Summary and Conclusion
Chapter 6  
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The present research work entitled “**Bioprospecting of traditional fermented foods and beverages of Himachal Pradesh for probiotics**” was carried out to characterize the traditional fermented food products of Himachal Pradesh for probiotics. The major findings reported in this thesis are summarized below:

1. Isolation of lactic acid bacteria

   Total 60 different fermented foods and beverages of various regions of Himachal Pradesh were explored for the isolation of LAB. Different morphological, biochemical and technological tests for the identification and characterization of LAB was done which resulted in selection of 40 LAB isolates from 221 different microbial isolates (yeast, bacillus and other micro-organisms) from various fermented foods.

2. Selection and characterization of potential probiotic LAB isolates

   The survival of 40 LAB isolates in acidic conditions at pH 2 and 3 was tested and isolates L6 and L22 were found to be the most acid tolerant isolates. Isolates L22, L23 and L29 were interesting, as they showed higher percentage of viability (>98 %) as compared to the reference probiotic strains i.e. P1 (97 %) and P2 (95 %). The *in-vitro* resistance of the 40 LAB isolates to different bile concentration (0.5, 1 and 2 %) were compared for 12 h. L6 and L22 retained the same level of viability at all the bile concentrations and thus these were considered to be the most bile tolerant isolate as no significant differences in the viability was found.

   Levels of cholesterol assimilation in the presence of different bile salts (cholic acid, taurocholic acid and oxgall) during 20 h of growth of the 40 LAB isolates were checked. In broth containing cholic acid, overall cholesterol removal was observed to be significantly higher for all LAB isolates than other bile salts. L1, L6, L9, L14, L16, L19 and L36 assimilated more than 30 μg/ml of cholesterol in the presence of cholic acid. Heat-killed and resting cells showed a small degree of cholesterol removal, ranging from 0.53 to 2.62 μg/ml for dead cells and 1.29 to 5.15 μg/ml for resting cells as compared with 2.53 to 47.70 μg/ml for growing cells.

   Resistance to the action of lysozyme by LAB isolates were evaluated in MRS at 30 °C for 24 h by varying lysozyme concentration from 50-150 μg/ml. No significant differences were observed in the loss of viability of 11 LAB isolates (L1, L7, L9, L15,
L18, L22, L25, L26, L30, L32 and L40) at different lysozyme concentration as compared to the control.

Variations in the susceptibility of LAB to different antibiotics were observed. All of the 40 LAB isolates displayed resistant to vancomycin. Most of them were sensitive to penicillin, ampicillin and erythromycin. LAB isolates were examined for their antibacterial activity against different foodborne pathogens and spoilage bacteria by bit disc and agar well diffusion method. All isolates showed good zone of inhibition against the various pathogens by bit disc assay method but variable results were found in agar well diffusion method.

Autoaggregation and co-aggregation abilities of the all LAB isolates including references probiotics strains (P1 and P2) for 3 h and 24 h of incubation was analysed. L9 after 3 h and L21, L16, L22, L31 and L40 after 24 h showed significantly (p<0.00001) higher autoaggregation over the P1 and P2. Twelve isolates (L1, L9, L14, L16, L19, L21, L22, L24, L29, L31, L36 and L40) exhibited highest coaggregation activity i.e. > 60 % at 3 h and 24 h. The hydrophobicity of the isolates was studied using different hydrocarbons i.e. n-hexadecane, xylene, octane and toluene and found that all the isolates exhibited a different degree of hydrophobicity. The isolates L9 (60.23 %), L21 (53.45 %) and L31 (62.35 %) showed the highest hydrophobicity index (HI) in n-hexadecane in comparison to P1 (49 %) and P2 (52.10 %). The HI of L21 (48.43 %) and L29 (43.42 %); L31 (58.64 %) and L21 (29.87 %) was highest in xylene, toluene and octane respectively.

Except for 5 isolates, all the LAB isolates produce EPS on skimmed milk-ruthedium red plates. All of the LAB isolates (40 isolates) were α-haemolytic (i.e. no haemolysis). Out of 40 isolates 19 isolates didn’t show any proteolytic activity on skimmed agar plates (no zone of hydrolysis). Highest proteolytic activity was recorded in L6 (21.2 mm in diameter) followed by L40 (16.2 mm) and L23 (15.5 mm). All the tested LAB isolates were negative for BSH activity as none of them showed precipitation (granulation) zones on agar plates. None of the LAB isolates showed phosphoketolase activity.

Tolerance of LAB to the different concentration of H2O2 (50, 100 and 150 μg/ml) was assayed. All the isolates were quite tolerant to the H2O2 concentration, only 0.5 log CFU/ml reduction in the viability count was observed.
3. Amplification of 16S rDNA of LAB using polymerase chain reaction

Statistical differences among the isolates were pointed out through the Principal Component Analysis (PCA) and 20 LAB isolates with promising probiotic potential were finally selected and carried forward for the 16S rDNA identification. The identification of the predominant microflora in the traditional fermented foods with probiotic potential by 16S rDNA revealed that *Lactobacillus brevis* and *Lactobacillus casei* were the main fermenting organisms. In addition *Lactobacillus buchneri* and *Lactobacillus paracasei* were the other lactic acid bacterial species associated with food fermentations having probiotic characteristics.

4. Detection of enzymatic activities of finally selected LAB isolates

Selected 20 LAB isolates were evaluated for their enzymatic activities for enzymes i.e. β-galactosidase, β-glucosidase, amylase, proteolytic, urease and phytase activity. 13 LAB isolates produced blue colour colonies on X-Gal plates that indicating the presence of β-galactosidase enzyme. High value of β-galactosidase activity was recorded in *L. casei* PLA12 and *L. brevis* PLA7 (ranging from 0.4 to 0.8 U/mg dcw). Maximum β-glucosidase activity was observed in *L. casei* PLA5 (14.97 U/mg dcw) and it was lowest in *L. casei* PLA10 (1.05 U/mg dcw). Highest proteolytic activity was detected in *L. casei* PLA5 (26 U/mg) followed by *L. casei* PLA10 (19.6 U/mg) and it was minimum in *L. casei* PLA8 (3.26 U/mg). The maximum amylase activity was recorded in *L. casei* PLA12 (0.5 U/mg), *Lactobacillus* sp. PLA17 (0.3 U/mg) and it was least (around 0.1 U/mg) in *L. brevis* PLA4, *L. casei* PLA10, *Lactobacillus* sp. PLA18, *L. brevis* PLA14 and *L. brevis* PLA15. The Phytase activity was observed to be highest in *L. paracasei* PLA11 (25 mm) followed by *Lactobacillus casei* PLA12 and *Lactobacillus* sp. PLA6 both showing 24 mm zones on sodium phytate plates. Urease activity was absent in all LAB isolates.

5. In-vitro adhesion assay of selected isolates

The five finally selected isolates (*L. brevis* PLA2, *L. paracasei* PLA8, *L. buchneri* PLA9, *L. casei* PLA10 and *L. brevis* PLA16) on the basis of highest hydrophobicity and co-aggregation properties were further examined for their ability to adhere to HT-29 cells. On comparative evaluation, *L. brevis* PLA2 (24.36±0.5), *L. paracasei* PLA8 (21.21±0.25) and *L. brevis* PLA16 (15.26±0.5) were the most adhesive isolates based on their respective per cent adhesion.
6. Screening of LAB isolates for inhibitory action against *Listeria monocytogenes*

Antimicrobial activity of finally selected LAB isolates against *Listeria monocytogenes* (food borne pathogen) was screened firstly by bit disc method and then by well diffusion assay method. *L. buchneri* PLA10 and *L. plantarum* PLA19 showed inhibitory effect after neutralizing the supernatant to pH 6.5 showing production of some bacteriocin like substances (BLS) by these two isolates.

7. Evaluation of efficacy of probiotic formulations (whey permeate) for inhibition of *Listeria monocytogenes* in raw vegetables and meat by dipping method

Two isolates (PLA10 and PLA19) showed significant inhibition zone by agar spot test, showing area of inhibition greater than 5 mm for 28 days of incubation at refrigeration temperature were finally selected as promising LAB isolates for the inhibition of *L. monocytogenes* at refrigeration temperature in various ready to eat food products (vegetable salads and meat).

LAB isolates (PLA10 and PLA19) possibly producing bacteriocin like substances were selected that effectively inhibited the growth of *L. monocytogenes* in cooked meat at 4 °C for a period of 28 days storage. 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 that limits further growth of the pathogen but cannot decrease the number of *L. monocytogenes*.

A study of the effect of LAB isolates on control of *L. monocytogenes* in fresh vegetable for a period of 14 days was also performed. It was revealed that the cocktail of isolates PLA10 and PLA19 were most effective against *L. monocytogenes* as 3.6 fold reductions in the pathogen count was observed.

8. Preparation of probiotic product

Soymilk was fermented with *L. casei* PLA5 for 24 h at 30 °C. Pasteurised soymilk was supplemented prebiotics including mannitol, fructo-oligosaccharides (FOS), inulin
and maltodextrin and growth of LAB was analysed for 24 h. The maltodextrin and FOS supplementation resulted in enhanced growth of *L. casei* PLA5 by 1.24 and 1.09 log CFU/ml respectively as compared to control (*p* < 0.05) after 24 h. The viability of *L. casei* PLA5 in soymilk was found to be more in comparison to standard probiotic strain i.e. *L. casei* Shirota. Moisture and ash content of the fermented soymilk at different fermentation time was analysed and ash content ranged between 0.31 and 0.42 %. All the samples had high moisture contents which ranged between 86.12 % - 88.5 % from 0-48 h. The total carbohydrates in fermented soymilk during fermentation decreased from 5.5 g/100 ml to 2.57 g/100 ml (w/v) on the 48 h of fermentation. Higher increase in calcium (4.89-20.32 mg/100g) and magnesium (220.03 to 245.48 mg/100g) levels was observed in soymilk fermented with *L. casei* PLA5 as compared to soymilk (control). There was not much difference in the zinc levels in soymilk fermented with *L. casei* PLA5.

The proteolytic activity of the *L. casei* PLA5 in fermented soymilk increased significantly (*p* <0.05) from 12 to 48 h. Highest β-glucosidase (3.40 U/mg) and β-galactosidase activity (0.10 U/mg dw) of *L. casei* PLA5 were observed after 24 h of fermentation.

Soy milk (control) exhibited highest H$_2$O$_2$-scavenging effect (13.89 %) and it decreased during fermentation. The DPPH (1, 1-diphenyl-1-picrylhydrazyl) values increased with the fermentation. Polyphenol content was found to decrease from 14.01 (mg/100 ml) at 12 h to 6.01 (mg/100 ml) at 48 h during fermentation while flavonoids content of soymilk was highest at 24 h of fermentation.

Viability of LAB in soymilk was evaluated for 14 days at refrigeration temperature and it was observed that the viable cell counts of *L. casei* PLA5 in fermented soymilk ranged from 8.01 to 7.51 log CFU/ml on 14$^{th}$ day of cold storage at 4 °C.
Conclusion

The study was undertaken with aim to isolate and characterise the LAB isolates from various fermented foods and beverages of Himachal Pradesh. Sixty fermented foods and beverages were subjected for the isolation studies resulting in the 221 different microbial isolates out of which 40 were the LAB isolates. *L. brevis* and *L. casei* were the most dominant microorganisms. These 40 LAB isolates were further exposed to various *in-vitro* tests (acid-bile tolerance, antibiotic susceptibility, antimicrobial activity, hydrophobicity, auto and co-aggregation and cholesterol assimilation) which are prerequisite for the selection of efficient probiotic isolates and twenty LAB isolates were finally selected as a promising probiotic candidates.

Two LAB isolates (*L. buchneri* PLA10 and *L. plantarum* PLA19) can be used as a biopreservatives to ensure the safety and quality of the RTE food products. These two LAB isolates have effective bacteriostatic and bactericidal activities against food borne pathogen i.e. *L. monocytogenes* which was tested on cooked meat and ready-to-eat vegetables for 28 days of refrigeration. LAB isolate (*L. casei* PLA5) showing good proteolytic, phytase and β-glucosidase activity was selected for the preparation of the probiotic soymilk. Addition of prebiotics resulted in improvement of the growth rate, fermentation time and viability of LAB isolates PLA5.

Based on the findings obtained from the present study, it was concluded that increased antioxidative activities and reduced polyphenol content of soymilk through fermentation with LAB isolate has great potential of developing a probiotic soymilk having health beneficial attributes. This isolates has promising use as a biopreservatives in food sector. Further investigations need to be conducted in animal experiments to assess the *in-vivo* efficacy of the isolates for the commercial and therapeutic benefits.