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Phenotypic and genotypic characterization of non-starter *Lactobacillus* species diversity in Indian Cheddar cheese

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ABSTRACT

A total of 243 non-starter lactobacilli were isolated from 12 premium quality Indian Cheddar cheese samples ripened for different periods and in different plant conditions. They were classified up to species level using mainly sugar fermentation assay and PCR. Based upon phenotypes, a maximum of 46.50% were classified as *Lactobacillus paracasei*, followed by 34.98% isolates as *Lactobacillus plantarum*. Only 3.29% were classified as *Lactobacillus rhamnosus* and 4.12% as *Lactobacillus delbrueckii* species, while 22 (9.05%) isolates (of which 16 *L. plantarum*/*Lactobacillus paraplantarum* and 6 *Lactobacillus delbrueckii* ssp. *lactis*/*Lactobacillus crispatus*) could not be designated to a single species. One isolate of *Lactobacillus coryniformis* ssp. *coryniformis* was isolated for the first time from Cheddar cheese (0.41%) while 1.65% isolates remained unidentified. Mostly, the tentative characterization based on phenotype, could be confirmed by PCR targeting rRNA. Those isolate groups which could not be tested in PCR, or resembled with more than one species in their phenotypic traits, could be resolved by the BLAST homology analysis of the partial *tuf* gene sequences of few representative isolates.

1. Introduction

Cheddar is one of the most consumed cheese varieties world over with characteristic taste and other sensory properties, understood mostly to be the result of the metabolic activity of various species of Non-starter (NS) lactobacilli inhabiting the cheese during the maturation process. Many studies have reported ripening acceleration and flavor enhancement in Cheddar cheese prepared with lactobacilli as starter adjuncts (Banks & Williams, 2004; Swearingen, O'Sullivan, & Warthesen, 2001; Trepanier, Simard, & Lee, 1991). The various *Lactobacillus* species reported from matured Cheddar cheese are *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus curvatus* and sometimes *Lactobacillus brevis* along with few others in minority (Banks & Williams, 2004; Chandry, Moore, & Hillier, 2002; Fitzsimons, Cogan, Condon, & Beresford, 1999; Williams, Choi, & Banks, 2002). The occurrence and prevalence of good or bad strains of NS lactobacilli during ripening can be related mainly with the quality of milk, particular plant atmosphere and handling practices during the processing. In India, different atmospheric conditions, use of mixed milk instead of cow milk for Cheddar making and inconsistent plant hygiene practices, could be determining for the indigenous microflora of premium quality Indian Cheddar. The routine biochemical tests are used successfully for species characterization within genus *Lactobacillus*, however they often fail to discriminate among closely related species occurring in the same ecological environments, like inside the ripening cheese. Substantiating the results based on biochemical tests with PCR can help in reliable characterization of NS lactobacilli associated with Cheddar cheese. However, it is important to note that *Lactobacillus* is the largest genus among Lactic Acid Bacteria and is widely heterogeneous containing a range of species isolated from diverse sources. Besides, it has a number of closely related species groups isolated from the same nutritional and physiological environment, sharing high resemblance not only in their phenotypic traits but in ribosomal RNA sequence also. This has led to taxonomic disputes and problems in characterization among closely resembling *Lactobacillus* species groups solely on the basis of 16S ribosomal RNA (Singh, Goswami, Singh, & Heller, 2009). Moreover, discrimination power of a number of housekeeping genes like *hsp* 60, *rpo B*, *rec* A, *tuf*, etc have been found higher than 16S rRNA and can differentiate even among closely related *Lactobacillus* species

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sometimes (Blaiotta et al., 2008; Shevtsov et al., 2012; Torriani, Felis, & Dellaglio, 2001; Ventura, Canchaya, Meylan, Klaenhammer, & Zink, 2003). Hence this study was aimed to isolate, identify and characterize the indigenous *Lactobacillus* species prevailing in the Indian Cheddar cheese samples at various maturity ages by phenotyping, PCR and partial *tuf* gene sequence comparisons. In our best of belief it is the first detailed study of the NS lactobacilli present in Indian Cheddar cheese.

2. Materials and methods

2.1. Processing of cheese samples

Twelve premium quality 500 g Cheddar blocks of ripening maturity ages ranging from 4 days to 7 months, were procured from Experimental Dairy Plant (National Dairy Research Institute, Karnal, Haryana), Verka Cheese Plant (Mohali, Punjab) and Modern Dairies (Karnal, Haryana) in evacuated polypacks and stored at 4 °C. All cheese samples were coded with alphabets as shown in Table 2. Manufacturing parameters important for Cheddar production like type of milk, pasteurization temperature, curd cooking temperature and storage temperature implemented in three plants were as follows: fresh cow milk, 72 °C, 30–37 °C and 10 °C in Experimental Dairy Plant, N.D.R.I.; mixed milk, 72 °C, 38 °C and 5–7 °C in Verka Cheese Plant; mixed milk, 72 °C, 39 °C and 10 °C in Modern Dairies Plant. After scraping the outer thin layers with a sharp sterile knife, the samples were grated aseptically and were stored in pre sterilized glass sample bottles for microbiological analysis.

2.2. Non-Starter Lactic Acid Bacteria (NSLAB) isolation and enumeration

The isolates were obtained by serial dilution and double layer plating of Cheddar cheese samples on MRS agar (Hi-Media) followed by incubation at 37 °C for 72 h. Gram positive, non-motile and catalase negative rods and coccobacilli isolates were stocked in 1:1 glycerol–MRS mixture in the National Collection of Dairy Cultures (N.D.R.I., Karnal, India) at −80 °C. Isolates from stocks were thawed, subcultured in MRS broth and streaked on MRS plates before use.

2.3. Type and reference strains used

A collection of 8 type and reference strains of six mesophilic *Lactobacillus* species were used in this study. Five strains were obtained from NCDC (*Lactobacillus paraplantarum*, NCDC 334 or DSMZ 10667^T; *L. paracasei*, NCDC 63 or ATCC 393^T; *Lactobacillus delbrueckii* ssp. *lactis*, NCDC 3 or ATCC 4797; *L. rhamnosus*, NCDC 18 or ATCC 7469 and NCDC 24 or ATCC 8014). Three more strains: Lpl (*L. plantarum*), Lpar (*L. paracasei*) and Lcur (*L. curvatus*) were received from Royal Veterinary & Agricultural University, Denmark through personal communication. These were used to corroborate the presumptive identification of cheese isolates.

2.4. Phenotypic characterization of reference strains and isolates

All reference strains and isolates were examined for growth at 15 and 45 °C after incubation in MRS broth for 2 days; for CO_2 production from glucose in MRS broth (lacking citrate). Ammonia production from arginine was detected by addition of Nessler's reagent to cultures grown in arginine broth at 37 °C for 48 h. CHL medium (a basal medium for carbohydrate fermentation) with bromocresol purple indicator was used to examine the fermentation pattern for 10 sugars namely glucose, galactose, fructose, raffinose, melibiose, mannitol, cellobiose, rhamnose, mannose and ribose. Glucose, being metabolized by all *Lactobacillus* species was included as positive control. Sugar discs (25 mg, Hi-Media) or filter sterilized sugar solutions of 0.6% conc. were added to 4 ml of sterilized CHL medium tubes aseptically and fermentation pattern was recorded after incubation at 37 °C for 7 days. The reference strains, which were showing variable sugar fermentation pattern and those isolates for which no conclusive species could be assigned on the basis of 10 sugar fermentation reactions were further subjected to a battery of 21 more fermentation reactions including adonitol, arabinose, arabitol, D amygdalin, dulcitol, erythritol, fucose, β gentibiose, glycogen, inositol, inulin, lactose, maltose, methyl α D glucopyranoside, methyl α D galactopyranoside, meleizitose, salicin, sorbitol, sucrose, trehalose, xylose and esculin hydrolysis reaction, to have more elaborate understanding of their phenotypes.

2.5. Genotypic characterization of isolates

2.5.1. DNA extraction

Genomic DNA isolation from all isolates and reference strains was carried out using the method of Pospiech and Neumann (1995).

2.5.2. Polymerase chain reaction

PCR primers used for the study were selected from the published literature and were synthesized from Sigma Aldrich, New Delhi and Bangalore Genei Pvt. Ltd., Bangalore, India (Table 1). All PCR reactions carried out in this study were performed in 25 μl reaction volumes containing 20 pm of each primer, 0.2 mM of each dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, 50 ng of DNA template, and 0.5–1 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). Amplification was carried out in a hot lid thermal cycler (Eppendorf, AG Hamburg, Germany) under cycling conditions as published previously in literature (Table 1). PCR products were stored at 4 °C. Aliquots of PCR products were separated by 2% agarose gel containing ethidium bromide (0.5 μg/ml) in TAE buffer (pH 8.0) and visualized under UV light with 100 bp DNA ladder O'Gene ruler™ (Fermentas, Maryland, USA) as reference marker to determine the size of products.

2.5.3. Partial tuf gene sequencing

2.5.3.1. Designing of degenerate primers. A total of 28 *tuf* gene sequences coding for translation elongation factor, from 12 different *Lactobacillus* species commonly occurring in dairy products were retrieved from GenBank. Their accession numbers are as follows

Table 1
Oligonucleotide primers used in study.

Specificity	Primer region	Primer sequence	Reference
Genus specific	16S rRNA	(F) 5′ CTCAAAACTAAACAAAGTTTC 3′ (R) 5′ CTTGTACACACCGCCCGTCA 3′	Dubernet et al., 2002
L. plantarum species	16S–23S Spacer region	(F) 5′GCCGCCTAAGGTGGGACAGAT3' (R) 5′TTACCTAACGGTAAATGCGA 3′	Walter et al., 2000
L. rhamnosus species	16S–23S Spacer region	(F) 5′ CAGACTGAAAGTCTGACGG 3′ (R) 5′ GCGATGCGAATTTCTATTATT3'	Walter et al., 2000
L. paracasei species	16S rRNA	(F) 5′CTAGCGGGTGCGACTTTGTT 3′ (R) 5′GGCCAGCTATGTATTCACTGA 3′	Song et al., 2000
L. crispatus species	16S–23S Spacer region	(F) 5′GTA ATG ACG TTA GGA AAG CG 3′ (R) 5′ACT ACC AGG GTA TCT AAT CC 3′	Walter et al., 2000
Degenerate primers	*tuf* gene	1 (F) 5′ CTG GTC GTG GKA CHG TTG 3′ 2 (R) 5′ GAA TGG VGT RTG ACG DSC 3′	This study