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MOLECULAR IDENTIFICATION OF *Lactobacillus* spp., ISOLATED FROM TRADITIONAL BULGARIAN DAIRY PRODUCTS

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ABSTRACT

Genus *Lactobacillus* constitutes a heterogeneous group of lactic acid bacteria (LAB) that currently has more than 180 species and subspecies, most of which play an important role in food fermentations. The increasing interest in these species is due to their ability to improve quality of dairy products, to ensure their safety and to promote consumers` health. The aim of this study was to perform a molecular identification of 45 newly-isolated LAB strains from traditional Bulgarian dairy products: artisanal curd, katak, yoghurt, white-brined and yellow cheese. They were characterized as *Lactobacillus* spp. The phenotypic and physiological similarity, observed from the studied dairy lactobacilli, created some difficulties in their identification. This required a combination of classical and genomic methods and new adapted protocols for dairy LAB. Therefore, the DNA from all LAB was extracted and several methods based on polymerase chain reaction (PCR) method were further applied: (i) Multiplex PCR with specific primers for the differentiation of closely related species from the group of *Lactobacillus plantarum;* (ii) 16S rDNA analyses and (iii) RAPD PCR - a high discriminative genotypic method for distinguishing strain-specific features of representatives of *L. plantarum* group. Twenty-three of the investigated strains were identified as *L. plantarum*. The non-identified lactobacilli were subjected to 16S rDNA PCR, the gold standard in bacterial taxonomy. According to the BLAST analysis of the obtained sequences, they have been identified as: *Lactobacillus fermentum* (3 strains), *Lactobacillus rhamnosus* (2 strains), *Lactobacillus delbrueckii* spp. *bulgaricus* (1 strain) and *Lactobacillus paracasei* (1 strain). A group of 4 strains with very low similarity to the presented *Lactobacillus* spp. in the GenBank – NCBI data was found, which could be new LAB species. Further characterizations, however are needed and they are still in progress.

KEYWORDS: *Lactobacillus* spp., Identification, PCR, Multiplex PCR, RAPD PCR.

INTRODUCTION

Consumer demand for the fermented dairy products is growing intensively worldwide. These products include yoghurt, curd, cheese, koumiss, kefir and others. In Bulgaria for centuries different home-made traditional dairy products also exist, such as katak and white (feta) cheese. They are part of the daily menu of Bulgarians with an average annual consumption of yoghurt - 29 kg per capita, white cheese - 12.4 kg, yellow hard cheese (kashkaval) - 3.5 kg and other dairy products - 2.1 kg.[1] Thanks to their nutritional value and beneficial impact on human health and physiology, they are highly recognized as functional foods.

In Bulgaria as well as in the world, traditional dairy products have a long history of safe use and proven record of excellent nutritional value and good taste. These properties thereof depend both on the raw material used, and the manufacturing technology including the type of primary cultures and the specific microbiota of the product generated in the course of its preparation and storage. The primary reason of their long history of safe consumption is the fact that several species, which are major components in their production, have GRAS (Generally Recognized As Safe)/QPS status.[2]

Literature data from the recent years show the essential role not only of starter cultures, but also nonstandard, so called secondary microflora, particularly in cheeses with long maturation period.[3] Important emphases in this direction are evidences collected for probiotic potential of various representatives of the LAB part of the autochthonous microbiota of these dairy products.[4] Critical for these beneficial properties (taste and

probiotics) are LAB. For Bulgarian white cheese active dynamism and broad representation of the genus *Enterococcus* and *Lactobacillus* (in the early stages) and genus *Lactobacillus* throughout the period of maturation in the finished product is proven. Relatively little studied is the microflora of "katak" a traditional fermented product with specific weak sour taste and consistency.

Stable domination of species from the groups of *L. plantarum* and *L. casei* is reported respectively in traditional Greek cheese and Spanish Armada.[5,6] It is noteworthy that cheeses made from raw milk differ greatly in terms of organoleptic properties and composition of LAB flora than those made from pasteurized milk. Microbiota of home-made dairy products prepared according to the traditional recipes without the use of industrial yeasts and improvers is much more diverse and has better probiotic characteristics. They often feature a naturally longer shelf life (with no added preservatives). Several papers show the potential role of Bulgarian cheese as a source of beneficial bacteria.[7,8,9]

Moreover, the interest of scientists is focused especially on development of new fermented products and supplements in response to consumers` needs. Traditional fermented dairy products are a part of people`s nutrition since ancient times and are a proven source of various lactic acid bacteria with pivotal role in the maintenance and recovery of a healthy state of humans. [10] However, the autochthonous microbiota, and the beneficial strains, which contribute not only to organoleptic but also to the beneficial properties of food, is poorly characterized. This certainly stimulates the interest of the scientific community of microbiologists and biotechnologists towards new research. In this direction are our studies on 23 samples of artisanal cheeses and 22 varieties of yoghurt collected from different geographical regions of Bulgaria. The aim of this work is to identify to what species and molecular genotyping belong the newly isolated strains of these dairy products prepared according to traditional recipes, without the addition of starter cultures.

MATERIALS AND METHODS

Food sample, microorganisms and culture conditions

Several home-made samples of traditional dairy products (curd, yoghurt, yellow cheese and white-brined cheese) have been collected from different rural regions of Bulgaria and were used for lactic acid bacteria isolation.[8] A collection of more than 70 isolates was created and initial characterization as members of the genus *Lactobacillus* has been proved. Forty-five newly isolated lactobacilli are a subject of the present study. Lactobacilli were grown in MRS broth (Merck, Germany) under anaerobic conditions (GasPak 100 Anaerobic system, BD Bioscience, USA) at 37 ºC for 24 h.

Isolation of genomic DNA from LAB isolates

Isolation of genomic DNA from pure cultures of the *Lactobacillus* strains was performed according to the method of Delley et al., (1990),[11] adapted for lactobacilli by Danova et al., (2005).[12] The obtained DNA samples were analyzed by agarose gel electrophoresis and the concentration and purity were checked by NanoDrop. They were stored at -20°C and were used as target in PCR assays.

PCR analyses

Multiplex PCR analysis for identification of the group of *L. plantarum*

Multiplex PCR analysis was performed using primers based on *rec-A* gene: paraF (5`-GTC ACA GGC ATT ACG AAA AC-3`), plantF (5`-CCG TTT ATG CGG AAC ACC TA-3`), pentF (5`-CAG TGG CGC GGT TGA TAT C-3`) and pReV (5`-TCG GGA TTA CCA AAC ATC AC-3`), (LKB Vertriebs GmbH, Austria). Amplification reactions were performed with 2XPCR TaqMixture kit (HiMedia, India), primers paraF, pentF, plantF and pREV (0.25 µM each) and 1 ng of DNA/µl. They were held on the apparatus Techne Progene FPR0G02Y DNA Thermal Cycler (Oxford, UK) with an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 10 s, elongation at 72 °C for 30 s, and a final synthesis at 72 °C for 5 min. The resulting PCR products were visualized using agarose gel electrophoresis.[13]

RAPD PCR for distinguishing representatives of the group of *L. plantarum*

RAPD PCR analysis was performed with primer M13V (5`-GAG GGT GGC GGT TCT-3`), (MWG-Biotech, Germany) according to the method of Ehrmann et al., (2003).[14] Amplification reactions were performed with 2XPCR TaqMixture kit (HiMedia, India), 0.2 µM primer M13V and 1 ng of DNA/µl.[15] They were conducted on the above-mentioned apparatus, following amplification programme: (i) 3 cycles of initial denaturation at 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min, 32 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, elongation and ultimate synthesis at 72 °C for 3 min, followed by cooling to 4 °C. PCR products were analyzed on agarose gel electrophoresis.

16S rDNA PCR

Amplification of 16S rDNA gene was carried out with the following pairs of primers: forward primer fD1 (5`-AGA GTT TGA TCC TGG CTC AG-3`) and reverse primer rD1 (5`-AAG GAG GTG ATC CAG CC-3`).[16] Amplification reactions were performed with 2XPCR TaqMixture kit (HiMedia, India), primers rD1 and fD1 (0.6 µM each) and 1 ng of DNA/µl. They were conducted on the above-mentioned apparatus under the following amplification conditions: initial denaturation at 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min, 32 cycles of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at

56.5 °C for 75 s, elongation at 72 °C for 75 s, and a final synthesis at 72 °C for 5 min.

Agarose gel electrophoresis of PCR products

Electrophoresis of the DNA/amplified PCR products were performed on Mini Gel II Complete Horizontal Electrophoresis System (VWR International, USA). DNA products, obtained by isolation of genomic DNA, were analyzed by agarose gel electrophoresis (1% (w/v) agarose type II-A (Sigma-Aldrich, Germany) in 0.5xTBE buffer (stock solution 10xTBE, pH 8.3). Electrophoresis was held for 30 min at a gradual decrease and a change of voltage from 100 to 80V within an interval of 10 min. Amplification fragments from 16S rDNA and Multiplex PCR were analyzed using 1.25% and 1.5% (w/v) agarose gel respectively, and those from RAPD PCR - 2% (w/v) agarose gel. Electrophoresis was performed for 40 min at a gradual decrease and change of voltage from 100 to 40 V within an interval of 5-10 min. After staining with ethidium bromide (0.5 $\mu g.ml^{-1}$), fragments were visualized using a transilluminator system GDS-8000 (UVP, UK) at $\lambda = 254$ nm.

Sequencing and analysis of the 16S rDNA gene sequence

The DNA of 15 LAB isolates was amplified using primers fD1 and rD1 for the preparation of unpurified 16S rDNA products according to Weisburg et al., (1991).[16] The amplified products of approximately 1550 bp were purified and sequenced using a 3730xl DNA analyzer (Thermo Fisher Scientific, USA) by Macrogen Inc. (Belgium). The obtained sequences were analyzed with Chromas 2.3 programme (Technelysium Pty Ltd., Australia) and species identification of strains was determined by BLASTN analysis.

RESULTS AND DISCUSSION

Lactobacilli are widely spread in a variety of fermented dairy products and in raw milk, different plants and plant-derived materials, as well as animals and human. Their quality as starter bacteria for various milk products is well known. Relatively little is known, however, about the so-called non-starter LAB (NSLAB) in traditional dairy products. Therefore, in our recent studies artisanal samples have been collected from traditional for Bulgaria and Balkans products: katak, yoghurt, white-brined and yellow cheese, curd and a collection of more than 70 pure cultures has been created.[8] All samples were home-made and prepared according to traditional recipes, preserved up to day in different regions of Bulgaria without the use of industrial starter cultures.

In the present paper a total of 45 newly isolated strains are characterised according to modern polyphasic taxonomy. The group was formed on the base of morphological characteristics, according to which all 45 isolates were determined as Gram-positive, catalase-negative and rod-shaped bacteria, with polymorphism of cells. They were anaerobic or aerotolerant and grew well in MRS broth (pH 5.4÷6.5) at 37 °C, actively acidifying the medium. Thus, they were determined as representatives of the genus *Lactobacillus.*

Accurate and definite identification of microorganisms is essential for biotechnological, industrial, biomedical, pharmaceutical and environmental studies.[17] Therefore, in parallel with the conducted classical morphological, physiological and biochemical tests, a variety of modern molecular methods such as Multiplex PCR, 16S rDNA PCR and sequencing of 16S rDNA gene and RAPD PCR analyses to identify the group of strains to species level were used. For their implementation, a total DNA for each strain of the formed group was derived. Experiments were carried out by the method of Dellay et al., (1990), [11] with modifications reported by Danova et al., (2005). [12] After checking nativity of DNAs by the agarose gel-electrophoresis, they were used for subsequent genetic analyses. Multiplex PCR analysis was performed with primers based on *rec*A-gene, which distinguish the species-specific strains *L. plantarum, L. paraplantarum* and *L. pentosus* according to the method of Torriani et al., (2001). [13] Results, obtained by the 1.5% agarose gel-electrophoresis, were presented in Fig. 1:

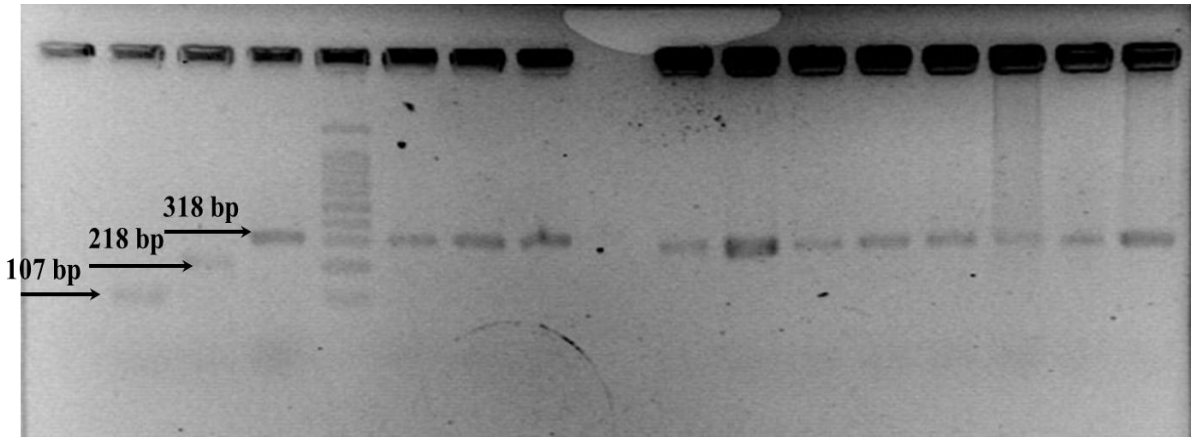

Figure 1: Photo-negative ethidium bromide stained agarose gel (2% w/v) showing Multiplex PCR products of *L. plantarum* strains, with primers based on *rec*A-gene (according to [13]).

Lines: 1 – L. paraplantarum ATCC 700211^T; 2 - L. pentosus ATTC 8041^T; 3 - L. plantarum ATCC 14917^T; 4 – Molecular marker 100 bp; 5 – Kz3; 6 – BS32; 7 – OC1; 8 – 3V; 9 – 10V; 10 – S9; 11 – 2V;

Using Multiplex PCR, the strains S3, S5, S6, S7, S8, S9, S10, S12, 1V, 2V, 3V, 7V, 8V, 10V, OC1, BS32, BS41, Kz1, Kz2, Kz3, Ko1, Ko2, Ko3 were identified as members of the species *Lactobacillus plantarum*, according to their identity with the type culture *L. plantarum* ATCC 14917^T (Fig. 1). Our results confirmed the study of Danova et al., (2009)[9] which also established the presence of *L. plantarum* in home-made yoghurt, as well as white-brined cheese and katak samples.

The number of LAB strains belonging to the species *L. plantarum*, is about 49% of all isolates, indicating the stable presence of the species in various domestic dairy products consumed in Bulgaria (Fig. 1).

Species *L. plantarum* constitutes the majority of non-starter LAB found in most ripened cheese varieties. While examining the microflora of Bulgarian white-brined cheese, it was found predominance of *L. plantarum*.[7,18] The species is reported as the predominant representative of the genus *Lactobacillus* in other classic home-made sheep hard cheeses and white-brined cheeses in the Balkans.[19]

L. plantarum was also reported in cheeses like Pecorino cheese, Morlacco cheese, Canestrato Pugliese cheese, Monte Veronese cheese, Spressa cheese, some Argentinean cheeses, etc. [20] In addition to yoghurt and cheese, *L. plantarum* is found in various vegetables (pickles, olives, sauerkraut, dough, etc.), meat and fish.[21]

16S rDNA PCR is a standard method for identifying bacteria and establishing diversity within a microbial group or outside it, and is widely applicable to the genus *Lactobacillus*. This is due to the highly conservative sequence of the 16S rDNA gene, together with the presence of highly variable regions that provide species-specific sequences useful for the study of phylogenetic diversity of bacteria.[22,23] As a result, the genomic DNAs of 9 newly isolated LAB are used as a template to perform PCR amplifications with following primers: fD1 (5`-AGA GTT TGA TCC TGG CTC AG-3`) and rD1 (5`-AAG GAG GTG ATC CAG CC-3`) according to the method, described in point 2.3.5 from Materials and methods. The amplification products were sequenced using a 3730xl DNA analyzer (Thermo Fisher Scientific, USA) by Macrogen Inc. (Belgium) and the obtained sequences were further analyzed with Chromas 2.3 programme (Technelysium Pty Ltd., Australia) in order to exclude the places where the primers were gripped. After a Blast analysis of the available sequences in GenBank, the species identification of strains was determined (Table 1).

Table 1: Identification of newly-isolated *Lactobacillus* strains from yoghurt, katak, white-brined cheese by the 16S rDNA sequence analysis.

№	*Isolates*	*Source*	*Closest similarity (based on 16S rDNA sequencing)*	*Probability, [%]*
1.	Ro33	Sheep yoghurt	*Lactobacillus rhamnosus*	94
2.	Ro34	Sheep yoghurt	*Lactobacillus delbrueckii ssp. bulgaricus*	95
3.	8V	Buffalo yoghurt	*Lactobacillus plantarum*	95
4.	S2	Sheep katak	*Lactobacillus rhamnosus*	95
5.	S4	Sheep katak	*Lactobacillus fermentum*	95
6.	BS31	Mixture: buffalo-cow cheese	*Lactobacillus fermentum*	98
7.	G7D	Cow cheese	*Lactobacillus fermentum*	92
8.	S11	Yellow cheese from cow milk	*Lactobacillus paracasei*	95
9.	Ko2	Sheep cheese	*Lactobacillus plantarum*	95

As a result of various methods combination and sequencing of the 16S rDNA gene, the 9 strains isolated from various dairy products were identified as *L. fermentum* (3 strains), *L. rhamnosus* (2 strains), *L. plantarum* (2 strains), *L. delbrueckii ssp. bulgaricus* (1 strain) and *L. paracasei* (strain 1). The results obtained for the two strains isolated from katak are among the first data from molecular genetic analysis for lactic acid microflora of katak.

At the same time it was demonstrated the presence of *L. fermentum* in samples of white cheese (strains G7D and BS31). *L. fermentum* is the main heterofermentative species of the genus *Lactobacillus* in human gastrointestinal tract and is widely used in industrial fermentations as a starter in dairy industry. This species, as well as *L. rhamnosus*, has proven effectiveness in treating and preventing urogenital infections of women. It has been reported in the literature the isolation of *L. fermentum* of different types white cheese: cheese Tulum, [24] Ras cheese [25] and Cheddar cheese. [26]

As a result of identification it was shown the presence of *L. paracasei* species in a sample of yellow cheese (kashkaval). *L. paracasei* was reported as the dominant species after *Lb. plantarum* in samples of feta cheese [27] and white-brined cheese.[28]

RAPD PCR is a precise method for strain distinction of lactobacilli from different ecological niches at species and subspecies level. RAPD analysis was applied in order to generate specific DNA profiles which could be compared and then grouped according to their degree of similarity with type cultures of relevant phylogenetic group. Identification to strain level is extremely important since most of the characteristics of lactobacilli are strain-specific. The first condition a newly isolated strain to be applied in production of dairy products is its species identification and strain genotyping. Therefore, all strains were further subjected to RAPD PCR analysis using a primer M13V. The results are presented in Fig. 2:

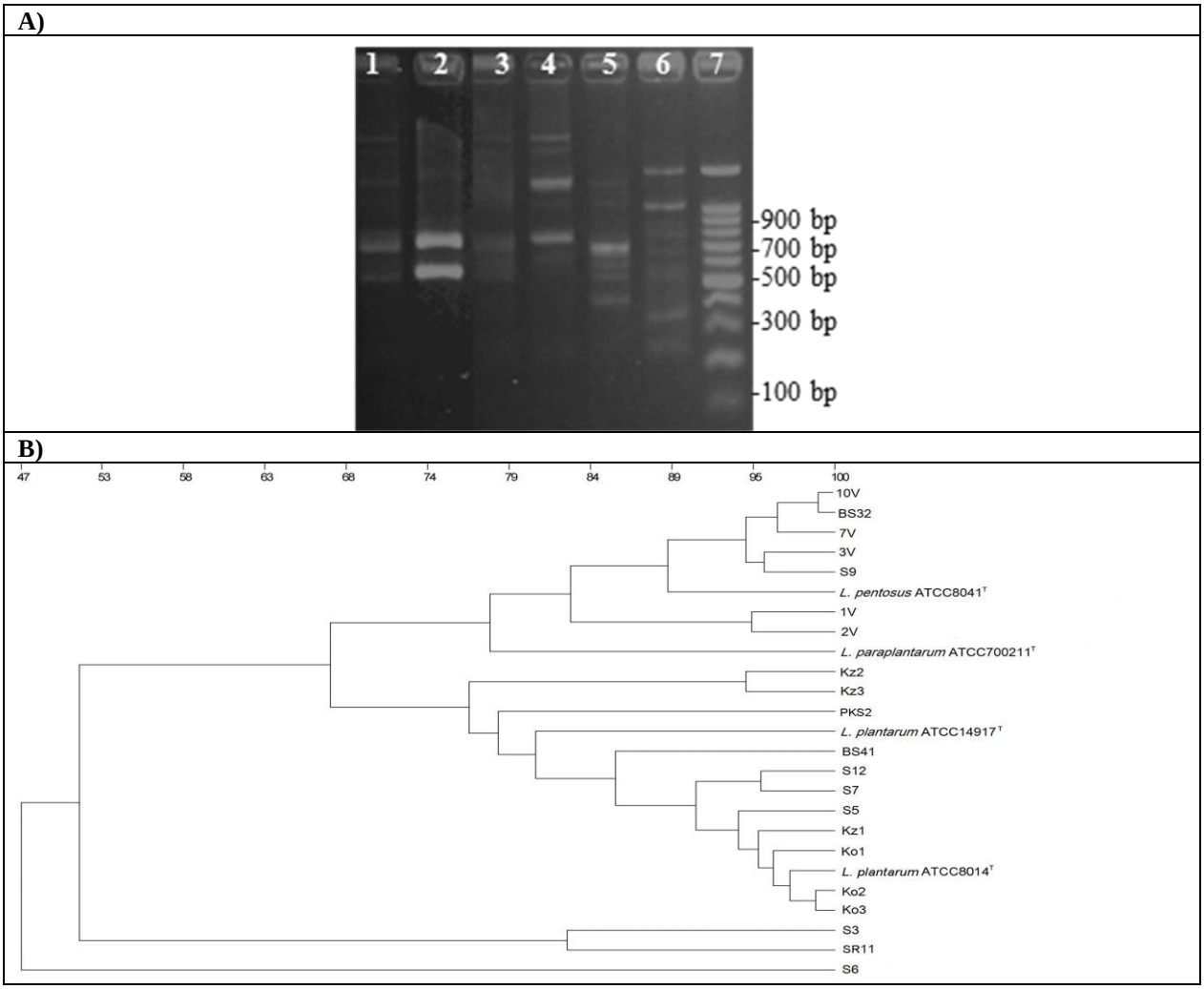

Figure 2: A) RAPD PCR analysis of strains from katak, yoghurt and white-brined cheese and type strains *L. plantarum* ATCC 14917^T, *L. paraplantarum* ATCC 700211^T and *L. pentosus* ATCC 8041^T with M13V primer (according to [14]); B) UPGMA analysis and dendrogram of *L. plantarum* fragments, obtained by RAPD PCR analysis with MVSP programme.

Legend: *L. plantarum* ATCC8014^T – type strain; PKS2, SR11 – previously identified strains [12]
Starts: 1 – S12; 2 – S6; 3 – S7; 4 - *L. plantarum* ATCC 14917^T; 5 - *L. paraplantarum* ATCC 700211^T; 6 - *L. pentosus* ATCC 8041^T; 7 – 100 bp DNA ladder (Fermentas, Thermo Fisher Scientific, USA)

All isolates were identified as members of the species *L. plantarum.* The obtained results, presented in fig. 2, showed a variety of strain-specific differences with the type cultures (*L. plantarum* ATCC 14917^T, *L. paraplantarum* ATCC 700211^T, *L. pentosus* ATCC 8041^T) and a high degree of genetic similarity between isolates. On one hand, a big part of strains isolated from different kinds of white-brined cheeses (strains Ko1, Ko2, Ko3, S12 from sheep milk, strain S7 from goat milk) and strain BS41 from buffalo milk), yellow cheese from goat milk (strain Kz1) and katak from sheep milk (strain S3) were clustered with type strain *L. plantarum* ATCC 14917^T with a high degree of genetic similarity (about 90%). On the other hand only a small group of the strains (strains 1V and 2V), isolated from buffalo yoghurt, showed characteristics of genetic similarity (about 80%) with type strain *L. paraplantarum* ATCC 700211^T. A part of the strains also showed about 95% of genetic similarity with type culture *L. pentosus* ATCC 8041^T.

As a result of the PCR analysis a phylogenetic tree was constructed and the newly isolated lactobacilli are assigned to the type cultures *L. plantarum*, *L. paraplantarum* and *L. pentosus* (Fig. 3).

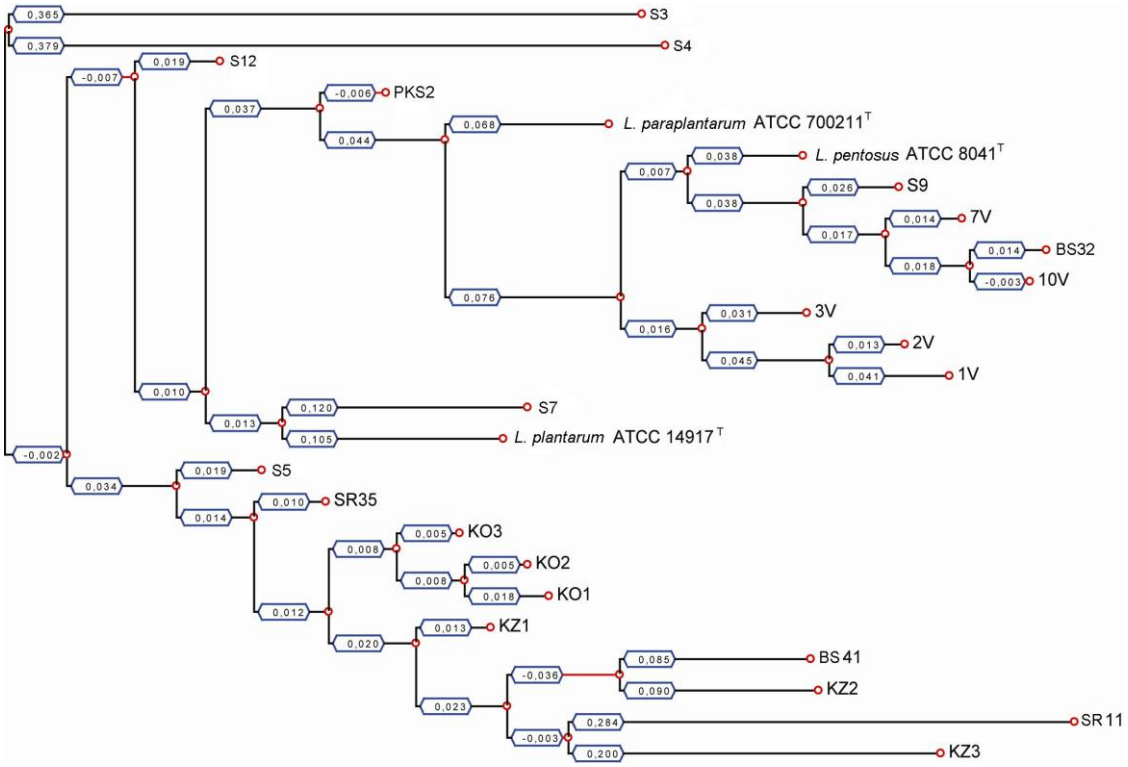

Figure 3: Phylogenetic tree of newly identified species of *Lactobacillus plantarum* group from yoghurt, katak and white-brined cheeses.

CONCLUSION

Representatives of *Lactobacillus* genus are widely used in the production of fermented dairy products. Forty-five LAB strains were isolated from traditional Bulgarian dairy products and initially characterized as representatives of genus *Lactobacillus*. The variety of methods used in the present study helped to identify the investigated LAB strains as representatives of *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, *Lactobacillus delbrueckii* spp. *bulgaricus* and *Lactobacillus paracasei*. A group of 4 strains with very low similarity to the presented *Lactobacillus* spp. in the GenBank – NCBI data was found, which could be new LAB species. The obtained results enhance our knowledge of autochthonous microbiota in dairy products but at the same time have shown the need of prompt and quick identification techniques in distinguishing between these candidate probiotic strains present in fermented foods. Our cognition in this area is still limited and needs further investigation.

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