Research article

DETECTION OF *LISTERIA* SPP. DURING PRODUCTION AND RIPENING OF *PETROVSKÁ KLOBÁSA*

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The aim of this study was to determine the survival of Listeria spp. during the preparation and ripening of an artisan Serbian sausage (Petrovská klobása) and to characterize the isolates by amplification of the hlyA gene. Results obtained by standard microbiological and molecular methods showed that during fermentation, drying, ripening and storage of Petrovská klobása, 17 out of 99 samples were positive for the presence of Listeria spp. Among them we detected a hemolytic, atypical, non pathogenic *L. innocua* strain FSL J1-023, whose genome sequence is important in understanding the role of horizontal gene transfer and recombination in the evolution of pathogenicity of Listeria. The most important product characteristics influencing the survival and growth of Listeria spp. during production and ripening of Petrovská klobása were: pH, water activity, temperature and starter culture.

Key words*:* hlyA*, Listeria* spp.*, L. monocytogenes,* PCR*,* Petrovská klobása*,* survival

INTRODUCTION

Listeria monocytogenes is a psychrotrophic, gram-positive, non-spore-forming, facultative anaerobic bacteria, that has become an important cause of human foodborne infections worldwide [1]. It is associated with meningoencephalitis, septicaemia and abortion in humans, especially in individuals at risk including pregnant women, newborn babies, the elderly and the immunocompromised [2,3]. Foodborne listeriosis, caused by the pathogen *Listeria monocytogenes*, is a relatively rare but serious disease with high fatality rates (20–30%) compared with other foodborne pathogens, such as *Salmonella* [4]. All *Listeria* species have been isolated from soil, decaying vegetable matter, silage, sewage, water, and animal feed [5].

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L. monocytogenes is recognized as a problem for the food industry mainly due to its environmental persistence, attributed in part to its ability to form biofilms. Various studies have indicated that certain strains of *L. monocytogenes* survive well within the food processing environment [6-8] and the persistence of such strains is of concern as they have the potential to act as a continuous source of contamination [8]. *L. monocytogenes* has the ability to adhere to and grow on a variety of surfaces found in food processing plants including stainless steel, rubber, glass and polypropylene [9]. The best environment for listeria growth is thermally untreated food, food that spent a long time during storage, food that has been produced in non-hygienic food plants as well as cooked-cooled meals "ready to eat" (RTE) [10].

The identification of *L. monocytogenes* based on standard microbiological methods is laborious, time consuming [11] and in food products requires up to a week for species identification [12]. Therefore, faster techniques have been applied, without the need for isolation of pure cultures.

The present study was conducted in order to:(a) detect *Listeria* spp. strains during the preparation and ripening of an artisan Serbian sausage (*Petrovská klobása*), in the ingredients used to make sausages and in the environment surface samples, (b) characterize the isolates by amplification of the *hlyA* gene and (c) analyze the influence of the physico-chemical properties on survival of *Listeria* spp.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The standard strains of *Listeria monocytogenes* 4b ATCC 19115 and *Listeria innocua* ATCC 33090 were obtained from the American Type Culture Collection (ATCC; Manassas, Va., USA). Standard strains were grown on brain-heart agar (BHA) (Merck, GmbH Darmstadt, Germany) and buffered peptone water (Merck) at 37°C.

Samples

Raw materials used for *Petrovská klobása* production (meat, spices and meat batter), environmental surfaces samples and pig carcass swabs were examined for the presence of *Listeria* spp.

The investigation covered a seven-month period of *Petrovská klobása* production, starting in February 2011 with slaughtering and sausage preparation, finishing with the end of the storage period in September 2011.

Preparation of Petrovská klobása

Petrovská klobása, traditional dry-cured sausage, is produced in rural households, in an area nearby the town of Bački Petrovac in the Autonomous Province of Vojvodina (Northern Serbia), according to the experience and traditional technology.

Petrovská klobása, were manufactured from a mixture of lean minced pork (80%) and pig fat (20%) obtained from carcasses of large white cross breed animals. After grinding the meat and the fat to a size of about 10 mm (with adjustable plate holder diameter set), raw materials were mixed with seasonings (2.50% red hot paprika powder, 1.80% salt, 0.20% raw garlic paste, 0.20% caraway and 0.15% sucrose) for about 10min. Two different formulations of sausages were prepared: one formulation with commercial meat starter culture (Quick- starter, Lay, Germany), while the other formulation was without the starter culture so the sausages were subjected to spontaneous fermentation. The commercial meat starter culture was added according to the manufacturer's recommendations: 15 g / 100 kg of meat batter. The seasoned batter was immediately stuffed in collagen casings (Cutisin, 55 mm in diameter) and the raw sausages with two different formulations were subjected to industrial controlled drying conditions (E1-sausages made in the household and dried in the industry, E2-sausages made in the household with the addition of commercial meat starter culture) ($t = 3.5 – 18.6°C$) until achieving a moisture content of 35% (60 days) and to uncontrolled traditional conditions (D1-sausages made in the household and dried in the traditional way, D2-sausages made in the household with the addition of commercial meat starter culture). After 60 days of drying and ripening, the sausages marked as E1 and E2, were stored unpacked, packed in chitosan and vacuum packaging. The traditional temperature ranged from -1.00 to 19.7°C and these sausages, cold smoked with specific kinds of wood during 10-15 days (with pauses), reached moisture content <35% after 90 days when they were moved to an industrial facility to complete the ripening process. One part of the sausages was stored unpacked and the other was packed in a vacuum packaging. Both groups of sausages were stored at t = 10°C, RH = 75%, for 7 months. Sampling was carried out before stuffing (at day 0) and on the 2nd, 6th, 9th, 15th, 30th, 60th, 90th, 120th day. Portions of 25 g were used for microbiological analyses.

Environmental surface samples

Environmental swabs taken from machines (mincing and stuffing), working tables, knives and cleavers, workers' hands and coveralls collected according to ISO 18593 [13]. The samples were labeled from 1-20, and kept refrigerated and analyzed within 2 h.

Pig carcass swabs

For the control of the general hygiene of food processing operations the swabs were taken from the following pig carcass sites: back, cheek, ham and stab. Sampling was performed by using a non-destructive method which is described in the international standard [14].

Detection and isolation of Listeria spp.

The standard microbiological method was carried out by two-step enrichment [15]. For primary enrichment, samples were homogenized in half-concentrated Fraser

broth and incubated at 30°C for 24 h. After that, 100 μl was transferred into 10 ml of Fraser broth and incubated for 48 h at 37°C. A loopful of the secondary enrichment broth culture was plated onto polymyxin-acriflavine-lithium chlorideceftazidime-aesculin-mannitol (PALCAM) agar (Merck) and OXFORD (Oxoid, UK) agar plates, and incubated for 48 h at 37°C. For each sample *Listeria* presumptive colonies were tested for catalase and oxidase reactions, Gram staining and motility.

Identification of Listeria isolates

Biochemical identification

Hemolysis was assessed by streaking isolated colonies on 5% sheep blood agar (Base: meat peptone 15 g l-1, liver digest 2.5 g l-1, yeast extract 5 gl-1, sodium chloride 5 g l-1, agar 9 g l-1. To 100 ml base, 5 ml of defibrinated sheep blood was added). After 24 h of incubation at 37°C the plates were examined for the presence of a zone of hemolysis.

Biochemical identification of the *Listeria* isolates was performed using the culture method in combination with API *Listeria* identification system (BioMerieux, France). API test was performed according to the manufacturer's instructions.

PCR identification

The strains identified as *Listeria* spp. were further confirmed by PCR amplification targeting the *hlyA* locus encoding listeriolysin O. Primers used in this study are listed in Table 1. Final species determination was performed by *hlyA* sequencing.

Table 1. Oligonucleotides used in this study for PCR detection of *Listeria monocytogenes*

Primer sequences orientation 5'→3'	PCR product (bp)	Target gene	Reference
LM1: CCTAAGACGCCAATCGAA LM2: AAGCGCTTGCAACTGCTC	702	hlyA	BORDER & al.[31]

Total DNA from isolates was extracted by using the DNeasy Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria. Lysates were kept on ice until PCR was performed. DNA solution (5 μl) was used as a template for PCR amplification (0.1-1 μg).

PCR was performed in a final volume of 50 μL containing 1 x PCR buffer (10 x PCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 2.5 μM of each primer, 1 U of *Taq* polymerase (Fermentas UAB, Lithuania) and 0.1-1 μg of DNA template. The samples were subjected to amplification in a DNA thermal cycler (Flexigene, Techne, UK). Amplification conditions, with primers targeting *hlyA* were as described previously [2].

The *hlyA* amplicons were purified by QIAquick PCR Purification KIT/250 (QIAGEN) and sequenced using the same primers as for PCR, by the IIT Biotech sequencing service in Bielefeld, Germany. The BLAST algorithm (http://www.ncbi.nlm.nih. gov/BLAST) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Physico-chemical analysis

Water activity of the sausages was measured during defined phases of ripening, using Testo 650 measuring instrument with a pressure – tight precision humidity probe (Testo AG, USA). The pH values of sausages were measured using a portable pH meter (Consort C931, Turnhout, Belgium) equipped with an insertion glass combined electrode (Mettler Toledo Greifensee, Switzerland).

RESULTS

Results obtained by standard microbiological and molecular methods showed that during fermentation, drying, ripening and storage of *Petrovská klobása*, 17 out of 99 samples were positive for the presence of *Listeria* spp.*,* including environmental samples (coveralls), pig carcass swabs, red hot paprika powder, 2 meat batters and 12 sausages.

Results obtained in our study indicated that the pig carcass was contaminated with *L. monocytogenes* which was isolated only from ham (1). The swabs from coveralls (9) were also contaminated with *L. monocytogenes* (Table 2).

Interestingly, sequencing of obtained *hlyA* DNA fragments demonstrated that swabs from the ham and coveralls were contaminated with the same strain of *L. monocytogenes* (*L. monocytogenes* strain NRRL B-33446).

As for the spices used in the preparation of the *Petrovská klobása*, red hot paprika powder (5L) was contaminated with nonpathogenic *Listeria* spp., *L. innocua*. Other nonpathogenic *Listeria* spp., *L. grayi* (from meat batters and sausages) and *L. welshimeri* (from sausage) were also isolated during production of *Petrovská klobása* (Table 2).

In order to ensure the safety of traditional fermented sausages, monitoring of *Listeria* spp. during their storage was carried out according to the current legislation. Based on the obtained results it can be concluded that this group of bacteria was present in sausages from 2nd (D11, D21, E11, E21), 6th (D12, D22, E12, E22) 9th (D13, D23, E23) and 15th (D14) days of production (Table 2).

The microbiological identification results show that *L. monocytogenes* isolates were obtained from 5 samples and these isolates were D11, D21, E11, D23 and E23. The isolates were further checked by PCR using *hly A* gene specific primers (LM1 / LM2). Results indicated that all 5 isolates were positive for the presence of *hlyA* genes (Fig. 1).

Sequencing of the obtained *hlyA* PCR fragments from these 5 samples showed a significant homology (100 % and 99 %) with *L. monocytogenes* strain NRRL B-33467 (Table 2), serogroup 1/2a.

Table 2. Detection of *Listeria* spp. during production and ripening of *Petrovská klobása*, traditional dry-fermented sausage from Northern Serbia

Sample code	Type of the sample	Classical method	PCR	Sequencing
1	swab from ham	*L. monocytogenes*	+	*L. m* NRRL B-33446
9	swab from coveralls	*L. monocytogenes*	+	*L. m* NRRL B-33446
3L	batter with starter	*L. grayi*	+	*L. innocua* FSL J1-023
4L	batter without starter	*L. grayi*	+	*L. innocua* FSL J1-023
5L	red hot paprika power	*L. innocua*	–	nd
D11	sausage (2nd day)	*L. monocytogenes*	+	*L. m* NRRL B-33467
D21	sausage (2nd day)	*L. monocytogenes*	+	*L. m* NRRL B-33467
E11	sausage (2nd day)	*L. monocytogenes*	+	*L. m* NRRL B-33467
E21	sausage (2nd day)	*L. grayi*	+	*L. innocua* FSL J1-023
D12	sausage (6th day)	unacceptable profile	+	*L. innocua* FSL J1-023
D22	sausage (6th day)	unacceptable profile	+	*L. innocua* FSL J1-023
E12	sausage (6th day)	unacceptable profile	+	*L. innocua* FSL J1-023
E22	sausage (6th day)	unacceptable profile	+	*L. innocua* FSL J1-023
D13	sausage (9th day)	*L. welshimeri*	–	nd
D23	sausage (9th day)	*L. monocytogenes*	+	*L. m* NRRL B-33467
E23	sausage (9th day)	*L. monocytogenes*	+	*L. m* NRRL B-33467
D14	sausage (15th day)	*L. grayi*	+	*L. innocua* FSL J1-023

nd – not determined; E1 – sausages made in the household and dried in the industry; E2 – sausages made in the household with the addition of commercial meat starter culture; D1 – sausages made in the household and dried in the traditional way; D2 – sausages made in the household with the addition of commercial meat starter culture
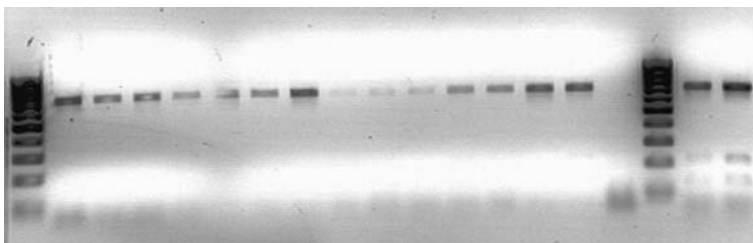
Figure 1. Agarose gel electrophoresis of the PCR products obtained by using LM / LM2 primers: M marker (MassRulerTM DNA Ladder), swab from ham (1), swab from coveralls (9), sausage - 2nd day (D11), sausage - 2nd day (D21), sausage - 2nd day (E11), sausage - 2nd day (E21), sausage -6th day (D12), sausage - 6th day (D22), sausage - 6th day (E12), sausage - 6th day (E22), sausage - 9th day (D23), sausage - 9th day (E23), sausage - 15th day (D14), *Lm - L. monocytogenes* 4b ATCC 19115, Nk - negative control, M marker (MassRulerTM DNA Ladder), batter with starter (3L), batter without starter (4L)

DISCUSSION

As seen in Table 2, the swabs from the pig carcass and coveralls were contaminated with *L. monocytogenes*. These results confirm that both animals and personnel can be a potential source of contamination by bacteria of the genus *Listeria* during the ripening period of dry fermented sausages.

A number of authors mention frequent findings of *Listeria* spp. both on poultry carcasses and in the slaughterhouse itself, particularly at the end of the slaughter line [16]. Miettinen *et al.,* [17] assumed that increased contamination with *L. monocytogenes* occurs after chilling when carcasses are cut up and, mainly, when their skin is removed. Previously, the authors [18,19] reported frequent findings on poultry cuts and especially, on the hands of slaughterhouse staff, slaughterhouse desks and equipment. It follows from the above that *Listeria* spp. can persist in the slaughterhouse environment, and the high prevalence of these bacteria may be a sign of insufficient hygiene practices.

According to our findings, *L. monocytogenes* isolates were obtained from 5 sausage samples and these isolates were labeled as: D11, D21, E11, D23 and E23. Direct sequencing of *hlyA* PCR fragments and nucleotide sequence analysis demonstrated significant homology with *L. monocytogenes* strain NRRL B-33467. Ward *et al.* [20] indicated that NRRL B-33467 is an atypical strain and suggest that the 1/2a serotype has multiple evolutionary origins or that the 1/2a serotype has been acquired by some strains with a 1/2c genomic background via genetic exchange.

As seen in Table 2, *L. innocua (*from red hot paprika powder), *L. grayi* (from meat batters and sausages) and *L. welshimeri* (from sausage), were also isolated and their detection is often used as an indicator of the presence of *L. monocytogenes* [21]. The isolates which were identified using the standard microbiological methods as *L. grayi* (meat batter with and without commercial starter culture and sausages) (3L, 4L, E21, D14) or were with a biochemical unacceptable profile (D12, D22, E12, E22) belong to a hemolytic, atypical, rare, natural, non pathogenic *L. innocua* strain FSL J1-023. To this conclusion we reached after PCR , based on *L. monocytogenes* specific *hlyA* encoding listeriolysin (Figure 1) and after sequencing of the obtained *hlyA* DNA fragment. Literature data indicate this aberrant strain is exceptional in that it contains the pathogenicity island [22] and a homologue of a surface protein, internalin *inl*A [23]. *L. innocua* strain FSL J1-023 is hemolytic-positive, rhamnose and xylose fermentation-negative *Listeria* strain which gives contradictory results in standard confirmatory tests [22]. The genomic sequence of this strain is of great importance because it will help us to understand the overall importance of homologue recombinations in the evolution of *L. innocua* and *L. monocytogenes*. For this reason, application of molecular methods allows for reliable monitoring of all *Listeria* species and it can be exploited for a better understanding of the occurrence and distribution of *Listeria* during the ripening of dry fermented sausages. Also, molecular subtyping is essential for *L. monocytogenes* outbreak detection and epidemiological investigations, which are further complicated by the long incubation time for invasive listeriosis and the difficulty in identifying appropriate

controls for case-control studies [24]. On the other hand, molecular methods can provide "same-day" identification, which represents a significant reduction in time compared to standard microbiological methods for identification of *Listeria* spp. from positive food samples. This assay represents a significant improvement over standard microbiological methods due to its rapid and non-tedious format [25]. Also, Lakicevic *et al.* [26] suggest the need to use combined methods in order to obtain an accurate identification of *Listeria* spp.

Nonpathogenic *Listeria* spp. from feed samples (*L. innocua*) and from the sausage – D13 (*L. welshimeri*), by using PCR method, were negative for the presence of *L. monocytogenes*, so it was not necessary to perform DNA sequencing.

pH and a_w

After 9 days of *Petrovská klobása* production, *Listeria* spp. were not detected in homemade sausages dried the industrial way (E 13). This can be explained by the fact that the water activity (a_w) was lower than 0.94 and the pH value was lower than 5. After 15 days of production, *Listeria* was detected only in homemade sausages ripening in uncontrolled traditional conditions (D14). On the same day, there has a been loss of *Listeria* populations for homemade sausages with the addition of commercial starter culture and dried in the industrial (E24) and traditional way (D24), although the food had properties (a_w and pH) favorable for its growth. This can be explained by the fact that the preservatives and protective microflora, especially the starters, have a significant impact on the survival and growth of *Listeria* [27]. Literature data indicate that different strains of *Lb. sakei* isolated from meat and meat products have the ability to produce secondary metabolic substances – bacteriocines with a strong antilisterial activity [28-30].

CONCLUSION

Listeria monocytogenes, a serious hazard in relatively closed environments such as food processing plants, survives extremely well in the processing plant environment. Analysis of the production of traditional and autochthonous fermented sausages have confirmed that animals, stuff and ingredients were potential sources of *Listeria* spp. *L. monocytogenes* also has the tendency to form biofilms when resident populations become established in niches in the processing plant. These resident populations and the biofilms they form to enhance their survival are not easily eliminated by general-purpose cleaners or sanitizers and normal sanitation procedures.

Furthermore the temperature, water activity, pH and starter cultures had affected the survival and growth of *L. monocytogenes* during the production of the traditional fermented dry sausage. It should be pointed out that at the end of the production cycle, *Listeria* spp were not determined, in spite of the fact whether commercial culture or natural fermentation was used.

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DETEKCIJA *LISTERIA* SPP. U TOKU PROIZVODNJE I ZRENJA PETROVAČKE KOBASICE

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Cilj ovog rada je bio da se utvrdi preživljavanje *Listeria* spp. u toku proizvodnje i zrenja tradicionalne srpske kobasice (Petrovačka kobasica) kao i da se okarakterišu izolati dobijeni umnožavanjem *hlyA* gena. Rezultati dobijeni standardnim mikrobiološkim i molekularnim metodama su pokazali da je tokom fermentacije, sušenja, zrenja i skladištenja Petrovačke kobasice, 17 od ukupno 99 uzoraka bilo pozitivno na prisustvo *Listeria* spp. Među njima, otkriven je hemolitični, atipični nepatogeni soj *L. innocua* FSL J1-023, čija je genomska sekvenca važna u rezumevanju uloge horizontalnog genskog transfera i rekombinacije tokom evolucije patogenosti. Najvažnije karakteristike proizvoda koje su uticale na preživljavanje i rast *Listeria* spp. tokom proizvodnje i zrenja Petrovačke kobasice bili su pH, aktivnost vode, temperature i starter kultura.