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Isolation and molecular characterization of lactobacilli from traditional fermented Dahi produced at different altitudes in Nepal

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Abstract Dairy products are an important part of daily food in the Himalayan country of Nepal. A wide variety of traditional fermented milk products are obtained in relation to different geo-climatic conditions of the country and different ethnic groups. Locally called Dahi is the most popular product, but little data are available on the autochthonous lactic acid bacteria (LAB) characterizing this yogurt-like product. Thirty-two replicate samples of indigenous Dahi were collected from four districts of Nepal at different altitude. In total of 193 isolates were obtained. Of these, the 120 rod-shaped isolates representing the dominant LAB population (62%) were further identified at the species level by using internal transcribed spacers (ITS) amplification, 16S rRNA gene sequence determination, and species-specific PCR. A further characterization at the strain level was carried out by combining analysis of repetitive elements and randomly amplified polymorphic DNA (RAPD) typing. Based on these analyses, the isolates were grouped in ten different species, among which *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. paracasei*, and *L. rhamnosus* represented the dominant species. A high degree of intraspecies diversity was also observed for all *Lactobacillus* species, except for *L. rhamnosus* isolates, which proved to give a single typing profile. Bacterial isolates represent a source of novel potential probiotics and starter cultures. The strain collection obtained from this study is a first step in the preservation of the natural biodiversity of bacterial population of the traditional Nepalese fermented Dahi.

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1 Introduction

Over the past few years, the microbiological characteristics of traditional fermented milk products have been studied in many countries, and original collections of lactic acid bacteria (LAB) have been constituted (Yu et al. 2011; Bao et al. 2012). They represent an important tool both for preserving the rich microbial biodiversity that characterizes naturally fermented food and for obtaining new cultures. It is known that traditional fermented foods have unique and different microbial populations dependent on the production technology as well as on the environmental characteristics of the localities where they have been produced (Leroy and DeVuyst 2004; Colombo et al. 2009). Moreover, many reports have shown that artisanal dairy products can represent interesting sources for the isolation of bacterial strains with useful probiotic traits (Heller et al. 2003; Taverniti and Guglielmetti 2010).

Dairy products are an important part of daily food intake in the Himalayan country of Nepal. As a consequence of different geo-climatic conditions of the country and of the diversity of ethnic groups, a wide variety of traditional fermented milk products are produced and consumed. Their local names are Dahi (curd), Mohi (buttermilk), Ghiu (butter), and Chhurpi (dried cheese). These products are made with milk from different animals that are reared in the different geographical regions for dairy production, such as cow and buffalo are reared in the Terai and Hilly regions (from 100 to 3,000 m) whereas yak and chauri at high altitudes (>3,000 m) in the Himalayan region.

Locally called Dahi (curd) is the most indigenous and popular product of Nepal. It is a yogurt-like product prepared in different parts of the country and used as either nutritional food, appetizer, or dessert, as well as for the preparation of other ethnic dairy fermented products, as Ghiu, Mohi, and soft Chhurpi (Tamang 2010). There are different conventional methods for the preparation of Dahi, comprising the use of starter cultures, even though this is not a routine practice. In most cases, Dahi is traditionally made at household level, without starter cultures but using a portion of previously produced Dahi or Mahi or cream as inoculum. Usually, after heating or boiling, the milk is cooled to 30–40 °C and then transferred to a wooden (locally named “Theki”) vessel, where it is left overnight at 25–30 °C. Fermentation is carried out spontaneously by natural microbiota of the milk, along with the microorganisms that persist on the surface of vessels and in the processing environment. In the Terai region and some Hilly regions, earthenware pots (natural red clay pot locally called “maato ko kataaro”) are more common. These pots have a porous surface, so moisture is absorbed by the container especially when the Dahi tends to exude some whey and also gives a “muddy” flavor and a thicker texture. This vessel is wrapped in cloth, in hay, in sawdust, or put in a straw box to maintain the suitable temperature for the souring and coagulation processes. This step is the most difficult to achieve in the regions of Nepal at high altitudes (>3,500 m), with consequent problems related to slow acidification and delay in coagulation. In Nepal, the traditional Dahi can also be obtained by a semi-continuous method. Boiled and cooled milk, inoculated with an indigenous natural starter culture (in many cases, previously made Dahi), is put in the container,

which is covered with clothes and kept warm. The next day, a further quantity of cooled boiled milk is added and the fermentation goes on. This topping up with cooled boiled milk is repeated daily until the container is full. After the last incubation, the Dahi may be either used for consumption or churned to obtain butter or other dairy products.

The present study aimed to isolate and identify the natural dominant lactobacilli in traditional Dahi of the Hilly and Himalayan regions of Nepal, by using phenotypic and molecular methods, with the final purpose of obtaining novel probiotic or starter culture candidates. To our knowledge, no similar investigation on this ethnic fermented product has been carried out so far.

2 Materials and methods

2.1 Collection of samples

A total of 32 replicate samples (altogether 64) of traditional fermented Dahi were collected from four districts, viz. Bhaktapur, Gorkha, Lalitpur, and Rasuwa. These districts lie in the Hilly (HL) and Himalayan (HM) regions, according to the geographical map of Nepal. Stratification of sampling sites were done based on total number of districts in each region, and proportional random sampling method was followed for sample collection. The collected samples were transported to the laboratory under refrigerated conditions (4 °C) and subjected to microbiological analysis within the following 24 h.

2.2 Isolation, enumeration, and phenotypic characterization

Samples (10 g each) were homogenized in 90 mL of 0.85% (w/v) sterile saline solution in a Vortex (Rexmed, Taiwan); serially diluted in the same diluents; plated in duplicate on de Man, Rogosa, and Sharpe (MRS) (Difco, Detroit, MI, USA); and incubated at 37 °C for 48 h. After incubation and counting, colonies were all picked up when they not exceeded the number of ten colonies per plate, otherwise they were selected randomly. Purity of the isolates was checked by streaking several times and subculturing on fresh MRS agar, as well as MRS broth, followed by microscopic examination. Isolates were Gram-stained and tested for catalase production. Identified isolates of LAB were preserved in MRS broth containing 25% (w/v) glycerol, at -20 °C.

2.3 Genotypic identification

Genomic DNA for all PCR reactions was extracted from a 100 µL of an overnight culture diluted with 300 µL of TE buffer (10 mmol.L⁻¹ Tris-HCl, 1 mmol.L⁻¹ Na₂EDTA, pH 8.0) as previously described (Mora et al. 2000). The DNA sequences for the primers used in this study, their corresponding specificities, and the thermal cycle parameters employed are reported in Table 1. PCR reactions were performed in a 25 µL reaction mixture containing 100 ng bacterial DNA, 2.5 µL 10× reaction buffer Dream TaqTM (Fermentas, Vilnius, Lithuania), 200 µmol.L⁻¹ of each dNTP,

Table 1 PCR primers and conditions used for isolates identification

Primer specificity and reference	Primer pair (5' to 3')	Thermal conditions	Amplicon (bp)
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (Torriani et al. 1999)	Fw: GTGCTGCAGAGAGAGTTTGATCTGGCTCAG Rev: ACCTATCTCTAGGTGTAGCGCA	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 1 min at 57 °C, 1 min at 72 °C	1,030
<i>L. paracasei</i> (Ward and Timmins 1999)	Fw: CACCGAGATCAACATGG Rev: CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 54 °C, 45 s at 72 °C	290
<i>L. rhamnosus</i> (Ward and Timmins 1999)	Fw: TGCATCTTGATTTAATTTTG Rev: CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 54 °C, 45 s at 72 °C	290
<i>L. fermentum</i> (Coton et al. 2008)	Fw: TGTACACACCCGCCGTC Rev: TTTTCTTGATTTTATTAG	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 45 s at 72 °C	460; 270
<i>L. parabuchneri</i> (Coton et al. 2008)	Fw: TGTACACACCGCCCGTC Rev: TGTTACTCCGGTCTGTGC	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 45 s at 72 °C	330
<i>L. helveticus</i> (Fortina et al. 2001)	Fw: CTGTTTTCAATGTGCAAGTC Rev: TTTGCCAGCATTAACAAAGTCT Fw: CGCTGATTTCTAAGTCAAGCT Rev: CGACTAAGAAAGTGGAAACATTA Fw: TCTTATTACGCAATGGACCAA Rev: AATACCGGTTCTTGAGGTTAGA	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C	918; 726; 524
<i>L. brevis</i> (Coton et al. 2008)	Fw: TGTACACACCCGCCGTC Rev: TAAATGATGACCTTGGCGTC	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 45 s at 72 °C	330
<i>L. plantarum</i> (Torriani et al. 2001)	Fw: CCGTTTTATGCGGAACACC Rev: TCGGGATTACCAAACATCAC	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 56 °C, 45 s at 72 °C	318
Internal Transcribed Spacer region (ITS) (Jensen et al. 1993)	Fw: GAAATCGTAACAAGG Rev: CAAGGCATCCACCGT	2 min at 94 °C, 5 cycles of 45 s at 94 °C, 1 min at 55 °C, 1 min at 72 °C and 30 cycles of 45 s at 94 °C, 45 s at 60 °C, 2 min at 72 °C	

0.5 mmol.L⁻¹ MgCl₂, 0.5 μmol.L⁻¹ each primer, and 0.5 U Dream TaqTM DNA polymerase. Amplifications were carried out using a PCR-Mastercycler 96 (Eppendorf, Milan, Italy). Amplification products were electrophoresed in 1.5–2.5% (w/v) agarose gel (with 0.2 μg.mL⁻¹ of ethidium bromide) in 1× TAE buffer (40 mmol.L⁻¹ Tris-acetate, 1 mmol.L⁻¹ EDTA, pH 8.0) and photographed. A GeneRuler DNA ladder mix (Fermentas) was used as a size marker.

The 16S ribosomal RNA (rRNA) gene was amplified by PCR, using primers P0 (5'-GAAGAGTTTGATCCTGGCTCAG-3') and P6 (5'-CTACGGCTACCTTGTACGA-3'). The PCR mixtures were subjected to the following thermal cycling: 2 min at 94 °C, then 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, followed by a 7-min final extension at 72 °C. UltraClean PCR Clean-Up Kit (MoBio, Cabru s.a.s, Arcore, Italy) was used to purify PCR products that were sequenced. A 500 bp portion of the 16S rRNA gene was sequenced for representative isolates. Sequencing reactions were performed using primer pA (5'-AGAGTTTGATCCTGGCTCAG; nucleotides eight to 28 of the 16S rRNA gene of *Escherichia coli*), which allowed to obtain the sequence of variable regions V1, V2, and V3 (Edwards et al. 1989). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (<http://www.ncbi.nlm.nih.gov/blast>). The taxonomic differentiation of *L. paracasei* and *L. rhamnosus* was obtained employing species-specific probes as described by Ward and Timmins (1999) (Table 1). The assignment of all *Lactobacillus delbrueckii* isolates to a precise subspecies was obtained through an intraspecies-specific PCR analysis according to Torriani et al. (1999) (Table 1).

2.4 Genetic typing of bacterial isolates

Genetic fingerprinting was carried out by combined repetitive extragenic palindromic PCR typing technique (REP-PCR) using primers (GTG)5 (5'-GTGGTGGTGGTGGT G-3'; annealing temperature (T_a)=42 °C) and BOXA1R (5'-CTACGGCAAGGCGA CGCTGACG-3'; T_a =48 °C) (Guglielmetti et al. 2008) and random amplification of polymorphic DNA-PCR (RAPD) typing with primers M13 (5'-GAGGGTGGCGGT TCT-3'; T_a =38 °C), AP02 (5'-AGTCAGCCAC-3'; T_a =32 °C), OPI17 (5'-CGAGGG TGGTGATG-3'; T_a =46 °C), OPI02 (5'-GCTCGGAGGAGAGG-3'; T_a =48 °C), and 1254 (5'-CCGCAGCCAA-3'; T_a =33 °C) (Torriani et al. 1999; Mora et al. 2000; Rossetti and Giraffa 2005). An amplification protocol of 35 cycles was used. The PCR products were separated by electrophoresis and photographed. Banding pattern similarity was evaluated by construction of dendrograms using the NTSYSpc software, version 2.11 (Applied Biostatistics Inc., Port Jefferson, NY, USA), employing the Jaccard similarity coefficient. A dendrogram was deduced from a similarity matrix using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm. The faithfulness of the cluster analysis was estimated by calculating the coefficient correlation value for each dendrogram.

3 Results and discussion

All samples were from indigenous Dahi products obtained by traditional methods from different geographical regions. The viable mesophilic counts of LAB on MRS varied in

the range of 6.3 to 10.4 \log_{10} CFU g^{-1} (Table 2). The samples of Dahi produced in the Lalitpur and Bhaktapur districts showed higher LAB count than the samples of Dahi produced in Gorkha district. Two hundred and five bacterial isolates were collected; among them, 193 isolates were considered as presumptive LAB by their positive Gram reaction, absence of catalase, and lack of mobility. The majority of isolates were rod shaped (120 isolates) and, therefore, plausibly belonging to the genus *Lactobacillus*; the remaining were cocci (73 isolates). Other reports also showed the dominance of lactobacilli in traditional dairy products of the Himalayan region (Tamang et al. 2000; Dewan and Tamang 2007).

The identification and characterization of autochthonous *Lactobacillus* strains are important for understanding their contribution to the sensorial characteristics of the final product and for providing new strains to be used as industrial starters. The genus *Lactobacillus*, in fact, has a dominant role over other bacterial genera in food fermentation technologies (e.g., *L. delbrueckii*, *L. helveticus*, *L. plantarum*) and probiotic applications (e.g., *L. paracasei*, *L. rhamnosus*). For these reasons, the 120 rod-shaped isolates were selected for further studies.

At first, the 120 *Lactobacillus* isolates were submitted to molecular analysis for their identification at species level. To this aim, the isolates were clustered in nine groups according to electrophoretic profiles obtained by PCR amplification of the 16S–23S rRNA spacer region (ITS) (Fig. 1). The taxonomic identification was then reached by 16S rRNA gene sequencing of representative isolates for each cluster and confirmed by species-specific amplifications of all strains belonging to the same cluster. For the majority of the groups, the ITS profile was characteristic of one *Lactobacillus* species with the exception of cluster 2, which included two related species, *L. paracasei* and *L. rhamnosus*. The taxonomic differentiation of *L. paracasei* and *L. rhamnosus* was obtained employing species-specific probes. Finally, an intraspecies-specific PCR analysis (Torriani et al. 1999) allowed the assignment of all *L. delbrueckii* isolates (cluster 1) to a precise subspecies.

Based on these analyses, the 120 isolates were classified as belonging to ten different species (Table 3): *L. delbrueckii* subsp. *bulgaricus* (46 isolates, ITS cluster 1), *L. paracasei* (30 isolates, cluster 2), *L. rhamnosus* (21 isolates, cluster 2), *L. fermentum* (12 isolates, cluster 3), *L. parabuchneri* (5 isolates, cluster 4), *L. helveticus* (2 isolates, cluster 5), *L. coryniformis* (1 isolate, cluster 6),

Table 2 Sampling location and enumeration of lactic acid bacteria (LAB) isolated from indigenous Dahi

No. of samples	Sampling location in Nepal				LAB counts on MRS at 37 °C [\log_{10} CFU g^{-1}]	
	District	Region	Altitude (m)	Milk from	Range	Average
8	Gorkha	Himalayan	3,615–3,791	Cow	6.3–8.1	7.94±0.90
4	Rasuwa	Himalayan	3,017–3,977	Chauri	6.9–8.2	7.85±0.72
7	Rasuwa	Hilly	1,717–1,731	Buffalo	7.2–9.4	7.88±0.35
4	Lalitpur	Hilly	1,330–1,366	Buffalo	9.2–10.4	9.25±1.20
9	Bhaktapur	Hilly	1,342–1,357	Cow and buffalo	7.3–8.4	8.10±0.80

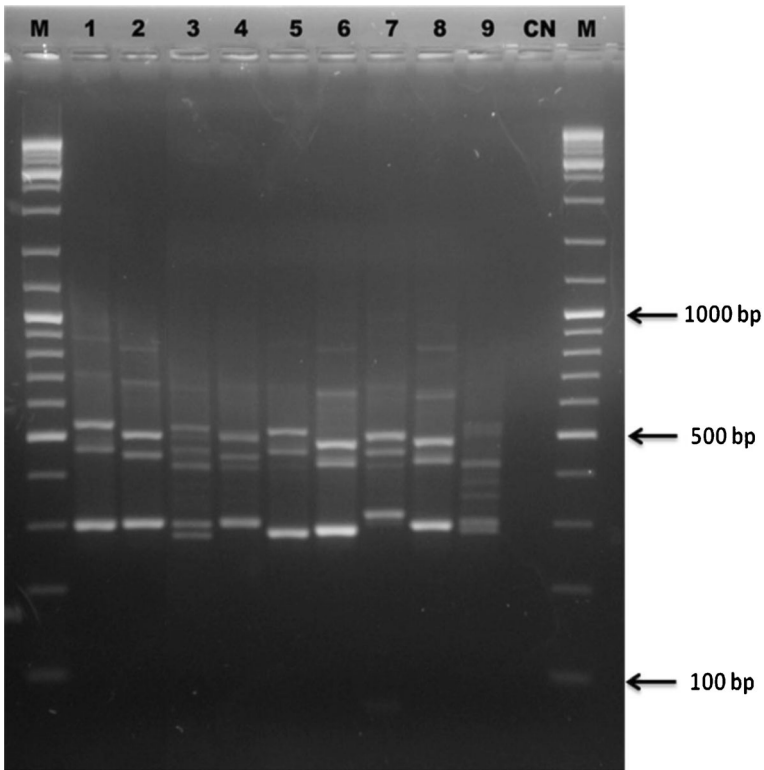


Fig. 1 ITS profiles obtained from representative lactobacilli isolates from traditional Dahi samples. *M* molecular weight

Table 3 Distribution of lactobacilli isolated from indigenous Dahi samples

Species	No. of isolates (%)	Samples				
		Gorkha-HM	Rasuwa-HM	Rasuwa-HL	Lalitpur-HL	Bhaktapur-HL
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	46 (38)	–	7	10	6	23
<i>L. paracasei</i>	30 (25)	23	4	–	3	–
<i>L. rhamnosus</i>	21 (18)	12	–	–	–	9
<i>L. fermentum</i>	12 (10)	4	–	1	1	6
<i>L. parabuchneri</i>	5 (4)	3	–	–	–	2
<i>L. helveticus</i>	2 (1.7)	–	–	2	–	–
<i>L. brevis</i>	1 (0.8)	–	1	–	–	–
<i>L. coryniformis</i>	1 (0.8)	–	1	–	–	–
<i>L. harbinensis</i>	1 (0.8)	–	–	1	–	–
<i>L. plantarum</i>	1 (0.8)	–	1	–	–	–
Total	120	42	14	14	10	40

HM Himalayan region, HL Hilly region

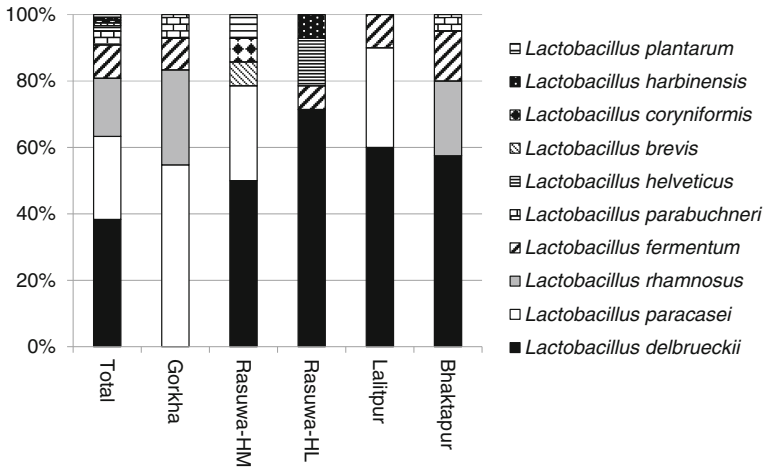


Fig. 2 Relative distribution of the *Lactobacillus* species identified in Dahi samples according to the geographical origin

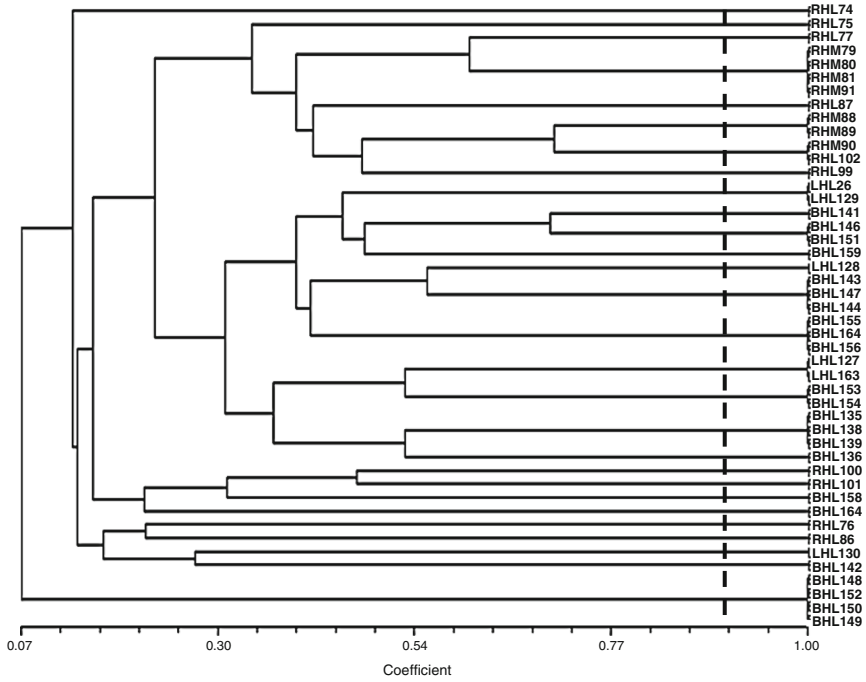
L. harbinensis (1 isolate, cluster 7), *L. brevis* (1 isolate, cluster 8), and *L. plantarum* (1 isolate, cluster 9).

According to our data, the homofermentative *L. delbrueckii* subsp. *bulgaricus* can be considered as one of the dominant lactobacilli in Dahi products. It accounted for 38% of the total isolates and was the predominant *Lactobacillus* population in the majority of the samples. The isolation of *L. delbrueckii* from the traditional Dahi was previously reported (Dellaglio et al., 2005; Watanabe et al., 2009). Particularly, *L. delbrueckii* subsp. *bulgaricus* was shown as the most abundant LAB species also in market Dahi products from Pakistan (Soomro and Masud 2007). Also the species *L. casei* or *L. paracasei* was already reported as a dominant group of lactobacilli in Dahi (Dewan and Tamang 2007; Soomro and Masud 2007). Particularly, we observed that the facultative heterofermentative *L. paracasei* (accounting for 25% of the total isolates) characterizes samples of Dahi produced in the Gorkha district. In general, we found a clearly different distribution of lactobacilli in the diverse dairy samples, which can be explained by the well-known importance of the environment on the relative distribution of different bacterial groups in the microbial ecosystems. Thus, animal origin of the milk, altitude, different technical conditions of product preparation, and any potential factor determining temperature shifts could have affected the bacterial competition during fermentation process.

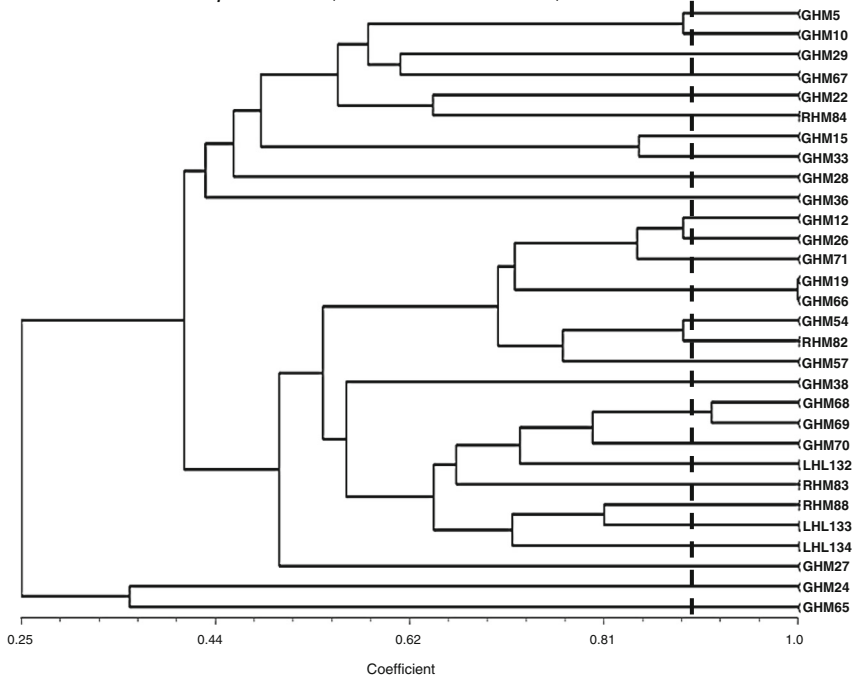
The other most abundant lactobacilli resulted *L. rhamnosus* (18% of isolates) and *L. fermentum* (10%), two species that were reported to be isolated from Dahi only in

Fig. 3 Unweighted pair group method using average linkages (UPGMA) dendrogram derived from similarity coefficients calculated by the Jaccard method (simple Jaccard (Sj) coefficients; shown on the scale at the bottom), showing the relationship among isolates, analyzed by RAPD and REP-PCR. Samples with a similarity coefficient higher than 0.9 (vertical line) have been included in the same genotype. **a** *Lactobacillus delbrueckii* subsp. *bulgaricus* isolates using combined OPI17, OPI02, and M13 fingerprints. **b** *Lactobacillus paracasei* isolates using combined 1254, M13, BOXA1R, and (GTG)5 fingerprints. **c** *Lactobacillus fermentum* isolates using combined OPI17, 1254, and M13 fingerprints. Origin of the isolates: LHL Lalitpur, HL; BHL Bhaktapur, HL; RHL Rasuwa, HL; RHM Rasuwa, HM; GHM Gorkha, HM

a *Lactobacillus delbrueckii* subsp. *bulgaricus* (matrix correlation: $r = 0.94$)



b *Lactobacillus paracasei* (matrix correlation: $r = 0.84$)



C *Lactobacillus fermentum* (matrix correlation: $r = 0.94$)

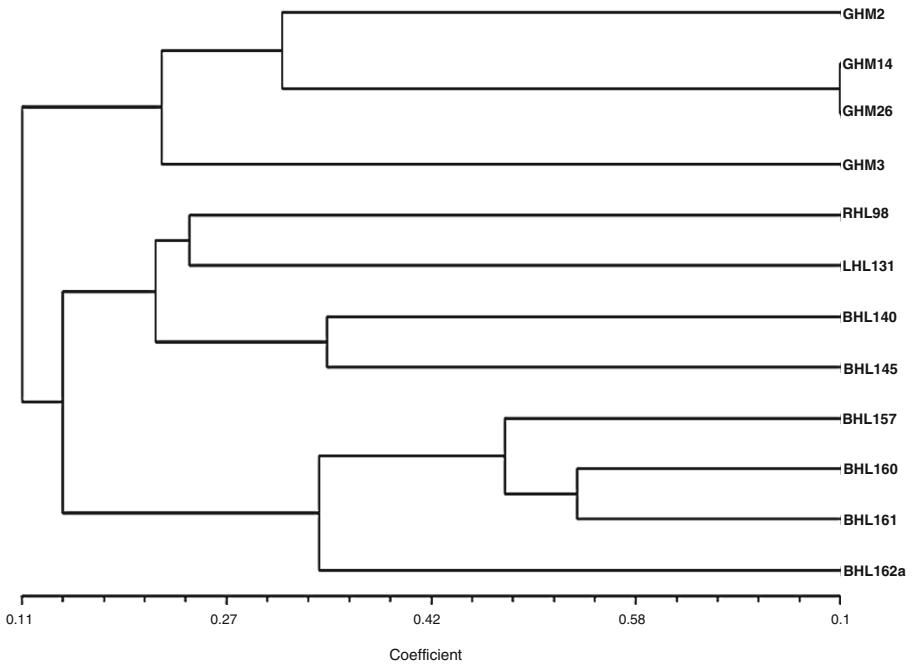


Fig. 3 (continued)

another published study, in which, however, the identification was based exclusively on sugar fermentation patterns (Bhardwaj et al., 2012).

In addition, a few other *Lactobacillus* species were found at very low frequency in some samples (Table 3; Fig. 2). Reportedly, also several other *Lactobacillus* species have been identified in traditional fermented Dahi, such as *L. bifementans* and *L. alimentarius* (Dewan and Tamang 2007). Our study indicates that the Himalayan ethnic fermented milk products are a rich source of different lactobacilli confirming previous results (Satish Kumar et al., 2013).

The technological properties and the probiotic potential of microorganisms are markedly strain specific; for this reason, we undertook the molecular fingerprinting of the main *Lactobacillus* species characterizing the traditional Dahi samples using RAPD and REP-PCR, which are techniques possessing intraspecies discriminatory power (Fig. 3). Notably, this analysis revealed that *L. rhamnosus* isolates, although originating from samples collected from different geographical regions, had identical/monomorphic fingerprints with all the tested primers. On the contrary, *L. delbrueckii* subsp. *bulgaricus*, *L. paracasei*, and *L. fermentum* isolates showed a high degree of biodiversity at the strain level. More specifically, the cluster analysis resulting from the combined patterns of the different primer sets revealed that most of the profiles within each species were unique (Fig. 3). Considering that technological and probiotic features of microorganisms are strain specific, this result is of interest in light of the possibility to select new potential probiotic strains or starter cultures to be used at industrial level.

4 Conclusion

The main aim of the present study was to isolate and genetically characterize the dominant lactobacilli in traditional Dahi collected from the different geographical regions of Nepal. The results obtained during this study highlight that rod-shaped bacteria were the dominant LAB population in traditional Nepalese Dahi samples (62% over 38% cocci). Within rod-shaped LAB, a wide diversity of *Lactobacillus* species was found, confirming that home-made traditional fermented products are a natural rich source of novel *Lactobacillus* strains. Among the identified isolates, there are species that have a known history of safe use and health-promoting attitude, such as *L. paracasei* and *L. rhamnosus*. The new Nepalese isolates of these species may provide a useful resource for further studies involving the selection of new cultures with potential novel probiotic characteristics. Furthermore, the strain collection obtained from this study represents a first step in the preservation of the natural biodiversity of bacterial population of the traditional Nepalese fermented Dahi.

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