

Effect of commercial starter cultures on survival of *Yersinia enterocolitica* and microbiological status of Sremska sausages

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Abstract: The aim of this study was to determine the survival of *Yersinia enterocolitica* (biotype 1, serotype O:8), and the microbiological status (lactic acid bacteria and Enterobacteriaceae), water activity and pH values of Sremska sausage (traditional dry-fermented sausage from Northern Serbia) during ripening (18 days). Four different groups of Sremska sausage were manufactured: CI group – control without starter culture; CII group – control with starter culture; EI group – was inoculated with 10^8 CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610, without starter culture and EII group – was inoculated with 10^8 CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610 and with starter culture. During ripening, microbiological examination was conducted according to ISO methods, on days 0, 3, 7, 12 and 18. In the inoculated sausages, *Y. enterocolitica* did not grow after day 12 of the ripening period. The results revealed that the use of starter cultures increased the number of lactic acid bacteria, while completely reducing the Enterobacteriaceae count compared with the Sremska sausage without starter culture. Also, the sausages manufactured with starter culture had lower pH values compared to the sausages without starter culture. In conclusion, the use of starter cultures contributes to improving the microbial safety of Sremska sausage.

Key words: *Yersinia enterocolitica*, Sremska sausage, food safety, microbiological status.

Introduction

Yersinia enterocolitica is a bacterium which belongs to the family Enterobacteriaceae, widely found in natural environments (EFSA Panel on Biological Hazards, 2014; Mitrović, 2016). This psychrotrophic bacterium has the capability to survive and multiply at low temperatures (Baltić et al., 2013; Ivanović, 2014; Baltić et al., 2016; Ivanović et al., 2016a). Also, *Y. enterocolitica* is zoonotic, causing yersiniosis, a frequently reported bacterial zoonosis in the European Union (EFSA and ECDC, 2013). Among the sources, pork is reported as a major reservoir for *Y. enterocolitica*. The bacterium is often present in the oral cavity of pigs especially tonsils, intestinal content, faeces and lymph nodes (EFSA Panel on Biological Hazards, 2014).

Sremska sausage is popular dry-fermented sausage in Serbia (and has a protected designation of origin). It is characterized by a specific hot taste, aromatic and spicy flavour, dark red colour and hard

consistency (Stanisic et al., 2014). Sremska sausage is one of the local fermented cured meat products that, until the mid-1950s, were produced exclusively on farms. According to the available data, production of Sremska sausage began in the middle of the 18th century. There are assumptions that the forerunner of today's Sremska sausage was the spicy and smoked Lucanica that Roman soldiers carried in their backpacks for encouragement before, and invigorating refreshment after battles (Stevanović et al., 2016). It is well known that the typical characteristics of fermented sausages are generated by chemical, biochemical, physical and microbiological changes occurring during fermentation, ageing and drying. Also, fermentation of raw materials improves the safety, shelf life and acceptability of food and it has a long tradition (Ivanović et al., 2015a; Domínguez et al., 2016). There are several lactic acid bacteria, mainly *Lactobacillus sakei* in Europe and *Pediococcus acidilactici* in the USA (Leroy et al., 2006), and some staphylococcal

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species, almost exclusively *Staphylococcus xylosum* and *Staphylococcus carnosus*, developed as commercial starters for the manufacture of dry sausages (Corbiere Morot-Bizot et al., 2007). The addition of starter cultures has become common in the manufacture of several types of fermented meat products in order to ensure safety, thus reducing the risk of pathogenic and spoilage bacteria, as well as to contribute to colour and flavour development and extend shelf-life (Lorenzo et al., 2012; Essid and Hassouna, 2013; Ciuciu Simion et al., 2014; Mitrovic, 2016).

The practice of utilising a short maturation period and storage of fermented sausage at refrigeration temperatures may result in unsatisfactory reductions of pathogens (*Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica*) if present. Thus, inclusion of a maturation period above refrigeration temperatures and using starter culture may increase the safety of fermented sausages (Lindqvist and Lindblad, 2009).

In Serbia, there is limited study on *Y. enterocolitica* and the bacterium is not routinely isolated. Therefore, the aim of this study was to evaluate effect of starter cultures on survival of *Y. enterocolitica* and microbiological status of Sremska sausages.

Materials and methods

Sausage production and sampling procedures

Four different batches of Sremska sausage were manufactured according to traditional techniques, two of them without starter culture (CI and EI) and the other two batches (CII and EII) with addition of commercial starter culture, Biostart Sprint (RAPS GmbH, Obertrum, Austria) at the level defined by the manufacturer in each case (20 g per 200 kg of meat). This product contains *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosum*, and starter cultures were added and the batter was mixed with gloved hands for 5 min. The EI and EII sausages were inoculated with *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610 (<http://www.atcc.org>), biotype 1, serotype O:8. The preparation of inoculum was according to Ivanovic et al. (2015b).

Sausage formulation comprised minced pork meat (35%), minced beef meat (23%), minced fat (20%), nitrite salt (2.3%, resulting in 175 ppm sodium nitrite), glucose (1%) and white pepper (0.2%). The mix was maintained at 4°C for 24 h and then stuffed into natural casings with a diameter of 34 mm. The sausages were fermented for 2 days at 20°C and 80–85% relative humidity and then transferred into a drying-ripening chamber where they

were kept for 18 more days at 17°C and 75–80% relative humidity. Sampling was performed by randomly selecting two links of each sausage on days 0, 3, 7, 12 and 18 of ripening.

Chemical composition, pH and a_w values

ISO recommended standards were employed to determine moisture (ISO, 1997), fat (ISO, 1992), protein (ISO, 1992b) and ash (ISO, 1999). The pH values were measured with a pH meter, TESTO 205 (Lenzkirch, Germany). The water activity (a_w) was measured using an aqualab water activity meter series 3 TE (Decagon Devices Inc., USA) on approximately 10 g of sausage according to the manufacturer's instructions.

Microbiological analyses

For microbiological analysis, 10 g of Sremska sausage was aseptically weighted into a sterile plastic bag, previously removing and discarding the outer plastic. Subsequently, samples were homogenized with 90 mL of a sterile solution of 0.1% (w/v) peptone water (Oxoid, Unipath, Basingtoke, UK), containing 0.85% NaCl and 1% Tween 80 as emulsifier, for 2 min at 20–25°C in a stomacher blender (Stomacher 400 Circulator, Seward, UK), thus making a 1/10 dilution. Serial 10-fold dilutions were prepared by mixing 1 mL of the previous dilution with 9 mL of 0.1% sterile peptone water. For enumeration of lactic acid bacteria, 1 mL of the appropriate 10-fold serial dilution was inoculated into Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK). The MRS plates were incubated at 30°C for 72 h, and all colonies were counted to enumerate lactic acid bacteria (ISO, 1998). Selective medium was used for enumeration of *Y. enterocolitica* (CIN – Cefsulodin-Irgasan-Novobiocin: *Yersinia* selective agar base CM0653 and *Yersinia* selective supplement SR0109, Oxoid) and was incubated at 30°C for 24 h (ISO, 2003). For an enumeration of *Enterobacteriaceae*, 1 mL of the appropriate 10-fold serial dilution was inoculated into violet red bile glucose agar (VRBG, Merck, Germany) (ISO, 2009). The VRBG plates were incubated at 30°C for 24 h. All purple colonies due to rapid fermentation of glucose surrounded by purple haloes of precipitated bile salts were counted (Fredriksson-Ahomaa et al., 2001). Lactic acid bacteria, *Y. enterocolitica* and *Enterobacteriaceae* counts were determined on days 0, 3, 7, 12 and 18 of ripening. After incubation, plates with 30–300 colonies were counted. The microbiological data were transformed into logarithms of the number of colony forming units (log CFU g⁻¹).

Statistical Analysis

A total of 80 sausages (ten sausages for each batch, four batches, two replicates) were analysed for the different parameters. Statistical analysis of the results was elaborated using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA; <http://www.graphpad.com>) and Microsoft Office Excel 2013 (Microsoft Corporation, Los Angeles, CA). The parameters were described by means and standard error of means (SEM) (*Y. enterocolitica* counts were described by means and standard deviation). One-way ANOVA with Tukey's *post hoc* test was performed to assess the significance of differences among control and experimental groups. Values of $P < 0.05$ and $P < 0.01$ were considered significant.

Results and discussion

Chemical composition and pH values

Values corresponding to moisture, protein, fat and ash at the beginning of the study are summarised in Figure 1.

The effect of starter cultures on chemical composition (moisture, fat, protein and ash) and pH values during the ripening period is presented in Table 1. At the beginning of the study, moisture, fat, protein and ash content was 55.36%, 25.71%, 15.45% and 3.54%, respectively (data not shown). The moisture content decreased during storage. There were no significant differences in protein values between the different sausage types. Our results are

in agreement with those obtained in another study, which also did not find significant differences in protein values between different sausages (Lorenzo *et al.*, 2014). However, in the current study, moisture, fat and protein content showed significant ($P < 0.01$) differences between treatments. Dalmis and Soyer (2008) also noticed that inoculated sausages had significantly lower moisture content compared to control sausages, without starter culture. In contrast, Essid and Hassouna (2013) found that the addition of *S. xylosum* and *Lactobacillus plantarum* starter culture did not affect the loss of sausage moisture. Santa *et al.* (2014) also found significant differences between moisture content at the end of ripening of Italian sausages. In that case, sausages inoculated with starter cultures had lower moisture and higher fat and ash values than control and experimental sausages without starter culture. Domínguez *et al.* (2016) found significant differences between protein content at the end of ripening of dry-cured foal sausage, unlike our findings.

The pH decreased sharply from initial mean value of 6.14 (data not shown) to reach, after 18 days of ripening, pH 5.32, 5.22, 5.30 and 5.27 for CI, CII, EI and EII sausages, respectively. So, the inoculation of the starter cultures resulted in greater acidification during the 18 days of production. The pH of CI sausages was significantly ($P < 0.001$) higher than those inoculated with starter cultures (Table 1). Other authors also reported similar results in dry-fermented sausages (Lorenzo *et al.*, 2014). These authors observed that all sausages inoculated with starter culture reached a lower pH than control sausages. The pH fall could be related to an accumulation of

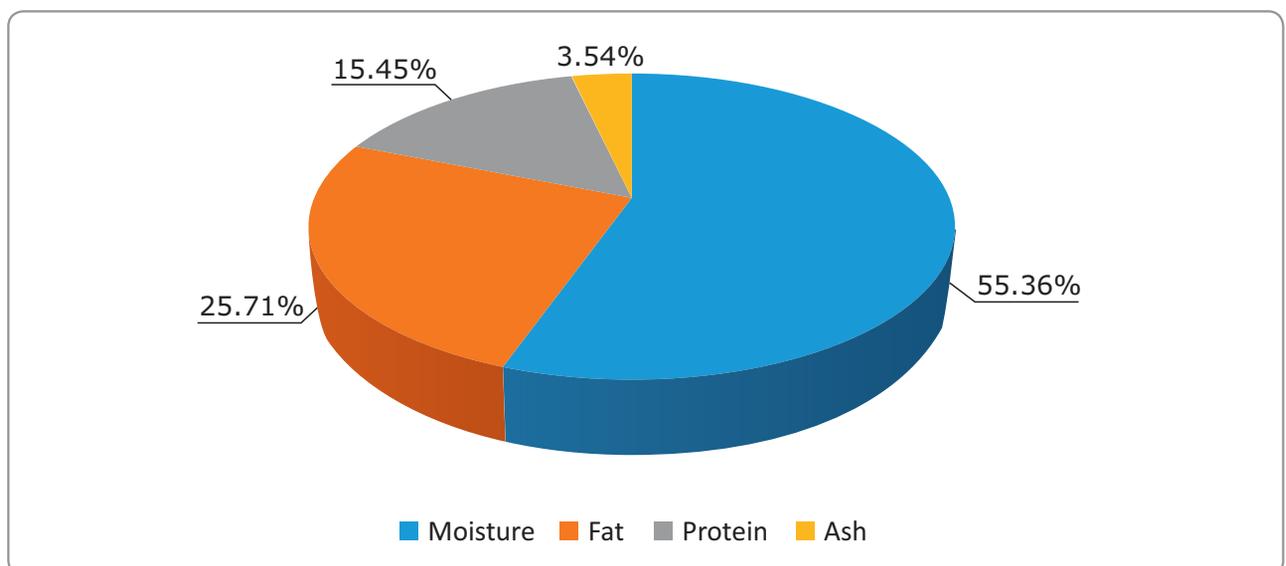


Figure 1. Chemical composition of the Sremska sausages at the beginning of the study

Table 1. Effect of commercial starter cultures on chemical composition (g 100 g⁻¹), pH and a_w values (at the end of ripening period)

Parameter	Sausage group				SEM	P value
	CI	CII	EI	EII		
	18. day					
Moisture	29.07 ^A	28.19 ^B	29.34 ^A	28.48 ^B	0.09	< 0.0001
Protein	25.49	25.67	25.42	25.63	0.05	0.18 (ns)
Fat	40.26 ^a	40.87 ^a	40.03 ^b	40.63 ^b	0.12	0.03
Ash	5.17 ^A	5.28 ^B	5.22 ^A	5.27 ^C	0.01	0.001
pH values	5.32 ^A	5.22 ^B	5.30 ^C	5.27 ^{BC}	0.009	0.0004
a _w values	0.911	0.910	0.921	0.914	0.02	ns

Legend: Within a row, means with a different letter are significantly different: ^{A-C}*P*<0.01; ^{a-b}*P*<0.05; ns – not significant. Groups: CI – control without starter culture; CII – control with starter culture; EI – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610 and with starter culture.

organic acids, mainly lactic, present in this type of sausage as a result of carbohydrate breakdown during fermentation (Zhao et al., 2011; Lorenzo et al., 2014). In contrast, Ciuciu Simion et al. (2014) did not observe differences in pH values between inoculated and non-inoculated Romanian Dacia sausage. However, it must be taken into account that the influence of starter cultures on pH values depends on the microorganisms present in the starter culture, and it is difficult compare the results between studies that used different starter cultures. In our study, a_w decreased throughout ripening in the four types of sausage studied. However, statistical analysis did not show significant differences (*P*>0.05) between the sausages inoculated with starter culture and the control sausages at the end of the study. Our result is in disagreement with those reported by Kaban and Kaya (2009), who observed that sausages with starter culture displayed lower a_w values in comparison to those of the control.

Microbial counts

Results concerning *Y. enterocolitica* levels detected during ripening of Sremska sausages produced with or without starter culture are reported in Table 2. Statistical analysis showed significant differences (*P*<0.01) between EI and EII sausages on *Y. enterocolitica* counts (on days 3 and 7 of ripening). The differences in *Y. enterocolitica* counts at the end of the process could be related to the addition of starter cultures. This outcome is in agreement with those reported by other authors (Ivanovic et al., 2015b; Mitrovic, 2016), who found the use of selective starter to produce sausages significantly affects

Y. enterocolitica levels. Initial counts of *Y. enterocolitica* were 6.17 log CFU g⁻¹ in our EI and EII sausages. On day 7 of ripening, counts of *Y. enterocolitica* decreased to 4.30 and 4.99 log CFU g⁻¹ for EI and EII sausages, respectively. At the end of the ripening period, *Y. enterocolitica* counts were not detected. The decrease in *Y. enterocolitica* counts during the 18 days of ripening for the inoculated sausages suggests the poor competitiveness of *Y. enterocolitica* due to the intensive growth of lactic acid bacteria and the associated decrease of pH, as reported by other works (Lindqvist and Lindblad, 2009; Ivanovic et al., 2015a; Ivanovic et al., 2015b). Most lactic acid bacteria are more tolerant to the antagonistic effects of lactic acid than *Y. enterocolitica*, and they produce large amounts of lactic acid. The lactic acid produced can exercise a negative influence on *Y. enterocolitica* (Ivanović et al., 2015b).

The effect of starter cultures on the *Enterobacteriaceae* counts of Sremska sausage is shown in Table 3. Initial counts of *Enterobacteriaceae* were 4.64 log CFU g⁻¹ in the control sausages and 5.29 log CFU g⁻¹ in the experimental sausages. The number of these bacteria depends mainly on the hygienic quality of the raw materials and the handling conditions during processing (Ivanovic et al., 2013). During ripening, this group of bacteria displayed a strong decrease in Sremska sausages inoculated with starter culture (CII sausages from 4.64 log CFU g⁻¹ to 2.47 log CFU g⁻¹ and EII sausages from 5.29 log CFU g⁻¹ to 3.03 log CFU g⁻¹). The inhibitory effect exerted by the starter cultures on the pathogenic microbiota was, therefore, evident, especially from day 7 of the ripening period. Our findings are similar to those reported by Essid and Hassouna

Table 2. *Y. enterocolitica* counts (log CFU g⁻¹) during the ripening period of naturally fermented Sremska sausages inoculated with starter culture (groups EI and EII) ($\bar{X}\pm\text{SD}$)

Day of ripening	EI Sausages	EII Sausages	P value
0	6.17±0.34	6.17±0.34	0.990 (ns)
3	4.84 ^A ±0.09	4.49 ^B ±0.04	< 0.0001
7	4.30 ^A ±0.04	4.99 ^B ±0.30	< 0.0001
12	nd	nd	
18	nd	nd	

Legend: Within a row, means with a different letter are significantly different: ^{A-B}*P*<0.01; ns – not significant.

Sausage groups: EI – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610 and with starter culture.

nd: Absent from 10 g sample.

(2013) and Ciuciu Simion *et al.* (2014), who observed lower final counts of *Enterobacteriaceae* in control sausages than in sausages inoculated with lactic acid bacteria strains. From the results of the present work, it seems that the inclusion of the starter culture substantially contributes to the decrease of the *Enterobacteriaceae* and *Y. enterocolitica* counts throughout the ripening. Also, a low number of *Enterobacteriaceae* is a very relevant indicator of food safety (Rubio *et al.*, 2013; Ivanovic *et al.*, 2013, Janjic *et al.*, 2015; Ivanovic *et al.*, 2016b).

Initial lactic acid bacteria counts in starter culture inoculated sausages (CII and EII, 5.21 log CFU g⁻¹ and 6.23 log CFU g⁻¹, respectively) were higher than those in the sausages without starter culture (CI and EI, 5.09 log CFU g⁻¹ and 6.20 log CFU g⁻¹, respectively) (Table 4). The maximum number of lactic acid bacteria was observed on day 12 of ripening period and then a slight decrease was observed and counts reached 9.03 log CFU g⁻¹, 9.12 log CFU g⁻¹, 9.06 log CFU g⁻¹, 9.01 log CFU g⁻¹, 9.20 log

CFU g⁻¹ for CI, CII, EI and EII sausages, respectively, at the end of the ripening period. This slight decrease of lactic acid bacteria during ripening is probably due to the decrease of fermentable carbohydrates (Lorenzo and Franco, 2012). Statistical analysis showed significant differences (*P*<0.01) between all groups of Sremska sausages. Nevertheless, Lorenzo *et al.* (2014) did not find significant differences between control and commercial starter cultures (with *Lactobacillus sakei*) groups, while Rubio *et al.* (2013) showed higher lactic acid bacteria counts (*P*<0.05) in control sausages compared to sausages inoculated with starter culture (*L. plantarum* and *Lactobacillus rhamnosus*).

Due to the good adaptation of lactic acid bacteria to the meat and their faster growth rates during Sremska sausage fermentation, they became the dominant microbe, as expected (Essid and Hassouna, 2013; Zhao *et al.*, 2011). Lactic acid bacteria belong to the desirable microbiota of fermented sausages (Ivanovic *et al.*, 2013, Mitrovic, 2016).

Table 3. *Enterobacteriaceae* counts (log CFU g⁻¹) during the ripening period of naturally fermented Sremska sausages ($\bar{X}\pm\text{SEM}$)

Day of ripening	Sausage Group				SEM	P value
	CI	CII	EI	EII		
0	4.64 ^A	4.64 ^A	5.29 ^B	5.29 ^B	0.050	< 0.0001
3	4.39 ^A	3.59 ^B	5.00 ^C	4.49 ^A	0.070	< 0.0001
7	3.01 ^A	2.47 ^B	3.93 ^C	3.03 ^A	0.040	< 0.0001
12	nd	nd	nd	nd	–	–
18	nd	nd	nd	nd	–	–

Legend: Within a row, means with a different letter are significantly different: ^{A-C}*P*<0.01

Groups: CI – control without starter culture; CII – control with starter culture; EI – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610 and with starter culture.

nd: Absent from 10 g sample

Table 4. Lactic acid bacteria counts (log CFU g⁻¹) during the ripening period of naturally fermented Sremska sausages ($\bar{X} \pm \text{SEM}$)

Day of ripening	Sausage Group				SEM	P value
	CI	CII	EI	EII		
0	5.09 ^A	5.21 ^B	6.20 ^A	6.23 ^C	0.020	< 0.0001
3	7.53 ^A	8.40 ^B	8.57 ^B	6.65 ^C	0.050	< 0.0001
7	8.71 ^A	9.56 ^B	8.06 ^C	8.70 ^A	0.040	< 0.0001
12	9.25 ^A	9.06 ^B	8.92 ^C	9.30 ^B	0.009	< 0.0001
18	9.03	9.12	9.06	9.20	0.007	0.23 (ns)

Within a row, means with a different letter are significantly different: ^{A-C}*P* < 0.01; ns – not significant.

Groups: CI – control without starter culture; CII – control with starter culture; EI – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with 10⁸ CFU mL⁻¹ of *Y. enterocolitica* ATCC 9610 and with starter culture. nd: Absent from 10 g sample

Also, lactic acid bacteria have a positive effect on the hygienic properties of the product, inhibiting pathogenic and spoilage microbiota by acidification or by the production of antimicrobials (Villani et al., 2007; Ivanovic et al., 2015; Lindqvist and Lindblad, 2009).

Conclusion

In general, the chemical composition parameters of the sausages evaluated in this study were affected by the use of starter cultures. Also, sausage pH seemed to be influenced by starter cultures, since

starter culture-inoculated Sremska sausage exhibited a stronger acidification than the control sausage groups during the 18 day ripening period. This acidification, together with the growth of desirable competitive microbiota, might explain the sharp decrease of *Enterobacteriaceae* and *Y. enterocolitica* counts observed in the sausages inoculated with starter culture. The inhibitory effect exerted by the starter cultures on *Y. enterocolitica* is, therefore, evident, especially from day 7 of the ripening period. Therefore, the use of starter culture can improve the microbial food safety of traditional Sremska sausages.

Conflict of interest. The authors declare that they have no conflicts of interest.

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