Phenotypic and Genotypic Identification of NSLAB from Raw Cow Milk

Jana Bezeková, Konrad J. Domig, Monika Lavová, Margita Čanigová

1Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76, Nitra, Slovakia
2BOKU – University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, A-1190 Vienna, Austria

Abstract
Non-starter lactic acid bacteria (NSLAB) were isolated from samples of raw cow milk (n=15). The LAB counts in raw cow milk varied between $1.25 \times 10^3$ and $2.64 \times 10^6$ cfu.ml$^{-1}$. Eighteen isolates of Lactobacillus spp. were phenotypically identified by their ability to ferment 49 carbohydrates using API 50CHL kits. The (GTG)5-PCR fingerprinting assigned all strains into well-differentiated clusters representing individual species. Identification of Lactobacillus species were confirmed using a species specific PCR. In accordance with biochemical identification (API 50 CHL) only 83.33 % strains were confirmed by PCR. Species specific PCR confirmed 6 strains of Lb. paracasei subsp. paracasei, 6 strains of Lb. plantarum, 3 strains of Lb. helveticus and 1 strain of Lb. rhamnosus. Two strains were not confirmed by PCR. These strains were identified by using 16S rRNA gene sequence data as Lb. inunguliei.

Keywords: Lactobacillus, identification, PCR, API 50 CHL, cow milk

1. Introduction
Non-starter lactic acid bacteria (NSLAB) are mostly facultative heterofermentative mesophilic lactobacilli including species such as Lb. casei, Lb. paracasei, Lb. rhamnosus and Lb. plantarum, as well as pediococci, Leuconostoc and micrococci. The main source of those microorganisms of cheese microflora is the raw milk [1]. It is generally considered that the milking machine and the bulk tank represent the major sources of raw milk contamination [2]. It has been shown that NSLAB can survive pasteurization at low numbers and slowly grow during cheese ripening up to $10^6$ – $10^7$ cfu.g$^{-1}$, depending on the cheese ripening period and temperature [3].

Post-pasteurization contamination due to a resident flora in the dairy plant has also been showed to contribute to NSLAB proliferation. NSLAB may overcome lactic acid starters in the cheese and become the dominant microflora [2]. The recognition of lactobacilli by simple morphological tests is often difficult because growth conditions and growth stage of the bacterial cells may seriously affect their morphology and many heterofermentative lactobacilli may exhibit coccobacilli morphology. Some traditional identification tests are based on the ability of lactobacilli to ferment various carbohydrates. Results of phenotypic methods of bacterial identification may be difficult to interpret or ambiguous, and require subsequent confirmation [4]. Many researchers have relied on the commercial identification kit API 50 CHL carbohydrate fermentation strips. However, it has been reported that the API 50 CHL system may not always be adequate for the reliable identification of Lactobacillus isolates due to the possible loss or acquisition of plasmids that
encode many carbohydrate fermentation traits. A comparative analysis of the 16S rRNA sequence is the commonly used molecular method for bacterial identification. Strains that generally show ≥ 97 % sequence similarity in the 16S rRNA are considered to be the same species. Unfortunately, high degrees of similarity (reaching 98.7 % – 99.9 %) have been observed for the 16S rRNA gene sequences among closely related Lb. casei group strains [5]. Alternatively, PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with the following characteristics: (i) a high discriminatory power, (ii) low cost, (iii) suitable for a high-throughput of strains and (iv) considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria [6].

The aim of this study was to determine presence of Lactobacillus spp. in raw cow milk and phenotypic and genotypic characterization these strains.

2. Material and methods

Isolation of mesophilic lactobacilli

The strains of lactobacilli (LAB) were isolated from the samples (n=15) of raw cow milk obtained from milk tanks and from milk vending machine. Sampling of milks was carried out under aseptically conditions. The counts of lactobacilli were determined on selective diagnostic MRS agar (HiMedia Laboratories, India). Samples were cultivated at temperature 30 ± 1 °C for 72 ± 2 hours under anaerobic conditions – GENbox generators (BioMerieux, France) [7].

Phenotypic characterization

The isolates were examined microscopically (cell morphology and arrangements) and checked by Gram staining and for catalase production using 2 % H2O2 on single colonies. Putative lactobacilli Gram-positive, catalase-negative rods from MRS plates were used for further analysis.

The ability to ferment carbohydrate substrates was studied using the API 50 CHL kits (BioMerieux, France), which enabled identification of the LAB isolates to species level. All the tests were performed according to the manufacturer's instructions as follows. A swab of each LAB isolates grown on MRS agar plates (incubated anaerobically at 37 °C for 48 h) was suspended in API 50 CHL medium. Using sterile pipette, homogenized suspension of the cells in the medium was distributed into each of the 50 wells on the strips. All wells were overlaid with sterile paraffin oil (BioMerieux, France) to affect anaerobiosis. The strips were moistened and covered as recommended by the manufacturer and incubated at 37 °C. The changes in color from violet to yellow were monitored daily for 2 days. Identification of Lactobacillus species were confirmed using a species specific PCR.

Genotypic Identification

Bacteria cells were grown in 10 ml MRS broth (Oxoid, Basingstoke, UK) for 24 h at their optimal temperature 37 ± 1 °C and 30 ± 1 °C under anaerobic conditions. One milliliter aliquot of each culture was centrifuged. Isolation of lactobacilli DNA was performed by commercial bacterial kit peqGOLD Bacterial DNA Kit (Peqlab, Germany). The purity and concentration of DNA was measured on the NanoDrop 2000c spectrophotometer (Thermo Scientific, Germany). Lactobacilli strains were subjected to rep-PCR as described Gevers et al. (2001) [6] using the (GTG)5 primer to determine their species status. The PCR products were loaded on a 2 % agarose gel, run at a constant voltage of 80 V for 110 min and stained with ethidium bromide in 1×TBE buffer for 30 min, destained for 15 min in distilled water and visualized under UV light. Results were evaluated using the software Bionumerics-Software 6.6.4. PCR products used for sequencing was prepared with using primers and protocols described Di Cello et al. (1997) [8]. Sequencing was carried by company Eurofins (Austria). Based on the results of rep-PCR and sequences which were compared with those available in the National Center for Biotechnology Information were lactobacilli strains identified by species specific primers (Fermentas, Germany).

The species specific PCR method conditions: reaction mixtures (25 µl) contained 200 µM of each desoxyribonucleoside triphosphate (dNTP), 1×PCR buffer (10 mM Tris–HCl, pH 8.8, 1.5 mM MgCl2, 50mM KCl, 1 g.L−1 Triton X-100), 0.5 μl DNA solution, 1U DyNAzyme Polymerase (Finnzymes, Finland), the specific primers and 1µl
The lactobacilli counts in raw cow’s milk varied between 1.25x10³ and 2.64x10⁶ cfu.ml⁻¹. Eighteen isolates (Lactobacillus strains) were rod shaped cells, Gram-positive, catalase-negative, non-motile and facultative anaerobic bacteria. Isolates were classified to the genus Lactobacillus, Kagkli et al. (2007) [12] determined numbers of lactobacilli 4.30 log₁₀ cfu.ml⁻¹ in raw milk.

The lactobacilli identification were used followed specific primers: Lb. paracasei (220 bp) F: 5´ CTAGCGGTTGCGACTTTGTT 3´ and R: 5´ GGCCAGCTATGTATTCACTGA 3´ [9]. Lb. paracasei subsp. paracasei (312 bp) F: 5´ CTAGCGGTTGCGACTTTGTT 3´ and R: 5´ GGCCAGCTATGTATTCACTGA 3´, Lb. rhamnosus (113 bp) F: 5´ CTAGCGGTTGCGACTTTGTT 3´ and R: 5´ GCGATGCGAATTTCTATTATT 3´ [10]. F: 5´ CTAGCGGGTGCGACTTTGTT 3´ and R: 5´ GGCCAGCTATGTATTCACTGA 3´, Lb. plantarum (220 bp) F: 5´ CTAGCGGGTGCGACTTTGTT 3´ and R: 5´ GGCCAGCTATGTATTCACTGA 3´, Lb. helveticus (524 bp) F: 5´ CTGTTTTCAATGTGGAATTC 3´ and R: 5´ TITGCGGATTACAACTGTC 3´ [11].

According to of results biochemical identification (API 50 CHL) were identified species of Lb. plantarum (n=6), Lb. paracasei subsp. paracasei (n=5), Lb. helveticus (n=3), Lb. rhamnosus (n=1), Lb. brevis (n=1) and Lb. fermentum (n=1). One strain no. 83 was identified as Lb. paracasei subsp. paracasei I or Lb. rhamnosus. This strain showed positive reaction with carbohydrates rhamnose. In accordance with biochemical identification (API 50 CHL) only 83.33 % strains were confirmed by PCR method – tab. 2.

**Table 1.** PCR conditions for amplification of lactobacilli species

<table>
<thead>
<tr>
<th>PCR conditions</th>
<th>Strain</th>
<th>Lb. paracasei</th>
<th>Lb. plantarum</th>
<th>Lb. helveticus</th>
<th>Lb. rhamnosus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatur.</td>
<td>94 °C, 2 min</td>
<td>94 °C, 4 min</td>
<td>94 °C, 2 min</td>
<td>94 °C, 4 min</td>
<td>94 °C, 2 min</td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Denatur.</td>
<td>94 °C, 30 s</td>
<td>94 °C, 1 min</td>
<td>94 °C, 30 s</td>
<td>94 °C, 1 min</td>
<td>94 °C, 30 s</td>
</tr>
<tr>
<td>Annealing</td>
<td>56 °C, 40 s</td>
<td>53 °C, 1 min</td>
<td>58 °C, 40 s</td>
<td>60 °C, 1 min</td>
<td>56 °C, 40 s</td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C, 72 °C</td>
<td>72 °C, 1 min</td>
<td>72 °C, 72 °C</td>
<td>72 °C, 1 min</td>
<td>72 °C, 72 °C</td>
</tr>
<tr>
<td>Final elongation</td>
<td>5 min, 7 min</td>
<td>8 min, 7 min</td>
<td>8 min, 7 min</td>
<td>8 min, 7 min</td>
<td>8 min, 7 min</td>
</tr>
<tr>
<td>Final hold</td>
<td>4 °C, 4 °C</td>
<td>4 °C, 4 °C</td>
<td>4 °C, 4 °C</td>
<td>4 °C, 4 °C</td>
<td>4 °C, 4 °C</td>
</tr>
</tbody>
</table>

**References** [9] [10] [11] [10]

PCR products were loaded on a 2 % agarose gel, run at a constant voltage of 80 V for 50 min and stained with ethidium bromide in 1x TBE buffer for 30 min, destained for 15 min in distilled water and visualised under UV light.

**3. Results and discussion**

The lactobacilli counts in raw cow’s milk varied between 1.25x10³ and 2.64x10⁶ cfu.ml⁻¹. Eighteen isolates (Lactobacillus strains) were rod shaped cells, Gram-positive, catalase-negative, non-motile and facultative anaerobic bacteria. Isolates were classified to the genus Lactobacillus, Kagkli et al. (2007) [12] determined numbers of lactobacilli 4.30 log₁₀ cfu.ml⁻¹ in raw milk.
uncommonly used fermentation tests are required and because the high number of species complicates this approach [13, 4, 14]. The (GTG)5-PCR fingerprinting assigned all strains into well-differentiated clusters (n=4) representing individual species – figure 1.

Figure 1. Dendrogram based on the numerical analysis of generated and digitized rep-PCR fingerprints of Lactobacillus species

Species specific PCR confirmed 6 strains of Lb. paracasei subsp. paracasei, 6 strains of Lb. plantarum, 3 strains of Lb. helveticus and 1 strain of Lb. rhamnosus. Two strains were not confirmed by PCR method and were identified by using 16S rRNA gene sequence data as Lb. ingluviei. Occurrence and isolation of these strains in environment is occasionally. Baele et al. (2003) [15] isolate Lb. ingluviei from the crop and intestines of pigeons. Lb. ingluviei may lead to weight increases in new-born ducks and chickens [16].

In this study, have been observed high degrees of similarity (reaching 99%) for the 16S rRNA gene sequences among closely related Lb. plantarum to Lb. pentosus and Lb. paracasei subp. paracasei to Lb. paracasei subsp. tolerans. In fact, it is widely acknowledged that Lb. plantarum and Lb. pentosus belong to the same 16S rRNA phylogenetic group and could only be distinguished using phylogenetic analysis of sequences of the 16S-23S large spacer region [17].

4. Conclusions

Some traditional identification tests are based on the ability of lactobacilli to ferment various carbohydrates. Results of phenotypic methods of bacterial identification may be difficult to interpret or ambiguous, and require subsequent confirmation. Phenotypic methods have some limitations, such as their poor reproducibility owing to the plasticity of bacterial growth and environmental conditions that may affect gene expression. Genotypic methods are increasingly applied for the identification of lactobacilli. Combination of phenotypic and genotypic methods is considered to provide the most reliable identification.

Acknowledgements

This work was supported by the VEGA grants from the Ministry of Education, Science, Research and Sport of the Slovak Republic, grant No. 1/0679/13.

References