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MOLECULAR TYPING OF LACTOBACILLI ISOLATED FROM DRY SAUSAGE "LUKANKA": COMPARISON OF WHOLE CELL PROTEIN (WCP) VERSUS DNA-BASED METHODS

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ABSTRACT

A collection of forty-two Lactobacillus isolates from "Lukanka" sausage, previously determined as belonging to the species L. plantarum, L. pentosus and L. paraplantarum, was subjected to phylogenetic analyses by protein- and DNA- based methods in order to investigate the diversity within the Lactobacillus microflora participating in the ripening of the final product, as well to compare the discriminatory powers of the methods used. Similar clusterings were obtained by all the four methods (whole cell protein electrophoresis, RAPD-PCR, Rep-PCR and ERIC-PCR), and consequent UPGMA analysis of the results showed that some of the methods are more applicable for differentiating closely related strains, while others are for species differentiation.

Keywords: ERIC-elements PCR (ERIC-PCR), random amplification of polymorphic DNA (RAPD), Rep-elements PCR (Rep-PCR), whole cell proteins electrophoresis (WCPE)

Introduction

Many laboratories worldwide still practice strains differentiation based upon the analysis of whole cell proteins separated on SDS-PAGE. However protein-based methods for studying strain relatedness are more and more replaced by molecular ones such as random amplification of polymorphic DNA (RAPD). In the last years new DNA-based methods emerged, for example repetitive elements PCR (REP-PCR), in particular amplification of enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (Rep) elements.

The main goal of this research was to estimate the discriminatory power of the different methods, as well as to characterize phylogenetically a collection of forty-two *Lactobacillus* isolates from the traditional Bulgarian dry sausage "Lukanka".

Materials and Methods

Bacterial strains and cultivation conditions

All *Lactobacillus* strains were grown on MRS broth – liquid or 1.5% agar at 30°C for 16–18 hours if not mentioned otherwise.

Whole cell protein preparation

Aliquots of 1 ml of MRS liquid broth were inoculated with 10 µl of overnight cultures of all strains, and allowed to develop for 18 hours at 37°C. The cells in the obtained cultures were precipitated by centrifugation for 90 sec at 10000 x g, washed twice with 1 ml of dH_2O, and finally resuspended in 1 ml H_2O. The optical density at 595 nm (OD_{595}) was measured. Equal quantities of cells – approximately 4.10^7 calculated on the basis of the OD_{595} (1 OD_{595} unit = $6{,}7.10^8$ cfu/ml (1)) were sedimented by centrifugation for 3 min at 10000 x g, resuspended in 200 µl

lysozime solution (10 mg/ml), and incubated for 1 hour at 37°C. The cells in the samples were sedimented once again by centrifugation for 3 min at 10000 x g, and resuspended in 50 µl 4x SDS-PAGE sample buffer containing 2-mercaptoethanol. At this stage the samples were stored frozen at – 20°C for several weeks. Immediately before the electrophoresis the samples were heated for 10 min at 99°C, allowed to cool, and 20 µl of each sample were loaded on the electrophoresis.

SDS-PAGE

Tris-glycine SDS-PAGE according to Laemmli (4) was performed under reducing conditions in V2-SET electrophoresis units (Scie-Plas) using 10% running gel and 3% stacking gel. The running gel size was 16 x 16 x 0.15 cm, and the electrophoresis was performed at constant current of 40 mA for about 5 hours. High Range molecular weight standards (Bio-Rad) were used. The gels were stained with Coomassie (5).

DNA isolation

Chromosomal DNA was isolated from single-colony inoculated 5 ml overnight cultures with GenElute™ Bacterial Genomic DNA Kit (Sigma – Aldrich Co.). From the obtained DNA, 20 µl aliquots were made and stored at – 20°C for several mounts until needed, or at 4°C for several days when used.

PCR techniques

All PCR primers and amplification conditions are listed in **Table 1**. All primers were synthesized by "Integrated DNA Technologies" (Belgium) at 25 nmol scale, and dissolved at final concentration of 100 mM for downstream applications. The PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems Co.) in a reaction volume of 20 µl. RPAD-PCR was performed with L1 primer according to Fitzsimons et al. (2), Rep-PCR was performed according to Jeršek et al. (3), and ERIC-PCR according to Matsumiya et al. (7). 8 µl of the reaction products were analyzed on agarose gels with concentrations ranging from 1.2 % to 1.8 % in TBE buffer system.

Phylogenetic analyzes

Phylogenetic analysis of the results of the WCPE, RAPD, Rep-PCR and ERIC-PCR were performed using GeneTools software (Syngene), and UPGMA dendrograms were constructed on the basis of the obtained similarity matrices.

TABLE 1

PCR primers used in this study

Application	Primer	Sequence 5'→ 3'
ERIC-PCR	ERIC1R	ATG TAA GCT CCT GGG GAT TCA C
	ERIC2	AAG TAA GTG ACT GGG GTG AGC G
Rep-PCR	REP1R-Dt	III NCG NCG NCA TCN GCC
	REP2-Dt	NCG NCT ATC NGG CCT AC
RAPD	L1	ACG CGC CCT

Results and Discussion

The results of the SDS-PAGE of the samples of whole cell proteins of the *Lactobacillus* strains are presented in **Fig. 1**. The analysis of the dendrogram obtained on the basis of the protein profile resulted in the formation of three clusters, two of them being well defined, and the third presenting more expressed phylogenetc distances.

The polymorph profiles obtained from the RAPD analysis (**Fig. 2**) led to the grouping of the strains again in three clusters, but in this case some strains (S14, S21) were not grouped within the clusters. Also a well defined subclustering was observed within some of the major clusters.

Similar results were obtained from the ERIC-PCR (**Fig. 3**) but in this case the calculated distances within the strains in each of the major clusters were greater. Once more the strain S21 was not clustered.

More expressed distances were obtained thanks to the Rep-PCR (**Fig. 4**). In this case all the three major clusters were divided into several subclusters, making this method more convenient for differentiation of closely related strains. If compared together, the results of the all four methods showed comparable results, the strains in the three major clusters probably reflecting the three predominant Lactobacillus species participating in the "Lukanka" ripening - *L. plantarum*, *L. paraplantarum* and *L. pentosus* (6).

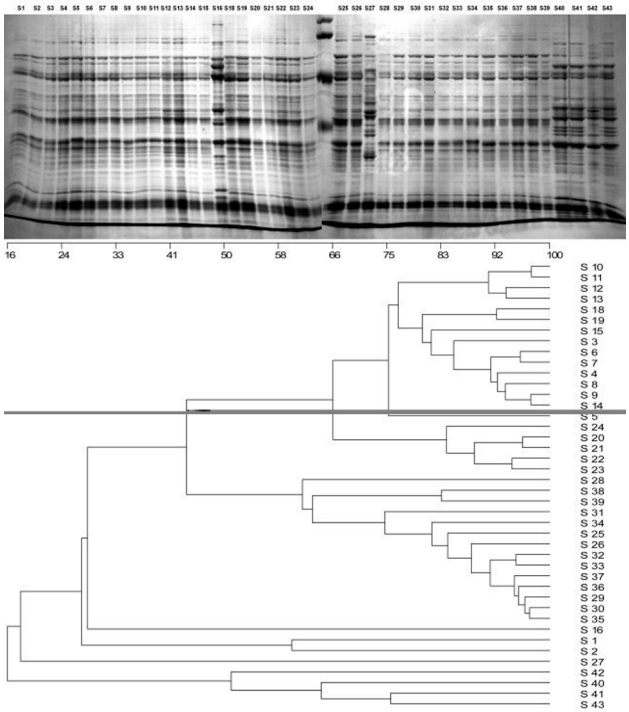

Fig. 1. Results of WCPE and dendrogram constructed on the basis of the upgma of the electrophoretical patterns

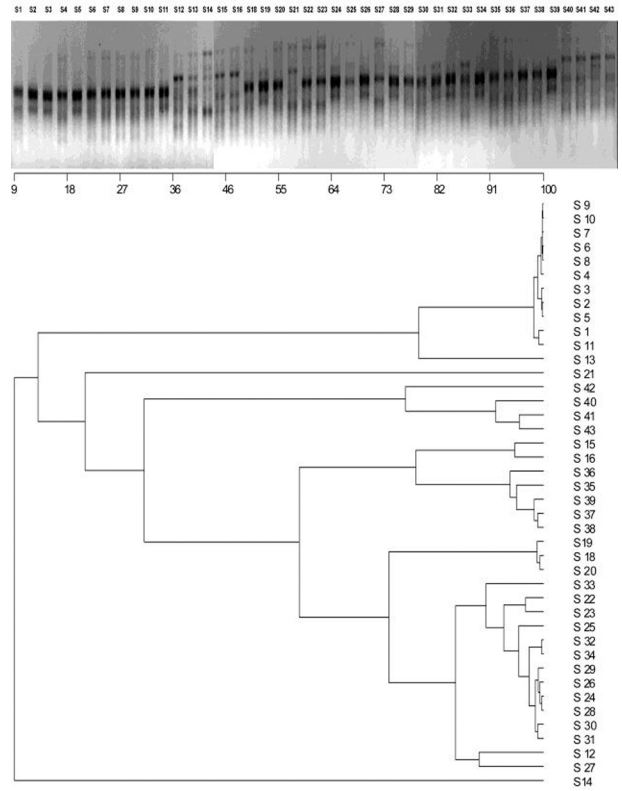

Fig. 2. Results of RAPD and dendrogram constructed on the basis of the upgma of the electrophoretical patterns

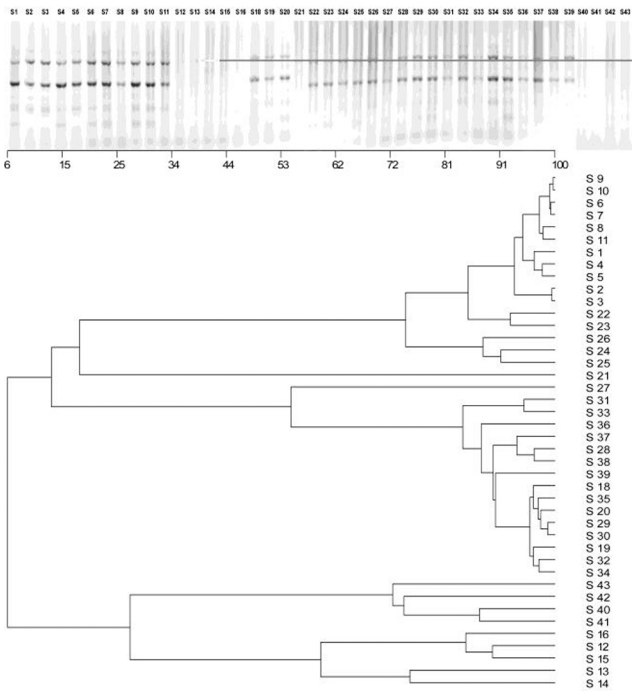

Fig. 3. Results of ERIC-PCR and dendrogram constructed on the basis of the upgma of the electrophoretical patterns

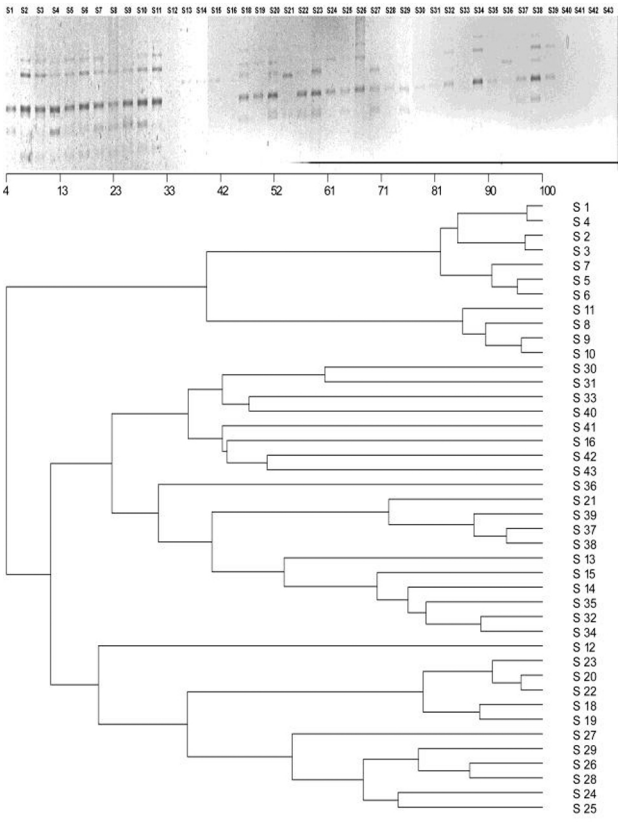

Fig. 4. Results of Rep-PCR and dendrogram constructed on the basis of the upgma of the electrophoretical patterns

Conclusions

In conclusion it was found that WCPE and Rep-PCR are more applicable for differentiating closely related *Lactobacillus* strains because of the greater values of the calculated distances within the clusters. On the other hand well defined clustering with smaller internal distances was obtained by RAPD and ERIC-PCR, making these methods more suitable for species grouping during phylogenetic analyses based on polymorphisms. In this case the calculated distances between strains belonging to the same species were less pronounced.

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