

**Diversity of Lactic Acid Bacteria and Their
Probiotic Properties in Some Naturally
Fermented Milk Products of Sikkim**

A Thesis Submitted

**To
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**By
Ranjita Rai**

Supervisor: Professor Dr. Jyoti Prakash Tamang

**Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
Gangtok 737102, India**

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SUMMARY

The major objectives of this PhD Thesis were to document native skill and knowledge of ethnic people of Sikkim for preservation of perishable fresh milk of cow and yak into different dairy products by spontaneous or natural fermentation using 'back-slopping method' and their microbiology and probiotics properties. Different types of naturally fermented milk (NFM) products of Sikkim viz. *dahi* (cow-milk), *dahi* (yak-milk), soft *chhurpi* (cow-milk), soft *chhurpi* (yak-milk), *mohi* (cow-milk), hard *chhurpi* (yak-milk), and *philu* (yak-milk) were well documented. The pH of NFM products collected from different places of Sikkim were mild acidic in nature. Lactic acid bacteria (LAB) were found dominant microorganisms with $>10^8$ cfu/g in all NFM products of Sikkim. A total of 272 LAB strains were isolated from 22 NFM samples. *Leuconostoc*, *Lactococcus*, *Enterococcus* and *Streptococcus* were tentatively identified on the basis of phenotypic characterization and biochemical tests. The 16S rRNA gene sequencing result based on Sanger method showed a complex microbial community with three genera and nine different species and sub-species viz. *Leuconostoc mesenteroides*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis*, *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*, *Enterococcus faecalis*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *tractae*, *Enterococcus italicus* and *Enterococcus pseudoavium*. In sample-wise distribution of lactic acid bacteria, *Lactococcus lactis* and *Leuconostoc mesenteroides* were dominated in *dahi* (cow-milk). In the other hand, *Leuconostoc* was found dominant in the *dahi* sample (yak-milk). In soft *chhurpi* (cow-milk), the genus *Leuconostoc mesenteroides* was found dominant followed by *Lactococcus lactis* subsp. *cremoris*. Additionally, two subspecies *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* and *Lactococcus lactis* subsp. *hordniae* were identified that have not been reported before. In soft *chhurpi* (yak-milk), *Enterococcus faecalis* was most dominant followed by

Leuconostoc mesenteroides. In *mohi* sample (cow-milk), *Lactococcus lactis* was predominant followed by *Leuconostoc mesenteroides*. In hard *chhurpi* (yak-milk), *Leuconostoc mesenteroides* dominated the sample and *Lactococcus lactis* subsp. *cremoris* was predominant bacteria in *philu* prepared from yak-milk. In terms of abundance and species richness, soft *chhurpi* prepared from yak milk exhibited highest species richness where as lowest species richness were recorded in *dahi* (yak-milk) and *philu* (yak-milk).

We applied high-throughput sequencing method to profile the bacterial community in NFM products of Sikkim. A total of 19 NFM samples were analysed and the analysis revealed *Firmicutes* was the dominant phylum followed by phylum *Proteobacteria*. The overall diversity, family *Streptococcaceae* was dominant followed by *Lactobacillaceae*, *Leuconostocaceae*, *Staphylococcaceae*, *Bacillaceae* and *Clostridiaceae* respectively. Similarly, under phylum *Proteobacteria*, family *Acetobacteraceae*, *Pseudomonadaceae* and *Enterobacteriaceae* was reported. A relative abundance of uncultured bacteria (9.8%) were also recorded. In this study, the overall diversity of NFM products at species level was dominated by *Lactococcus lactis*, *Lactobacillus helveticus*, *Pseudomonas fluorescens*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactococcus piscium*, *Lactococcus raffinolactis*, *Lactobacillus delbrueckii*, *Leuconostoc lactis* and *Lactobacillus gasseri*. In the other hand, species belonging to the acetic acid bacteria such as *Acetobacter lovaniensis*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Acetobacter syzygii* and *Hafnia alvei* were found. Interestingly, a relatively high abundance of *Lactobacillus helveticus* was found in soft *chhurpi* (cow-milk) which is not reported in earlier studies. Nonetheless, we found a fairly equal distribution among *Acetobacter*, *Lactococcus*, *Lactobacillus* and *Leuconostoc* at genus level in different NFM products of Sikkim. Analysis of alpha diversity revealed that significance

difference was observed between two samples, *chhurpi* and *dahi* ($p=0.0152$), however, there were no significance differences observed between milk sources.

We screened the probable probiotics attributes of LAB strains isolated from NFM products of Sikkim. About 33.8% of bacterial isolates showed positive result for beta-galactosidase enzyme activity on MRS plates. LAB isolates demonstrated variable degree of hydrophobicity assay, among them three bacterial strains *Enterococcus faecalis* (YS4-14), *Lactococcus lactis* subsp. *cremoris* (SC3) and *Enterococcus faecalis* (YS4-11) which showed hydrophobicity more than 85%. For acid tolerance, two strains *Lactococcus lactis* subsp. *hordniae* (SC17) and *Leuconostoc mesenteroides* (YS7-8) showed maximum acid tolerance in the study. Similarly, strain *Leuconostoc mesenteroides* (DY36) showed good tolerance to lysozyme environment. For the bile hydrolysis analysis, 16 LAB strains were capable of hydrolyzing both sodium cholate and sodium tauroglycocholate, however, only four strains were capable of hydrolyzing sodium taurocholate. Bile plays a vital role in gut defence mechanisms. Antimicrobial activity assessment of LAB strains showed varying zone of inhibition depending upon the tested pathogens, two strains *Leuconostoc mesenteroides* (SC11) and *Leuconostoc mesenteroides* (SC26) showed maximum inhibition zones against *E.coli* MCC2413, strain *Lactococcus lactis* subsp. *cremoris* (YS8-10) showed good inhibition against *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC3223, *Leuconostoc mesenteroides* (SC4) against *Staphylococcus aureus* subsp. *aureus* MTCC740 and *Lactococcus lactis* subsp. *cremoris* (YS8-7) against *Bacillus cereus* MTCC1272. Tested pathogen *Staphylococcus aureus* subsp. *aureus* MTCC740 was found most sensitive towards antimicrobial compounds while *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC3223 and *E. coli* MCC2413 showed less sensitivity. The probiotic culture *Lb. plantarum* MCC2034 also showed similar type of inhibition against tested

pathogens. Therefore, we can predict that LAB strains isolated from NFM products of Sikkim may have enzyme properties that may be applicable in the food industry.

Genes involved in survival of low pH such as *tdc*, *agu*, *clpL* and Ir1516 were detected in 41.1%, 52.9%, 82.3% and 55.8%, respectively. However, gene *hdc*, *odc*, LBA1272, *dltD*, La995, *gtf*, and *groEL* were not detected. For the binding capability, variability of genes *mub*, *map* and *msa* was detected. Binding gene *msa* was detected more than 50% in the study. However, no other binding genes *fbp*, *sor*, and *sbp* were detected. Generally, BSH activity was observed in gastrointestinal tract where bile salts are present. Bacterial genes *bsh*, LBA1432, LBA1679, LBA1446, Ir0085, Ir1584, LBA0552, and LBA1429 were analysed in this study. LBA1446, Ir1584 and *bsh* were widely distributed among bacterial strains with 45.5%, 29.4% and 22%, respectively. However, genes LBA1432, LBA1679, Ir0085, LBA0552, and LBA1429 were not detected in tested bacterial strains. For bacteriocin producing gene *Ent A*, *Ent B*, *Ent P*, Enterocin AS-48, *Nisin*, Lactococcin A, Lacticin 481 and *mes Y* were evaluated. Gene *mesY* were less frequently detected, it codes for Mesenteriocin Y, which is an anti-listeria bacteriocin produced by *Leuconostoc mesenteriodes*. However, no enterocin encoding genes were detected in the present study. Additionally, the gene encoding β -glucosidases for various LAB species were also detected in the study. Previous studies in β -glucosidase activity were mainly reported from milk and milk products. Based on screening of probiotic properties and gene detection, 20 probable probiotic strains of LAB were selected. The probiotic screening result indicates that LAB isolates derived from NFM products of Sikkim have potential probiotic properties that may be used in food industry. This research therefore provides the basic information for selecting possible strains for further evaluation of gene expression and its enzyme activity.

We also analysed the gene sequence obtained from high-through sequencing result using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSTs) software. Based on Kyoto Encyclopaedia of Genes and Genomes (KEGG) database, various predictive metabolic pathways and functional profiles were inferred from OTUs of bacteria from naturally fermented milk products of Sikkim. NFM samples showed high metabolic signatures, mainly, carbohydrate, amino acid, energy metabolism and some other minor metabolic activities. Casein-based products (*dahi* and *chhurpi*), and fat-based product (*gheu*) revealed a significant variation of metabolism among the two groups. Acetic acid dominance in *gheu* contributes to significant difference to that of the casein-based products. In *chhurpi*, significantly higher metabolic pathways were also observed particularly, biosynthesis of ansamycins, lipopolysaccharide biosynthesis, pentose phosphate pathway, thiamine metabolism, homologous recombination, glycolysis/gluconeogenesis pathways. Similarly, *gheu* showed a significant increase in metabolic pathways associated with valine, leucine and isoleucine biosynthesis, pantothenate and CoA biosynthesis, synthesis and degradation of ketone bodies, lysine biosynthesis, propanoate metabolism. Interestingly, the casein-based products, *dahi* and *chhurpi* showed a significant increase than *gheu*, particularly in the pathways associated with biotin metabolism, folate biosynthesis, seleno compound metabolism, cysteine and methionine metabolism, and vitamin B6 metabolism. It is said that some LAB synthesis vitamins in fermented milk products. Contrastingly, pathways associated with pyruvate metabolism, glycine, serine and theonine metabolism was shown to be significantly. In addition, KEGG database predicted some lactic acids bacteria present in NFM products of Sikkim have functional features and health promoting benefits to consumers, and should be a good source for mining probiotic bacteria for functional foods development.

This study has provided the complete information on lactic acid bacterial communities of NFM products of Sikkim, evaluated by culture-dependent technique (phenotypic characterization and 16S rRNA); culture-independent methods (NGS techniques by high-throughput amplicon sequencing) and screening of their probiotic attributes. The application of culture-independent NGS methods to research the microbial ecology of fermented foods is of great importance in understanding the products, where Illumina sequencing was shown to be one of the effective tools in this analysis.

This is the first study on insight analysis of the bacterial diversity of NFM products of Sikkim by culture-independent method as well as on the screening of probiotic properties of isolated LAB strains from NFM products. This study may provide basic information on composition of indigenous microflora in the NFM products and may be used to promote the development of starter culture. Moreover, data generated from this study can be used as reference data base for the future research.

Highlights of findings

- Based on 16S rRNA gene sequencing result, *Leuconostoc mesenteroides*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis*, *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*, *Enterococcus faecalis*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *tractae*, *Enterococcus italicus* and *Enterococcus pseudoavium* were isolated from NFM products of Sikkim.
- Based on high-throughput sequencing data; *Firmicutes* was the dominant phylum followed by phylum *Proteobacteria*. At species level, the following bacteria were detected in NFM samples: *Lactococcus lactis*, *Lactobacillus helveticus*, *Pseudomonas fluorescens*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactococcus piscium*, *Lactococcus raffinolactis*, *Lactobacillus delbrueckii*, *Leuconostoc lactis* and *Lactobacillus gasserii*, *Acetobacter lovaniensis*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Acetobacter syzygii* and *Hafnia alvei*.
- On the basis of *in vitro* screening and gene detection 20 probable probiotic strains were selected among *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Enterococcus faecalis*.
- KGGE database predicted metabolic pathways and functionality in NFM products of Sikkim with some health promoting benefits to consumers.

Schematic Diagram and Pictorial Presentation of Complete PhD Work

