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Identification of lactic acid bacteria isolated from dry fermented sausages

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ABSTRACT

The lactic acid bacteria (LAB) have a very important role in the formation of the specific organoleptic characteristics of dry sausages, as well as the prevention of growth of pathogenic microorganisms in sausages during the fermentation process. The aim of this study was to identify the strains of LAB involved in the fermentation of Croatian dry fermented sausages produced under industrial conditions without starter cultures. Among lactobacilli, the predominant strain was *L. plantarum* followed by *L. brevis.*

Key words: Croatian dry fermented sausage, lactobacilli, PCR

Introduction

Fermentation is a method of preservation and prolongation of the shelf life of meat. During fermentation, ripening and drying of fermented sausages, many complex microbial, biochemical and physicochemical processes take place and influence the quality and safety of the final products. Different microorganisms, derived from raw materials and the environment, naturally contaminate dry sausage mixtures. Among them, lactic acid bacteria (LAB) are found to be the most active microorganisms in fermented sausages (SAWITZKI et al., 2007). They ferment the sugars to acid and thus lower the pH, improving the texture of the products, providing prolonged stability against the proliferation of food pathogens and producing some aromatic compounds. The most common isolated LAB

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in fermented sausages are lactobacilli such as *L. sakei*, *L. plantarum* and *L. curvatus* (PARENTE et al., 2001; COCOLIN et al., 2004; AMMOR et al., 2005; DROSINOS et al., 2005; COCOLIN et al., 2009). From a hygienic and technological point of view, it is important to determine their influence on the natural microbial flora, and physicochemical and sensorial changes during the ripening of the sausages. Little information is available on the isolation and characterisation of LAB from Croatian dry fermented sausages

The aim of the study was to identify the indigenous strains of LAB employed in the fermentation of Croatian dry fermented sausages produced under industrial conditions without starter cultures.

Materials and methods

Sausage production. The sausages were manufactured in the meat industry according to the standard practice applied there without commercial starter cultures. Sausages were produced using lean pork (60%), beef (10%), pork back fat (24%), salt with 0.5% $NaNO_2$, sugar and ground black pepper, and minced red pepper and garlic. The fat used was kept at -15 °C. The meat used (lean pork and beef) was frozen and pre-tempered at -2 °C. All ingredients were mixed in a cutter. Initially, the meat was ground at low speed until coarse pieces of about 12 mm in diameter were produced. Then, all other ingredients were added sequentially. The cutting was continued until the fat particles were about 2 mm in diameter. The mixture was filled into 32-34 mm natural casings under vacuum. The temperature of the mixture during filling was 0 to -2 °C. After vacuum stuffing, sausages were allowed to equilibrate at room temperature (12 h at 20 °C and 95% RH), then cold smoked for 48 h at the same temperature and 85-90% RH. Then, sausages were ripening in a fermentation chamber until the 28th day. During this period, the temperature and RH were gradually reduced from 20 °C and 90 % to 16-18 °C and 75 %, respectively, after which they were ready for consumption. Samples were taken at the end of ripening to determine the species present in the sausages.

Microbiological analysis. Strains of lactobacilli were isolated from sausages produced under industrial conditions without the addition of starter cultures. The lactic acid bacteria count was determined by microbiological analysis (MRS agar; *Lactobacillus* agar according to De Man, Rogosa and Sharpe; manufacturer Merck, Germany). The isolates confirmed as gram-positive and catalase-negative bacilli were biochemically identified using the API 50 CHL (BioMerieux) test and the computer program APILAB Plus. All the isolates with fermentative profile confirmed by the API test as excellent (ID>99%; 50 isolates) were considered as lactobacilli and were subjected to further analyses. The reference strain was *L. brevis*, American Type Culture Collection, ATCC B287 (Oxoid).

DNA extraction for RAPD PCR from pure cultures was performed by SAMBROCK et al. (1989.) After the DNA extraction of the bacterial cells, the DNA concentration of each sample was determined by spectrofotometry using Helios Beta (England).

RAPD PCR protocol. All the bacterial strains confirmed as lactobacilli by the API 50CHL test were further identified by RAPD procedure with the primer M13 (5' - 3'; GAGGGTGGCGGTTCT) (ANDRIGHETTO et al., 2001). Amplification reactions were performed in a 25 μL volume containing 10 mM buffer (pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 1 uM primer M13, 2 U Taq polymerase and 80-100 ng DNA. After an initial step of 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 20 s, ramp to 72 °C at 0.6 °C s^{-1} and elongation at 72 °C for 2 min were carried out. A final extension was performed for 5 min. at 75 ºC. RAPD-PCR products were analysed by electrophoresis on 1.5 % (w/vol) agarose gel. LAB isolates were subjected to RAPD-PCR analysis at least twice. According to the specific imprint in the gel after RAPD-PCR, the sequences were grouped and there were two dominant groups among them. Samples of each specific group were subjected to a further PCR with species-specific primers.

Specific PCR protocol. Species-specific PCR was carried out with lactobacilli colonies taken directly from the agar, according to MASSI et al. (2004). The primers R16-1 (5'-CTCAAAACTAAACAAAGTTTC-3') and LbLMA1-rev (5'-CTTGTACACACCGCCCGTCA-3') were used in the tests. Amplification reactions were performed in a 25 μL volume containing 10 mM buffer (pH 8.3), 2 mM $MgCl_2$, 200 mM dNTPs, 0.5 uM each primers, 1 U Taq polymerase and 1 colony of lactobacillus strain. After a 5 min initial denaturation at 95 °C, 39 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 30s and elongation at 72 °C for 2 min were carried out. A final extension was performed for 5 min. at 75 ºC. Reference strain, *L. brevis* ATCC B287 was used to validate specific primers. The PCR products obtained were approximately 250 bp.

Sequence analysis. DNA sequences obtained after the specific PCR were purified using a Macherey-Nagel purification set. The DNA sequences obtained in this way were sent to MWG Biotech, Germany for analysis of the 16S rDNA nucleotide sequence. After the analysis, the nucleotide sequences were compared with the known data stored in the NCBI gene databank (National Centre for Biotechnology Information; www.ncbi.nlm.nih.gov) by standard methods of comparison. A definite confirmation of the strain was obtained on the basis of these results.

Results

After 28 days of fermentation, 57 bacteria strains were isolated from dry sausages on MRS agar. According to their morphological features (thin, short, flat or slightly twisted or coccoid respectively) a total of 50 isolates, belonging to the genus *Lactobacillus*, were

further identified by the API 50 CHL system. The most frequent isolates of LAB in the fermentation process of dry fermented sausages in our samples were *L. brevis* (1) (15 isolates, 30 %), *L. plantarum* (1) (14 isolates, 28 %) and *L. plantarum* (2) (8 isolates, 16 %). In addition, sporadic isolates of *L. pentosus, L. mesenteroides* subsp. *mesenteroides*, *L. lactis* subsp. *lactis, L. curvatus* were identified.

According to specific imprint in gel after RAPD-PCR, the DNA sequences obtained were grouped into two profile clusters. To eliminate any doubt related to mistakes in the identification of strains by RAPD-PCR, strains from every group were subjected to PCR with the primer pair LbLMA1-rev/R16 and sent for sequencing to MWG Biotech, Germany, for final confirmation of strains. The sequences of nucleotides, after specific-specific PCR, were compared with the nucleotide sequences available in the NCBI gene databank. *L. plantarum* was confirmed as the strain with the highest level of agreement (cluster 1; access number U97139; 99% agreement in nucleotide sequence), *L. brevis* respectively in case of cluster 2 (access number AJ937772; 95% agreement in nucleotide sequence).

Discussion

One of the techniques based on PCR technology, developed in research, is the RAPD (Random Amplified Polymorphic DNA, RAPD) procedure. It is based on the fact that as many as 70% of the 16S RNA gene sequence of all prokaryotes are "preserved", i.e. identical, whilst other gene parts are highly genus- or species-specific. Analysis of the 16S and 23S rRNA sequence is one of the commonly used methods of identification and differentiation of lactobacilli (VERSALOVIC et al., 1994; CHAGNAUD et al., 2001; ESCALANTE et al., 2001). The construction of a universal primer is thus possible (ZOETENDAL et al., 1998; RANDAZZO et al., 2002; MOZZI et al., 2010). RAPD-PCR is a well-established method for strain characterisation (ANDRIGHETTO, 2001; BOUTON, 2002) and it has proved itself to be an acceptable method, very suitable as a preliminary reaction in cases of a large number of samples (TORRIANI et al., 1999). For strain identification and determination, the product of RAPD-PCR should be subjected to polymerase chain reaction with primers specific for the strain to be confirmed. The nucleotide sequence should be determined in products obtained after specific PCR, and the comparison of the obtained sequence with a known sequence from the gene databank either confirms or refutes the hypothesis (AYMERICH et al., 2003). Lactobacillus species are the dominate microflora in fermented sausages (ERKKILA et al., 2001). The composition of autochthonous dry sausages differs from region to region, and there are also differences in the technological production process. This might have led to the dominance of *L. plantarum* over *L. curvatus* and *L. sakei* in the dry sausages investigated in the Mediterranean region, as confirmed by the results of our studies (PAPAMANOLI et al., 2003; AMMOR et al., 2005; KOZAČINSKI et al., 2008). The most frequent isolates of LAB in the fermentation process of dry sausages

from European countries are *L. sakei*, *L. curvatus* and *L. plantarum* (GURAKAN et al., 1995; SANTOS et al., 1998; SAMELIS et al., 1998; ERKKILA et al., 2001; AYMERICH et al., 2003; PAPAMANOLI et al., 2003; DROSINOS et al., 2005; GASPARIK-REICHARDT et al., 2005; RANTSIOU et al., 2006; DROSINOS et al., 2007; KOZAČINSKI et al., 2008; MOZZI et al., 2010). The majority of the strains isolated from the Croatian dry fermented sausages were assigned to the species *L. plantarum* and *L. brevis*. It is known that in fermented meat products, the dominant LAB species are lactobacilli belonging to the *L sakei* and *L curvatus* species. Other lactobacilli such as: *L. plantarum*, *L. casei*, *L. brevis* or *L. alimentarius* can be isolated as well (RANTSIOU and COCOLIN, 2008). The results of our investigation are very close to the data of the DURAKOVIĆ et al. (2002), who also pointed out that lactic acid bacteria are the significant component of the microflora in the pork, where *L. plantarum, L. brevis* and *L. viridenscens* are dominant, and *Leuconostoc* spp. and *Pediococcus* spp can also be isolated from the sausages. They also noticed that the presence of these probiotic or protective strains as major organisms in the sausages after fermentation and ripening is connected with flavour profiles, similar to that produced by the commercial meat starter culture and commercial dry sausage recipe.

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SAŽETAK

Bakterije mliječne kiseline značajne su u smislu tvorbe specifičnih organoleptičkih svojstava trajnih kobasica, a posebno sprječavanja rasta i razmnožavanja patogenih mikroorganizama u nadjevu tijekom zrenja. Cilj ovoga rada bio je identificirati sojeve laktobacila koji sudjeluju u fermentaciji autohtonih trajnih kobasica proizvedenih u industrijskim uvjetima bez dodatka starter kultura. Kao najčešći izolati laktobacila u procesu zrenja trajnih kobasica utvrđeni su *L. plantarum* i *L. brevis*.

Ključne riječi: hrvatske trajne kobasice, *Lactobacillus*, lančana reakcija polimerazom