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Effect of starter cultures on survival of *Listeria monocytogenes* in Čajna sausage

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Abstract. The aim of the study was to evaluate the survival of *Listeria monocytogenes* during the production of Čajna sausage with short maturation time. Sausage batter was inoculated with three different serotypes 4b and serotype 1/2a of *L. monocytogenes*. Control sausages were without any starter culture added; the second batch was inoculated with strains of *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus*, and the third batch was inoculated with strains *of Debaryomyces hansenii*, *Lactobacillus sakei*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Staphylococcus carnosus* and *Staphylococcus xylosus*. After 18 days of ripening, *L. monocytogenes* was not detected in any of the sausages, but during this fermentation and drying, the numbers of this pathogen was lower in the sausages inoculated with starter cultures.

1. Introduction

Čajna sausage is a traditional dry, fermented meat product, widely produced and consumed in Serbia [1]. Sausages are prepared by mixing ground meat, fat with combinations of spices, flavorings, salt, sugar, additives, and frequently, starter cultures [2]. During dry sausage manufacture, the fermentation and ripening processes lead to pH and water activity (a_w) decreases, which remains the main manner of achieving the safety of this type of fermented product [3].

Raw meat for sausage production and the final product tend to be consumed without prior cooking, which is why fermented sausages are a ready-to-eat product [2,4]. Thus, even if dry, fermented sausages are generally recognized as microbiologically safe, when initial contamination of the raw materials is high or there is insufficient control, the safety of these products can become compromised [5]. In past years, several outbreaks of food-borne illness associated with fermented meats have been reported and *Listeria monocytogenes* have been often detected in finished fermented sausages [6].

According to a USDA risk assessment [7] dry, fermented sausages are classified in a group designated as moderate risk for *L. monocytogenes* presence. Due to its ubiquitous nature, *L. monocytogenes* can contaminate meat and meat products during slaughter and processing operations, including cutting, slicing and packing [2,8]. Furthermore, this pathogen can persist and grow at low pH values, at low water activity, and at refrigeration temperatures [8,9] and pose a serious risk to human health.

Insufficient data is available on the survival of *L. monocytogenes* in Čajna sausages even though the product is widely consumed. Therefore, the aim of present study was to evaluate if *L. monocytogenes* is able to survive during the production of short maturation Čajna sausages produced with autochthonous and commercial starter cultures.

2. Materials and methods

2.1. Inoculum preparation

L. monocytogenes serotype 4b ATCC 19115, serotype 4b NCTC 11994, and serotype 4b and 1/2a were previously isolated from smoked herring and smoked salmon, respectively. All isolates were resuscitated twice in Brain Heart Infusion (BHI) broth (Oxoid, UK) and incubated at 37°C for 24 h. Then bacterial cultures were mixed in approximately equal proportions to produce the cocktail inoculum, and an appropriate volume of the inoculum was added to sausage batter to produce approximately 10^6 CFU of *L. monocytogenes* per gram of batter.

2.2. Sausage preparation and inoculation of pathogens

Čajna sausage was prepared from a mixture of lean pork (75%) and pig fat (25%) obtained from carcasses of Yorkshire x Landrace crossbreed pigs. Sausage batter was prepared by grinding frozen lean meat and fat tissue to 8 mm size and mixing with 2.1% salt containing 0.6% sodium nitrite and the spice mixture, Čajna nova (Prima commerce, Serbia) (4 g/kg) in a commercial meat processing plant and transported under refrigerated conditions to the faculty workshop. The batter for fermented sausage was inoculated with the cocktail of *L. monocytogenes* strains. After mixing, the batter was divided into three batches: the first batch was control (C) without any starter culture added, the second batch (EI) was inoculated with strains of *Lactobacillus sakei, Staphylococcus carnosus* and *Staphylococcus xylosus* (Biostart Sprint, RAPS GMBH, Obertrum, Austria), and the third (EII) was inoculated with strains of *Debaryomyces hansenii, Lactobacillus sakei, Pediococcus acidilactici, Pediococcus pentosaceus, Staphylococcus carnosus* and *Staphylococcus xylosus* (BACTOFERM™ B-LC-007, The Craft Butchers Pantry, US). The prepared mixtures were stuffed into 34mm-diameter collagen casing, to produce 30 sausages of approximately 450 g each for each group. Čajna sausage is a rapidly fermented sausage, and the ripening process (fermentation and drying) for the sausages lasted 18 days. First, the sausages were cold smoked for 8h during 3 days at 21-23°C and 80-85% relative humidity and, then were dried for 18 days in a climate chamber at 17°C with a relative humidity of 75%.

2.3. Microbiological analyses

Samples were analyzed for *L, monocytogenes*, lactic acid bacteria (LAB) count and total *Enterobacteriaceae* count on day 0 and on days 3, 7, 14 and 18 of storage. For bacterial enumeration, 10g of sausage samples were added to 90 ml of Buffered Peptone Water (BPW) (Merck, Germany) and homogenized in a Stomacher blender (Stomacher 400 Circulator, Seward, UK) for 2 min. Serial decimal dilutions were prepared and 0.1 ml or 1 ml of appropriately diluted suspension was inoculated directly on the surface of the appropriate media for enumeration of the different bacteria. *L. monocytogenes* was enumerated on Agar Listeria acc. to Ottaviani and Agosti (ALOA, Oxoid) and plates were incubated for 24-48h at 37°C according to ISO 11290-2:1998. LAB were enumerated on MRS (Merck, Germany) following incubation at 30°C for 72h according to ISO 15214:1998 and *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBGA, Merck, Germany) after incubation at 37°C for 24h according to ISO 21528-2:2004. After an appropriate period of incubation for each type of bacteria, plates were examined visually for typical colony types and morphological characteristics associated with each growth medium, number of colonies was counted, and results were recorded as colony forming units per g (CFU/g).

2.4. pH and a_w values

The pH was measured using a pH meter, TESTO 205 (Lenzkirch, Germany). Water activity (a_w) was measured with an aqualab water activity meter series 3 TE (Decagon Devices Inc., USA) according to the manufacturer's instructions.

2.5. Statistical analysis

Statistical analysis of the results was conducted using the software GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The results were expressed as mean±standard deviation. The effects of different starter cultures during ripening period were appraised by one-factor analysis of variance (ANOVA) with Tukey's multiple comparison test at 95% confidence level (difference considered significant if $P<0.05$).

3. Results

L. monocytogenes counts ranged between 6.30 and 6.35 log CFU/g at the beginning of the study without significant differences between sausages (P>0.05). Slight but non-significant decreases (0.13-0.17 log CFU/g) were noted within the first three days of fermentation. Then, *L. monocytogenes* counts decreased significantly in all sausage types and were below the detection limit on day 18. From day 7 until day 14, the decrease of *L. monocytogenes* was significantly ($P< 0.05$) slower in the sausages without starter cultures added. No significant differences were found in *L. monocytogenes* numbers among treatments with starter cultures added, except on day 14 when significantly lower numbers of this pathogen were measured in the sausages inoculated with strains of *Debaryomyces hansenii, Lactobacillus sakei, Pediococcus acidilactici, Pediococcus pentosaceus, Staphylococcus carnosus* and *Staphylococcus xylosus.* Even so, at the end of the ripening process, *L. monocytogenes* was below the detection limit in all types of sausages, regardless of whether or not they were inoculated with the starter cultures.

Table 1. *L. monocytogenes* counts (log CFU/g) during the fermentation and drying of Čajna sausage.

	0	3	7	14	18
C	6.35 ± 0.05^{A}	6.17 ± 0.04^{A}	4.90 ± 0.05^{A}	2.78 ± 0.02^{A}	nd
EI	6.33 ± 0.03^{A}	6.20 ± 0.02^{A}	4.50 ± 0.06^{B}	2.32 ± 0.02^{B}	nd
EII	6.30 ± 0.02^{A}	6.16 ± 0.01^{A}	4.35 ± 0.04^{B}	2.02 ± 0.03^{C}	nd

Within a column, means with a different letter are significantly different: A-C, P<0.05; nd-below detection limit
C – control sausages
EI – sausages with Biostart Sprint commercial starter
EII – sausages with BACTOFERM™ B-LC-007 commercial starter

The initial number of LAB was 4.52 log CFU/g in control sausages and 5.09 and 5.37 log CFU/g in sausages with starter cultures added (Table 2). As expected, LAB counts increased rapidly in all sausages during processing. During the ripening and fermentation process, in control sausages, LAB counts increased by 3.42 log CFU/g, while in the sausages with starter cultures added, the measured increase was 3.74 and 4 log CFU/g. On day 14, LAB had reached their final population level and slightly, but not statistically significant ($P>0.05$) decreased on day 18.

Table 2. LAB counts (log CFU/g) during the fermentation and drying of Čajna sausage.

	0	3	7	14	18
C	4.52 ± 0.09^{A}	6.06 ± 0.07^{A}	7.23 ± 0.36^{A}	8.13 ± 0.05^{A}	7.94 ± 0.15^{A}
EI	5.37 ± 0.07^{B}	7.33 ± 0.06^{B}	8.96 ± 0.20^{B}	9.19 ± 0.07^{B}	9.11 ± 0.06^{B}
EII	5.09 ± 0.07^{B}	6.97 ± 0.09^{C}	8.82 ± 0.05^{B}	9.14 ± 0.07^{B}	9.04 ± 0.06^{B}

Within a column, means with a different letter are significantly different: A-B, P<0.05
C – control sausages
EI – sausages with Biostart Sprint commercial starter
EII – sausages with BACTOFERM™ B-LC-007 commercial starter

Initial *Enterobacteriaceae* counts ranged between 4.44 and 4.47 log CFU/g (Table 3). The numbers of *Enterobacteriaceae* did not change significantly during the first 3 days of fermentation but decreased thereafter in all sausages and were below the detection limit at the end of the ripening process.

Table 3. *Enterobacteriaceae* counts (log CFU/g) during the fermentation and drying of Čajna sausage.

	0	3	7	14	18
C	4.44 ± 0.07^{A}	4.36 ± 0.05^{A}	2.91 ± 0.06^{A}	1.91 ± 0.04^{A}	nd
EI	4.48 ± 0.05^{A}	4.34 ± 0.04^{A}	2.80 ± 0.05^{A}	1.36 ± 0.03^{B}	nd
EII	4.47 ± 0.06^{A}	4.38 ± 0.05^{A}	2.87 ± 0.05^{A}	1.59 ± 0.06^{C}	nd

Within a column, means with a different letter are significantly different: A-C, $P<0.05$; nd-below detection limit
C – control sausages
EI – sausages with Biostart Sprint commercial starter
EII – sausages with BACTOFERM™ B-LC-007 commercial starter

Changes in pH of the sausages during fermentation and drying are presented in Table 4. During this period, the pH decreased in all sausages, indicating a normal sausage making process, but decreases occurred significantly ($P < 0.05$) faster in sausages inoculated with the starter cultures than in sausages without starter culture added. Decreases in the pH values by 0.77-0.89 coincided with the increases in LAB counts.

Table 4. pH during the fermentation and drying of Čajna sausage.

	0	3	7	14	18
C	6.05 ± 0.07^{A}	5.86 ± 0.06^{A}	5.51 ± 0.06^{A}	5.34 ± 0.03^{A}	5.28 ± 0.03^{A}
EI	6.03 ± 0.08^{A}	5.78 ± 0.08^{A}	5.42 ± 0.04^{B}	5.20 ± 0.03^{B}	5.18 ± 0.02^{B}
EII	6.05 ± 0.05^{A}	5.81 ± 0.05^{A}	5.41 ± 0.03^{B}	5.19 ± 0.02^{B}	5.16 ± 0.03^{B}

Within a column, means with a different letter are significantly different $P<0.05$
C – control sausages
EI – sausages with Biostart Sprint commercial starter
EII – sausages with BACTOFERM™ B-LC-007 commercial starter

The addition of 2.1% salt produced an initial a_w of about 0.968 in the initial sausage batters (Table 5). During fermentation and drying, a_w decreased in all sausages to levels of 0.909-0.912, and decreases were significant from day 7. Statistical analysis did not show significant differences ($P>0.05$) between the sausages inoculated with starter culture and the control sausages.

Table 5. Water activity (a_w) during the fermentation and drying of Čajna sausage.

	0	3	7	14	18
C	0.968 ± 0.001^{A}	0.961 ± 0.001^{A}	0.948 ± 0.004^{A}	0.928 ± 0.001^{A}	0.909 ± 0.001^{A}
EI	0.968 ± 0.001^{A}	0.963 ± 0.001^{A}	0.952 ± 0.001^{A}	0.929 ± 0.001^{A}	0.912 ± 0.001^{A}
EII	0.968 ± 0.001^{A}	0.962 ± 0.002^{A}	0.952 ± 0.001^{A}	0.929 ± 0.001^{A}	0.912 ± 0.002^{A}

Within a column, means with a different letter are significantly different $P<0.05$
C – control sausages
EI – sausages with Biostart Sprint commercial starter
EII – sausages with BACTOFERM™ B-LC-007 commercial starter

4. Discussion

Serotypes 1/2a, 1/2b, and 1/2c of *L. monocytogenes* are frequently isolated from food products and serotypes 1/2a, 1/2b, and 4b cause 95% of the human cases of listeriosis [10,11], which is the reason these serotypes were used in the present study.

The final a_w and pH values and the concentration of salts, nitrites, and spices should suppress or inhibit the growth of pathogenic microorganisms in the fermented sausages, but *L. monocytogenes* could survive these conditions [12]. This is why bacteriocin-producing starters are often added. The addition of starter cultures is of particular importance for the microbial stability of quick-ripened fermented sausages, which are not greatly dried [13], like Čajna sausage. *Lb. sakei* presented in both commercial starter cultures can produce bacteriocins that showed broad inhibitory activity against pathogenic microorganisms including *L. monocytogenes* [14,15]. The results from the present study indicated that *Lb. sakei* as starter cultures for sausages played an important role in the control of *L. monocytogenes*. Furthermore, *Pediococcus acidilactici* pediocin-producing strain was added to one of the commercial starter cultures, and *L. monocytogenes* counts in the EII sausages inoculated with this starter culture were the lowest. Nonetheless, *L. monocytogenes* was inhibited, not only in the sausages with starter cultures, but also in non-inoculated sausages. This can be attributed to the fact that during spontaneous fermentation, LAB rapidly dominated the total microbiota in the present study. Autochthonous LAB are recognized as good competitors and exhibit a bioprotective or inhibitory effect on fortuitous microbiota as a result of the competition for nutrients and/or of the production of bacteriocins or other antagonistic compounds such as organic acids, hydrogen peroxide and enzymes [16].

Furthermore, Mataragas et al. [3] suggested that inactivation of *L. monocytogenes* is observed when pH and a_w values are within the range that does not support growth of the pathogen ($pH \leq 5.0$ and $a_w \leq 0.94$). In the present study, final a_w values were in within this limit. The pH decreased faster in sausages inoculated with the starter cultures than in sausages without starter culture added. This result suggests that the starter cultures played an active role in the fermentation process. Even so, on day 18, the pH still was above pH 5. pH and a_w are important factors for *L. monocytogenes* inactivation in fermented sausages; however, fermentation and drying temperatures have a significant role. It is suggested that temperatures at or above 20°C are needed, especially during the first 48 h of fermentation, for rapid inactivation of *L. monocytogenes* [6,17]. The significant reduction of this pathogen in the present study could be attributed to the high temperatures used during first three days of fermentation.

In accordance with present results Drosinos et al. [18] reported that *L. monocytogenes* was rapidly inactivated, decreasing by 3-4 log CFU/g during 28 days and by 4-5 log CFU/g in in fermented products from Croatia and Bosnia and Herzegovina. Contrary to results from present study, Ducic et al. [5] reported only reductions of 0.8 and 0.5 log CFU/g *L. monocytogenes* in fermented sausages without starters, even though the pathogen was inoculated at similar levels to those we used. Differences in the results could be explained by characteristics of isolates used and the production process.

A low *Enterobacteriaceae* count is crucial to obtaining high-quality hygienic sausages [19]. In the present study, the initial number of this bacterial group was relatively high. This could be attributed to the poor hygienic status of incoming raw materials or the processing environment in the meat processing plant where stuffing for sausages was prepared [5]. Nonetheless, the number of *Enterobacteriaceae* decreased during storage and was below 2 log CFU/g in all sausages at the end of the rapid fermentation. These results indicate the inhibitory effect of the inoculated probiotic cultures but also the acidification which was observed in control sausages as result of the activity of autochthonous LAB against *Enterobacteriaceae*.

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