Available online at www.jibresearch.com; ISSN: 2348-6473 (online)

Original Research

Journal of Innovative Biology

September 2014 Vol. 1, Issue 3, P. 168-174

Biodiversity of *Lactobacillus* from traditional Indian Cow milk Churpi cheese

Dhiraj Kumar Nanda[1, 2], Reeti Chaudhary[1]*, Karan Veer Singh[2], Dinesh Kumar [2, 3]

[1]Department of Biotechnology, D C R University of Science & Technology, Murthal - 131039, Haryana
[2]National Bureau of Animal Genetic Resources, Karnal – 132001, Haryana
[3]Centres for Agricultural Bioinformatics, Indian Agricultural Statistics Research Institute, New Delhi -110012

Abstract: Churpi cheese is one of the traditional varieties of fermented dairy product prepared from cow milk, and consumed by ethnic groups of people residing in alpine regions of India, Sikkim and Darjeeling. The aim of current study was to investigate the biodiversity of *Lactobacilli* in indigenous Churpi cheese prepared from cow milk. Twenty strains of *Lactobacilli* were isolated from eight different samples of Churpi cheese procured from different regions of Sikkim (India), and were analysed by phenotypic and genotypic methods. For molecular characterization of these isolates 16 S r DNA sequencing was carried out which confirmed species of these isolates. Nine isolates were confirmed as *Lactobacillus casei*, followed by four as *L. plantarum*, three as *L. delbrueckii*, two as *L. paracasei*, and two as *L. brevis*. The results of this study shows a high level of biodiversity among *Lactobacilli* isolated from Churpi cheese and offered a remarkable reservoir of 'natural' microbes. Isolates obtained in this study can be potentially used for the development of defined strain starter for Churpi cheese and other dairy products.

Keywords: Cow milk; Churpi cheese; Lactobacillus; 16S rDNA

Received: 03 September 2014 / Accepted: 27 September 2014 / Published Online: 30 September 2014

© 2014 jibresearch.com

Churpi is a traditional variety of cheese like dairy product which is mainly prepared and consumed in the Himalayan regions of Darjeeling and Sikkim, India (Dewan and Tamang, 2007; Tamang et al. 2000). Churpi can be prepared from milk of cow and yak, churpi prepared out of cow milk is softer than yak Churpi and has a mild to strong flavoured taste and is consumed as curry mix, pickles and condiments. It has been reported that per capita average consumption of soft-variety churpi in the Darjeeling hills is 6.9 g/day, and in Sikkim is 9.9 g/day (Yonzan and Tamang, 1998). Traditional process of Churpi preparation involves fermentation of boiled or unboiled cow milk, such traditional dairy products have their natural microflora is from non-pasteurized milk and raw materials which can be the sources of new strains of microbes (Weerkamp et al. 1996; Wouters et al. 2002). The fermentation in such food products is carried out by a class of microbes known as lactic acid bacteria (LAB), comprising a group of numerous bacterial genera including *Lactobacillus, Lactococcus, Streptococcus,* and *Leuconostoc* (Holzapfel et al. 2001). LAB are commonly found in foods and fermented products (O'sullivan et al. 2002) including dairy products (Beresford et al. 2001). Pure microbial community isolated from traditionally fermented foods exhibits diverse metabolic activities which can be compared with commercial strains used as industrial starters (Klijn et al. 1995; Randazzo et al. 2002). Thus, efforts are needed to isolate, characterize and pre-serve the microbial diversity of raw milk and traditional dairy products.

Lactobacillus have been characterized by phenotypic methods as morphology, Sugar fermentation analysis (Kandler and Weiss, 1986; Coeuret et al. 2003) fatty acid methyl ester (FAME) analysis (Klein et al. 1998; Giraffa and Neviani, 2000) and whole-cell protein profiling for certain lactobacilli at both species and sub-

Quick **R**esponse **CODE**:
Nanda et al., 2014

The article may be access online @
http://www.jibresearch.com

Corresponding Author:
Chaudhary R, Department of Biotechnology, D C R University of Science & Technology, Murthal - 131039, Haryana
Email: reeti.malik@gmail.com

species level (Giraffa and Neviani, 2000). These phenotypic methods include poor reproducibility, ambiguity of some techniques, extensive logistics for large-scale investigations and poor discriminatory power (McCartney, 2002). Thus these phenotypic protocols have been supplemented by using various DNA fingerprinting techniques like RAPD-PCR, RFLP, ribotyping, and rep-PCR (Nguyen et al. 2013; Massi et al. 2004; Gevers et al. 2001). Sequencing of 16S rRNA gene have been found more reliable for molecular ecology and diversity analysis study because it is difficult to identify minor components of microbiota by other methods (Kim and Chun, 2005; Lane, 1991; Collins and Wallbanks, 1992; Babalola, 2004).

In the context of Churpi cheese studies on diversity of *Lactobacilli* in Yak Churpi has been carried out (Prashant et al. 2009) but report concerning the genotypic and phenotypic characterization of the microflora from Churpi prepared from cow milk is lacking behind. Hence, the current study was taken up with the objective to isolates, characterize, and study the diversity within the *Lactobacillus* from cow milk Churpi cheese. Natural biodiversity of *Lactobacilli* from Churpi can be of immense importance and can be used to formulate defined starter strains for promoting and commercialization of Churpi cheese.

MATERIALS AND METHODS

Collection of cheese samples

Eight samples of four different varieties of Churpi cheese samples **A**: Dry powder Churpi cheese, **B:** Wet Churpi cheese farmer made, **C:** Wet Churpi cheese machine made, **D:** Dry Churpi cheese garland were collected from Local market of Gangtok, Sikkim, India. During transportation the cheese samples were kept in refrigerated condition and brought to lab, and kept in same condition till analysis.

Isolation of *Lactobacillus*

In order to recover a majority of Lactobacillus diversity for subsequent characterization purpose, cheese samples were subjected to microbiological analysis for total bacterial counts. Ten gram of cheese sample was homogenized with 90 mL of 2% sodium citrate solution, which was further serially diluted (10^{-1} – 10^{-8}) in normal saline solution (0.85% NaCl) and one mL of decimal dilutions of the samples were pour plated with culture media De Man Rogosa Sharpe (De Man et al. 1960) (MRS) agar (Himedia, Bombay, India) and incubated for 48 h at 37 °C. Colonies grown on the plates were counted, the suitable plates with well isolated discrete colonies were then selected for isolation and 10–15 individual colonies were picked randomly from each sample plate with help of sterile toothpick and transferred into MRS broth tubes and incubated at 37 °C. After incubation for 24-48 hours, the MRS broth cultures were examined microscopically for purity. The tubes showing negative for catalase test and gram positive rods were selected and further purified by successive streaking on MRS agar plate. All colonies thus isolated were presumptively screened on the basis of gram reaction, morphology, and catalase test.

All these isolates were preserved as glycerol stocks (MRS broth with 15% glycerol) and stored at −20 °C, for long term preservation freeze dried ampoules were prepared by lyophilization using freeze dryer (Edwards High Vacuum International, Sussex, England) and were stored for further characterization by biochemical and molecular methods.

Phenotypic characterization

Negative staining and gram staining were used to check the morphology of Isolates. For Biochemical characterization of these putative *Lactobacillus* isolates a battery of few biochemical tests was carried out as per the scheme suggested in Bergey's Manual (Kandler and Weiss, 1986). The ability of cultures to ferment and produce acid from various sugars was tested by using HiCarbohydrate™ Kit KB009 (Hi Media Laboratories Ltd., Mumbai) comprising of 35 different carbohydrate sources, Cultures were inoculated to the kit as per instruction provided by manufacturer. This fermentation profile obtained was used to prepare a dendrogram of isolates by using NTSYSpc 2.02 software package (Applied Biostatistics Inc., NY, USA).

Molecular characterization

DNA extraction

Genomic DNA of all isolates was extracted from 2 mL samples of overnight cultures grown in MRS broth at 37 °C as previously described (Pospiech and Neumann, 1995) with slight modification.

Genus-specific PCR

Lactobacillus genus-specific primer (Dubernet et al. 2002) as in Table 1, targeting 16S rRNA gene was used for the confirmation of *Lactobacillus* genus. The Polymerase chain reaction (PCR) was performed in 25 µL of reaction volume, containing 50–100 ng of genomic DNA, 1X Taq buffer, 1.5 mmol/L $MgCl_2$, 10 mmol/L of each deoxyribonucleotide tri phosphate (dNTP), 50 ng of each primer, and 1 unit of Taq DNA polymerase (Fermentas, Germany). Amplification was performed on Eppendorf Mastercycler (Hamburg, Germany) according to the program of earlier published literature (Dubernet et al. 2002). Amplification was verified by electrophoresis on 1.5% (w/v) agarose gel in 1X TAE buffer using GeneRuler 100 bp DNA ladder (Fermentas, Germany) as a molecular weight marker, and stained with ethidium bromide (1 mg/mL).

16S rRNA gene (partial) Sequencing and phylogenetic analysis

For sequencing of 16S rRNA gene (partially) PCR was carried out for all isolates by using primer 7F (Lane, 1991) and S-G-Lab-0677-R (Heilig et al. 2002) as in Table 1. The PCR products of 750 bp size (25 µL) amplified were custom sequenced by using primer 7F (Lane, 1991) by Automated DNA Sequencing Services provided by Vimta Labs Pvt. Ltd, Hyderabad, India. The

© 2014 jibresearch.com

Table 1 List of primer used in this study for Lactobacillus identification

Target	Primer Sequence 5'-3'	Annealing Temp.(°C)	Product size (bp)	Reference
***Lactobacillus* Genus**	**F**- CTCAAAACTAAACAAAGTTTC **R-** CTTGTACACACCGCCCGTCA	55	250	Dubernet *et al.* 2002
16 S rDNA	**7F -**AGAGTTTGAT(C/T)(A/C)TGGCTCAG **S-G-Lab-0677-R** -CACCGCTACACATGGAG	57	750	Lane 1991; Heilig *et al.* 2002

chromatogram of sequences obtained from the service provider were analysed, trimmed and converted to FASTA format by using BioEdit Sequence alignment editor Version 7.0.9.0. Basic Local Alignment Search Tool (BLAST) analysis was performed to check the identity of DNA sequence in the database and for species determination. The sequences thus generate after analysis with species identity were submitted to the GENBANK database NCBI (Bethesda, MD. USA) by using Sequin Application Version 12.10 (NCBI, NIH). The phylogenetic analysis of these sequences was done with help of MEGA 5.0 (Tamura et al. 2011), and a phylogenetic tree was constructed with reference sequences from GenBank database NCBI, by using unweighted pair-group method with arithmetic averages (UPGMA).

RESULTS

In all of the collected samples LAB as well as aerobic mesophilic counts were found to be in the range of 1.6 x 10^5 – 4.0 x 10^5 CFU/ gm of cheese. Yeast and mold were detected only in samples D. A total of 103 colonies were picked up initially from MRS plates, these isolates were presumptively screened for genus *Lactobacillus.* Finally only 20 isolates (2 from Churpi cheese-A, 7 from Churpi cheese-B and 11 from Churpi cheese-C) were confirmed to be *Lactobacillus* on basis of preliminary screening strategies, all of the 20 isolates conformed to the general phenotypic characteristics of genus *Lactobacillus i.e.* Gram positive, rod shaped, nonmotile, non-sporulating, and catalase negative. None of isolate from Churpi cheese-D sample could be confirmed to be *Lactobacillus*. The generic status of isolates was further confirmed by PCR, all these 20 isolates gave a specific band of 250 bp with *Lactobacillus* genus specific PCR (Fig 1) as described by (Dubernet et al. 2002), confirming for *Lactobacillus* at genus level.

Phenotypic characters especially sugar fermentation pattern of all the isolates was used to prepare a dendrogram (Fig. 2) with help of NTSys software using SM coefficient and clustered by UPGMA method, based on sugar fermentation profile these isolates were clustered into 2 major groups at 0.75 coefficient level the cluster-I was further divided into 3 subgroups mainly comprising of isolates from Churpi cheese-C and B, cluster-II was divided in 2 subgroups and comprised isolates from sample B and A as in figure 2. These isolates were found ability to ferment Lactose, Maltose, Fructose, dextrose, Trehalose, Mannose, Inulin, Salicin and Cellobiose.

For species confirmation, partial sequencing of 16S rDNA (V1–V3 region) was performed. For sequencing all the 20 isolates gave a positive PCR product of 750 bp (Fig. 3). BLAST analysis of the sequences obtained was carried out to find similarity of our sequence with the sequence deposited in GENBANK, and species of all

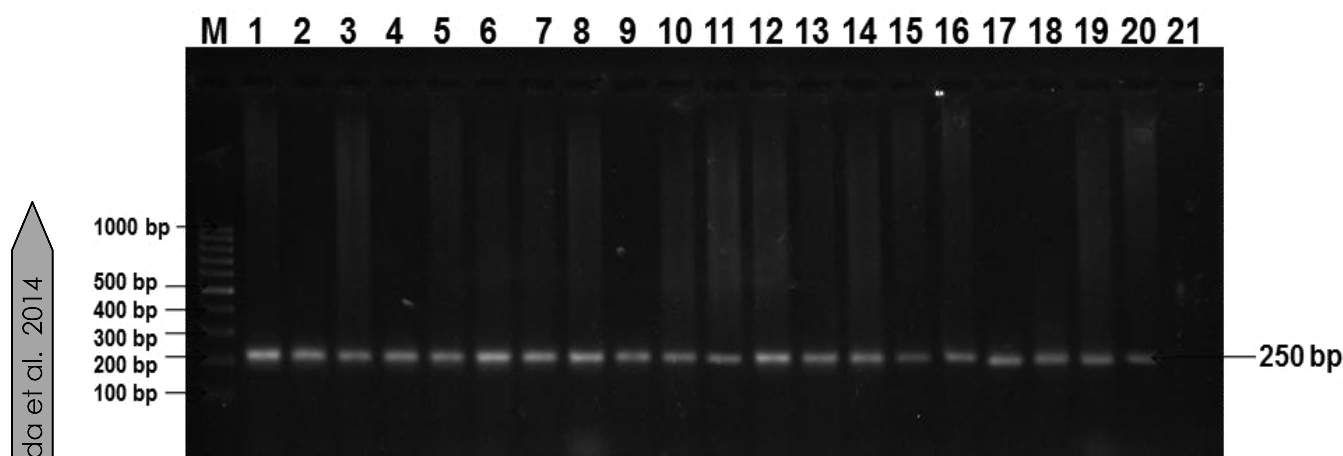

Figure 1 Agarose gel electrophoresis pattern of PCR amplified product of *Lactobacillus* genus-specific primer (250 bp). M: 100bp ladder, lane 1 to 20: *Lactobacillus* isolates, lane 21: -ve control

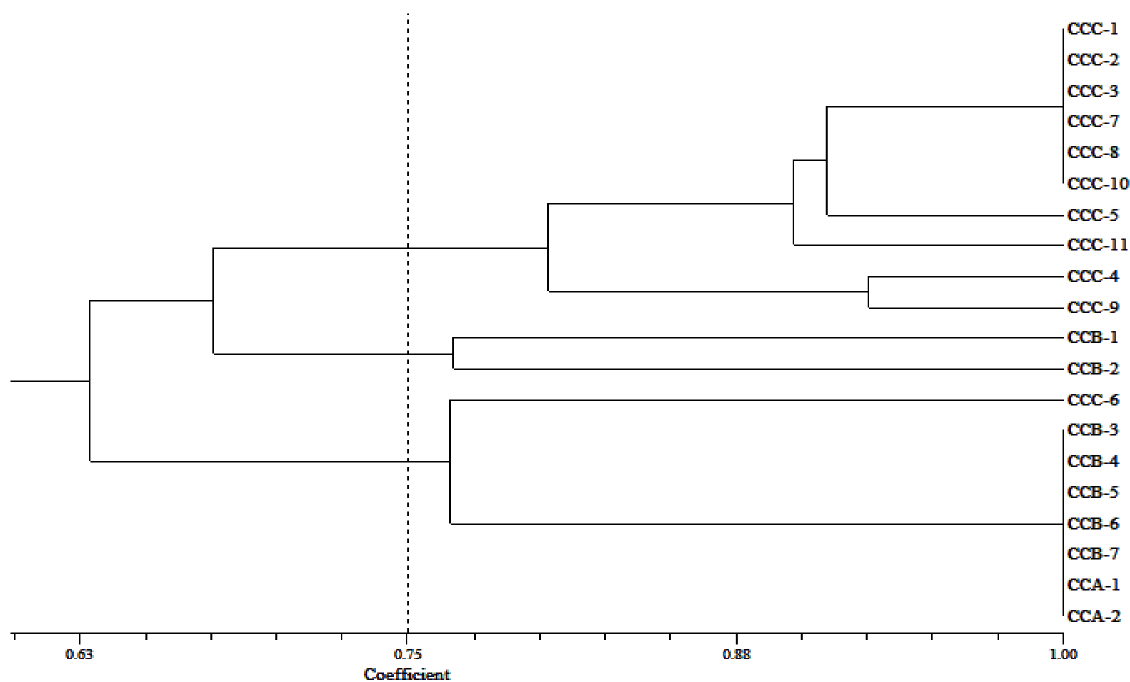

Figure 2 Dendrogram discriminating isolates on the basis of their sugar fermentation profiles constructed using NTSYSpc 2.02

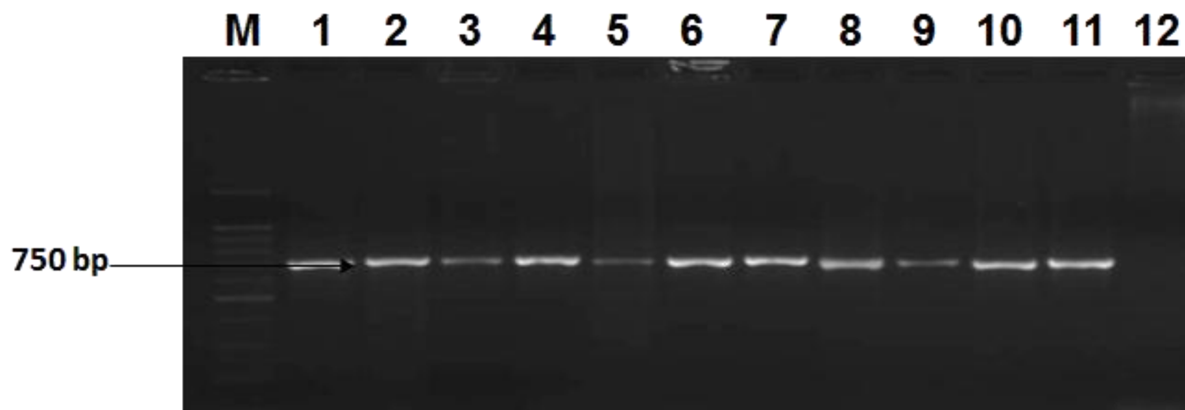

Figure 3 Agarose gel electrophoresis pattern of PCR amplified product of 16S r DNA (750 bp). M: 100bp ladder, lane 1 to 11: *Lactobacillus* isolates, lane 12: -ve control

isolates was confirmed. The sequence data generated have been submitted to GENBANK database under accession numbers as in Table 2. Among the isolates *Lactobacillus casei* (9) was found to be most prevalent in Churpi cheese samples followed by *L. plantarum* (4), *L. delbrueckii* (3), *L. paracasei* (2) and *L. brevis* (2).

DISCUSSION

Fermentation of any traditional food products is carried out by the natural, wild-type LAB which can come from the raw materials or from the environment. During studying the natural microbial diversity of Churpi cheese in the present study, *Lactobacilli* were found to be dominating other LAB with predominance of *L. casei* in sample C followed by *L. plantarum* in sample B, *L. delbrueckii* in samples A and B, *L. paracasei* in sample C and *L. brevis* in sample B. These species *L. casei, L. plantarum,* and *L. brevis* have been reported to be predominating *Lactobacilli* of nonstarter lactic acid bacteria (NSLAB) in Cheddar cheese (Peterson and Marshall, 1990) our result supports the similar predominance in Churpi cheese. Similarly earlier studies on Churpi cheese of yak milk clearly supports the

© 2014 jibresearch.com

Table-2: Accession numbers of sequences deposited to GENBANK

Species	Accession number	Sample
Lactobacillus casei	HM163461, JX104095, HM163462, HM163463, HM163464, JX104096, JX104097, HM163467, HM163468	C
Lactobacillus paracasei	HM163465, HM163466	C
Lactobacillus delbrueckii	JX104090, JX104091, JX104094	A, B
Lactobacillus brevis	JX104092, JX104093	B
Lactobacillus plantarum	HM163457, HM163458, HM163459, HM163460	B

presence of *L. paracasei* and *L. plantarum* as the dominating LAB species (Tamang et al. 2000; Prashant et al. 2009). *L. delbrueckii* has been found dominating *Lactobacilli* in various types of artisanal cheese as Ragusano cheese (Randazzo et al. 2002) Italian hard and semi-hard cheeses (Giraffa et al. 2004) Serbian cheese (Begovic et al. 2011) and camel cheese (Nanda et al. 2011). Thus the species of *Lactobacilli* obtained from Churpi cheese are in agreement with the findings of other workers in different varieties of cheese. The *Lactobacilli* grow as secondary microflora particularly during maturation process and influence the organoleptic properties of any cheese (Veljovic et al. 2007), thus the peculiar flavour of Churpi can be correlated with these isolates. *Lactobacillus* species, viz. *L. brevis, L. fermentum, L. rhamnosus, and L. coryniformis*, are less commonly found in cheese where NSLAB densities are initially lower and build up with time during maturation (Crow et al. 2001). The presence of *L. brevis* in sample B (farmer made Churpi cheese) may be due to maturation of cheese.

In the present study it was tried to isolate and identify the isolates genotypically by comparing their partial DNA sequence of 16S r RNA gene with the sequences with the existing sequences in the public database GENBANK (NCBI). The sequence results were able to successfully characterize the species of isolates. The dendrogram derived from the sequences of isolates along with reference sequences retrieved from database clearly grouped the isolates with reference sequence representing the species (Fig 4). But the two isolates of *L. paracasei* and reference sequence could not be grouped separately and were grouped along with *L. casei,* this result was obtained because *L. casei* and *L. paracasei* are phylogenetically closely related to each other (Felis et al. 2001; Diancourt et al. 2007) and they have similarities in 16 S r DNA sequences.

No discrepancy was detected in phenotypic traits and molecular data. By comparing the dendrogram generated by phenotypic and genotypic data Fig. 2 and Fig. 4 it was found that the isolates were clustered in similar pattern except one isolates CCC-6. A polyphasic approach involving the combination of biochemical and molecular techniques is a method of

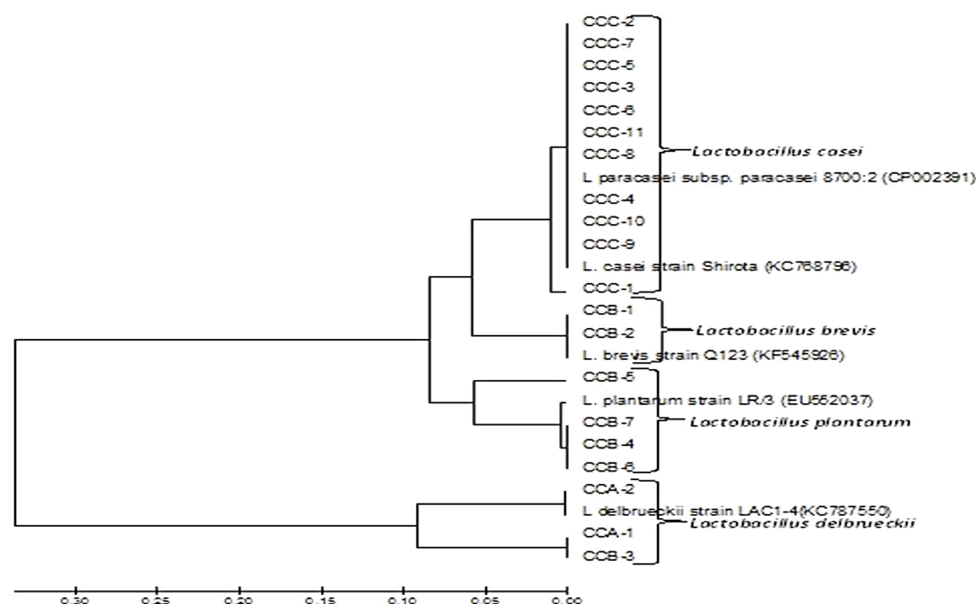

Figure 4 Phylogenetic analysis of 20 strains of *Lactobacillus* using 16 S r DNA sequences along with standard sequences retrieved from database constructed using the UPGMA method by Mega 5.0 software

choice for characterization of *Lactobacilli* from dairy and non-dairy sources (Vandamme et al. 1996; Gancheva et al. 1999; Lombardi et al. 2002; Nanda et al. 2011) in the current study also polyphasic approach allowed the identification of *Lactobacilli* in Churpi cheese and establish the biodiversity within these isolates. These strains of *Lactobacilli* can be further explored for the development of primary and adjunct starter cultures for Churpi and other varieties of cheeses and dairy products.

Conclusion

In the current study, 20 isolates of *Lactobacillus* were identified and characterized by a combination of conventional and molecular techniques. The study provides data on microflora of Churpi cheese. The technological properties of these isolates can further be explored with good starter activity and flavour production for industrial use as a novel starter culture for the preparation of cheese and other fermented dairy products. These isolates could also be studied for their possible probiotic applications.

Acknowledgements

The help provided by Chairman, Department of Biotechnology DCRUST is sincerely acknowledged. Authors also acknowledge the help provided by Directors of NBAIM, NDRI and NBAGR. Fellowship to DKN and financial support by Indian Council of Agriculture Research (ICAR), Government of India, New Delhi in the form of Network Project Application of Microbes in Agriculture and Allied Sectors (AMAAS) is thankfully acknowledged.

References

Babalola OO (2004) Molecular techniques: An overview of methods for the detection of bacteria. African J Biotechnol 2(12):710-713

Begovic J, Brandsma, JB, Jovcic B, Tolinacki M, Veljovic K, Meijer WC, Topisirovic L (2011) Analysis of dominant lactic acid bacteria from artisanal raw milk cheeses produced on the mountain Stara Planina, Serbia. Arch Biol Sci 63(1): 11-20

Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM (2001) Recent advances in cheese microbiology. Int Dairy J 11(4):259-274

Coeuret VR, Dubernet SGN, Bernardeau M, Gueguen M, Vernoux JP (2003) Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products. Le Lait 83(4):269-306

Collins MD, Wallbanks S (1992) Comparative sequence analyses of the 16S rRNA genes of *Lactobacillus minutus*, *Lactobacillus rimae* and *Streptococcus parvulus* : Proposal for the creation of a new genus *Atopobium*. FEMS Microbiol Lett 95(2):235-240

Crow V, Curry B, Hayes M(2001) The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. Int Dairy J 11(4):275-283

De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Microbiol 23(1):130-135

Dewan S, Tamang JP (2007) Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. Antonie van Leeuwenhoek 92(3):343-352

Diancourt L, Passet V, Chervaux C, Garault P, Smokvina T, Brisse S (2007) Multilocus sequence typing of Lactobacillus casei reveals a clonal population structure with low levels of homologous recombination. Appl Environ Microbiol 73(20):6601-6611

Dubernet S, Desmasures N, Guéguen M (2002) A PCR-based method for identification of lactobacilli at the genus level. FEMS Microbiol Lett 214(2):271-275

Felis GE, Dellaglio F, Mizzi L, Torriani S (2001) Comparative sequence analysis of a recA gene fragment brings new evidence for a change in the taxonomy of the Lactobacillus casei group. Int J Syst Evol Microbiol 51(6):2113-2117

Gancheva A, Pot B, Vanhonacker K, Hoste B, Kersters K (1999) A Polyphasic Approach towards the Identification of Strains Belonging to *Lactobacillus acidophilus* and Related Species. Syst Appl Microbiol 22(4):573-585

Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. FEMS Microbiol Lett 205(1):31-36

Giraffa G, Andrighetto C, Antonello C, Gatti M, Lazzi C, Marcazzan G, Lombardi A, Neviani E (2004) Genotypic and phenotypic diversity of *Lactobacillus delbrueckii* subsp. *lactis* strains of dairy origin. Int J Food Microbiol 91(2):129-139

Giraffa G, Neviani E (2000) Molecular identification and characterization of food-associated lactobacilli. Italian J Food Sci 12(4):403-423

Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, De Vos WM (2002) Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 68(1):114-123

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. The American J Clin Nutr 73(2):365-373

Kandler O, Weiss N (1986) Genus Lactobacillus. Bergey's Manual of Syst Bacteriol 2:1209-1234

Kim M, Chun J (2005) Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. Int J Food Microbiol 103(1):91-96

Klein G, Pack A, Bonaparte C, Reuter G (1998) Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol 41(2):103-125

Klijn N, Weerkamp AH, De Vos WM (1995) Detection and characterization of lactose-utilizing Lactococcus spp. in natural ecosystems. Appl Environ Microbiol 61(2):788-792.

Lane DJ, 1991. 16S/23S rRNA sequencing. In Goodfellow et al. *Nucleic acid techniques in bacterial systematics.* J. Wiley, pp 115-148

Lombardi A, Dal Maistro L, De Dea P, Gatti M, Giraffa G, Neviani E (2002) A polyphasic approach to highlight genotypic and phenotypic diversities of Lactobacillus helveticus strains isolated from dairy starter cultures and cheeses. J Dairy Res 69(1):139-149

Massi M, Vitali B, Federici F, Matteuzzi D, Brigidi P (2004) Identification method based on PCR combined with automated ribotyping for tracking probiotic Lactobacillus strains colonizing the human gut and vagina. J Appl Microbiol 96(4):777-786

Mccartney AL (2002) Application of molecular biological methods for studying probiotics and the gut flora. British J Nutr 88:29-37.

Nanda DK, Tomar SK, Singh R, Mal G, Singh P, Arora DK, Joshi BK, Chaudhary R, Kumar D (2011) Phenotypic and genotypic characterisation of Lactobacilli isolated from

© 2014 jibresearch.com

camel cheese produced in India. Int J Dairy Tech 64(3):437-443

Nguyen HKT, Ha DL, Doan TTV, Quach TT, Nguyen HK (2013). Study of Lactobacillus Acidophilus by Restriction Fragment Length Polymorphism (RFLP) Analysis. In: 4th International Conference on Biomedical Engineering held at Vietnam. Springer, pp. 195-197

O'sullivan L, Ross RP, Hill C (2002) Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84(5):593-604

Peterson SD, Marshall RT (1990) Nonstarter lactobacilli in Cheddar cheese: a review. J Dairy Sci 73(6):1395-1410.

Pospiech A, Neumann BR (1995) A versatile quick-prep of genomic DNA from gram-positive bacteria. TIG 11(6):217-218

Prashant, Tomar SK, Singh R, Gupta SC, Arora DK, Joshi BK, Kumar D (2009) Phenotypic and genotypic characterization of lactobacilli from Churpi cheese. Dairy Sci Tech 89(6):531-540

Randazzo CL, Torriani S, Akkermans a DL, de Vos WM, Vaughan EE (2002) Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Appl Environ Microbiol 68(4):1882-1892

Tamang JP, Dewan S, Thapa S, Olasupo NA, Schillinger U, Wijaya A, Holzapfel WH (2000) Identification and enzymatic profiles of the predominant lactic acid bacteria isolated from soft variety Chhurpi, a traditional cheese typical of the Sikkim Himalayas. Food Biotechnol 14:99-112

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* 28(10):2731-2739

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60(2):407-438

Veljovic K, Terzic-Vidojevic A, Vukasinovic M, Strahinic I, Begovic J, Lozo J, Ostojic M, Topisirovic L (2007) Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese. J Appl Microbiol 103(6):2142-2152

Weerkamp aH, Klijn N, Neeter R, Smit G (1996) Properties of mesophilic lactic acid bacteria from raw milk and naturally fermented raw milk products. Nederlands melk en Zuiveltijdschrift 50(2):319-332

Wouters J, Ayad EHE, Hugenholtz J, Smit G (2002) Microbes from raw milk for fermented dairy products. Int Dairy J 12(2):91-109

Yonzan H, Tamang JP (1998) Consumption pattern of traditional fermented foods in the Sikkim Himalaya. J Hill Res 11:112-115