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CHARACTERIZATION OF LACTIC ACID BACTERIA FROM DRY SAUSAGES

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ABSTRACT

Eight genera (Lactobacillus, Leuconostoc, Staphylococcus, Enterococcus, Lactococcus, Micrococcus, Streptococcus) are most commonly used meat starter cultures. The Lactobacilli associated with meat fermentation and the efforts to accurate classification and identification of them are becoming more important as various isolates of LAB become more commonly used as starter cultures.

The present work deals with characterization of the microflora associated with naturally fermented Bulgarian sausages (Lukanka) and sausages (Lukanka) with defined starter culture produced by "Tandem". More then 200 strains of genera Lactobacillus were isolated during the different stages of the fermentation process of the naturally fermented sausages. The morphological, cultural and physiological and biochemical analysis were performed. In such a system such as meat with a wide variety of complex substrates serving as sources of peptoses, organic acid and other fermentable compounds different end products are formed. Research on the production of lactic acid, determination of pH, screening for producers of antimicrobials was performed.

Keyword: lactobacilli, dry sausages, bacteriocins

Introduction

Dry and semi dry sausages represent the largest category of fermented meat products. Traditionally, dry sausages acquired their particular sensory characteristics from exposure to salt, indigenous gram positive microorganisms such as LAB, staphylococci, micrococci. Lactic acid bacteria (LAB) have been used in fermentations to preserve the nutritive qualities of various foods. The lactobacilli associated with meat fermentations are members of the genus Lactobacillus. most common lactobacilli found in dry sausages .However, attempts to identify lactobacilli isolated from meat are usually less than successful because most of documented descriptions and schemes of identification are based on isolates from other food sources .A series of investigation on atypical lactic acid streptococci isolated from fermented meats resulted in their identification as *Lb.sakei* and *Lb.curvatus* (1). These species outnumbered the typical lactobacilli, identified as *Lactobacillus delbrueckii* subsp.*lactis*, *Lb.brevis*, *Lb.farciminis*, *Lb.buchneri*, *Lb.plantarum*, *Lb.curvatus*, *Lb.alimentarius* ,*Weissella virdescens*, unspecified leuconostocs, and pediococci 1,000-fold and were typically psychrotrophylic and less acid tolerant.(1,2) Efforts to accurate classification and identification of them are becoming more important as various isolates of LAB become more commonly used as starter cultures.

Bacteriocins, produced by LAB have attracted a great interest in food industry due to their applicational potentiality in food preservation. It was documented that such species as *Lb.sake*, *Lb.curvatus*, *Lactobacillus plantarum* produce bacteriocins which may found broader application in starter cultures for meat fermentation in the future.

The present work deals with characterization of the microflora associated with naturally fermented bulgarian sausages (Lukanka) and sausages (Lukanka) with defined starter culture produced by "Tandem". More then 200 strains were isolated during the different stages of the fermentation process of the naturally fermented sausages. In this paper, we

report on the screening of antimicrobial compounds of 7 lactic acid bacteria isolated from a small scale facility producing traditional dry sausages and as well their taxonomic determination.

Materials and methods

The 7 LAB strains considered of in this study were isolated from a small-scale facility producing dry sausage "lukanka". The strains with numbers SM3, SM6, SM7, SM9, SM11, SM12 and SM28 were used. All the used strains were identified to belong to genera *Lactobacillus*. Initial identification of all the strains was performed by API 50CHL system (BioMerieux, France), according to the manufacturer's instructions.

The isolates were subcultured twice (10% inoculum, 24 hours, 30 ˚C) in 15 ml MRS broth and kept frozen at –20 ˚C in MRS supplemented with 10% glycerol. The isolates were tested against *L.innocua* F (ENITIAA, Ecole Nationale des Ingénieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France) and *E.coli* NBIMCC 3398. An agar diffusion assay was used for the detection of the antagonistic activity (7).

Total DNA from strains SM3, SM6, SM7, SM9, SM11 and SM12, was isolated with *GeneElute™ Bacterial Genomic DNA Kit* (*Sigma-Aldrich*) according to the manufacturer.

GS-PCR was performed according to (3,4) using two Lactobacillus–specific primers sets **(Table 1)**.

TABLE 1

Genus-specific PCR with Lab0677F/Lact71R primers set (Pane A) and LactoF/LactoR primers set (Pane B). 100 bp ladder was used as molecular weight marker

Lab0677F Lact71R	5-'CTCCATGTGTAGCGGTG-3' 5'-TCAAAACTAAACAAAGTTTC-3	Moura et al.
LactoF LactoR	5'-TGGAAACAGRTGCTAATACCG-3' 5'-GTCCATTGTGGAAGATTCCC-3'	Byun et al.

RAPD PCR was performed according to (5) and (6) with oligonucleotide primers L9 and L10 respectively **(Table 2)**.

The results were analyzed with GeneTools v. 4.00 software (Syngene). The amplification was analyzed on 1,2 % agarose gel with TAE buffer system in Hoeffer HE 33 electrophoresis system (GE Healthcare). The gels were visualized with ethidium bromide and documented with DigiGenius system (Syngene)

TABLE 2

RAPD oligonucleotide primers L9 and L10

L9	GCAGCCGG	Sohier et al.
L10	AGTCAGCCAC	Tynkkynen et al.

Characterization of the antimicrobial compounds. Isolates exhibiting antagonistic activity against pathogenic microorganisms were investigated for their antimicrobial compounds. These isolates were grown overnight at 30 ˚C in 10 ml MRS broth. A cell free solution was obtained by centrifugation (15min at 4 ˚C). The samples were adjusted to pH 6.5 to rule out acid inhibition. Sensitivity to heat of the antibacterial compounds was investigated by treatment the culture supernatant in water bath at 80 ˚C for 10 min and for 1 hour. The residual activity was determined by agar diffusion assay. Sensitivity to proteolytic enzymes of the antibacterial compounds was investigated by addition of trypsin and proteinase K at final concentration of 1mg to the culture supernatants. The samples were incubated for 3 hours and the residual activity determined by agar diffusion method.

Results and discussion

Lactic acid bacteria originally isolated from traditional sausages are probably the best candidates for improving the microbiological safety of these foods, because they are well adapted to the conditions in the sausages and should therefore be more competitive that LAB from other sources.

LAB strains were screened for exhibition of antagonistic activities against indicator microorganisms as *E.coli* and *L.innocua*. Results were presented on fig.1a and 1b and table 3. Therefore, characterizing the antibacterial compounds responsible for the inhibition of these pathogens was of interest.

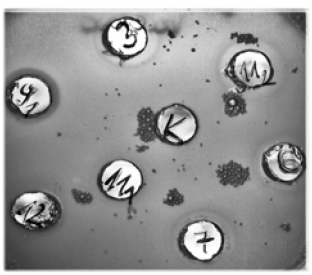


Fig. 1a Antibacterial activity against *E.coli*

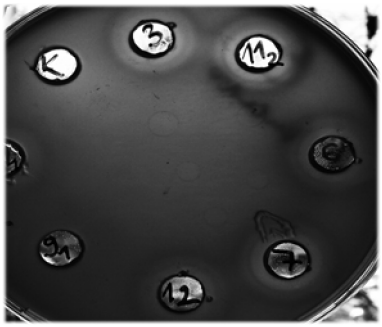


Fig. 1b Antibacterial activity against *L.innocua*

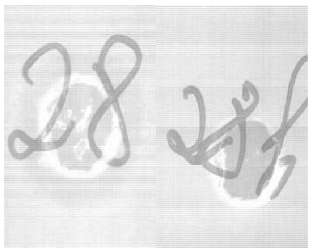


Fig. 2a Antibacterial activity against *L.innocua* F

Fig. 2b Antibacterial activity against *E.coli*

TABLE 3

Effect of enzymes and heat treatment on inhibitory activity of Lactobacillus strains, cell-free supernatants

Strain	Activity against *L.innocua* (mm sterile zone)			Activity against *E.coli* (mm sterile zone)		
	control	Temperature	Trypsin /proteinase K	control	Temperature	Trypsin /proteinase K
SM3	9	-	-	17	-	-
SM6	12	12	10	18	18	17
SM7	12	-	-	18	-	-
SM9	12	12	12	18	17	16
SM11	12	-	-	18	-	-
SM12	12	11	11	18	17	16
SM28	12	-	-	20	-	-

Regarding the proteinaceous nature, the thermostability at 80˚C for 10 minutes of the antibacterial compounds of strains SM3, SM7, SM11 and A28 and their residual activities, it was concluded that these antimicrobials were bacteriocin like. The most promising strain from the examined ones seemed to be strain SM28. Its antimicrobial activity against *E.coli* is higher in comparison with the other strains.

These antibacterial compounds may constitute interesting weapons to fight against biofilm growth and implementation of undesirable microorganisms on the processing surfaces. The next task of study was the affiliation of the strains.

The genus affiliation of the strains investigated was determined by the Lactobacilllus-specific primers sets (3,4) **Fig. 3a and 3b.**

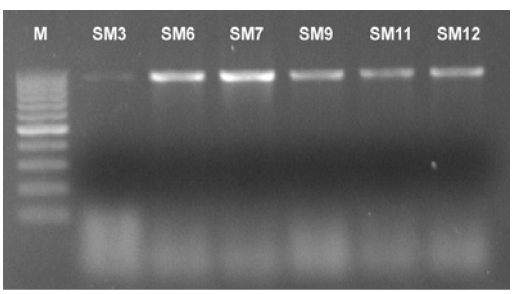


Fig. 3a Genus-specific PCR with Lab0677F/Lact71R primers set. 100 bp ladder was used as molecular weight marker

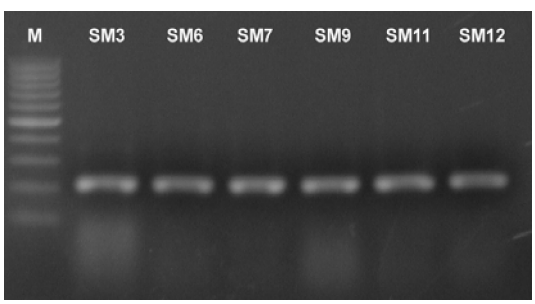


Fig. 3b Genus-specific PCR with LactoF/LactoR primers set. 100 bp ladder was used as molecular weight marker

The electrophoretic patterns obtained with the RAPD-PCR were subjected to UPGMA analysis with GeneTools v. 4.00 software, and dendrograms reflecting the phylogenetic relatedness of the investigated strains were constructed.

In both cases, with L9 and L10 oligomer primers, clustering in two concordant groups was obtained, the first group comprising strains SM3, SM7, SM9 and SM11 and the second one strains SM6 and SM12 **(Fig.4a and 4b)**.

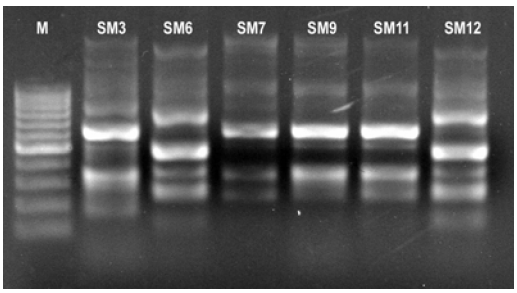
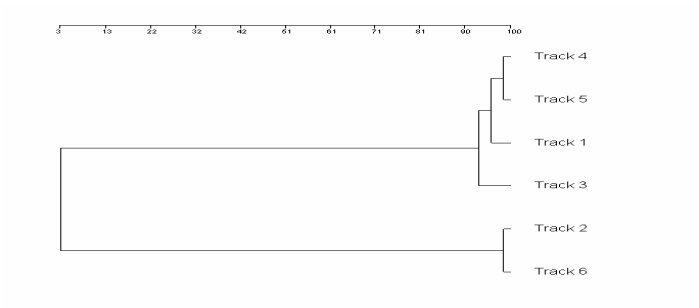


Fig. 4a RAPD analysis with L9 primer



UPGMA analysis of the obtained RAPD band patterns with L9 primer

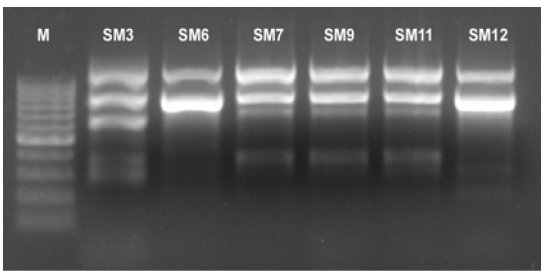
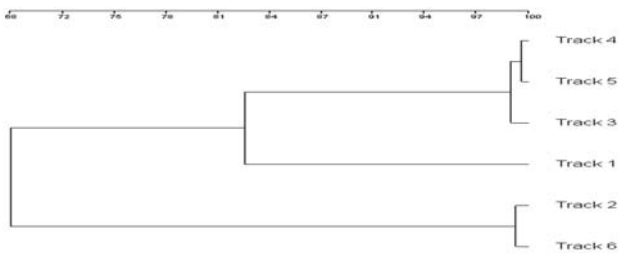


Fig. 4 b RAPD analysis with L10 primer



UPGMA analysis of the obtained RAPD band patterns with L10 primer.

The results from API test indicate that strain SM7 belong to species *Lactobacillus pentosus* 99 % and strain SM9 belong to species *Lactobacillus brevis* 96.7 % respectively

It is the first report on bacteriocin-like activities of strains that occur within the microbial ecosystem of dry bulgarian sausage “lukanka” produced from a small scale facility and their preliminary taxonomic determination.

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