LACTIC ACID MICROFLORA OF BULGARIAN MILK PRODUCTS FROM MOUNTAIN REGIONS

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ABSTRACT

This study aimed to isolate and characterize viable lactic acid bacteria (LAB) from the most popular in Bulgaria fermented milk products. Different samples from home-made cheeses, yoghurt and katak, from ecological regions of Stara Planina, Rila and Rodopi mountains were collected. A total of 25 LAB cultures (coci and rods) were isolated and polyphasic taxonomic characterization was performed. Eight of the strains from yoghurt were phenotypically similar to Lactobacillus delbrueckii. Other seven isolates from cheese and yoghurt were identified as Lactobacillus plantarum, according to classical tests and API LAB profiles. Their species affiliation was confirmed by species-specific and multiplex PCR. The molecular-based methods allowed reclassifying two cheese isolates determined as L. paracasei. L. plantarum was the predominant species in cheese samples and referred as member of non-starter lactic acid bacteria. Two of the new isolated L. plantarum strains showed anti-E. coli activity.

Keywords: Artisanal milk products, L. plantarum, lactic acid bacteria

Introduction

Lactic acid bacteria (LAB) play an essential role in the majority of food fermentations. Except starter cultures, employed in the manufacture of the dairy products, other LAB called non-starter (NSLAB) are also presented in the natural microflora of these products. Predominantly NSLAB are mesophilic lactobacilli and pediococi (Rehman et al., 2000). Most frequently found species are L. casei ssp. casei, L. paracasei ssp. paracasei, L. rhamnosus, L. plantarum, L. pentosus, L. fermentum, L. brevis, L. buchneri, L. curvatus and L. acidophilus (5; 10, 16). Nowadays, an increased scientific interest on the microflora presented in the traditional dairy products is occurred and a wide variety of strains are under intensive studies. Different lactobacilli have been found to produce metabolic products that play important role in controlling the microbial balance in the hosts (19). Certain LAB from milk products have been selected for their healthy beneficial effects (20). They prevent the growth of pathogenic bacteria, in different ecosystems, by production of antimicrobials such as organic acids, hydrogen peroxide and bacteriocins (18). Thus, the new data on lactic microflora from widely consumed fermented foods is important from scientific and practical point of view.

The purpose of this study was to isolate and characterize viable NSLAB from Bulgarian home made milk products, such as yoghurt, white cheese and katak. A group of new isolated strains was identified according to modern polyphasic taxonomy and their inhibitory activity against E. coli was estimated.

Materials and methods

Isolation and phenotypic characterization of lactic acid bacteria

Twenty five pure LAB cultures were isolated from samples of home-made cheeses, yoghurt and katak, collected from Rila, Stara planina and Rodopi Mountains. The cheeses were made from ovine and goat raw milks and are fully ripened for two months in 10% w/v brine at 10°C. Katak is a specific national fermented product, prepared from raw ewe’s milk, with high concentration of NaCl and is ripened for a short period at 10-15°C. The isolation of LAB was done in parallel on four agar media- MRS (Scharlau, Spain), Rogosa (Difco, USA), M17 (Scharlau, Spain) and TG (Fluka Chemie, Buchs, Switzerland) after cultivation at 37°C and 42°C in anaerobic conditions.
conditions (BBL® Gas Pak Anaerobic System Envelopes). All strains were isolated by morphotype, as randomly selected colonies, from countable plates on MRS and Rogosa agar, following incubation at 37°C for 48 h under anaerobic conditions. The isolates were subcultured in MRS broth at 37°C for 24 h, checked for purity by streaking on PCA agar, and maintained in MRS broth supplemented with 20% (v/v) glycerol at −80°C until further tests.

The strains were tested for cell morphology, Gram, oxidase and catalase reactions, growth in MRS broth at 37°C and 42°C, and milk coagulation ability. Initial identification was done by the API 50CHL system (bioMérieux, France), as recommended by the manufacturer. The fermentation profiles obtained after 48 h cultivation at 37°C in anaerobiosis were facilitated by API 50CHL, ver. 4.0 identification software.

**PCR analyses**

Total DNA from new isolates and reference L. plantarum ATCC 14917 strain was extracted according to Delley et al. (8). All PCR reactions were done in a Progene cycler (Techne, UK) in 25 μl volume, using Ready To Go™ PCR beads (Amersham Biosciences). Species-specific PCR with primers for L. plantarum were performed according to Chagnaud et al. (3). Multiplex-PCR analysis was performed, using recA-targeted primers (21), obtained from LKB Vetriebs Gmbh.

**Antimicrobial activity testing**

The inhibitory effect of the strains identified as L. plantarum (see Results) was determined against the test-culture Escherichia coli HB101 (Collection of Institute of microbiology, BAS), by a well-diffusion assay, as described previously (9). Filtered (0.22 μm, Millipore) cells-free supernatants (100 μl of each), from 24 h cultures were used as control samples. In order to eliminate the putative effect of produced lactic acid they were additionally buffered with NaOH (5M) to pH 5.5-6.0. When the inhibition zone was determined around the wells of both control and buffered samples, the inhibitory effect (more than 10 mm zone) was assumed to be due to bacteriocin-like substances (BLS) or H2O2.

**Results and Discussion**

Fermented foods and especially yoghurt and white cheese, constitute a substantial part of the diet of Bulgarian consumers. However, limited data exists on biodiversity and biological activity of NSLAB of these popular milk products (4). In the present study we characterize viable LAB microflora in Bulgarian yoghurt, white brined cheese and katak, from ecological regions of Rila, Stara planina and Rodopi Mountains. All artisanal samples were prepared according to traditional technology without addition of starters.

**Isolation and identification of lactic acid bacteria**

Initial quantitative analyses of collected food samples showed the presence of high number viable bacteria, often in association with yeast. Twenty-five bacterial cultures were randomly selected from countable MRS and Rogosa agar plates. Their initial identification at genus/species level was done according to established phenotypic criteria (13). All isolates were considered LAB, based on their growth ability on selective media and phenotypic characteristics: Gram-positive, catalase and oxidase-negative reactions; non-motile; non-sporeforming rods and cocci. All cheese isolates grew well at 37°C in MRS broth and milk, while the majority of yoghurt and katak-originated strains grew better at 42°C and coagulated 10% (w/v) skim milk. A predominance of regular rod forms was observed in all collected samples from fully ripened cheese and yoghurt (Fig. 1). These isolates corresponded well to the typical characteristics of the genus Lactobacillus (13) and were subjected to present investigation. Isolation of lactobacilli in predominance was also reported for alberquilla cheese, made from a spontaneously fermented mixture of raw goat and sheep milk in the Alpujarra mountains region of Granada, SE Spain (1).

The classical identification scheme of Lactobacillus species includes morpho-phisiological characteristics and the ability to utilize various carbohydrate substrates. Eight of the strains from yoghurt were phenotypic similar to the species L. delbrueckii. Their identification should be confirmed by species-specific PCR and the analyses are still in progress. According to results obtained with API LAB 50CHL system seven Lactobacillus cheese isolates were identified as species L. plantarum and two as species L. paracasei subsp. paracasei (Table 1). Conventional microbiological study on microflora of Feta cheese concluded L. plantarum as the most common non-starter LAB during ripening (14). By classical isolation methods Van Hoorde et al. (23) revealed the predominance of L. plantarum, L. paracasei, L. brevis, L. rhamnosus and Pediococcus pentosaceus in two Flemish artisan raw milk Gouda-type cheeses after 8 and 12 weeks of...
Identification of *Lactobacillus* strains isolated from home-made milk products by API LAB 50 CHL mini kits (bioMérieux, France) and anti-*E. coli* activity determination.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolated from</th>
<th>Origin</th>
<th>Species affiliation</th>
<th>Identification authenticity (%)</th>
<th><em>Activity (mm zone)</em> with CFS</th>
<th>nCFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1</td>
<td>White ovine cheese</td>
<td>Stara planina</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.9 %</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>PS2</td>
<td>White ovine cheese</td>
<td>Koprinka-</td>
<td><em>L. paracasei</em></td>
<td>Excellent -98.7 %</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>PS3</td>
<td>White ovine cheese</td>
<td>Rodopi -Gela</td>
<td><em>L. paracasei</em></td>
<td>Excellent -99.8 %</td>
<td>13</td>
<td>(+/-)</td>
</tr>
<tr>
<td>PS4</td>
<td>White ovine cheese</td>
<td>Gela</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.8 %</td>
<td>12</td>
<td>(+/-)</td>
</tr>
<tr>
<td>PS5</td>
<td>White ovine cheese</td>
<td>Gela</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.8 %</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>PKS1</td>
<td>White goat cheese</td>
<td>Stara planina</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.6 %</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>PKS2</td>
<td>White goat cheese</td>
<td>Zverino</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.9 %</td>
<td>13</td>
<td>(+/-)</td>
</tr>
<tr>
<td>PKS3</td>
<td>White goat cheese</td>
<td>Zverino</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.9 %</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>CDM4</td>
<td>Ewe’s yoghurt</td>
<td>Rila</td>
<td><em>L. plantarum</em></td>
<td>Excellent -99.9 %</td>
<td>15</td>
<td>13</td>
</tr>
</tbody>
</table>

*CFS-cells-free supernatants without pH correction; nCFS- neutralized cells-free supernatants with NaOH (5M) to pH 5.5-6.0*

Carbohydrate fermentation has been used as a method to identify *Lactobacillus* for many years, but it is not very accurate because some strains belonging to different *Lactobacillus* species display the same fermentation results (17). We also observed very similar API LAB 50 CHL profiles into the group of the strains. Therefore, identification of new isolates, mainly based on phenotypical and biochemical characters, might not be sufficient for species identification. More discriminative and reliable methods are needed and the PCR analysis has been included as a fast and effective approach in taxonomic studies of microorganisms (12).

All strains were firstly subjected to *L. plantarum* specific PCR amplification. A single fragment (~900 bp) was detected for the type strain *L. plantarum* ATCC 14917 and the isolates, only with exception of the strain PS4 (Fig. 2A). The isolates PS2 and PS3 identified by API LAB 50CHL mini-kit as *L. paracasei*, also gave PCR amplification products (Fig. 2A) and should be reclassified as *L. plantarum*. The correct species affiliation was confirmed by multiplex PCR analysis.
L. plantarum has been the major strain involved in the production of mozzarella cheese (7). While in cheeses, made from raw goat and sheep milk in South Spain, was found as most abundant species L. paracasei, followed by considerably less quantities of L. plantarum and L. brevis (1). From traditional milk-based products, made and consumed in Africa, such as “Amasi” and “kule naoto”, as well as from Mongolian kumis (6) also were isolated L. plantarum strains (2). The presence of L. plantarum in Bulgarian yoghurt was unexpected. The normal microflora of this popular milk product is presented by species L. delbrueckii subsp. bulgaricus and subsp. lactis, L. helveticus in combination with Streptococcus thermophilus (4, 15). Probably the stain CDM4 was part of NSLAB of home-made yoghurt, viable in fermented milk till consumption time. Thus, the species L. plantarum is evident part of lactic microflora of different fermented products.

Anti- Escherichia coli activity of new characterized lactobacilli

Additional step in characterization of new isolated L. plantarum strains was determination of their inhibitory activity against E. coli. The agar diffusion method was used, as described previously (9). The growth of test-culture E. coli HB101, inoculated in agar plates (~10^8 CFU/ml), was strongly inhibited in all tests with acid spent supernatants (Table 1). Probably, this effect was due to the produced lactic acid, which is reported as detrimental for many Gram (-) bacteria. However the organic acids do not seem to be the sole active inhibitory metabolite in studied group of strains. After elimination of the putative effect of lactic acid, two of isolates (PKS1 and CDM4) were strong inhibitors, while the strains PS3, PS4 and PKS2 expressed only a bacteriostatic effect in vitro (Table 1). Therefore, a possible production of bacteriocin-like or other low-molecular antimicrobials was estimated for the group of new isolated strains. Bacteriocinogenic L. plantarum strains, originated from different fermented products, have been reported (9, 22). The presence of such strains, with a broad spectrum of activity, including E. coli and other food-spoilage and food-born pathogens, may contribute to the safety and storage ability of the food products (22).

Conclusions

This work proved the presence of viable non-starter LAB microflora in the most popular Bulgarian milk products - yoghurt and white cheese. Some of the isolates expressed antagonistic activity against E. coli. Thus, the artisanal fermented products, from ecological mountain regions of the Balkans could be a promising source for isolation of new active strains, which remain alive till consumption. The polyphasic taxonomic characterization, allows the correct determination of the species affiliation of eight strains as L. plantarum. This species has several applications in food industry and contribute significantly to the final quality and safety of many products (22). The assessment of the presence of L. plantarum in traditional fully ripened white cheese could be completed with more detailed biological and taxonomic studies and they are still in progress.

Acknowledgment

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REFERENCES


