ABSTRACT: In present study, Lactobacilli were isolated and characterized from various milk products, where out of 25 isolates, 5 lactobacilli strains were screened for probiotic properties viz. tolerance to low pH, high bile salt concentration, cholesterol assimilation, cell aggregation and heat tolerance etc. All the 5 isolates showed comparable cell-surface hydrophobicity, and inhibitory activity against B. cereus, E. coli, S. aureus, and Salmonella spp. However, the values for L. plantarum M5 were significantly higher than that of other isolates. Besides these attributes, it could also withstand pH 2.5, and 50°C up to 20 min. The results of storage studies showed that storage at -20°C and 5-7°C had no effect on viable count, and it had good viability after 6 weeks of storage. The results suggest that L. plantarum M5 and L. helveticus L3 could be exploited for potential probiotic foods for better nutrition and health.

KEYWORDS: Functional Foods, Gut Health, Probiotics, Lactic Acid Bacteria and Lactobacillus

INTRODUCTION

Probiotics are gaining a significant interest for human consumption due to the growing evidences associated with their use to alter the composition or metabolic activities of the gut microbiota, or to their benefits health effects such as improvement of the normal gut microflora, prevention of infectious diseases and food allergies, reduction of serum cholesterol, anti-carcinogenic activity, stabilization of the gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms and improvement in the digestion of lactose in intolerant hosts (Roberfroid, 2000; Ouwehand et al., 2002; Salminen et al., 2005; Nagpal et al., 2007; 2010). Moreover, these days’ people are becoming more aware by adopting preventive measures rather than curative ones. Although, several probiotic strains have been identified, and probiotic products using these cultures are available on the market’s shelves, there is a need to investigate the biodiversity of more potential probiotic wild strains from the environmental sources or indigenous food sources so as to observe their natural occurrence in the society.

In India, especially in northern states viz. Haryana and Punjab, annual milk production as well as consumption is quite high, and there are many indigenous varieties of dairy products that are consumed as an integral part of daily diets. In Punjab state, annual milk production is about 51.5 MT (about 10% of total milk production in India), and per capita availability of milk is also the highest in India (unpublished data). Therefore, the present investigation was executed in Jalandhar where about 25.5 liters of milk is consumed per family per month (unpublished data). Still, not much information is available regarding occurrence of probiotic lactobacilli in food or milk products in Jalandhar or nearby places of Punjab. Hence, the present investigation was aimed to explore the occurrence of such beneficial microbes in these regions where milk and milk products are an integral and prevalent part of daily food habits. Additionally, successful isolation of potential indigenous strains of probiotic lactobacilli could provide an opportunity to exploit these microbes for making probiotic products that could prove to be a natural remedy for a population of higher age groups to treat disorders such as diabetes, lactose intolerance, and dysentery.

MATERIALS AND METHODS

Isolation of lactobacilli

The milk and milk product samples were procured from the local markets of Jalandhar and nearby cities of Punjab, viz. Phagwara and Kotkapura, in sterile plastic bags and homogenized by dissolving in 100 ml of sterilized phosphate buffer saline. Samples from all the sources were diluted serially 10-fold in PBS and then
inoculated on deMan Rogosa and Sharpe (MRS, Hi-Media) agar plates by pour plate method (Awan and Rahman, 2005). MRS agar plates were incubated at 37°C for 48 hours anaerobically. Morphologically distinct and isolated colonies were picked and transferred to new MRS agar plates by streaking. Finally, pure colonies were obtained.

**Identification of Lactobacillus species**
Macroscopic appearance of all the colonies was examined for cultural and morphological characteristics by Gram’s staining. Size, shape, color and texture of the colonies were recorded. Bacterial isolates were tested for catalase production by catalase test and by growth at 15°C and 45°C. Sugar fermentation test was performed for acid production (Harrigan and McCance, 1976). Identification of genus and species was confirmed by performing various biochemical tests (Table 1), as mentioned in Bergey’s Manual of Systematic Bacteriology using a standard commercial identification systems viz. API-50 CHL (Biomerieux®, France) and Pibwin Software (Dr. Trevor Bryant, University of Southampton; File Version: 4.1.222.0).

**Screening of isolated Lactobacillus species for probiotic attributes**
To confirm the probiotic attributes, the isolates were subjected for tests recommended as per WHO standards (FAO/ WHO, 2002; Ashraf et al., 2009)

**Cell Surface Hydrophobicity:** The method of Rosenberg et al. (1983) with slight modification was adopted to measure the cell surface hydrophobicity. The bacterial cells were grown in MRS broth at 37°C for 18 h and harvested. The bacterial cell pellet was washed twice with PBS buffer at 10,000 rpm for 10 min, and resuspended in buffer. The absorbance was adjusted to 0.8 at 600 nm using spectrophotometer. Three ml of the bacterial suspension was added in acid washed test tubes followed by addition of 1 ml hexadecane and then incubated at 37°C for 10 min. The two phases became separated and again mixed well. The mixed phases were again kept at 37°C for 1 h for phase separations. The aqueous phase was gently taken out with the help of micropipette and absorbance of the aqueous phase was measured at 600 nm. The fraction of adherent cell was taken as percent decrease in absorbance of the aqueous phase after mixing and phase separations relative to that of original suspension.

\[
\text{% Surface Hydrophobicity} = \frac{(\text{Abs } \text{Final})}{(\text{Abs } \text{Initial})} \times 100
\]

**Antimicrobial Activity:** The antimicrobial activity of the isolated strains was determined by the method introduced by Pinto et al. (2006). The culture was grown in MRS broth for 18-24 h at 37°C and the cells were removed by centrifugation at 12000 rpm for 20 minute at 5°C. The culture supernatant thus obtained was sterilized by passing through a sterile 0.22mm membrane filter. 20 ml of the fresh culture of indicator bacteria was grown for 16-18 h in BHI at its optimum growth temperature was mixed with 5 ml of soft agar melted and cooled to 45°C and poured into Petri-plates containing 15-20 ml MRS agar. The soft agar was allowed to solidify in the plates followed by transfer to 5°C for 1 h before wells were made in the agar. The wells were then filled with 100 μl of sterile supernatant. The plates were refrigerated for 3-4 h to facilitate the diffusion of supernatant without growth of the indicator strains, which was followed by incubation at 37 °C for 24 – 48 h. A clear zone around the well was considered as positive inhibition.

**Bile Salt Hydrolysis:** It was done by the method of Tsai et al. (2005). The selected cells were grown in MRS broth at 37°C for 18-24 h. 40 μl of the culture was overlaid on to the agar medium containing the bile salts (Sodium deoxycholic acid and Sodium taurocholate at a concentration of 0.3% each), and was incubated at 37°C for 5 days. BSH activity was considered when halos of precipitated free bile acids (cholic acid or deoxycholic acid) surrounding the colonies were formed due to hydrolysis and acidification of the medium.

**Cholesterol removal:** Freshly prepared MRS broth was supplemented separately with 0.3% bile salts viz. cholic acid and taurocholic acid, and was inoculated with each isolate at 1% level and incubated anaerobically at 37°C for 24 h. After incubation, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a modified colorimetric method as described by Rudel and Morris, (1973).

**Acid Tolerance:** The pH of the MRS broth was adjusted to 1.5, 2.5, 4.5 and 6.5 with 1 N HCl. Overnight grown cultures of lactobacilli were inoculated in MRS broth at different pH and incubated at 37°C. One ml of the culture was taken after 1, 2, 3 and 4 h, and cell count was measured at 600 nm.

**Cell Aggregation:** The cells grown overnight in MRS broth at 37°C were harvested and the cell pellet was washed twice with PBS and resuspended again to a final absorbance of 0.5 at 600 nm. The suspension was centrifuged and the pellet was resuspended by vortexing in equal volume of broth removed at first stage after the overnight growth. The mixture was allowed to stand at 37°C for 2 h. After incubation, 1 ml of the upper suspension was taken to measure the absorbance against culture supernatant as the blank. The percent difference between the initial and final absorbance gave an index of cell aggregation that was expressed as follows:

\[
\text{Aggregation (\%)} = \frac{(\text{Abs } \text{Final})}{(\text{Abs } \text{Initial})} \times 100
\]

**Heat Tolerance:** The cells grown overnight in MRS broth at 37°C were incubated at 45°C, 55°C, 60°C, 65°C for 20 min, and the samples were plated on MRS agar and incubated overnight at 37°C. The plates were observed for the colonies next day.

**Bacterial viability during storage**
The storage viability of isolated Lactobacilli was recorded weekly at -20°C, refrigeration (5-7°C) and room temperature. The test tubes were inoculated with 10^6 CFU of each culture suspension. These inoculated test tubes were stored at -20°C (with 10% v/v glycerol), 5-7°C and room temperature for 6 weeks. The growth was monitored weekly by plate count method.
Reference Strain

Reference strain of *L. casei* was obtained from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana, India.

Statistical Analysis

The data were expressed as mean (± standard deviation) of three replicates using randomized factorial design of analysis of variance (ANOVA) according to the General Linear Models procedure of SYSTAT Version 6.0.1 (1996, SPSS Inc.) for test of significance (P<0.05), as per the method of Snedecor and Cochran, (1980).

RESULTS AND DISCUSSION

Collection of samples and isolation of lactobacilli

Initially, a total of 65 samples of milk, curd, cheese, dahi and lassi were collected from different places of Jalandhar, Phagwara, Kotkapura and nearby locations of Punjab. From these samples, 217 colonies that showed appearance typical of lactobacilli, were picked from the agar plates, and were subjected to gram staining. Of these 217 colonies, only 91 showed the presence of gram-positive rod-shaped cells (data not shown here). These colonies were transferred into MRS broth tubes and were again checked for the presence of gram-positive, non-sporing, non-motile and catalase-negative rods, after which, only 28 colonies confirmed the results (Table 1).

Biochemical identification of lactobacillus isolates

These selected 28 isolates were further subjected to different genus-specific and species-specific biochemical tests for confirming the identification (Table 1). All the 28 samples were Catalase-negative, and did not reduce nitrate; while 20 cultures showed positive results for Arginine hydrolysis and 18 cultures were positive for the production of gas from glucose. All the isolates were able to grow at 45°C and were positive for Bile Esculin hydrolysis. Fermentation of 6 different sugars produced varying results. Among all 28 isolates, 22 cultures were positive for sucrose, 16 cultures were positive for Mannitol, 21 were positive for Cellobiose; while 14 cultures showed positive result for Arabinose, 18 cultures showed positive results for Mellobiose and 20 cultures were positive for Trehalose. NaCl tolerance was carried out at three different concentrations of NaCl i.e. 2, 4 and 6%.

Genus and species specific identification was done by comparing the results with those mentioned in Bergey’s manual (Mundt, 1986) and Pilbwin Software (Dr. Trevor Bryant, University of Southampton; File Version: 4.1,222.0). As per the results obtained, 15 isolates were tentatively identified as *Lactobacillus plantarum*, 6 isolates each were of *Lactobacillus brevis* and *L. casei*, and one isolate belonged to *Lactobacillus helveticus*. It was observed that curd and cheese samples primarily contained *Lactobacillus plantarum*, *L. brevis* and *L. casei*, and milk had only *L. plantarum* and *L. casei*, whereas lassi contained *L. plantarum*, *L. casei* and *L. helveticus* (Table 1).

Screening of isolates for Probiotic Attributes

To qualify as a probiotic, a microorganism should show all or some of the attributes so that it can survive the acidic pH in the stomach, and multiply and colonize in the intestine of the host. Therefore, after the genus and species-specific identification, several important properties like cell-surface hydrophobicity, bile salt hydrolysis/ tolerance, acid tolerance etc. were studied to assess the potential of these isolates as potential probiotic cultures. These attributes in a culture would enable them to survive the harsh acidic environment and toxic bile salts while passage to the intestine and bind with the gut epithelial layer due to non-specific interactions like hydrophobic effect and specific interactions between the cell surface carbohydrate moieties, surface receptors or cell surface binding proteins.

Cell-surface hydrophobicity: An important aspect, which is widely considered in the bacterial adhesion, is the cell surface hydrophobicity (Rosenberg et al., 1983), which is based upon the nature of their cell surface involved in interaction with phagocytes, adherence to non-wettable solid surfaces, and partitioning at liquid: liquid and liquid: air interfaces. Hence, all the samples were tested for cell surface hydrophobicity (Fig. 1) by using hydrocarbon n-hexadecane, which is widely used for evaluating the surface hydrophobicity of microbial cells. Isolates showed a variation in cell-surface hydrophobicity of which maximum was 55% and lowest 13.8%. The hydrophobicity of reference strain was observed to be 54.4%. Five isolates i.e. C2, L1, L3, M5, and Ch1 showed the maximum hydrophobicity of 55, 49.7, 54, 48.4 and 47.4%, respectively, which was comparable to the hydrophobicity of the reference strain. However, some isolates viz M4, M7, Ch2, Ch3, and Ch6 showed very low hydrophobicity of 13.8, 17.3, 22, 22 and 21.2%, respectively. Cell surface hydrophobicity of *L. johnsonii* and *L. acidophilus* have earlier been reported as high as 74-95% and 23-88%, respectively, while *L. plantarum* from goat have shown surface hydrophobicity of 47-69% depending upon the solvent used (Drakslor et al., 2004). On the other hand, some strains of lactobacilli have shown surface hydrophobicity as low as 3%. Though, adhesion and colonization are prerequisite events for a probiotic organism, these attributes greatly depends upon factors like presentation, orientation, substratum, host cell receptor specificity and microorganism cell surface proteins. Hydrophobic strength could be involved in bacteria-epithelial cell interactions and therefore, it serves a good index of binding of a potential probiotic microorganism in the gut. However, it has also been observed that lactobacilli with similar hydrophobicity values may or may not have adhesive property depending upon the microbial species and strain type. Using hexadecane, percentage cell surface hydrophobicity has been reported from 5 to 95% for different probiotic bacteria (Pinto et al., 2006), suggesting that hydrophobicity may not be the only criteria but an important component in a complex interplay between several factors that enable a microorganism to bind and colonize the
host gut for successful propagation. Therefore, all the 5 isolates having maximum hydrophobicity along with the reference strain of L. casei were selected and subjected to further probiotic tests.

**FIGURE 1.** Cell-surface hydrophobicity of different Lactobacillus isolates. Values are mean ± S.D. (n= 3).

**Antimicrobial Activity:** The five isolates viz C2, L1, L3, M5, and Ch1 that exhibited high hydrophobicity were tested for antimicrobial activity using four indicator bacteria i.e. E. coli, B. cereus, S. aureus and Salmonella spp. (Table 2). The zone of inhibition (diameter) varied from the minimum of 10.9 mm to the maximum of 14.5 mm. Isolates C2, L3, M5 showed the maximum zone of inhibition of 14.2 mm, 13.8 mm, and 13.4 mm, respectively, against the Salmonella spp. which was followed by L3 (13.3 mm) and M5 (12.6 mm) against S. aureus, while isolate C2 showed 13.7 mm against E. coli. Isolates L1 and Ch1 showed the lower range of zone of inhibitions against all the indicator bacteria. GI tract is the major port of infectious diseases in animals; therefore, it is beneficial for animals to have a protective layer in the gut in the form of antimicrobial agents secreted by body or other beneficial microflora residing in the GI tract. The GI tract contains a large number of bacterial species (~500 microbial species), and some of them have important health protecting and promoting effect (Bengmark, 1998). Enteric LAB is one of the most important groups of bacteria helping to protect the host system against external invaders. Therefore, antibacterial activity is a desirable attribute for selection of probiotic organism. The ability to produce certain proteinaceous antimicrobial substances, known as bacteriocin, is an extremely beneficial property and hence can be used as a bonus for the probiotic culture to enhance its commercial value. These compounds inhibit related and unrelated organisms, thus providing an upper hand not only in survival in the gut environment but also causing a shift in the microbial balance towards their side. Apart from the bacteriocins, LAB produces lactic acid, acetic acid and hydrogen peroxide (Klaenhammer, 1993) that can serve as potent antibacterial agents against pathogens as well as non-pathogens. Among lactobacilli, L. acidophilus, L. gasseri, L. casei, L. reuteri and L. plantarum showed beneficial effect such as suppression of undesirable and pathogenic microorganism by competitive exclusion and antagonism in the GI tract and other organs (Korasapati, 1998). L. plantarum has been shown to produce antibacterial peptide known as plantaricin (Telemo et al., 1999), which is primarily active against Pseudomonas, Aeromonas sobria and Aeromonas cavice. A strain of L. plantarum isolated from grass silage showed broad-spectrum antifungal activity against food- and feed-borne filamentous fungi and yeasts (Broberg et al., 2002). L. plantarum in combination with prebiotics has been reported to strongly inhibit the growth of pathogens (Gibson, 2004).

In present study, antibacterial studies were carried out against only a few pathogens; nevertheless the results indicated the potential of isolates L3 and M5 to possess inhibitory activity, which is a much desired attributes of enteric or vaginal LAB for prophylactic and therapeutic applications against the common pathogens. The results were also comparable with those of Kelley et al. (2003) and Wilson et al. (2005) that showed the antibacterial activity of L. plantarum against Listeria monocytogenes, E. coli and range of other pathogens. Therefore, isolates L3, M5 and C2, showing maximum zone of inhibition against each indicator bacteria, could serve as a potential probiotic organism with health-promoting functional attribute like antimicrobial activity against several pathogens. Hence, based on the results, these three isolates were further selected for bile salt hydrolysis test.

**Bile Salt Hydrolysis:** The selected isolates were also tested for BSH activity at different concentrations i.e. 0.3, 0.5, 1.0, 2.0 % of bile salts (Table 3). Isolate M5 and reference strain were positive for all the concentrations while, L3 and C2 were positive upto 1% of bile salt concentration. L. acidophilus, L. fermentum as well as bifidobacteria (Tahri et al., 1996; Pereira et al., 2003) have been observed to remove cholesterol much more efficiently from the medium in the presence of bile salts. Bile salts are water-soluble end product of cholesterol and are synthesized in the liver during normal enterohepatic circulation. Conjugation increases the aqueous solubility of bile salts under physiological conditions. Therefore, deconjugation can lead to decreased solubility and hence lower reabsorption in the enterohepatic system, thereby resulting in increased demand for cholesterol as a precursor of bile salts. The clearance of the cholesterol leads to hypocholesterolemia and hence improving the health of the host suffering with cardiovascular diseases. Lactic acid bacteria with BSH activity are prescribed in prophylactic and therapeutic applications against the common pathogens (Gibson, 2004).

**Cholesterol assimilation:** Since, high level of serum cholesterol has been associated with risks of coronary heart disease, the use of
probiotic bacteria in reducing serum cholesterol levels has attracted much attention. Levels of cholesterol assimilation during 24 h of growth of the strains are shown in Fig. 2. Cholesterol removal varied among strains and ranged from 9.1 to 28.7 μg/ml. In general, both *L. plantarum* (M5) and reference strain *L. casei* assimilated more cholesterol in the presence of cholic acid as well as taurocholic acid, compared to the control. In broth containing cholic acid as well as taurocholic acid, overall cholesterol removal was observed to be higher for isolate M5 compared to those of *L. casei*, L3 and C2. Isolate M5 assimilated more than 28 μg/mL cholesterol in the presence of cholic acid, and was better than isolates L3, C2 and *L. casei* which assimilated 23.8, 14.3 and 27.5 μg/mL cholesterol.

Cholesterol assimilation in the presence of different bile sources showed good relationship with bile tolerance of the strains studied (Table 3). Strains showing greater tolerance towards bile salts exhibited overall higher cholesterol assimilation in the presence of cholic acid and taurocholic acid. Also, most strains exhibited better growth in the presence of cholesterol, indicating that cholesterol stimulated their growth.

Growth observed in the presence of different bile sources suggested that conjugated bile, taurocholic acid was more inhibitory towards strains of lactobacilli as compared to deconjugated bile i.e. cholic acid. The resistance of the lactobacilli towards deconjugated bile may be due to the fact that conjugated bile salts have greater solubility and detergent activity, and may, therefore be more toxic than their deconjugated counterpart. This was supported by the fact that the cholic acid added to the fermentation broths was far less soluble than taurocholic acid based on the solubility index. Taurocholic acid was not accumulated by *Lactobacillus salivarius* JCM 1044 due to its hydrophilicity (Kurdi et al., 2000). Such preference was also supported by previous reports (Kurdi et al., 2000; Yokota et al., 2000) that showed *Lactobacillus* species actively accumulated cholic acid in an ATP-dependent manner, or when they were energized by glucose. Our results suggest that under high concentration of conjugated bile, strains of *L. plantarum* and *L. casei* are likely to survive best. Three possible mechanisms for removal of cholesterol from media by lactobacilli may be proposed: assimilation of cholesterol during growth, incorporation of cholesterol into the cellular membrane of cells, and binding of cholesterol to cell-surface.

**Acid Tolerance:** The isolates C2, L3, M5 were tested for acid tolerance by subjecting to a range of pH 1.5, 2.5, 4.5, and 6.5 (Fig. 3). Acid tolerance is one of the major attributes of a probiotic organism. They must survive the harsh acidic environment in the gut to remain there for a while in good number and express their health promoting functions (FAO/WHO, 2002). Lactobacilli that are to be used as dietary adjunct, must survive the pH of human stomach. In present study, all the isolates along with the reference strain of *L. casei* were exposed to different pH and for 4 hours and readings were observed after the interval of one hour. The results indicated that the isolate C2 took little longer either to survive the low pH shock or to adapt (which may involve expression of genes involved in acid tolerance) to low pH. Isolates L3 and M5 also showed similar rate of growth as shown by known probiotic culture *L. casei*. At appreciably higher pH value of 4.5, the rate of growth of L3 and M5 was similar to that of standard probiotic culture of *L. casei*. The results were found consistent with those reported by Lankaputhra and Shah (1995); it showed a strain dependent acid-tolerance in lactobacilli and bifidobacteria at a pH level of 1.5 to 3.0, with viability ranging from 6 log cfu/ml to 8 log cfu/ml of culture for different strains.
Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant in order to survive in the human gastrointestinal tract. Survival at pH 3.0 for 2 h and in bile concentration of 1000 mg/L is considered optimum for acid and bile tolerance for probiotic strains (Usman and Hosono, 1999). All strains of lactobacilli studied survived the acidic condition and bile concentration to a variable extent. Similarly, all strains had varying capabilities to remove cholesterol in-vitro. Strains of lactobacilli used in this study also showed varying levels of viability at pH 2.5 and 1.5 (Fig. 3). Isolates M5 and L. casei survived best under the acidic conditions, while viability of strains C2 and L3 was lower. In general, L. plantarum M5 survived better under acidic conditions than L. casei. Moreover, since these two isolates also showed good bile tolerance and high cholesterol removal from media, these strains could certainly be exploited as potential candidates for use as a dietary adjunct to lower serum cholesterol in-vivo.

**Cell Aggregation:** It has been suggested that cellular aggregation could be important to promote the colonization of beneficial microorganisms, as suggested for lactobacilli in the GI or urogenital tract (Tomas et al., 2005). The cellular aggregation help not only in the colonization but also in providing a protective shield to the host system due to formation of a bacterial biofilm (Rickard et al., 2003) over the host tissue and thus protecting against the infection by pathogens. Therefore, the three selected isolates viz. C2, L3 and M5 were further tested for their cell aggregation ability (Table 4). The cell aggregation of all the three isolates varied from 52 to 60% that was comparable with that of reference strain that showed aggregation of 58%. The maximum cell aggregation of 60% was showed by L3 that was 2% more than that of L. casei. It has been reported that some bacteria like L. gaseri (Ventura et al., 2002) produce an aggregation-promoting proteins. Similar factors have been reported from other species like L. johnsonii, L. reuteri, chicken lactobacilli (Roos et al., 1999; Ventura et al., 2002). L. plantarum 299v have been shown to adhere to enterocytes of the human intestinal mucosa and colonize the human gut mucosa, and also to the ileal epithelial cells of albino rat (Oyetayo et al., 2004). Successful probiotic bacteria should be able to colonize the mucosal surfaces, at least temporarily, and to prevent the attachment of pathogens such as E. coli (Lee et al., 2000), Helicobacter pylori (Mukai et al., 2002) and other intestinal or food-borne pathogens (Todoriki et al., 2001).

**Heat Tolerance:** The isolates were also subjected to different temperatures i.e. 45°C, 55°C, 60°C and 65°C for heat tolerance ability in order to check their technological attributes (Table 5). All the three isolates showed varying results. The colonies of isolate C2 were observed only at 45°C, whereas L3 showed positive results at 45°C and 55°C. Heat tolerance capacity of M5 was upto 60°C while the reference strain tolerated heat up to 65°C.

**Storage studies**

The results of storage studies showed that storage at -20°C and 5-7°C had no effect on viable count of all isolated lactobacilli species, and all species had good viability after 6 weeks of storage. Weekly, little decline in the viable count of all the species was observed when stored at -20°C or at refrigeration temperature (Fig. 4). However, as anticipated, a decline was observed in viable counts of all lactobacilli after 6 weeks of storage at room temperature. This high viability during storage under freezing conditions could be helpful while exploiting these lactobacilli as probiotics in various industries or institutes etc. The results are well in agreement with the reports of Pascual et al. (1999) and Ashraf et al. (2009).
CONCLUSION
The present investigation appears to be the first attempt to explore the occurrence of wild probiotic strains of lactobacilli such as *L. plantarum*, *L. helveticus* and *L. casei* etc. among various indigenous fermented milk products prevalent in this northern part of the country where milk production as well as consumption is highest. Also, the occurrence of such isolates in these products could reflect, to some extent, the considerable existence of these beneficial microbes in the gut of human population within these northern regions of the country. Moreover, these wild strains of lactobacilli were also found to exhibit significant probiotic attributes. Especially, isolates M5 (*L. plantarum*) found to exhibit significant probiotic attributes. Especially, country. Moreover, these wild strains of lactobacilli were also accumulated spontaneously, driven by membrane pH in many lactobacilli. *Journal of Bacteriology* 182, 6525-6528.


