Dairy Sci. Technol. 89 (2009) 531–540
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DOI: 10.1051/dst/2009029

Available online at:
www.dairy-journal.org

Original article

Phenotypic and genotypic characterization of lactobacilli from Churpi cheese

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Received 8 January 2009 – Revised 20 May 2009 – Accepted 10 June 2009
Published online 25 July 2009

Abstract – The aim of this study was to investigate the diversity of lactobacilli in Churpi cheese, a traditional variety of cheese made from yak milk. Thirty-five lactobacilli isolated from five different samples procured from different parts of Arunachal Pradesh (India) were analyzed by phenotypic and genotypic methods. The 16S rDNA sequencing for all the isolates was performed. Five different species of lactobacilli were isolated from Churpi cheese with the predominance of *Lactobacillus paracasei* followed by *Lactobacillus plantarum*. All the isolates were further evaluated for their technological properties such as citrate utilization, exopolysaccharide (EPS), bacteriocin, and acid production. Forty-three percent isolates were found positive for citrate utilization, while 14% were found good acid producer, and 11% for EPS production. *Lactobacillus coryniformis* was an uncommon species found in Churpi cheese. Isolates obtained in this study can be potentially used for the development of defined strain starter for Churpi cheese.

Churpi cheese / *Lactobacillus* / 16S rDNA / yak

摘要 – Churpi 干酪中乳杆菌的表型和遗传型特性分析。本研究目的是调查由牦牛乳制作的传统干酪—— Churpi 干酪中乳杆菌的多样性。本研究选取了来自印度 Arunachal Pradesh 不同地区的 5 种不同的 Churpi 干酪样品，通过表型和遗传型方法分析，从中共获得了35 株乳杆菌。所有分离菌株均进行了 16S rDNA 序列分析。从 Churpi 干酪中获得了 5 种不同的乳杆菌，其中 *Lactobacillus paracasei* 为优势菌，其次是 *Lactobacillus plantarum*。对所有分离的菌株的技术特性，如柠檬酸盐利用能力、产胞外多糖、细菌素的能力和产酸能力等进行了进一步评价。43% 菌株能够利用柠檬酸盐，14% 的菌株具有较好的产酸性能，11% 的菌株可产胞外多糖。*Lactobacillus coryniformis* 是 Churpi 干酪中罕见的菌种。当前研究所获得的菌株，具有用于生产 Churpi 干酪发酵剂的潜力。

Churpi 干酪 / *Lactobacillus* / 16S rDNA / 牦牛

Résumé – Caractérisation phénotypique et génotypique de lactobacilles de fromage Churpi. Le but de cette étude était d'évaluer la diversité des lactobacilles du fromage Churpi, une variété

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traditionnelle de fromage fabriqué à partir de lait de yak. Trente-cinq lactobacilles isolés de cinq échantillons différents obtenus de différents endroits d'Arunachal Pradesh (Inde) ont été analysés à l'aide de méthodes phénotypiques et génotypiques. Le séquençage de l'ADNr 16S de tous les isolats a été réalisé. Cinq espèces différentes de lactobacilles ont été isolées du fromage Churpi avec une prédominance de *Lactobacillus paracasei* suivi par *Lactobacillus plantarum*. Tous les isolats ont ensuite été évalués pour leurs propriétés technologiques telles que l'utilisation du citrate, la production d'exopolysaccharides (EPS), de bactériocine et d'acide. 43 % des isolats se sont avérés positifs pour l'utilisation du citrate, 14 % bons producteurs d'acide et 11 % producteurs d'EPS. *Lactobacillus coryniformis*, espèce rare dans le fromage, a été trouvé dans le fromage Churpi. Les isolats obtenus dans la présente étude pourraient servir au développement de levains de souches définies pour la fabrication de fromage Churpi.

fromage Churpi / *Lactobacillus* / ADNr 16S / lait de yak

1. INTRODUCTION

Yak (*Bos grunniens*; now *Poephagus grunniens*) is one of a few domesticated animals capable of surviving in extreme environmental conditions. It is mainly found in the highlands of the Nepalese Himalayas, India (Kashmir and Arunachal Pradesh), China (Tibetan highlands), Mongolia, and Bhutan. The composition of yak milk is 16.9–17.7 $g{\cdot}L^{-1}$ dry matter, 49–53 $g{\cdot}L^{-1}$ protein, 55–72 $g{\cdot}L^{-1}$ fat, 45–50 $g{\cdot}L^{-1}$ lactose, and 8–9 $g{\cdot}L^{-1}$ minerals [20]. Yak milk is processed into a number of dairy products such as butter, fermented milk (e.g. *Kurut*), and cheese (e.g. Churpi). Churpi, the so-called in Tibetan and Nepali languages and *byaslag* in Mongolia, is a delicious cheese having a consistency ranging from soft to slightly hard, color white to orange, taste and odor, sour to pungent, pH 5.97–7.0. It is traditionally made from yak milk in an indigenous cylindrical churner made of wood and bamboo strips. Churpi cheese is an artisanal cheese manufactured from raw yak milk on a small scale and at a household level with the common practice of backslopping. Cheese blocks are brined and aged for 4–5 months at ambient temperature for the development of a good flavor. The chemical composition of yak cheese is around 68.2% of total solid (TS), 49.4% of butterfat on a dry matter basis, and 1.37% of salt [20]. It is largely consumed in the Himalayan highland and its industrial production is not yet standardized.

The biodiversity of commercial starters has become limited due to the large-scale use of starter culture for industrial fermentation process. Traditional dairy products have their natural microflora from non-pasteurized milk and raw materials are still the source of new strains [19]. Pure cultures isolated from complex ecosystems of traditionally fermented foods exhibit a diversity of metabolic activities that diverge strongly from the ones of comparable strains used as industrial bulk starters [11]. Thus, efforts should be made to preserve the microbial diversity of raw milk and traditional dairy products. Knowledge of the prevalence of lactic acid bacteria (LAB) in the natural flora of Churpi can pave the way for the formulation of defined strain starter. Microbial diversity has been studied using various DNA fingerprinting techniques like RAPD-PCR, RFLP, ribotyping, and DGGE. However, sequencing of 16S rRNA gene is more reliable for molecular ecology and diversity analysis study because it is difficult to identify minor components of microbiota by other methods [10]. In the context of Churpi, no previous report concerning the genotypic and phenotypic characterization of the microflora has been found. Hence, this study was taken up with the objective of phenotypic, genotypic, and technological characterization of *Lactobacillus* isolated from Churpi cheese.

2. MATERIALS AND METHODS

2.1. Samples

Five Churpi cheese samples were analyzed in this study, of which two were obtained from the National Research Centre for Yak (NRCY, Arunachal Pradesh) and three samples were procured from domestic sources and local markets of Arunachal Pradesh. All samples were collected aseptically in sterile NASCO sampling bags kept in an ice box and transported to the laboratory for analysis.

2.2. Isolation of *Lactobacillus*

Ten grams of cheese sample were homogenized with 90 mL of 2% trisodium citrate, serially diluted (10^{-1}–10^{-8}) in saline solution, plated into DeMan, Rogosa and Sharpe (MRS) agar medium (Oxoid, Hampshire, England), and incubated under anaerobic conditions in CO_2 incubator (New Brunswick Scientific Co., NJ, USA) at 37 °C for 48–72 h. Twenty colonies were randomly picked from each plate and purified by streaking on MRS agar plates. A total of 200 colonies were screened on the basis of Gram reaction, morphology, and catalase test; 35 putative *Lactobacillus* isolates so obtained were further characterized by biochemical and molecular methods. The isolates were preserved by lyophilization (Edwards High Vacuum International, Sussex, England) and also as glycerol stocks (MRS broth with 30% glycerol) at −20 °C for further characterization.

2.3. Reference strains

The reference strains viz. *Lactobacillus plantarum* NCDC 221 (NCDO 340), *Lactobacillus delbrueckii* ssp. *lactis* NCDC 3 (ATCC 4797), *Lactobacillus fermentum* NCDC 155 (ATCC 8289), *Lactobacillus paracasei* NCDC 63 (ATCC 393), *Lactobacillus rhamnosus* NCDC 24 (ATCC 8014), *Lactobacillus helveticus* NCDC 5 (ATCC 8018), and *Lactobacillus brevis* NCDC 337 (ATCC 14869) were obtained from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana, India.

2.4. Phenotypic characterization of isolates

Isolates were Gram-stained and tested for catalase activity. All isolates were tested for their ability to grow at 10, 15, and 45 °C, at different concentrations of NaCl (4%, 6.5%, and 8%), at pH 3.9 and 9.6, and their ability to produce CO_2 by the fermentation of glucose and NH_3 production from arginine. Sugar fermentation patterns were determined using the CHL as basal medium.

2.5. Microbiological analysis

For enumerating LAB, 10 g of each cheese sample was dissolved in 90 mL of 2% sodium citrate buffer, which was serially diluted using physiological saline (10^{-5}, 10^{-6}, and 10^{-7}). LAB were enumerated by pour plating the dilutions on lactic agar (Oxoid, Hampshire, England) and incubating for 72 h at 37 °C.

2.6. Genotypic characterization of *Lactobacillus*

Genomic DNA was extracted from 2 mL samples of overnight cultures grown in MRS broth at 37 °C as previously described [13]. *Lactobacillus* genus-specific primer (Tab. I) targeting 16S rRNA gene was used for the confirmation of *Lactobacillus* genus as previously described [7]. Polymerase chain reaction (PCR) was performed for the identification of isolates in 25 μL of reaction volume, containing 50–100 ng

Table I. List of primers used in this study for *Lactobacillus* identification.

Target	Primer	Primer sequence 5′–3′	Ta (°C)	Product size (bp)	Reference
Lactobacillus genus	Genus-F Genus-R	CTCAAAACTAAACAAAGTTTC CTTGTACACACCGCCCGTCA	55	250	[7]
7F S-G-Lab-0677-R	7F S-G-Lab-0677-R	AGAGTTTGAT(C/T)(A/C)TGGCTCAG CACCGCTACACATGGAG	57	700	[8, 12]
L. casei	Y1 *L. casei*	TGGCTCAGAACGAACGCTAGGCCCG TGCACTGAGATTCGACTTAA	52	290	[19, 23]
L. paracasei	Y1 *L. paracasei*	TGGCTCAGAACGAACGCTAGGCCCG CACCGAGATTCAACATGG	52	290	[18, 22]
L. pentosus	16 *L. pentosus*	GCTGGATCACCTCCTTTC GTATTCAACTTATTAGAACG	53	220	[5]
L. plantarum	16 *L. plantarum*	GCTGGATCACCTCCTTTC ATGAGGTATTCAACTTATG	53	220	[5]
prtP	P6 P7	CAACACGGCATGCATGTTGC CTGGCGTTCCCACCATTCA	55	393	[11]

of genomic DNA, 1 X Taq buffer, 1.5 mmol·L^{-1} $MgCl_2$, 10 mmol·L^{-1} of each dNTP, 50 ng of each primer, and 1 unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India). Amplification was performed on an Eppendorf Mastercycler (Hamburg, Germany) according to earlier published literature [5, 7, 8, 11, 12, 18, 22]. Amplification was verified by electrophoresis on 1.5% (w/v) agarose gel in 1 X TAE buffer using a 100 bp ladder (Bangalore Genei, Bangalore, India) as a molecular weight marker. Gels were stained with ethidium bromide (1 mg·mL^{-1}).

Sequencing of 16S rDNA and phylogenetic analysis were performed as follows.

16S rDNA gene of all 35 isolates was amplified using primer 7F [12] and primer S-G-Lab-0677-R (Tab. I) [8]. The amplified PCR products from 35 isolates were purified with sodium acetate-ethanol precipitation. The PCR products (10 μL) were directly sequenced using primer 7F [12] with the help of Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem, Foster City, USA) on an automated genetic analyzer (ABI 3100 Applied Biosystem, USA) to obtain partial sequence of the 16S rDNA. Sequences so obtained were analyzed using the Chromas software (version 1.45, http://www.technelysium.com.au/chromas.html). BLAST analysis was performed to check the identity of DNA sequence in the database and for species determination.

For the differentiation of closely related species, *Lactobacillus casei* and *L. paracasei*, *Lactobacillus pentosus* and *L. plantarum*, species-specific PCR [5, 18] was performed (Tab. I).

2.7. Technological characterization of isolates

The isolates were evaluated in triplicates for various technological attributes.

In order to determine the lactic acid-producing ability of all isolates, tubes containing 20 mL of heat-treated (90 °C, 10 min) reconstituted skimmed milk (Modern Dairies, Karnal, India) having 11.5% TS were inoculated with active culture (1% v/v) and incubated at 37 °C for 6 and 24 h. The percentage of lactic acid was estimated of samples by titrating curd with 0.1 N NaOH to an end point of pale pink using phenolphthalein as indicator and the pH of acidified milk was measured using a pH meter (Thermo Electron, Madison, WI, USA) in triplicate.

Exopolysaccharide (EPS) production of *Lactobacillus* isolates was screened by copper sulfate staining [1]. The isolates were microscopically examined for capsule formation.

Citrate utilization by *Lactobacillus* isolates was screened qualitatively on modified differential agar medium in which the formation of Prussian blue colonies shows positive citrate fermenting colonies as previously described [9].

To screen isolates for bacteriocin production, an assay was performed against two indicator organisms, *Pediococcus acidilactici* NCDC 252 and *L. brevis* NCDC 01, using agar well diffusion assay [4].

All isolates were examined for the presence of the prtP gene coding for cell envelope proteinase. In order to amplify this gene, primers P6 and P7 (Tab. I) were used according to PCR conditions previously described [11].

3. RESULTS

Mesophilic lactic count of cheese samples was found to vary between 6.1×10^6 and 7.1×10^6 g^{-1}. Of 200 colonies initially screened on the basis of microscopic examination and catalase test, 35 isolates appeared to belong to the *Lactobacillus* genus and were further characterized by biochemical and molecular methods. All 35 isolates conformed to the general phenotypic characteristics of genus *Lactobacillus*,

Table II. Biochemical and technological characteristics of *Lactobacillus* strains isolated from Churpi cheese (Fig. 2).

Isolates	Growth at								Arginine	Citrate utilization	prtP gene	EPS	pH 6 h[a]	pH 24 h[a]	Acidity 6 h[a]	Acidity 24 h[a]
	4% NaCl	6.5% NaCl	8% NaCl	pH 3.9	pH 9.6	10 °C	15 °C	45 °C								
L. brevis Y-1-3	−	−	−	−	−	−	+	−	+	+	−	−	4.34	4.13	0.90	1.17
L. brevis Y-2-10	+	−	−	+	−	−	+	−	+	+	−	−	4.26	4.03	0.85	1.05
L. coryniformis Y-1-17	+	−	−	−	−	+	+	+	−	−	−	−	6.56	5.33	0.20	0.46
L. coryniformis Y-1-18	−	−	−	−	−	+	+	−	−	−	−	−	6.56	5.23	0.24	0.45
L. coryniformis Y-3-1	−	−	−	−	−	+	+	−	−	−	−	−	6.44	5.41	0.38	0.42
L. coryniformis Y-3-8	+	−	−	−	−	+	+	−	−	−	−	−	6.46	4.17	0.94	1.01
L. paracasei Y-1-10	+	−	−	−	−	−	+	−	−	+	+	−	5.71	5.14	0.45	0.55
L. paracasei Y-1-19	+	+	−	−	−	−	+	−	−	−	+	−	6.39	5.0	0.24	0.43
L. paracasei Y-1-20	+	−	−	−	−	−	+	−	−	+	+	−	6.36	5.24	0.25	0.50
L. paracasei Y-1-23	+	−	−	−	−	−	+	−	−	−	+	−	6.37	4.84	0.30	0.70
L. paracasei Y-3-2	+	+	−	−	−	+	+	−	−	+	−	−	5.55	5.40	0.24	0.46
L. paracasei Y-3-5	+	−	−	−	−	+	+	−	−	−	+	−	4.30	4.10	0.87	0.97
L. paracasei Y-3-6	−	−	−	−	−	+	+	−	−	−	+	−	6.52	5.30	0.23	0.5
L. paracasei Y-3-10	−	−	−	−	−	+	+	−	−	−	+	−	6.39	4.44	0.23	0.97
L. paracasei Y-3-11	−	−	−	−	−	+	+	−	−	−	−	−	4.25	4.01	0.23	1.01
L. paracasei NRC-1	−	−	−	−	−	+	+	−	−	+	+	−	5.70	5.43	0.26	0.42
L. paracasei NRC-2	−	+	−	−	−	+	+	−	−	−	−	−	5.98	5.02	0.35	0.67
L. paracasei NRC-3	+	+	−	−	−	+	+	−	−	+	−	−	6.47	4.76	0.23	0.67

Table II. Continued.

Isolates	Growth at								Arginine	Citrate utilization	prtP gene	EPS	pH 6 h[a]	pH 24 h[a]	Acidity 6 h[a]	Acidity 24 h[a]
	4% NaCl	6.5% NaCl	8% NaCl	pH 3.9	pH 9.6	10 °C	15 °C	45 °C								
L. paracasei NRC-4	+	−	−	−	−	+	+	−	−	−	−	+	5.07	4.90	0.72	0.87
L. paracasei NRC-5	+	−	−	−	−	+	+	−	−	+	+	−	4.44	4.20	0.93	1.07
L. paracasei NRC-6	−	−	−	−	−	+	+	−	−	−	+	−	5.56	5.21	0.62	0.53
L. paracasei NRC-7	−	−	−	−	−	+	+	−	−	−	+	−	5.74	5.13	0.31	0.64
L. paracasei NRC-11	−	−	−	−	−	+	+	−	−	−	+	+	5.20	5.14	0.54	0.60
L. plantarum Y-2-1	+	−	−	−	−	−	+	−	−	+	−	−	5.41	5.12	0.43	0.65
L. plantarum Y-2-5	+	+	−	−	−	−	+	−	−	−	−	+	5.63	5.17	0.46	0.56
L. plantarum Y-2-7	+	−	−	−	−	−	+	−	−	−	−	−	6.44	5.36	0.22	0.44
L. plantarum Y-2-8	+	+	−	−	−	−	+	−	−	+	−	−	6.46	5.26	0.22	0.54
L. plantarum Y-2-9	+	+	−	−	−	−	+	−	−	+	−	−	6.45	5.94	0.22	0.42
L. plantarum Y-2-11	+	+	−	−	−	−	+	−	−	+	−	−	6.51	5.44	0.22	0.46
L. plantarum Y-2-14	+	+	+	−	−	+	+	−	−	−	−	−	4.99	4.32	0.83	0.96
L. plantarum Y-2-15	+	−	−	−	−	−	+	−	−	+	−	−	6.43	5.40	0.22	0.50
L. plantarum Y-2-16	+	−	−	−	−	+	+	−	−	+	−	−	6.47	5.37	0.22	0.43
L. plantarum Y-2-17	+	+	−	−	−	−	+	−	−	−	−	−	6.53	5.28	0.22	0.54
L. plantarum Y-3-3	+	−	−	−	−	+	+	−	−	+	−	+	4.25	4.03	0.45	0.59
L. helveticus NRC-12	−	−	−	−	−	−	+	−	−	−	−	−	5.42	5.05	0.43	0.75

[a] Mean value of triplicate.

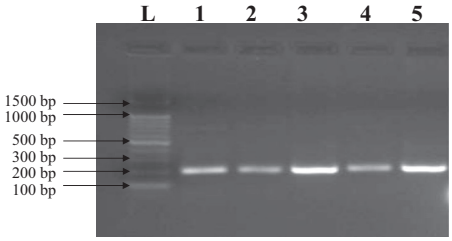

Figure 1. Genus-specific PCR of *Lactobacillus* (250 bp) L = 100 bp ladder, lane 1 = *L. brevis* Y-1-3, 2 = *L. paracasei* Y-1-10, 3 = *L. coryniformis* Y-1-17, 4 = *L. plantarum* Y-2-1, and 5 = *L. helveticus* NRC-12.

i.e. Gram positive, rod shaped, nonmotile, non-sporulating, and catalase negative (Tab. II). The generic status was further confirmed by PCR as all the isolates showed amplification product of expected size (250 bp) using genus-specific primers (Fig. 1). Based on the phenotypic characters, the isolates were tentatively designated as *L. casei* (17), *L. plantarum* (11), *Lactobacillus coryniformis* (4), *L. brevis* (2), and *L. helveticus* (1). For species confirmation, partial sequencing of 16S rDNA (V1–V3 region) was performed. The species level identification could only be done for *L. brevis*, *L. helveticus*, and *L. coryniformis* isolates on the basis of 16S rDNA sequencing. The species designation of other isolates could not be resolved by the 16S rDNA sequencing as the BLAST analysis results showed equal resemblance to the closely related species, *L. casei* and *L. paracasei*; *L. plantarum* and *L. pentosus.* To resolve the issue further, a species-specific PCR was performed and it finally confirmed the isolates to be *L. paracasei* and *L. plantarum.* Sequence data generated for all isolates were submitted to the GenBank under the following Accession Nos. EU637371–EU637403, EU886737, and EU886738. Except the two isolates NRC-1 and NRC-2, the partial sequencing of 16S rDNA demonstrated high identity (99–100%) with 16S rDNA sequence respective to the members of *Lactobacillus* from the GenBank database.

4. DISCUSSION

The fermentation of traditional foods is usually affected by natural, wild-type LAB originating from the raw materials and the environment. This study shows the predominance of *L. paracasei* and *L. plantarum* in Churpi cheese. This cheese is made from raw milk of yak in indigenously designed churners made from wood and bamboo strips. The churners and the bamboo strips may contribute to the microflora of cheese as young bamboo shoots have been shown to harbor lactobacilli. Tamang and Sarkar [16] showed the predominance of *L. plantarum* and *L. brevis* in young bamboo shoots. In a recent study on fresh and fermented yak milk, Wu et al. [21] reported the presence of *L. fermentum*, *L. helveticus*, and *Lactobacillus curvatus* showing that lactobacilli could play an important role in the fermentation of yak milk.

L. paracasei, *L. brevis*, and *L. plantarum* have been reported to be the predominant members of the nonstarter lactic acid bacteria (NSLAB) community of mature Zlatar and Spanish goat cheese [14, 17], which are also artisanal type of cheese. The lactobacilli grow as secondary flora particularly during the maturation process and influence the organoleptic properties of the cheese [17]. *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 [6]. *L. coryniformis* is an uncommon species found in low densities in cheeses and we obtained the same result in the case of Churpi cheese also. In this study, we tried to identify the isolates genotypically by comparing the partial sequence of 16S rDNA as the majority of lactobacilli can be identified by sequencing of the first half region, i.e. from V1 to V3

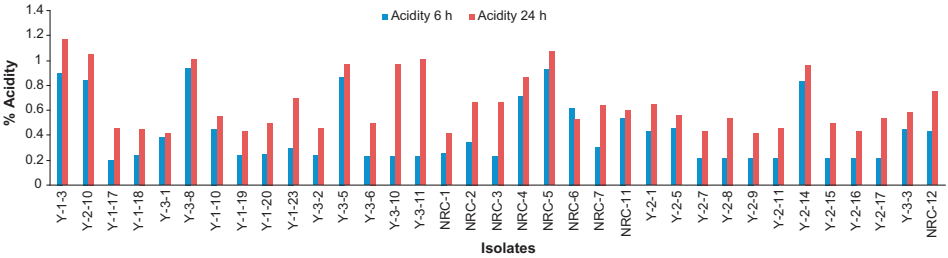

Figure 2. Percentage acidity (mean value of triplicate) produced after 6 and 24 h by different isolates of *Lactobacillus* as listed in Table II.

of 16S rDNA [3]. However, it was not able to resolve some of the closely related species, as *L. casei*, *L. paracasei*, *L. plantarum*, and *L. pentosus* showed a very high degree of sequence similarity in the amplified region. In such cases, the use of species-specific PCR or complete sequencing of 16S rDNA is preferable [15]. The identity of closely related species was successfully ascertained by the use of species-specific PCR. Genotypic heterogeneity could be seen in *L. paracasei* as isolates NRC-1 and NRC-2 showed only 95–96% sequence similarity, with the existing sequences in the GenBank database.

Technological properties of starter and nonstarter bacteria are known to affect the cheese quality. The most acidifying strains found were *L. coryniformis* (Y-3-8), *L. paracasei* (NRC-5), *L. brevis* (Y-1-3), *L. paracasei* (Y-3-5), *L. brevis* (Y-2-10), and *L. plantarum* (Y-2-14), which produced between 0.83% and 0.94% lactic acid after 6 h at 37 °C, while other isolates produced lower amounts of lactic acid in milk. Among strains of different LAB isolated from traditional Egyptian dairy products, Ayad et al. [2] also reported overall lower acidifying activity in the case of wild-type lactococci. A total of 15 citrate utilizing isolates were identified belonging to *L. plantarum* and *L. paracasei*. Crow et al. [6] also reported NSLAB from Cheddar cheese to be citrate fermenters. The citrate-positive isolates can influence the organoleptic attributes of cheese due to the production of carbonyl compounds and carbon dioxide. The production of EPS influences the textural and rheological properties of fermented milk products. It contributes to water retention in the final product, may impart a higher flavor intensity due to carbohydrate masking, and improve the mouth feel. The proteolytic activity of LAB has a role in cheese maturation. In this study, only *L. paracasei* isolates (12) were found positive for the prtP gene. As all technological properties were estimated only qualitatively, hence no relationship could be established among different technological traits.

A polyphasic approach involving the combination of biochemical and molecular techniques allowed the identification of lactobacilli in Churpi cheese. No discrepancy was detected in phenotypic traits and molecular identification. These strains of lactobacilli can be further explored for the development of primary and adjunct starter cultures for Churpi and other artisanal cheeses.

Acknowledgments: We sincerely acknowledge the financial assistance of the Indian Council of Agriculture Research (ICAR), Government of India, New Delhi in the form of Network Project on Application of Microbes in Agriculture and Allied Sectors (AMAAS). The authors also acknowledge the help provided by the Directors of NBAIM and NBAGR. Senior Research Fellowship of Prashant by ICAR is also acknowledged.

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