol, hydrogen peroxide, oxygen, pressure, starvation or osmotic environment can affect the tolerance capacity of the organisms (Toit et al., 2013). The stress adaptation is a species or even strain-specific characteristic involving the fatty acid composition of the membrane (Alvarez-Ordonez et al., 2008). Hydrogen peroxide diffuses rapidly through cell membranes; concentrations of H₂O₂ between 0.1 and 1.0 mM have been shown to have toxic effects on many other species of bacteria (Dowds and Hoch, 1991; Imlay and Linn, 1986). Some lactic acid bacteria formed detectable amount of H₂O₂ and some did not, regardless of their preference or requirement for aerobic or anaerobic conditions (Whittenbury, 1964). Probiotics food product should contain significant numbers of viable lactic acid bacterial counts during the passage time in gastrointestinal system. So the
lactic acid bacteria should survive in the hydrogen peroxide stress condition. In the present study, all the isolates were quite tolerant to the H₂O₂ concentration with 0.5 log CFU/ml reduction in the viability counts.

5.4 Amplification of 16S rDNA of LAB using polymerase chain reaction (PCR)

The beneficial properties of probiotics and the increased human consumption of these products have augmented efforts to identify potential probiotic strains (Quezada et al., 2013). Selection and identification criteria for probiotic strains are now considered essential. In selecting potential probiotic strains, species identification by 16S rDNA sequence analyses and the evaluation of their physico-chemical, safety and functional properties, such as resistance to gastric acid and tolerance to bile salts, are highly important (FAO/WHO, 2002).

There are many molecular tools for the identification of microorganism species. Among them, 16s rRNA gene sequencing is the most frequently used, because it is extremely useful for determining phylogenetic relationships among organisms from the level of domains to the level of moderately closely related species (Lane, 1991). Diversity of LAB in traditional foods was investigated by PCR amplification of specific gene regions. By comparing the 16S ribosomal DNA sequences of the isolated strains with the sequences available in NCBI/BLAST (100% homology), the isolated isolates were identified as *L. brevis, L. paracasei, L. casei, L. buchneri* and *L. plantarum*.

5.5 Detection of enzymatic activities of finally selected LAB isolates

Utilization of indigestible fibres and oligosaccharides, has been recognized as an important attribute of probiotics (Alazzeh et al., 2009; Gyawali and Ibrahim, 2012; Song et al., 2012). Species of *Lactobacillus* that produce functional enzymes such as β-galactosidase, β-glucosidase, amylase, protease and phytase could have an important impact on human health. However, the production capacity of such hydrolysing enzymes by *Lactobacillus* is strain specific (Ibrahim et al., 2010; Zotta et al., 2009).

β-Galactosidase is an enzyme produced by some of bacteria, especially lactobacilli in dairy products like yoghurt, cheese and milk (Gheytanchi et al., 2010). Lactose intolerance has been recognized for many years as a common problem in many children and most adults throughout the world (Heyman, 2006). Therefore, by addition of lactobacilli producing β-galactosidase as probiotic to milk and cheese and other dairy products could help to relieve lactose intolerance symptoms. Colonies growing on X-gal
medium with blue colour were regarded as bacteria containing β-galactosidase enzyme (Favier et al., 1996). Our results detected by different biochemical methods (ONPG, X-gal methods) confirmed each other which were similar to the previous reports (Favier et al., 1996; Gheytanchi et al., 2010). In conclusion, LAB possesses β-galactosidase, and utilizes lactose slowly and weakly because of the possible slow transport of lactose into the microorganism.

Through the hydrolysis of plant metabolite glucoconjugates, β-glucosidase activities of lactic acid bacteria make a significant contribution to the dietary and sensory attributes of fermented food (Michlmayr and Kneifel, 2014). β-D-glucosidase activity is widespread among LAB and presumably plays a substantial role in the interaction with the human host as well, as β-glucosidases release a wide range of plant secondary metabolites from their β-D-glucosylated precursors (Hayek et al., 2013). β-glucosidase activity was observed in most of the tested isolates which was determined by monitoring the rate of hydrolysis of p-nitrophenyl-β-D-glucopyranoside (pNPG). Similar results were reported by the Mahajan et al. (2010); Otieno et al. (2005); Hayek et al. (2013).

LAB possesses a complex system of proteinases and peptidases which enable them to use casein as a source of amino acids and nitrogen (Kirilov et al., 2009). The first step in casein degradation is mediated by cell wall located proteases, which cleave casein to oligopeptides. Further degradation to smaller peptides and amino acids that can pass through the cell membrane is performed by peptidases (Sandine et al., 1972). Evaluation of proteolytic activity of LAB isolates were evaluated and best activity was observed in *L. casei* PLA5. The proteolytic activity of LAB isolates were reported by many researchers (Kirilov et al., 2009; Scolari et al., 2006).

Amylolytic LAB utilizes starchy biomass and converts it into lactic acid in single step fermentation. Although most LAB are unable to degrade starch because of the lack of the amylolytic activity, a few exhibit this activity and are qualified as amylolytic lactic acid bacteria (ALAB) which are able to decompose starchy material through the amylase production during the fermentation processes (Asoodeh et al., 2010). Some of the LAB isolates tested exhibited amylase activity and same was reported by Fossi and Tavea, (2013). Phytate is a common fiber that found in cereals, legumes, and nuts, and acts as an antinutrient binding with proteins, lipids, carbohydrates, and metal ions like zinc, iron, calcium, and magnesium (Raghavendra and Halami, 2009). Phytate degrading activity in humans is relatively low mainly in the small intestine (Iqbal et al., 1994), so other sources
of phytate degrading enzymes are required. Microbial sources of such functional enzymes could be the most promising sources for human health. Although microbial phytases are considered of a great value in upgrading the nutritional quality of plant foods, very few studies have dealt with lactic acid bacteria. Twenty LAB isolates were screened for the production of phytase. Thirteen of them exhibited this enzyme activity on sodium phytate plates which was similar to the studies reported by Raghavendra and Halami (2009); Angelis et al. (2003). Enzymatic activities of LAB isolates were studied and it should be extended to maximize the production of specific enzymes so as to use the potential of these enzymes in food, feed and pharmaceutical industry. Further extensive research is needed to investigate the optimum concentrations and possible combinations of essential media components that could be used to maximize the enzymatic activities of the promising LAB isolates.

5.6 In-vitro adhesion assay

LAB may provide beneficial health effects by modifying the host immune system by reducing the colonization of pathogenic microorganisms and promoting healing of damaged mucosa during bacterial adhesion to the epithelium (Ouwehand et al., 2002). HT-29 cell line in vitro model for probiotic adherence studies has been extensively used to screen putative probiotic cultures (Argyri et al., 2013; Jensen et al., 2012; Mera et al., 2012). The organisms must adhere to mucosal epithelial cells lining the gut to be designated as probiotic (Boonaert and Rouxhet, 2000) which also depends on the number of bacteria added (Tuomola and Salminen, 1998).

In the present investigation, the numbers of bacteria adhering to HT-29 cell line were measured examining them directly under microscope after staining and also by colony count on agar after trypsinization. Good adhesion properties of Lactobacillus isolates were reported by many authors which is in agreement with our present study (Ouwehand et al., 2001; Wang et al., 2008).

The adhesion mechanisms are not fully understood; however bacterial cell-surface associated proteins with mucus and intestinal cell binding properties have been identified and characterized in probiotic strains (Sanchez et al., 2008; Velez et al., 2007). Probiotic bacteria compete with invading pathogens for binding sites to epithelial cells and the overlying mucus layer in a strain-specific manner (Morrow et al., 2012).
5.7 Screening of LAB isolates for inhibitory action against *Listeria monocytogenes*

*Listeria monocytogenes* is a gram positive, psychrotropic, facultative anaerobic bacterium and causes listeriosis, a severe food borne disease (Campos et al., 2011). It causes serious invasive illness, mainly in certain well-defined high-risk groups, including elderly and immune-compromised patients, pregnant women, newborns and infants (Rebagliati et al., 2009). The bacterium can survive under relatively extreme conditions such as low or high temperatures, low pH, reduced water activity and high salt content. Due to its psychrotropic character it is a pathogen of concern in refrigerated food products. The objective of this work is to screen the most promising LAB isolate, concerning the use of natural antimicrobials to control the growth of *L. monocytogenes* in food systems.

*Lactobacillus* isolates may be effectively used in various foods to protect against pathogenic bacterial contamination that may occur during the manufacturing processes. Use of LAB as biopreservatives has been approved in several studies, owing to their antagonistic activities toward various foodborne pathogens such as *L. monocytogenes*, *S. aureus*, and *E. coli*, etc (Pinto et al., 2006).

The antimicrobial ability of 20 lactobacilli isolates against *L. monocytogenes* was assayed. Simsek et al. (2006) conducted a similar study and reported the antibacterial activity of lactobacilli isolated from sourdough against *L. monocytogenes*. *L. plantarum* and *L. buchneri* showed antibacterial activity against *L. monocytogenes* at 30 °C and it was confirmed that anti-listerial activity was due to the production of bacteriocin like substances. Same results were reported by Lim and Im (2012); Yildirim and Yildirim (2001).

5.8 Efficacy of probiotic formulations (whey permeate) for the inhibition of *Listeria monocytogenes* in raw vegetables and meat by dipping method

*Listeria monocytogenes* is a foodborne pathogen of particular concern in ready-to-eat (RTE) meat products (CDC, 1999; Lou and Yousef, 1999) because of its ability to grow at refrigeration temperatures (Glass and Doyle, 1989; Juntila et al., 1988), its ubiquitous character (Samelis and Metaxopoulos, 1999), and its capacity to tolerate high concentrations of salt and levels of sodium nitrite typically used in these products (Buncic et al., 1997). Various control measures have been proposed to prevent and control the growth of *L. monocytogenes* in meat and ready to eat vegetables. Some of them include
the use of various additives, such as sodium lactate and sodium acetate (Blom et al., 1997), liquid smoke (Messina et al., 1988), monoglycerides (Wang and Johnson, 1997), and lysozyme (Hughey et al., 1989). Other physical methods are mainly post processing pasteurization (Roering et al., 1997), irradiation (Tarte et al., 1996), and high hydrostatic pressure which have been used in the control of the pathogen but these are very costly techniques. Despite the use of these non-traditional additives in recent years, outbreaks of foodborne listeriosis associated with RTE meat products have continued to occur (Trias et al., 2008). Occasional allergic reactions in sensitive individuals and the formation of potentially carcinogenic by-products (e.g. nitrosamines from nitrite) which has been reported by the use of non-traditional preservation methods have increased the interest in biopreservation (Roller, 2003).

The use of natural preservatives may be effective to retain the quality of minimally processed products by having an antimicrobial effect, inhibiting spoilage and avoiding oxidative processes (Meyer et al., 2002). Biopreservation systems that use LAB as a method of control of the pathogen have also been investigated and are becoming more popular as these are mainly focussed in achieving food safety without compromising the sensory and nutritional qualities of foods (Gandhi and Chikindas, 2007). Several studies have reported the inhibition of *L. monocytogenes* by microbial antagonism of LAB in laboratory media (Usmiati and Marwati, 2009; Das and Goyal, 2013) as well as in different meat systems (Amezquita and Brashears, 2002; Zdolec et al., 2007) and in different plant based systems. The availability of carbohydrate reservoir of lactose in whey (dairy waste product) and presence of other essential nutrients (soluble proteins, lipids and minerals) for the growth of microorganisms makes the whey one of the potential substrate for the production of different bio products (Panesar et al., 2010). Use of whey in the growth of LAB serves two purposes: one is the suitability of whey as a low cost substrate for lactic acid production and reduces the wasteful and costly disposal of whey which creates environmental pollution problem. *Lactobacillus* isolates used to decontaminate food borne pathogen from ready to eat foods were grown in the whey permeate supplemented with yeast extract (2.5 g/L) for 36 h. Yeast extract adds suitable nutrients like minerals, vitamins and peptides that promote optimum growth of lactic acid bacteria in low nitrogen nutrient media (Alonso et al., 2010). Growth of LAB in the whey permeate was reported by the Panesar et al. (2010); Alonso et al. (2010).
5.8.1 Efficacy of selected LAB isolates for inhibition of \textit{L. monocytogenes} in cooked meat

The LAB isolates have been characterized for having fast growth rates at refrigeration temperatures, so that they can overcome the growth of \textit{L. monocytogenes} by nutrient depletion or by enhancement of the production of bacteriocins which has been reported in various studies.

In our study, two isolates of LAB possibly producing bacteriocin like substances were selected that effectively inhibited the growth of \textit{L. monocytogenes} in cooked meat at 4 °C for the period of 28 days of storage. During this time, the numbers of LAB increased by only about 1 log\textsubscript{10} cycle in all cases, and no visible signs of spoilage like colour change, undesirable aromas and stickiness or texture change were seen which was in agreement to the work done by the Amezquita and Brashears, (2002).

In the case of PLa19 and cocktail of PLa10 and PLa19 bactericidal antilisterial activity was observed which was possibly due to the production of bacteriocin like substances which may either generate more bacteriocin or continuously excrete bacteriocin so as to replenish the inhibitor by nutrient depletion or organic acid production (in cocktail) over the entire period of storage. Conversely bacteriostatic antilisterial activity was observed in the PLa10 which was mainly due to the production of organic acids which limits the further growth of the pathogen but cannot decrease the number of \textit{L. monocytogenes}.

When cocktail of LAB isolates were used significant reduction in the growth of pathogen occurred. Hence, we hypothesized that the use of a combined culture of the selected LAB isolates might have a potential synergistic effect enhancing the activity of the bacteriocin, favoured by the stronger acid production by isolates. This is in agreement with the studies reported by Janes et al., (2002); Concha-Meyer et al., (2011) who reported that more effective inhibition against \textit{L. monocytogenes} was observed when combination of two isolates than each strain separately was taken. Duffes et al. (1999) reported the inhibition of \textit{L. monocytogenes} in cocultures with bacteriocin-producing strain of \textit{Carnobacterium} sp. in a simulated cold-smoked fish system during the first 18 days of storage at 4 °C.

According to Montville et al. (1995) cell wall damage in \textit{L. monocytogenes} due to low temperature means that nisin molecules can cross the cytoplasmic membrane more easily causing cell death. Buchanan and Bagi (1997) suggested that the inhibition of \textit{L.}
monocytogenes by bacteriocin producing strains of Carnobacterium piscicola was due to nutrient depletion rather than to the production of bacteriocins because of the rapid growth of organism. In that case, the use of bacteriocin producing LAB could play a role in controlling colonization by pathogenic and spoilage bacteria in food processing facilities (Ammor et al., 2006).

5.8.2 Efficacy of selected LAB isolates for inhibition of L. monocytogenes in ready to eat vegetables

Ready-to-eat fresh vegetable and fruit products demand has continuously increased in the last decades reflecting the consumer's interest for fresh and healthy foods with an easy way of preparation (Trias et al., 2008). Minimally processed vegetables and sliced fruit belong to the low-acid foods, and exhibit a characteristic high humidity. These facts together with the high number of cut surfaces can provide ideal conditions for microbial growth, including foodborne pathogens and spoilage (Ongeng et al., 2006). Various methods such as chlorine washing, irradiation, and modified atmosphere packaging have been applied to preserve and increase the shelf life of meat, fresh vegetables and fruits (Jacxsens et al., 2002; Kim et al., 2005). However, most of these techniques do not show a complete effectiveness. So the preservation technique using bioprotective microorganisms have already shown its potential for practical application in various foods, such as meat (Holzapfel et al., 1995; Vermeiren et al., 2004) and plant derived products (Trias et al., 2008). Lactic acid bacteria (LAB) have been used to preserve meat and dairy products (Stiles and Holzapfel, 1997) and fermented vegetables or fruit juices (Ruiz-Barba et al., 1994).

A study of the effect of LAB isolates on control of L. monocytogenes in fresh vegetable for the period of 14 days was performed. It was revealed that the cocktail of isolates PLa10 and PLa19 were the most effective against L. monocytogenes as 3.6 fold reductions in the pathogen count was observed. In fact, it has been reported that the inhibitory effect of bacteriocins can be enhanced when they are used in combination (Hanlin et al., 1993; Mulet-Powell et al., 1998). Organic acids production and bacteriocin like substances were detected as main inhibitory antilisterial mechanisms. It was observed that the low pH might have exerted a combined antimicrobial effect with the bacteriocins. A reduction in pH has been previously reported as a mechanism for inhibiting food-borne pathogens (Brashears and Durre, 1999; Allende et al., 2007).

Several bacteriocins produced by LAB with a wide spectrum of activity against
bacteria have been earlier reported that are effective against *L. monocytogenes* (Ennahar et al., 2000; Osmanagaoglu, 2007). The spectrum of activity of the bacteriocins showed not only an effect against *L. monocytogenes*, but also against other LAB which are found in fresh fruits and vegetables. Inhibition of other non-pathogenic competitors which may compromise the protection ability of the strain in the site of action or cause spoilage reactions by the bioprotective strain has been reported (Trias et al., 2008) which strongly support our result as no spoilage of vegetables and the growth of other undesirable organisms was observed. Isolates PLa10 and PLa19 have a bacteriolytic mode of action, which is in accordance with work reported for other lactic acid bacterial isolates (Klaenhammer, 1998).

The presence of sugar in the medium enhanced production of the bacteriocins was reported in previous reports (Parente and Ricciardi, 1999) and fresh fruits and vegetables are considered to be the rich in carbohydrates which support the production of bacteriocins like substances by LAB isolate (PLa19) hence imparting bactericidal antilisterial activity in the raw vegetable during the storage period of 14 days.

5.9 Preparation of probiotic product

Soybean is well known vegetarian food, an inexpensive, cholesterol and lactose-free source of protein and calories for human consumption (Scalabrini et al., 1998). Soy has been reported to reduce the risk of postmenopausal symptoms, osteoporosis and prostate cancer (Setchell and Cassidy, 1990). Various attempts have been made to develop soy-based probiotic products as dietary adjuncts which could be substituted for dairy products considering the nutritional content of soy and soy products (Liong and Yeo, 2009; Shah and Otieno, 2007; Wang et al., 2003).

5.9.1 Microbiological and chemical analysis of probiotic product

To exert beneficial effects in the host, it is essential that lactic acid bacteria should be alive and abundant in the product at the time of consumption. Malyoth et al. (1968) indicated that to exert some dietetic and therapeutic benefits, a cultured soymilk should contain at least $10^6$ CFU/ml when consumed. Therefore, viability and survival of lactic acid bacteria in the fermented soymilk drink during course of fermentation and storage was investigated.

Fermented soymilk samples had a viable count above $9.0 \log \text{CFU/ml}$ at the end of fermentation in both the *L. casei* PLa5 and *L. casei* Shirota (control). Soymilk was
considered to be better substrate for the growth of the probiotic strains as growth of the LAB strains were compared in milk and soymilk, soymilk proved that probiotic strains grow more quickly in it than in cow’s milk (Li et al., 2012). It has been demonstrated that probiotics are capable of utilizing sucrose, a major disaccharide in soymilk (Wang et al., 2003).

The texture, physical stability, flavor, and aroma of the fermented soymilk were related to pH (Granata and Morr, 1996). In general, coagulation of sterilized soymilk occurs at pH 5.7 (Chou and Hou, 2000). Previous research has shown that a common problem associated with fermented soymilk is low acidity and flavor intensity (Karleskind et al., 1991). The reported optimum pH of fermented soymilk is 4.2 to 4.3 (Oberman, 1985).

Significant increases in TA (0.01-0.09 %) and decreases in pH (6.79-4.13) in soymilk during fermentation were observed among the two probiotic strains which were similar to the work reported by Rekha and Vijayalakshmi (2008); Wang et al. (2002). This is due to the production of acids (acetic mainly lactic acids) during the fermentation. LABs, being saccharolytic, derived the energy for growth from substrate-level phosphorylation, by converting carbon source to lactic acid and other compounds (Modler et al., 1990).

5.9.2 Effect of prebiotics on the viability of *L. casei* PLa5 during fermentation

Prebiotics are defined as ‘non-digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of colonic microflora’ (Manning and Gibson, 2004). Fructooligosaccharides (FOS) contains 2-10 fructose units linked by glycosidic bonds, while inulin includes a broad range of fructans with chains of 3-60 units (Rossi et al., 2005). Another oligosaccharide with prebiotic properties is maltodextrin. Maltodextrins are malt oooligosaccharides with a degree of polymerisation ranging from 3 to 9 units of maltose and often act as flavour enhancers, fat replacers and bulking agents in foods (Oliveira et al., 2009). Recently, polyols such as mannitol have been included in the prebiotic group owing to their indigestibility properties (Liong and Shah, 2006).

In our studies growth and viability of isolate PLa5 (*L. casei*) was evaluated with all the four prebiotics i.e. FOS, inulin, maltodextrin and mannitol. Maximum growth of *L. casei* PLa5 was observed in FOS and maltodextrin which was similar to the work done by Yeo and Liong (2010). In agreement with our finding Oliveira et al. (2009) demonstrated that the growth of probiotics (*L. acidophilus, L. bulgaricus, L. rhamnosus and B. lactis*)
was stimulated in skim milk supplemented with FOS.

Supplementation with maltodextrin increased the growth of \textit{L. casei} PLa5. Considering that probiotics can produce various glycosyl hydrolases in order to utilise oligosaccharides (Amaretti et al., 2006), we postulate that the enhanced growth of \textit{L. casei} PLa5 in the presence of maltodextrin was probably due to the ability of this strain to produce the enzyme that hydrolyses maltodextrin to glucose for growth.

Supplementation with FOS also increased the growth of \textit{L. casei} PLa5. FOS is a mixture of fructose moieties linked by $\beta$ (2-1) glycosidic bonds with a terminal glucose unit (Rossi et al., 2005). In order to utilise FOS, a strain must possess the $\beta$-fructofuranosidase enzyme to hydrolyse the complex oligosaccharides (Meulen et al., 2004). $\beta$-fructofuranosidase is responsible for the hydrolysis of $\beta$ (2-1) bonds to form sugar monomers prior to fermentation and metabolism by the organism (Liong and Shah, 2006). Thus, it is supposed that the increased growth of our strains in the presence of FOS was probably due to their ability to produce $\beta$-fructofuranosidase enzyme.

5.9.3 Moisture and ash content

The ash content in a product is a reflection of the mineral compositions (Alganesh et al., 2009). This was an indication that all the samples investigated had micronutrients. However, ash content increased after fermentation it may be due to the bioavailability of micronutrients after fermentation (Rekha and Vijayalaksmi, 2010). These results showed that fermentation increases the ash content of the product which may be due to the contribution from fermentation microorganisms as reported by Oyeleke et al. (2012). The soymilk samples after different times of fermentation had high moisture contents which is $>86\%$. It is believed that it could affect the stability and safety of food with respect to microbial growth and proliferation hence the products will require cold storage (Ladokun and Oni, 2014).

5.9.4 Total Carbohydrates

The reduction of carbohydrate content during the fermentation process was possibly due to the breakdown of more complex components by enzymes produced by fermenting microorganisms (Yirmaga, 2013). Scalabrini et al. (1998) found that the stachyose and raffinose (major carbohydrates) content of soymilk reduced after 24 and 48 h of fermentation with LAB to at least half the original concentration. Fermentation might be also associated with the reduction of crude fiber content, which also advanced the
5.9.5 Minerals content

The phytic acid, the major anti-nutritional factor that blocks the availability of minerals in soya bean decreases during fermentation because of the action of phytase enzyme. LAB are the major source of this enzyme, as it hydrolyses phytate into myo-inositol and phosphate during fermentation (Nam and Man, 2009). There was an increase in calcium and magnesium levels and a decrease in iron in fermented soymilk compared to the control. Similar results were observed by Lopez et al. (2000); Rekha and Vijayalakshmi (2010) in whole-wheat flour and soymilk, where phytic acid was degraded by LAB leading to the increase in Ca and Mg bio-availability.

5.9.6 Determination of enzymatic activity of fermented soymilk

5.9.6.1 Proteolytic activity

Probiotic organisms are rich in proteolytic activity. This activity was determined by measuring free NH₃ groups using the o-phthaldialdehyde (OPA) method. An increase in proteolytic activity was observed during the time of fermentation in our experiment which was found to be similar to the pattern observed by Donkor et al. (2007); Omafuvbe et al. (2001); Rekha and Vijayalakshmi (2008). Some amino acids and peptides were used by the organisms during fermentation for cell growth and survival (Nielsen et al., 2001). Therefore high proteolytic activity of these organisms in soymilk may have contributed to appreciable cell growth, in addition to their ability to metabolize disaccharides present in soymilk as energy sources.

5.9.6.2 β-Glucosidase and β-Galactosidase activity of the L. casei PLa5 in fermented soymilk

β-Glucosidases (β-d-glucoside glucohydrolase, EC 3.2.1.21) comprise a heterogeneous group of enzymes that are able to cleave the β-glucoside linkages of di-oligosaccharide or other glucose conjugates. Probiotic micro-organisms have been found to possess β-glucosidases (Otieno et al., 2005) and can play an important role in improving the biological activity of soymilk. Previous study has demonstrated that fermentation of soymilk by LAB can enhance the bioconversion of isoflavone glucosides to biologically active aglycones by the action of β-glucosidase from the bacteria (Yeo and
Liong, 2010). In the present study, all isolates possessed varying levels of intracellular β-glucosidase specific activity ranging from 1.19 to 3.40 U/mg dcw and the highest activity was observed at 24 h of fermentation. There was an increase in β-glucosidase activity up to 24 h followed by a decline as fermentation progressed and same pattern of β-glucosidase activity was observed by Sumarna (2010).

The two endogenous enzymes (β-glucosidase and β-galactosidase) were responsible for the hydrolysis of β-1-6-glycosidic bonds in the conjugated isoflavone glycoside forms (Otieno et al., 2007) which are predominant in unfermented soymilk. Determination of the activities of the two enzymes is therefore important as an indicator of the hydrolysing potential of the micro-organism in releasing the bioactive isoflavone aglycone forms (Otieno and Shah, 2006). On the other hand, β-galactosidase activity (0.03-0.14 U/mg dcw) was much lower than β-glucosidase in the soymilk during fermentation time.

It is not clear why β-glucosidase activity would be high (more than 3 fold) as compared to that of β-galactosidase in soymilk as they appear to hydrolyse similar bonds. One possible reason for the lower β-galactosidase activity could be related to the concentration of β-D-galactopyranosides (such as lactose) found in the soymilk. As there is no lactose in soymilk, this could be a direct limiting factor in exhibiting β-galactosidase activity and there is no substrate challenge limiting production of the enzyme. β-galactosidase being in the crude form is of low specificity hence could possess some β-glucosidase activity. As there is a certain occurrence of β-D-glycopyranoside substrate such as glycosides in soymilk, it could stimulate higher β-glucosidase activity than β-galactosidase activity (Otieno and Shah, 2006).

**5.9.7 Determination of antioxidative activity**

Free radicals and other reactive oxygen species are generated by exogenous chemicals or endogenous metabolic processes in food systems or the human body. The radicals may cause oxidative damage by oxidizing biomolecules and results in cell death and tissue damage (Kehrer, 1993). Atherosclerosis, cancer, emphysema, cirrhosis, and arthritis have been correlated with oxidative damage (Kehrer, 1993; Jacob, 1994). Therefore, oxidative damage plays a significant pathological role in human disease. However, ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on the human body (Lin and Yen, 1999). Beans contain phenolic compounds that exhibit antioxidative activity (Drumm et al., 1990). Previous
research has demonstrated that the antioxidative activity of fermented soyfoods, such as *miso*, *natto*, and *tempeh* was remarkably stronger than unfermented steamed soybeans (Berghofer et al., 1998; Sheih et al., 2000).

### 5.9.7.1 Hydrogen peroxide (H$_2$O$_2$) scavenging effect

Hydrogen peroxide can be generated in biological and food systems. Being a non-radical oxygen-containing reactive agent, it can form a hydroxyl radical (the most highly reactive oxygen radical known) in the presence of transition metal ions and participate in free-radical reaction (Halliwell et al., 1995). Soymilk without fermentation (control) was noted to exhibit an H$_2$O$_2$ scavenging effect of 13.89%. On the other hand, scavenging effect keep on decreasing till the fermentation of 48 h. This may be attributed to the formation of H$_2$O$_2$ by the LAB as suggested by various investigators (Collins and Aramaki, 1980; Kaneko et al., 1985; Teraguchi and Ono, 1987). These investigators indicated that some lactic acid bacteria might produce NADH oxidase that forms H$_2$O$_2$ in oxidizing NADH. Since LAB is devoid of catalase, a key enzyme for the breakdown of H$_2$O$_2$, thus it has to rely on enzymes such as NADH oxidase and NADH peroxidase to scavenge environmental oxygen (Amanatidou et al., 2001).

### 5.9.7.2 DPPH radicals scavenging assay

DPPH is a stable lipophilic free radical that is used to determine the proton-scavenging activity of the various soybean extracts. Proton radical scavenging is reportedly an important mechanism for antioxidation (Wu et al., 2011). The decrease in absorbance of DPPH radicals is caused by antioxidants through the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radicals by hydrogen donation. This is visually noticeable as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the activity of antioxidants (Alkan et al., 2008).

The DPPH radical scavenging activity increases (3.65-43.65 %) with fermentation time as compared to the soymilk (control). These results suggested that each fermentation extract might react as free radical scavengers by contributing hydrogen from their phenolic hydroxyl groups thereby forming stable free radicals that do not initiate or propagate further oxidation of lipids.

It was believed that the degradation of polymeric phenolic structures after being fermented by microorganisms may explain the increase in soluble phenolic content.
thereby decreasing antioxidant activity (McCuea and Shetty, 2005). Kevers et al. (2007) explained that antioxidant capacity (DPPH) decrease drastically during storage that at low temperature due to reduction in phenol content.

**5.9.7.3 Polyphenol and flavonoid content**

Polyphenols are present in considerable amount in soymilk. These anti-nutrients interfere with mineral bio-availability and digestibility of proteins (Serraino et al., 1985) and carbohydrates. Reduction in polyphenol content through fermentation may imply improve digestibility of proteins and carbohydrates and also enhance bioavailability of minerals in the fermented product, thereby improving the nutritive value of the product (Sindhu and Khetarpaul, 2003). Probiotic lactic acid bacteria when grown in soymilk have the ability to utilize phenolic components producing phytase enzyme and reduce the polyphenol content after fermentation. Polyphenol content decreased from 14.01 mg/100 ml to 6.01 mg/100 ml during fermentation period of 48 h. The diminishing effect of fermentation on polyphenols may be due to the activity of polyphenol oxidase present in the food grain or microflora. Similar observation was reported by Rekha and Vijayalakshmi (2008); Subrota et al. (2013). Soybean has been found to contain high contents of \( \tau \)-tocopherol, isoflavones, flavonoids and anthocyanins which possess biological and antioxidative activity (Correa et al., 2010; Jeng et al., 2010; Kumar et al., 2010). Due to the action of \( \beta \)-glucosidase produced by LAB during fermentation which catalyses the release of total phenolics and flavonoids from the soybean substrate leading to an increase in the content of flavonoid compounds (Dajanta et al., 2013). Increase in flavonoid content (1.46-3.52 mg/ml) was observed in 24h of fermentation which was in accordance with the observations of Lee et al. (2008); Juan and Chou (2010).

**5.9.8 Viability of LAB during storage**

Probiotic products should contain minimum count of \( 10^6 \) CFU/ml of viable bacteria at the time of consumption (Tamime et al., 2005). To maintain beneficiary effect of probiotic products, it is important to demonstrate a good survival of bacteria in the products throughout the shelf-life of the products. It is usually considered that the acceptable final population of the probiotic organisms in fermented product at the end of the shelf life, should be anywhere between 5-8 log CFU/ml (Svensson, 1999). Stable LAB counts and pH was observed during the 14 days storage at refrigeration temperature. It is concluded that the viability and stability of *L. casei* PLa5 in fermented product could be maintained at 4 °C for 14 days storage.