6. SUMMARY AND CONCLUSION

Fermented foods constitute a substantial part of the diet in India. LAB play an important role in the majority of food fermentations, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetables and bakery products. The preparation of this indigenous fermented food generally depends on a spontaneous or chance inoculation by naturally occurring LAB and the use of starter cultures is still at very early stages. One of the major contributions of these microorganisms is the extended shelf life of the fermented product by comparison to that of the raw substrate. Among the bacteria producing antimicrobials, LAB has attracted researchers very much as they are considered GRAS.

Eight most promising lactobacilli were chosen from twenty two isolates based on their spectrum of activity against other LAB and pathogens. The eight lactobacilli were characterized based on the various classical phenotypic tests, physiological tests and biochemical tests including carbohydrate utilization profiles. All isolates were homo fermentative, catalase and gelatin negative. However, the conventional methods of characterization were not sufficient for intra-species differentiation. Therefore, molecular characterization was performed by RAPD, 16S rRNA analysis, 16S ARDRA and Multiplex PCR for species identification. RAPD was carried out using the primer R2 and M13. Five different clusters were obtained based on RAPD indicating strain level variation. 16S rRNA analysis showed 99 to 100 % homology towards Lactobacillus plantarum. The restriction digestion pattern was similar for all the isolates with the restriction enzyme AluI. The subspecies were identified by performing Multiplex PCR using species specific primer. Among the five clusters, three clusters were clearly identified as Lactobacillus plantarum subsp. plantarum, Lactobacillus
pentosus and Lactobacillus plantarum subsp. argentoratensis. Therefore, it is evident that in addition to various classical physiological, biochemical and sugar utilization profile, a combination of molecular methods can be used successfully for sub-species level identification of Lactobacillus isolates from fermented idli batter. Lactobacillus plantarum is a very versatile microorganism being explored around the world. However, the lactobacilli isolated from idli batter have been least explored as well as delineation of the isolates to sub-species level has not been reported.

The primary antimicrobial effect exerted by lactobacilli is by the production of lactic acid and reduction of pH. However, there are other metabolic products such as hydrogen peroxide, diacetyl, propionic acid, acetic acid, carbon dioxide, reuterin and bacteriocins reported to contribute to its antimicrobial activity. Lactobacillus plantarum JJ18 and Lactobacillus plantarum subsp. plantarum JJ60, probiotics from idli batter have produced bacteriocins JJ18 and JJ60 having wide spectrum of activity. After optimizing the environmental conditions for bacteriocin production the effect of various media components was determined. The maximum bacteriocin production was observed in MRS broth, pH 6.4 at 37 °C after 36 h. Bacteriocin production is known to be altered in different environmental conditions and optimum production may require a specific combination of environmental parameters. The production is often regulated by microbial growth, pH and temperature. Tryptone (as nitrogen source) and glucose (as carbon source) are required for optimal production of bacteriocins JJ18 and JJ60. Although bacteriocins can be produced during food fermentation, production in much higher amounts is observed under optimal physical and chemical conditions. The higher in vitro production is due to the absence of limiting factors, such as strong diffusion limitations, inactivation by proteases, and the adsorption to food particles. In general,
the cultivation conditions directly affect bacteriocin production and indirectly through biomass production.

Many purification techniques are being carried out worldwide for bacteriocins. Laboratory purification protocols usually include precipitation step, followed by various combinations of gel permeation, ion-exchange and hydrophobic interaction chromatography, with a final RP-HPLC purification step. Several protocols based on adsorption/desorption or on phase partitioning have been developed for large scale recovery and purification of bacteriocins. The bacteriocins JJ18 and JJ60 in this study were partially purified by precipitation with acetone and gel permeation chromatography. The bacteriocins JJ18 and JJ60 in tris tricine SDS-PAGE electrophoresis depicted a single band less than 3.5 kDa. However, the strain *Lactobacillus plantarum* JJ18 was inhibited by bacteriocin JJ60 and *Lactobacillus plantarum* JJ60 by bacteriocin JJ18, whereas no inhibition was observed against the respective producer strains indicating that the two bacteriocins are different. However further purification and sequencing of bacteriocins is necessary for confirming the results.

Activity was not affected by surfactants like Triton X-100, Tween 80 and Tween 20 as well as on treatment with NaCl, Urea and EDTA. Protease treatment resulted complete loss of activity of the partially purified bacteriocins JJ18 and JJ60, while lipase and amylase had no effect indicating that the bacteriocins belonged to Class II. The bacteriocins remained active at wide range of pH and temperature. Bacteriocins JJ18 and JJ60 were thermostable, which is a very useful characteristic if it is to be used as a food preservative, because many food-processing procedures involve the heating step. The bacteriocins were able to adsorb onto producer and target cells, *Lactobacillus*
plantarum and Listeria monocytogenes and differentially in the presence of various surfactants, salts and solvents. A bactericidal mode of action was observed against Listeria monocytogenes. Bacteriocins are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. The modes of action of bacteriocins are generally by targeting the cytoplasmic membrane. They dissipate the proton motive force through the formation of pores in the phospholipids bilayer. The bacteriocins JJ18 and JJ60 having wide spectrum of activity against various pathogens can be applied as a bio-preservation in food industry.

The bacteriocins from lactobacilli isolated from other traditional foods of India have proven its applicability in biopreservation. A number of bacteriocins have been described for Lactobacillus plantarum isolated from fermented meat products, other food sources and fermented beverages. According to the recent trends in food preservation, the improvement of microbiological safety of foods can be attained with the help of bacteriocin-producing strains of LAB isolated from the same food which needs to be preserved. Since the majority of bacteriocinogenic lactobacilli are natural food isolates, their antimicrobial peptides could be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner. Currently, nisin and pediocin are the only bacteriocins commercially exploited to date.

Recent research has revealed that LAB can produce low-molecular weight (LMW) antibacterial substances, e.g., phenyl lactic acid, p-hydroxyphenyllactic acid, cyclic dipeptides such as cyclo (Gly-L-Leu), cyclo (L-Phe-L-Pro), and cyclo (L-Phe-trans-4-OH-L-Pro), benzoic acid, methyl hydantoin, mevalonolactone, and short-chain fatty acids. The biologically active, non-proteinaceous LMW compounds produced by LAB are poorly characterized, although the existence of such compounds has frequently been reported. The difficulties in purification and identification of this type of LMW
compound are reported. The main difficulty was that several compounds were involved in the co-operative action and that the concentrations of the compounds were extremely low. They have not yet been effectively exploited in commercial applications because of their inadequate structural characterization.

The wide spectrum of antimicrobial activities reported for these molecules suggest high potential in medical applications. *Lactobacillus plantarum* JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60 isolated from *idli* batter also produced low molecular weight compound in addition to the bacteriocins. The low molecular weight compound showed wide spectrum of activity against various pathogens. The mechanism of action of these compounds which leads to death of the target cells is not yet clear. The molecular weight of the compound was around 204 daltons which was clearly revealed in LC-MS analysis. Based on the various structural elucidation studies, the compound produced by the isolates *Lactobacillus plantarum* JJ18 and JJ60 was indole-3-propanamide. Various studies are now being explored for isolation and purification of LMW substances. Synergistic activity among these compounds is also promising for variety of application in industries.

The increase in bacterial resistance to various antibiotics has stimulated investigations around the world to improve disease control strategies which led to the discovery of new vaccines and non-specific immunostimulants. Even though there has been a long history of safe consumption of probiotic lactobacilli in traditional food, several criteria must be examined before they are used as probiotic agents or in industrial grade food products. Probiotics may provide a number of potential health benefits mainly through maintaining good balance and composition of intestinal flora, helping to increase the body’s ability to resist the invasion of pathogens and maintain the host’s well being. An experimental focus on bacteriocin production by probiotic
LAB strains has indicated that this potential might play an important role during in vivo interactions occurring in the human GIT. The eight potential probiotic *Lactobacillus plantarum* strains from fermented *idli* batter were screened for probiotic properties using in vitro assays such as bile tolerance, acid tolerance, transit tolerance in the upper human GIT, auto-aggregation, co-aggregation, hydrophobicity, susceptibility to various antibiotics, bile salt hydrolase assay, cholesterol assimilation and hemolysis. The isolates were able to tolerate up to 0.3% of bile for 4-6 hours and pH 2.5, 3.5, 4.5, 6.5, 7.5 and 8.5. The isolates were able to resist growth against gastric and intestinal fluid.

Survival of bacteria in the GIT is in partly due to their ability to sense other members and signals within their environment and awareness to the presence of the host and microbial competitors. Tolerance to the extreme gastrointestinal conditions (acid, bile, enzymes, low levels of oxygen), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens have been some important criteria for probiotic selection. The auto-aggregation of the different *Lactobacillus plantarum* strains ranged from 65 to 80% in all the isolates. The co-aggregation with pathogens were higher when compared to the LAB. The isolates showed resistance towards antibiotics like gentamycin, ciprofloxacin, nalidixic acid and norfloxacin. All the isolates showed bile salt hydrolase activity with cholesterol lowering capacity, the highest being 73% by *Lactobacillus plantarum* JJ18. The isolates possessed β-galactosidase activity exhibiting 322 – 1000 MU of enzyme activity. No isolates showed hemolysis activity.

There are several evidences supporting potential clinical applications of probiotics in the prevention and treatment of diseases of the gastrointestinal, respiratory and urogenital tracts. Other clinically proven functional aspects of probiotic include cholesterol reduction, diarrhea prevention, enhancement of lactose intolerance symptoms, anticancer effects and immunomodulatory effects. Hence, the different
*Lactobacillus plantarum* isolates exhibiting probiotic potential would attribute beneficial effect to mankind.