STUDY OF THE DIVERSITY OF THE LACTOBACILLUS MICROFLORA IN RAW DRY SAUSAGE LUKANKA DURING RIPENING

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ABSTRACT

In this study from 7 samples from different stages of the ripening of Bulgarian traditional raw dry sausages of Lukanka. Totally 43 strains were isolated and identified as *Lactobacillus* spp. based on their growth, Gram stain, catalase and oxidase activity. Their affiliation to this genus was confirmed by PCR with genus specific primers, 16S ribosomal RNA The results show different Lactobacillus species (*Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus sakei*).

Key words: *Lukanka , Lactobacillus spp, dry sausage.*

I. Introduction

Meat fermentation is an ancient process originally used to extend the shelf life of perishable raw materials. During fermentation, complex biochemical and physical reactions take place that result in a significant change of the initial characteristics. Moreover, production of aromatic substances during fermentation define the sensorial characteristics of the final product that are significantly different from the ones of the raw materials used. Low acid fermented meat products (final pH 5.3 to 6.2) (Aymerich et al.,2003) are a group of traditional Mediterranean products with a great diversity between different countries and between different regions of the same country. The first evidence of sausages production date back to the period of the Roman empire (Lücke, 1974).

II. Materials and Methods

1. Sample

A total of 7 samples from different stages of the ripening of Bulgarian traditional raw dry sausages of Lukanka- were collected. All samples were stored at 4^{o}C until their analysis.

2. Isolation of lactic acid bacteria

Ten grams of Bulgarian traditional dry sausage-Lukanka. samples were diluted in 90 ml saline solution, homogenized, serially diluted in the same solution and plated on MRS agar (De Man, Rogosa and Sharpe Agar, Merck Darmstadt Germany). The plates were then incubated at 42^{0}C for 24 – 48h. The colonies which were morphologically different were picked up and inoculated as stab. Were isolated 43 strains. (Table .1)

3. Identification of strains

3.1 DNA Isolation

Chromosomal DNA was isolated from single-colony inoculated @,,GenElute ^{TM},, Bacterial Genomic DNA kit (Sigma Aldrich Co). From the obtained DNA, 20µm aliquots were made and stored at -20 ^{0}C for several mounts until needed, or at 4^{0}C for several days when used.

3.2 PCR - amplification with primers specific for the genus Lactobacillus

The Genus- specific PCR with the primer sets Lact71R/Lab0667F (Moura.P et al., 2007) and LactoF/R(2) was performed according to the authors. The amplification was performed in a 2720 Thermal Cycler (Applied Biosystemes Co) in a reaction volume of 20 µl.

3.3 Sequencing of the 16S rRNA genes.

Isolated DNA by the Sigma kit was used as template for a PCR amplification employing universal primers (by Weisburg et al. 1991): fD1 (5'-AGAGTTTGATCCTGGCTCAG-3 ') and rD1 (5'-TAAGGAGGTGATCCAGGC-3') 16S rRNA gene, in the following conditions:

Ready toGO PCR kit was used (Amercham, Austria), and each reaction was performed with primers fD1 and rD1 (0.6 µl each), 0.025 U Taq / µl, with 10X PCR buffer (Polymed) and ~ 5-10 ng of DNA / µl with Raina concentration 3 mM $MgCl_2$.

The PCR amplification was performed under the following conditions: the program included: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation for 1 min at 94°C, to 15 min at 56ºC, elongation of 1.15 min at 72°C and elongation of terminal 5 min at 72°C.

The resulting amplification products were visualized on 1% agarose gel after staining with ethidium bromide. Sequencing was performed on the ABI PRISM®310 DNA Genetic Analyzer, (PE Applied Biosystems), and the resulting sequences were further processed by the Program Chromas 2 · 3 (Chromas version 2 · 3; http://www.technelysium.com.au/chromas.html) to exclude areas where grip primers.

III. Results and Discussion

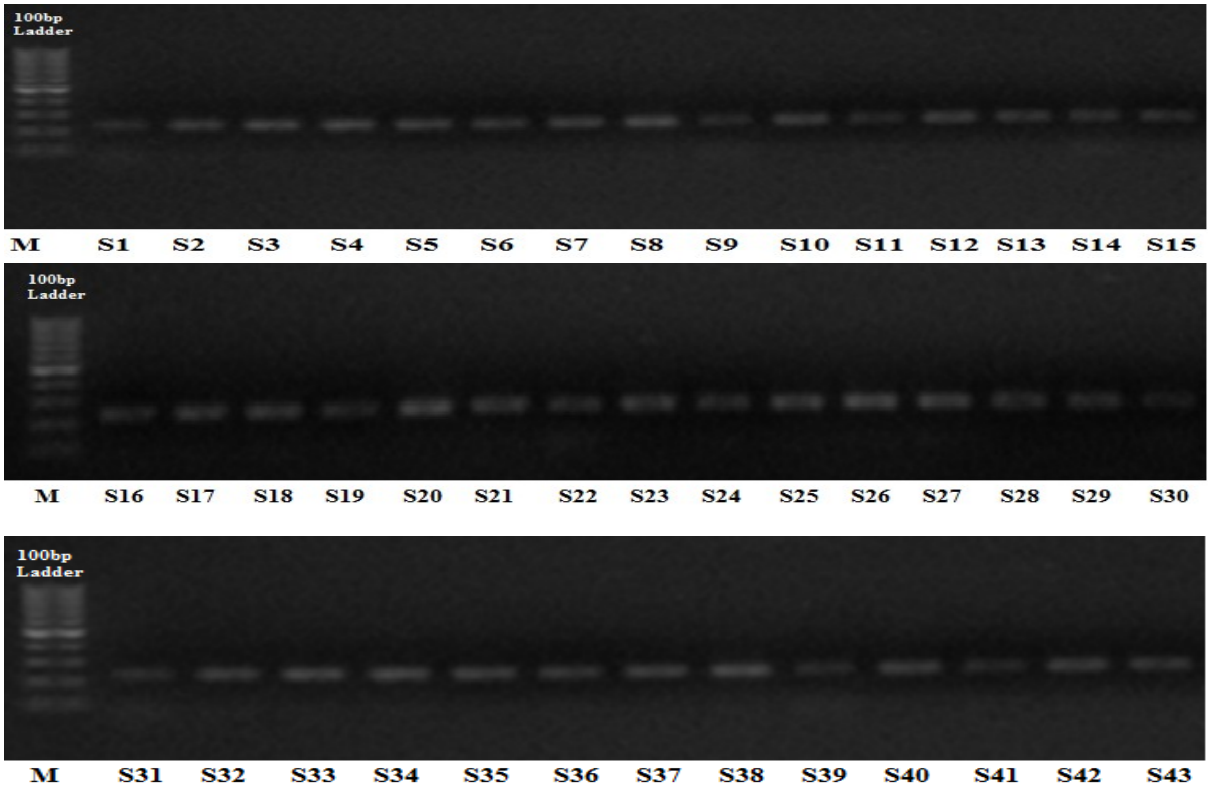
With using classical microbiological methods from seven differens samples were isolation and identified 43 strains (Table1). Based on morphological characteristics , the strains were identified as belonging to the genus Lactobacillus.

Table.1 Isolated strains from different stages of fermentation Lukanka- sausage

Sample A1	Sample A2	Sample A3	Sample A4	Sample A5	Sample A6	Sample A7
Grinding and maturation of the minced meat 72h	Mix with spices and filling	Squeezed	Intermediate sample	After first pressing	After second pressing	Before vacuuming
S6	S4	S31	S17	S1	S16	S2
S8	S19	S18	S24	S14	S27	S3
S9	S22	S34	S35	S20	S36	S5
S10	S29	S37	S38	S25		S15
S11	S33	S42	S41	S32		S21
S12				S40		S26
S13				S43		S28
S23						S30
S38						S39

S- LAB strains isolated from raw dry sausage Lukanka

Based on the criteria, which was made on strains (Morphological and physiological signs) the isolated strains showed us a possible belonging to the genus Lactobacillus. For confirmation of their belonging to the genus, DNA was isolated from them, which was used in the PCR amplification with specific primers for the genus Lactobacillus.



Figures 1 Determination of the affiliation of the newly isolated strains to the Lactobacillus genus by genus-specific PCR primers.

The analysis results are shown in Fig. 1. This shows that in the 43 isolates a product of the expected length of 232 bp was amplified, which indicates their belonging to the genus Lactobacillus.

Definition of family strains (Fig 1.) were subjected to species identification by 16S rRNA sequencing analysis. The total isolated DNA, whose nativeity and purity was checked beforehand was used as a template PCR amplification of the charter on the 16S rRNA gene with a pair of universal primers.

The resulting amplification products with a size of 1500 bp were purified and subjected to sequence analysis. The subsequent comparative analysis of the obtained sequences with the data available at the National Center for Biotechnology Information (NCBI) blast library enables species determination of the strains. The results are summarized and presented in Fig. 2.

№	Strains	Identification	Max score	№	Strains	Identification	Max score	№	Strains	Identification	Max score
1	S1	*Lactobacillus plantarum*	2481	16	S16	*Lactobacillus sakei*	2475	31	S31	*Lactobacillus plantarum*	2480
2	S2	*Lactobacillus plantarum*	2471	17	S17	*Lactobacillus plantarum*	2478	32	S32	*Lactobacillus plantarum*	2476
3	S3	*Lactobacillus plantarum*	2478	18	S18	*Lactobacillus plantarum*	2470	33	S33	*Lactobacillus plantarum*	2479
4	S4	*Lactobacillus plantarum*	2480	19	S19	*Lactobacillus plantarum*	2474	34	S34	*Lactobacillus plantarum*	2478
5	S5	*Lactobacillus plantarum*	2477	20	S20	*Lactobacillus plantarum*	2478	35	S35	*Lactobacillus plantarum*	2481
6	S6	*Lactobacillus plantarum*	2475	21	S21	*Lactobacillus plantarum*	2480	36	S36	*Lactobacillus brevis*	2473
7	S7	*Lactobacillus plantarum*	2474	22	S22	*Lactobacillus plantarum*	2479	37	S37	*Lactobacillus plantarum*	2475
8	S8	*Lactobacillus brevis*	2477	23	S23	*Lactobacillus plantarum*	2481	38	S38	*Lactobacillus brevis*	2478
9	S9	*Lactobacillus plantarum*	2470	24	S24	*Lactobacillus plantarum*	2475	39	S39	*Lactobacillus plantarum*	2474
10	S10	*Lactobacillus plantarum*	2473	25	S25	*Lactobacillus plantarum*	2477	40	S40	*Lactobacillus plantarum*	2456
11	S11	*Lactobacillus plantarum*	2479	26	S26	*Lactobacillus plantarum*	2479	41	S41	*Lactobacillus plantarum*	2472
12	S12	*Lactobacillus sakei*	2475	27	S27	*Lactobacillus brevis*	2474	42	S42	*Lactobacillus plantarum*	2476
13	S13	*Lactobacillus sakei*	2476	28	S28	*Lactobacillus brevis*	2471	43	S43	*Lactobacillus plantarum*	2478
14	S14	*Lactobacillus sakei*	2481	29	S29	*Lactobacillus plantarum*	2476				
15	S15	*Lactobacillus sakei*	2472	30	S30	*Lactobacillus plantarum*	2477				

Fig.2. Results of 16S rRNA sequence analysis of strains of the genus Lactobacillus, isolated from sausage

Thirty-three of the strains were identified as *L. plantarum*, five as *L. brevis* and five - as L. sakei.

Through the use of molecular methods it has been found that the lactic acid bacteria that are identified in the traditional fermented dry sausages are *L. sakei*, *L. curvatus*, and *L. plantarum* (Leroy et al., 2007). *L. sakei* often dominates and is represented in more than 42% of the isolates (Comi et al., 2005; Coppola et al., 2000; Greco et al., 2005; et al 2003 Papamanoli; Urso et al., 2006).

In the research Aymerich, (2006) was shown that *L. sakei* is found in all Spanish sausages and represents 89% in"chorizo" and 76% in the traditional product "fuet".

In the French dry sausages the type represents 100% of the isolates of the final product, except in minor amounts in the raw material (Ammor et al., 2005). L. curvatus is the second type identified. It dominates in some Greek and Italian dry sausages (Comi et al., 2005, Rantisiou et al., 2005). *L. plantarum* is the third type, which dominates the lactic flora in Greek sausage (Drosinos et al., 2005).

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