Chapter 6

SUMMARY AND FUTURE SCOPE
The present research work entitled "Characterization of probiotic cultures from fermented foods of Himachal Pradesh and their efficacy against colonization by Cronobacter sakazakii in Caenorhabditis elegans model system" was carried out with an aim to isolate and identify new probiotic strains from traditional fermented food products of Himachal Pradesh and to check their efficacy against infection of C. sakazakii in Caenorhabditis elegans model. The major findings reported in this thesis are summarized below:

The study targeted milk and cereal based food products for the isolation of probiotic lactic cultures. For this, a total of 150 samples (fermented cereal-based dough and fermented dairy samples) were collected from different places and districts of Himachal Pradesh. The primary isolation was done on MRS agar where from these samples we obtained 101 distinct isolates based on their colony characteristics. The colonies were opaque, white and creamish colonies. All these isolates were identified as Gram-positive rods or cocci, facultative anaerobes and catalase negative. Subjecting to safety attributes depicting hemolytic activity on sheep blood agar plates, out of 101, 51 isolates were found to be non-hemolytic as these isolates did not produce any zone of clearance on blood agar plates. Rest of the isolates resulted in hemolytic reaction. The non-hemolytic isolates were further screened for their survival under GI tract conditions which is a prerequisite for a probiotic candidate.

Out of 51 isolates 15 isolates were able to pass through the acidic pH of stomach and bile salt conditions of intestine. These 15 isolates were further investigated for simulated GI tract conditions and further checked for in vitro and in vivo probiotic attributes as per joint protocols given by “Department of Biotechnology (DBT), Govt. of India and Indian Council of Medical Research (ICMR), Govt of India”.

These fifteen isolates were subjected for identification using standard biochemical assays and molecular approaches targeting 16SrRNA region. These isolates were identified using the 16S rDNA region: Brevibacillus thermoruber (three strains), Lactobacillus gastricus, Brevibacillus aydinogluensis, Enterococcus sp., L. paracasei, Weisella confuse, Lactobacillus fermentum (three strains), L. plantarum (two strains), and Pediococcus acidilactici (two strains).
The most promising strains (14 strains) were identified after testing their resistance, by using subtractive screening, to digestive gastrointestinal system barriers (acid and digestive fluid salts). The isolates were screened for their survival at highly acidic conditions (pH 2.0), digestive fluid salts (1%), and pancreatin (1mg/L) for different time intervals. The survival was determined using viable cell count method.

The cultures were also tested for their adhesion potential using in vitro methods for cell autoaggregation, cell surface hydrophobicity, and adhesion to Caco-2 cell line and mucin. All the isolates were able to adhere to Caco-2 cell lines and resulted in varying degree of autoaggregation and hydrophobicity to n-hexane. Among the tested strains, the maximal tolerance to a simulated gastric environment was observed in *L. paracasei* CD4 and *L. gastricus* BTM7 with higher scores in adherence studies.

After confirming the probiotic activities of these strains, the investigation was done to analyse their benefits towards functioning of *C. elegans* (*Caenorhabditis elegans*) as in vivo model. The protective effect of probiotics was assessed by increase in life expectancy of worms by colonizing inside the bowel. All the fifteen cultures were administered individually to age synchronized worms and their impact on mean life expectancy of *Caenorhabditis elegans* was calculated. The impact on pharyngeal pumping, normal reproduction and chemotactic conduct (binary choice assay) was conjointly determined. The colonization potential of these probiotics was also determined at different time intervals after the administration of the probiotics to the worms using microscopic fluorescent observations and population of microbes. The examination of survival was done by percent survival of the worm which demonstrated that the probiotic cultures enhances the survival possibility when compared with the control strain *E.coli* OP50. Feeding probiotics does not have any consequence on physiology of the worm like the reproduction behavior and pharyngeal pumping. The strain displays superior colonization and adherence within the worm’s gut and expands the life expectancy upto five days when compared with control *E. coli* OP50. However, the extension of life span of the worms was strain specific with maximum expectancy was observed for *L. paracasei* CD4. The worms also preferred probiotic cultures as their foods in comparison to standard *E. coli* OP50.

The in vivo studies in *C. elegans* established the fact these probiotic cultures are beneficial for the worm therefore, further study was targeted on the antimicrobial potential of these strains
using *in vitro* as well as *in vivo*. The challenge studies were performed in *Caenorhabditis elegans* infected with *C. sakazakii* strain. The antimicrobial activity was assessed by agar well diffusion assay and anti-biofilm forming potential was determined by standard Crystal Violet microtiter plate assay. The probiotic cultures exhibited varying degree of antimicrobial activities against the *C. sakazakii* by different preparations of CFS (heat inactivated and neutralized). The cell free supernatant (CFS) of *L. gastricus* BTM7 and *L. plantarum* K90 exhibited maximum antimicrobial activity against *C. sakazakii*. No inhibitory activities were observed in neutralized CFS indicating the potential role of organic acids or other metabolites displaying antimicrobial activity. A 40 µl concentration of CFS was found to possess highest biofilm inhibition potential as indicated by light and fluorescent microscopic images.

The pathogenic potential of *C. sakazakii* was determined using liquid media in *C. elegans*, where the *C. sakazakii* strain resulted in complete killing of the worm in 5 days. When compared to standard food of *E. coli* OP50 with a mean life span (MLS) of 16 days. The pathogen also resulted in impaired pharynx, distorted intestine, poor valval growth and internal hatching of the eggs in the worms.

The effects of probiotics against the infection of *C. sakazakii* in *C. elegans* were determined via competitive exclusion assays. Three assays were conducted in CE: competition, displacement and the significant increase in MLS of two to three days was observed for the worms in competition and displacement assays. A pretreatment with probiotic isolates “was found to result in better protection of the worm against infection with *C. sakazakii* by extending the life of the worm by five to six days indicating that preconditioning with probiotic lactic acid bacteria can be taken as an effective measure to overcome the invasion and colonization by the pathogens”.

In conclusion, the results obtained in this study contribute to the knowledge of the diversity of LAB microbiota present in traditional fermented foods and dairy products of Himachal Pradesh. The dairy and cereal based fermented foods are an important reservoir of beneficial bacterial cultures mostly belonging to the group of lactic acid bacteria. Not all lactic acid bacteria present in these foods could serve as probiotics as most of the isolates were not able to tolerate the simulated gastrointestinal fluid conditions. The strain specific activities were obtained for adhesion potential of the strains in vitro as well as in vivo. These probiotics could be used as pro-phylactic measures to prevent infections with *C. sakazakii*. The study also
established the fact that the *C. elegans* can be used as an efficient *in vivo* model to screen the probiotics for their effect on life span and intestinal colonization.