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ScienceDirect

Procedia Food Science 5 (2015) 14 – 17

Procedia Food Science

International 58th Meat Industry Conference "Meat Safety and Quality: Where it goes?"

Molecular characterization of lactic acid bacteria in *levačka* sausage

Branka Borovic[a,]*, Branko Velebit[a], Slavica Veskovic Moracanin[a], Brankica Lakicevic[a], Tatjana Baltic[a]

[a]*Institute of Meat Hygiene and Technology, Kacanskog 13, 11040 Belgrade, Serbia*

Abstract

Levačka sausage is traditionally fermented dry sausage which is produced in central Serbia. It is made of beef and pork with the addition of solid fat and natural spices. The whole manufacturing process lasted for 21 days. The goal of this study was to create a collection of lactic acid bacteria isolated during the fermentation and identify them using molecular methods. A total of 50 isolates from different stages of fermentation were identified by molecular methods. *Pediococcus pentosaceus*, *Ln. mesenteroides* were predominant microorganisms in *levačka* sausage. *Lb. curvatus*, *Lb. sakei* and *Lb. carnosum* were also identified.

Peer-review under responsibility of scientific committee of The 58th International Meat Industry Conference (MeatCon2015)

Keywords: fermented dry sausages; LAB; molecular methods

1. Introduction

For the last few decades, increasing attention has been paid to research related to the study of epiphytic microbiota in traditionally produced dry sausages, with particular emphasis on lactic acid bacteria (LAB). Traditional fermented sausages with a specific geographical origin have unique sensory characteristics and are generally of high quality[1].

Quality of traditionally fermented sausage is influenced by many factors such as selection of raw material, metabolic activity of epiphytic flora and physico-chemical properties developed during ripening, smoking and

* Corresponding author. Tel.: +381-11-2650-655; fax: +381-11-2651-825.
E-mail address: branka@inmesbgd.com

Peer-review under responsibility of scientific committee of The 58th International Meat Industry Conference (MeatCon2015)
doi:10.1016/j.profoo.2015.09.004

drying of meat[2]. The microorganisms that are most often responsible for these transformations are LAB, coagulase negative cocci (CNC) and yeasts[3]. Lactic acid bacteria are essential for dry sausage production. Their ability to lower the pH of the mixture by producing acid from sugars leads to the development of the desirable organoleptic properties, prevents the growth of pathogens and ensures the stability and safety of the final product[4,5]. Even if their initial levels varied their final levels were close to the one of industrial products manufactured with starter cultures[6].

LAB isolated from traditional dry sausages belong to different genera, such as *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Weissella*, *Pediococcoccus*, *Lactococcus*[7,8,9,10,11,12]. In traditionally fermented European sausages, facultative homofermentative lactobacilli constitute the predominant flora through ripening. *Lb. sakei* or/and *Lb. curvatus* generally dominate the fermentation process[13,14,15]. Other lactobacilli that may be found, albeit generally at minor levels, include *Lb. plantarum*, *Lb. bavaricus* (now reclassified as *Lb. sakei* or *Lb. curvatus*), *Lb. brevis*, *Lb. buchneri* and *Lb. paracasei*[13,14]. In order to protect the traditional approach to sausage manufacturing, it is essential to understand the microbial diversity and to select autochthonous starter cultures that can be used in the production of innovative foods with a geographical origin[16]. In Serbia, one of the traditional fermented dry sausages is *levačka* which is produced in the central Serbian region, Šumadija. Due to scarce literature data regarding microbiological profiles of this sausage, the goal of this paper was to create a collection of LAB isolated during the fermentation and complete their molecular identification.

2. Materials and methods

2.1. Levačka sausage

Levačka sausage was composed of pork, beef and fat in a percentage ratio 47%:20%:33%, respectively. Meat and fat were ground to the size of 3 mm and mixed with nitrite curing salt (2.5%), sucrose (0.33%) and spice mixture (0.25%; composed of sweet and hot red peppers, black pepper and garlic). Prepared stuffing was filled into pig small intestine (diameter of 34-36 mm). Sausages were cold smoked for 4 days. The whole manufacturing process (smoking, fermentation/ ripening and drying), in the traditional way, lasted for 21 days.

2.2. Microbiological investigation

Sausage samples for microbiology examinations were taken at 0, 2nd, 4th, 7th, 14th and 21st day. The experiment was repeated three times. Three samples at each step of sampling were collected and used for analysis.

Each sample weighing 25 g was homogenized in 225 ml of MRD (Oxoid, UK) in a stomacher (AES, France) for 90 s. Serial dilutions (10-fold) were plated onto MRS agar (Oxoid, UK) in duplicate, and incubated for 48 h at 30°C under microaerophilic conditions. From each plate, single colonies were randomly picked and streaked on new agar plates in order to obtain pure cultures. The LAB isolates from MRS agar were checked by Gram straining and catalase reaction. A total of 50 Gram-positive and catalase-negative isolates were further identified and characterized by molecular methods.

Total DNA from LAB was extracted from a single colony by using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria.

PCR was performed in a final volume of 50 µL containing 1× PCR buffer (10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM $MgCl_2$, 10 µM dNTP, 200 nM of each primer, 1 U of Taq polymerase (Fermentas, Lithuania) and 100 ng of DNA template.

The samples were amplified in a thermal cycler (Techne, UK) using primers P1V1 (GCGGCGTGCCTAATACATGC) and P4V3 (ATCTACGCATTTCACCGCTAC), complementary to the V1-V3 region of the 16S rRNA, 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 42°C, 2 min at 72°C and the final extension of 5 min at 72°C. PCR products were purified by QIAquick PCR purification kit (Qiagen, Germany) and sent for sequencing to IIT Biotech (Bielefeld, Germany). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://blast.ncbi.nim.nih.gov).