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A survey of Lactobacilli isolated from traditional fermented Dahi at different altitudes in Nepal

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A survey of *Lactobacilli* isolated from traditional fermented Dahi 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 yoghurt-like product. Thirty-two replicate samples of indigenous Dahi were collected from four districts of Nepal, at different altitude. In total, 193 strains of LAB were isolated. The rod-shaped isolates, which represented the dominant LAB population (62%), were further identified at the species-level by using Internal Transcribed Spacers (ITS) amplification, 16S rRNA nucleotide 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 120 isolates were grouped in ten different species, among which *L. delbrueckii* subsp. *bulgaricus*, *L. paracasei* and *L. rhamnosus* represented the dominant species. A high degree of intra-species biodiversity and a partial correlation with the geographical areas of sampling was also found.

Keywords: Traditional fermented Dahi, *Lactobacillus*, PCR, Molecular fingerprinting

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 (Harun-ur-Rashid et al. 2007; Kongo et al. 2007; Jokovic et al. 2008; 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 safe cultures. It is known that traditional fermented foods have unique and different microbial population dependent on the production technology as well as on the ecological localities where they have been produced (Fortina et al. 2003; Leroy and De Vuyst 2004; Colombo et al. 2009). The identification and characterization of these autochthonous LAB strains are important for understanding their contribution to the sensorial characteristics of the final product, and for providing new strains for industrial starters. 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).

The purpose of this research was to study ethnic fermented milk products of the Hilly and Himalayan regions of Nepal, for which no data have so far been reported in the literature. Dairy products are an important part of daily food 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 3000 m) whereas Yak and Chauri at high altitudes (> 3000 m) in the Himalayan region.

Locally called Dahi (curd) is the most indigenous and popular product of Nepal. It is a yoghurt-like product prepared in different parts of the Country and used both as nutritional food, appetizer, or dessert, and for the preparation of other ethnic dairy fermented products, such as Ghiu, Mohi, soft Chhurpi (Tamang 2010). There are different conventional methods for the preparation of Dahi, comprising the use

of starter cultures, but 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 at 30-40 ℃ 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 process. This step is the most difficult to obtain in the regions of Nepal at high altitudes (> 3500 m), with consequent problems related to slow acidification and delay in coagulation. In Nepal, 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, Dahi may be either used for consumption, or churned to obtain butter or other dairy products (Zamfir et al. 2006; Tamang 2010).

The main aim of the present study was to explore the natural microbial population of the dominant lactobacilli in traditional Dahi collected from different geographical regions of Nepal. Four districts lying in two different geographical regions of Nepal were followed to study the diversity of the lactobacilli involved in this process. Molecular methods, such as Internal Transcribed Spacers (ITS), 16S rRNA gene sequence analysis, species-specific PCR and RAPD analysis were applied for obtaining rapid and reliable identification and fingerprinting. To the best of our knowledge, this is the first survey of the natural microbiota of the traditional Nepalese fermented Dahi.

Materials and methods

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 Hilly (HL) and Himalayan (HM) regions, according to 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. Geographical Positioning System (Garmin Ltd., Olathe, KS, USA) was used for the measurement of altitude, longitude and latitude of each sampling point. The collected samples were transported to the laboratory under refrigerated conditions (4 °C) and subjected to microbiological analysis within the following 24 h.

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 Sharp (MRS) (Difco, Detroit, MI, USA) and incubated at 37 °C for 48 h. After incubation and counting, randomly selected colonies were isolated from plates of the highest sample dilutions. Purity of the isolates was checked by streaking several times and sub-culturing on fresh MRS agar, as well as MRS broth, followed by microscopic examination. Isolates were Gram-stained and tested for catalase production. Identified strains of LAB were preserved in MRS broth containing 25% (w/v) glycerol, at -20 °C.

PCR-amplification

Genomic DNA for all PCR reactions was extracted from a 100 µl of an overnight culture diluted with 300 µl of TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, 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 25 µl reaction mixture containing 100 ng bacterial DNA, 2.5 µl 10 × reaction buffer Dream Taq™ (Fermentas, Vilnius, Lithuania), 200 µM of each dNTP, 0.5 mM MgCl2, 0.5 µM each primer, and 0.5 U Dream Taq™ 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 of ethidium bromide) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and photographed. A Gene-Ruler DNA ladder mix (Fermentas) was used as a size marker.

DNA sequence analysis

The 16S rRNA gene was amplified by PCR, using primers P0 (5' GAAGAGTTTGATCCTGGCTCAG-3') and P6 (5'-CTACGGCTACCTTGTTACGA-3') (Guglielmetti et al. 2010). The PCR mixtures were subjected to the following thermal cycling: 2 min at 94 °C, then 5 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, with 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. Amplification was performed as previously reported using the primer (5'-AGAGTTTGATCCTGGCTCAG-3') position 8-27 of *Escherichia coli* (Lane 1991). 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).

Genetic typing of bacterial strains

Genetic fingerprinting was carried out by combined analysis of repetitive element (REP) typing using primers (GTG)5 (5′-GTGGTGGTGGTGGTG-3′; annealing temperature T_a= 42 °C) and BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′; T_a= 48 °C) (Versalovic et al. 1994; DeUrraza et al. 2000; Guglielmetti et al. 2008) and random amplification of polymorphic DNA-PCR (RAPD) typing with primers M13 (5′-GAGGGTGGCGGTTCT-3′; T_a= 38 °C), AP02 (5'-AGTCAGCCAC-3'; T_a= 32 °C), OPI17 (5'-CGAGGGTGGTGATG-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 Biostatics 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.

Results and discussion

LAB enumeration and isolation

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 (Table 2). The samples of Dahi produced in the Lalitpur and Bhaktapur districts showed higher LAB count than the samples of Dahi produced at high altitude. 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 isolated strains were rod-shaped (120 isolates) and the remaining were cocci (73 isolates). Thus, rod-shaped were the dominant LAB population

167 in Dahi samples (62% over 38% cocci). Other reports also showed the dominance of lactobacilli in
168 traditional dairy products of the Himalayan region (Tamang et al. 2000; Dewan and Tamang 2007). The
169 120 rod-shaped isolates were selected for further studies.
170
171 Identification and distribution of lactobacilli in Dahi products
172
173 The 120 *Lactobacillus* isolates were submitted to molecular analysis for their identification. A first
174 clustering step was reached by PCR amplification of the 16S-23S rRNA spacer region (ITS).
175 Altogether nine different profiles characterized by three dominant bands migrating at similar molecular
176 weight were obtained (Fig.1). The taxonomic identification of the isolates was reached by 16S rRNA
177 sequencing of representative strains for each cluster and confirmed by species-specific amplifications of all
178 strains belonging to the same cluster. For the majority of the clusters obtained, the ITS profile was
179 characteristic of one *Lactobacillus* species, with the exception of cluster 2, grouping two related species, *L.*
180 *paracasei* and *L. rhamnosus*, whose taxonomic differentiation was only obtained employing species-
181 specific probes (Ward and Timmins 1999). For *L. delbrueckii* group, representing the cluster 1, a suitable
182 specific PCR analysis (Torriani et al. 1999) allowed a further distinction into subspecies. Based on these
183 analyses, the 120 isolates were classified as belonging to 10 different species (Table 3; Fig. 2):
184 *Lactobacillus delbrueckii* subsp. *bulgaricus* (46 strains, cluster 1), *L. paracasei* (30 strains, cluster 2), *L.*
185 *rhamnosus* (21 strains, cluster 2), *L. fermentum* (12 strains, cluster 3), *L. parabuchneri* (5 strains, cluster 4),
186 *L. helveticus* (2 strains, cluster 5), *L. coryniformis* (1 strain, cluster 6), *L. harbinensis* (1 strain, cluster 7), *L.*
187 *brevis* (1 strain, cluster 8) and *L. plantarum* (1 strain, cluster 9).
188 The homofermentative *L. delbrueckii* subsp. *bulgaricus* could be considered one of the representative
189 lactobacilli in Dahi products. It accounted for 38% of the total isolated strains (Fig. 2A), and was the
190 predominant *Lactobacillus* population in the majority of the samples. The facultative heterofermentative *L.*
191 *paracasei* (accounting for 25% of the total isolates) seemed to characterize samples of Dahi produced at
192 high altitude (Gorkha district) in association with *L. rhamnosus*, also found in Bhaktapur samples in

193 significant amount (Fig. 2B). *L. fermentum* was scattered among samples, at low frequency. In addition, a
194 few other *Lactobacillus* species were found at very low frequency in some sample.
195
196 Molecular typing
197
198 The molecular fingerprinting of the main *Lactobacillus* species characterizing the traditional Dahi samples
199 were determined using RAPD and REP-PCR with BOXA1R and (GTG)5 primers (Fig. 3-5). These
200 methods, which use short arbitrary primers or primers targeting short repetitive sequences interspersed
201 throughout the genome, are an established approach for delineation of bacteria at the strain-level
202 (Randazzo et al. 2009; Guglielmetti et al. 2010; Švec et al. 2010). It is interesting to note that, although
203 isolated from different samples collected from different geographical regions, *Lactobacillus rhamnosus*
204 strains showed identical/monomorphic fingerprints with all the tested primers. On the contrary, *L.*
205 *delbrueckii* subsp. *bulgaricus, L. paracasei*, and *L. fermentum* isolates showed a high degree of biodiversity
206 at the strain-level. More specifically, the cluster analysis resulting from the combined patterns of the
207 different primer sets revealed that the majority of the profiles within each species were unique.
208 Furthermore, a possible relationship with the geographical origin of the isolates could be highlighted only
209 for *L. fermentum* (Fig. 5).
210
211 **Conclusion**
212
213 The results obtained highlight a wide diversity of *Lactobacillus* species in traditional Nepalese Dahi
214 samples, whose distribution, in some cases, can be related to the geographical region of production. Among
215 the isolates identified, there are species that have a known history of safe use, such as *L. paracasei* and *L.*
216 *rhamnosus*. The new Nepalese isolates of these species may provide a useful resource for further studies
217 involving the selection of new starter cultures with potential novel probiotic characteristics. Moreover, the

strain collection obtained from this study represents a first step in the preservation of the natural biodiversity of bacterial population of the Nepalese dairy products.

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Table 1 PCR primers and conditions used for isolates identification

Primer specificity and reference	Primer pair (5'to 3')	Thermal conditions	Amplicon (bp)
L. delbrueckii subsp. *bulgaricus* (Torriani et al. 1999)	Fw: GTGCTGCAGAGAGAGTTTGATCCTGGCTCAG Rev: ACCTATCTCTAGGTGTAGCGCA	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 1 min at 57 ^{0}C, 1 min at 72 ^{0}C	1030
L. paracasei (Ward and Timmins 1999)	Fw:CACCGAGATTCAACATGG Rev:CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 54 ^{0}C, 45 sec at 72 ^{0}C	290
L. rhamnosus (Ward and Timmins 1999)	Fw:TGCATCTTGATTTAATTTTG Rev:CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 54 ^{0}C, 45 sec at 72 ^{0}C	290
L. fermentum (Coton et al. 2008)	Fw:TGTACACACCGCCCGTC Rev:TTTTCTTGATTTTATTAG	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 48 ^{0}C, 45 sec at 72 ^{0}C	460; 270
L. parabuchneri (Coton et al. 2008)	Fw:TGTACACACCGCCCGTC Rev:TGTTACTCCGGTCTGTGC	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 48 ^{0}C, 45 sec at 72 ^{0}C	330
L. helveticus (Fortina et al. 2001)	Fw:CTGTTTTCAATGTTGCAAGTC Rev:TTTGCCAGCATTAACAAGTCT Fw:CGCTGATTCTAAGTCAAGCT Rev:CGACTAAGAAGTGGAACATTA Fw:TCTTATTACGCAATGGACCAA Rev:AATACCGTTCTTGAGGTTAGA	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 58 ^{0}C, 1 min at 72 ^{0}C	918; 726; 524
L. brevis (Coton et al. 2008)	Fw: TGTACACACCGCCCGTC Rev:TAATGATGACCTTGCGGTC	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 48 ^{0}C, 45 sec at 72 ^{0}C	330
L. plantarum (Torriani et al. 2001)	Fw:CCGTTTATGCGGAACACC Rev:TCGGGATTACCAAACATCAC	2 min at 94 ^{0}C, 35 cycles of: 45 sec at 94 ^{0}C, 45 sec at 56^{0}C, 45 sec at 72 ^{0}C	318
Internal Transcribed Spacer region (ITS) (Jensen et al. 1993)	Fw: GAAGTCGTAACAAGG Rev: CAAGGCATCCACCGT	2 min at 94°C, 5 cycles of: 45 sec at 94^{0}C, 1 min at 55°C; 1 min at72°C and 30 cycles of: 45 sec at 94 ^{0}C; 45 s at 60°C, 2 min at 72°C	

Table 2 Sampling location and enumeration of viable LAB on MRS agar in the indigenous Dahi

No. of samples	Sampling location in Nepal				LAB count [log (10) cfu/g]	
	District	Region	Altitude (m)	Milk from	Range	Average
8	Gorkha	Himalayan	3615-3791	Cow	6.3 – 8.1	7.94 ±0.90
4	Rasuwa	Himalayan	3017-3977	Chauri	6.9 – 8.2	7.85±0.72
7	Rasuwa	Hilly	1717-1731	Buffalo	7.2 – 9.4	7.88±0.35
4	Lalitpur	Hilly	1330-1366	Buffalo	9.2 – 10.4	9.25±1.20
9	Bhaktapur	Hilly	1342-1357	Cow and Buffalo	7.3 – 8.4	9.04±0.80

Table 3 Distribution of lactobacilli isolated from indigenous Dahi samples

Species	No. of identified strains	Samples				
		Gorkha-HM	Rasuwa-HM	Rasuwa-HL	Lalitpur-HL	Bhaktapur-HL
Lactobacillus delbrueckii subsp. *bulgaricus*	46	/	7	10	6	23
Lactobacillus paracasei	30	23	4	/	3	/
Lactobacillus rhamnosus	21	12	/	/	/	9
Lactobacillus fermentum	12	4	/	1	1	6
Lactobacillus parabuchneri	5	3	/	/	/	2
Lactobacillus helveticus	2	/	/	2	/	/
Lactobacillus brevis	1	/	1	/	/	/
Lactobacillus coryniformis	1	/	1	/	/	/
Lactobacillus harbinensis	1	/	/	1	/	/
Lactobacillus plantarum	1	/	1	/	/	/
Total	120	42	14	14	10	40

Fig. 1 ITS profiles obtained from representative lactobacilli isolates from traditional Dahi samples.

M, Molecular weight marker

Fig. 2 Incidence of *Lactobacillus* species isolated from Dahi samples

Fig. 3 Cluster analysis of the *Lactobacillus delbrueckii* subsp. *bulgaricus* strains using combined OPI17, OPI02, and M13 fingerprints (UPGMA). Strain origin: brown = Lalitpur, HL; red = Bhaktapur, HL; yellow = Rasuwa, HL; blue = Rasuwa, HM

Fig. 4 Cluster analysis of the *Lactobacillus paracasei* strains using combined 1254, M13, BOXA1R, and (GTG)5 fingerprints (UPGMA). Strain origin: brown = Lalitpur, HL; blue = Rasuwa, HM; green = Gorkha, HM

Fig. 5 Cluster analysis of the *Lactobacillus fermentum* strains using combined OPI17, 1254, and M13 fingerprints (UPGMA). Strain origin: brown = Lalitpur, HL; green, = Gorkha, HM; yellow = Rasuwa, HL; red = Bhaktapur, HL

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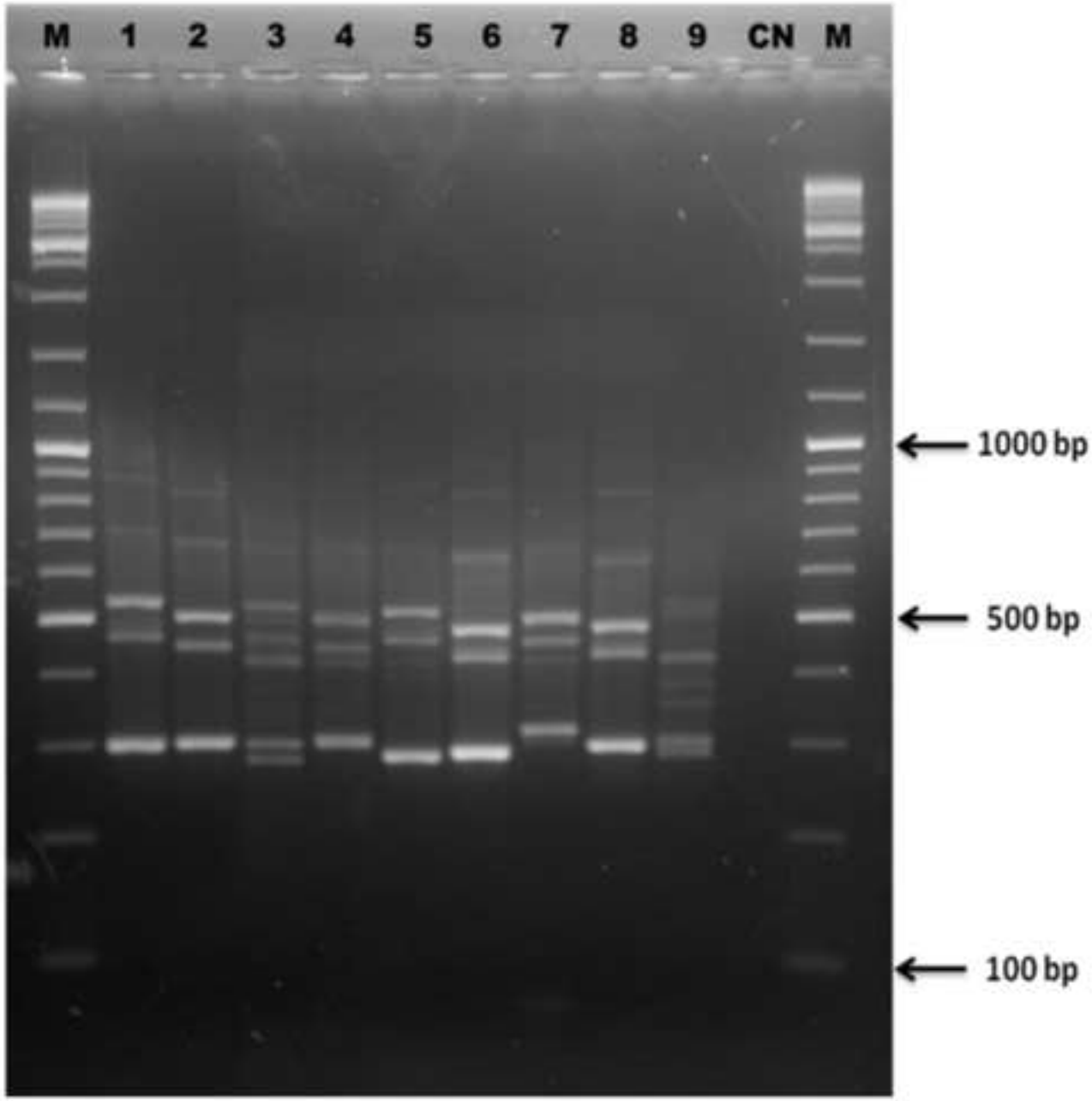

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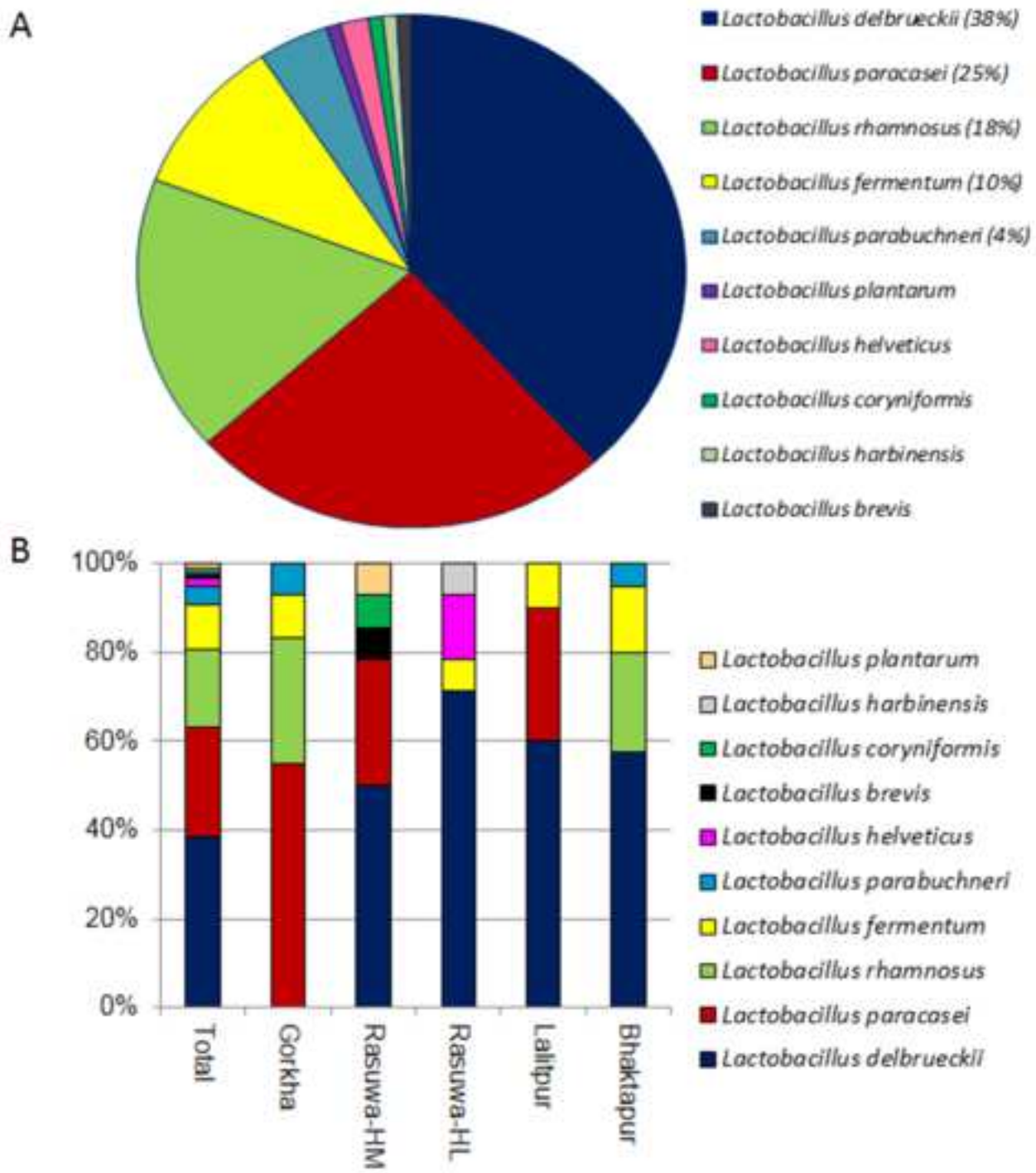

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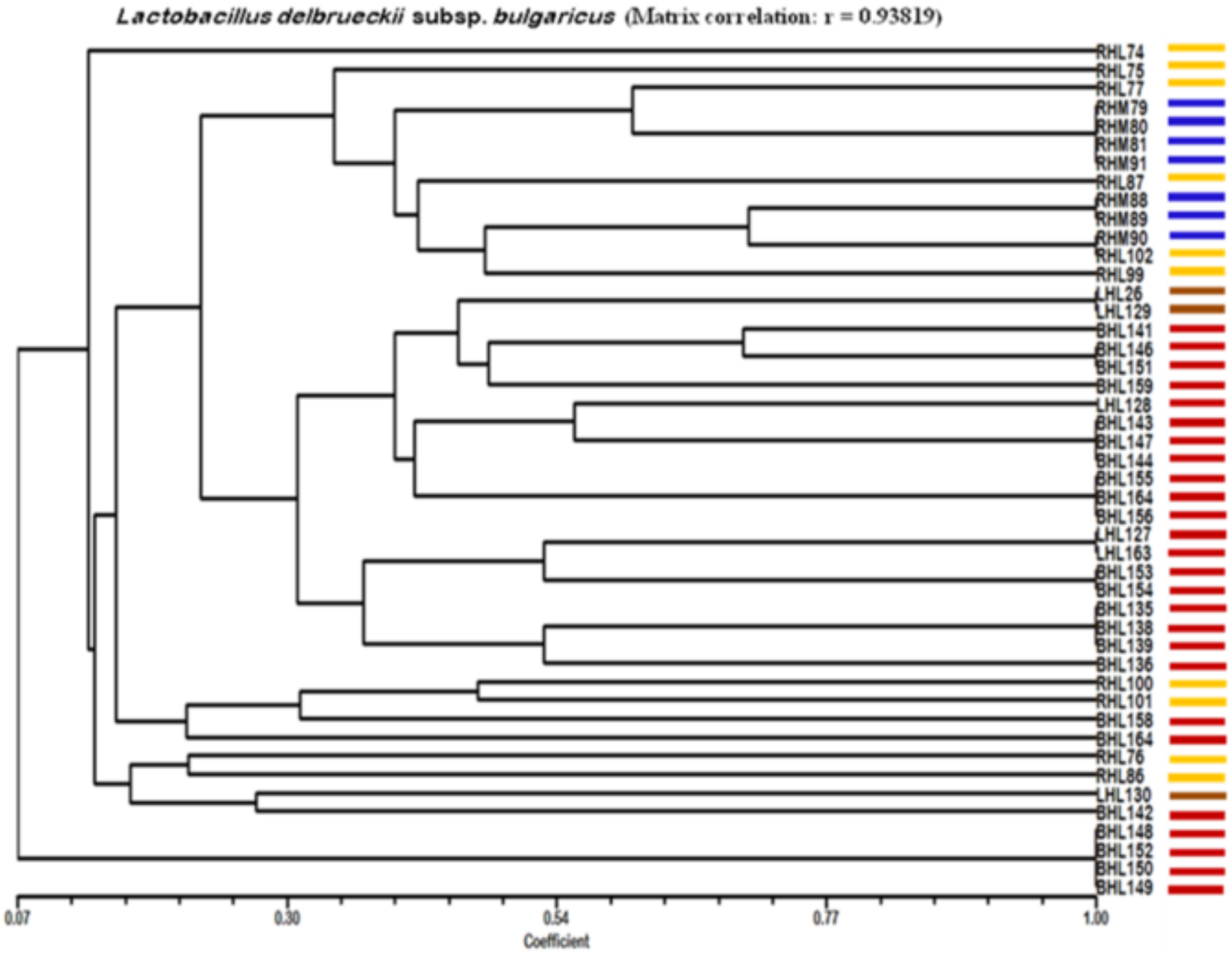

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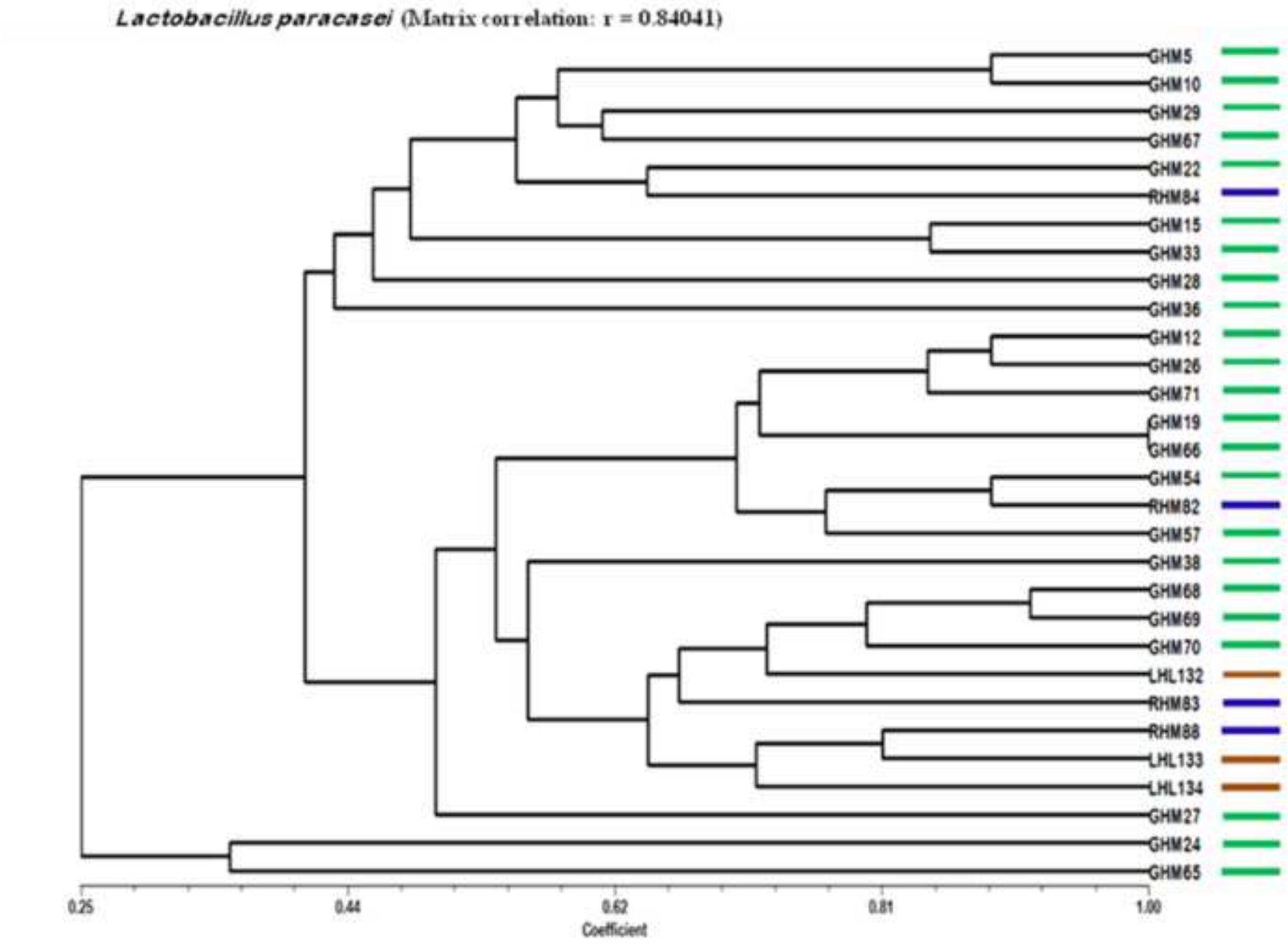

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