Inactivation of *Yersinia enterocolitica* in Fermented Sausages during Fermentation

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Abstract: The purpose of this study was to evaluate the storage conditions as a way to increase the safety of fermented sausage, with the specific objectives to investigate the effect of storage time, pH value, water activity and microbiological status (*Lactobacillus* spp. and Enterobacteriaceae) on the levels of *Yersinia enterocolitica* in fermented sausages. For this experiment, meat was divided into four equal portions of 10 kg each. The first portion was control group (CI group) without addition of *Y. enterocolitica* and starter culture. The second portion (CII group) was inoculated starter, the third portion (EI group) was inoculated with *Y. enterocolitica* and the fourth portion (EII group) was inoculated *Y. enterocolitica* and starter culture. Sampling was performed by randomly selecting two links of each sausage preparation at the 0, 3rd, 6th, 9th, 15th, 25th and 35th day of drying. Results show that *Y. enterocolitica* and Enterobacteriaceae were not detected after 25 d of storage. The results indicate that practice utilizing starter culture is satisfactory to reduction of pathogen if present. Thus, inclusion with starter cultures in a maturation period may increase the safety of fermented sausages.

Key words: Growth inhibiton, lactic acid bacteria, pH value, sausages, *Y. enterocolitica*.

1. Introduction

The pathogenic bacteria *Yersinia enterocolitica* has become increasingly important as a food contaminant [1, 2]. Of special significance in food hygiene is the ability of *Y. enterocolitica* to grow in refrigerated foods. Yersiniosis is a typical foodborne disease. *Y. enterocolitica* has been frequently isolated from a variety of foods, like untreated milk, chocolate milk, dairy cream and ice cream, vegetables like carrots, tomatoes, lettuce, celery and mushrooms, raw hare, beef and lamb. Thus, pigs (meat and meat products) have been considered to be the primary reservoir for the human pathogenic types of *Y. enterocolitica* [3, 4].

Fermented dry sausage is defined as a mixture of comminuted fat and lean meat, salt, nitrate and/or nitrite, sugar and spices (mostly oregano and black pepper), which is stuffed into casings, subjected to fermentation and then allowed to dry [5]. The quality of the final product is closely related to the ripening that takes place during the drying. Also, fermentation of raw materials improves the safety, shelf life and acceptability of food and it has a long tradition [6].

The meat fermentation research is based mainly on starter cultures (*Lactobacillus* spp.) of rapid acid-producing lactic acid bacteria (LAB) [7]. The addition of selected strains of starter cultures produce substances, such as lactic acid [8], bacteriocins, hydrogen peroxide, which are antagonistic towards spoilage and pathogenic organisms, like *Y. enterocolitica*, *Escherichia coli* and *Listeria monocytogenes* [6]. Appropriate culture has to be selected according to the specific formulation of the batter and technology of fermentation, since environmental factors will interact the selection of a limited number of strains that are competitive enough

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to dominate the process [9]. *Pediococcus acidilactici, P. pentosaceus* and *L. plantarum* species sometimes found in commercial starter cultures for meats, are rarely detected in large amounts in spontaneously fermented sausage because of their inferior competitiveness, compared to, for instance, *L. sakei* and *L. curvatus* [10]. *L. sakei, L. curvatus, L. plantarum, L. pentosus, L. casei, P. pentosaceus* and *P. acidilactici* are the most used species as commercial meat's LAB starter cultures [11, 5]. The first stage in designing a starter culture for a meat commodity is to characterize the LAB strains isolated from the meat product in question, and then select those best suited. In meat fermentations, the main function of LAB is to obtain a rapid pH drop of the batter, which in turn favours product safety by inactivating pathogens, product stability and shelf life by inhibiting undesirable changes caused by spoilage microorganisms or abiotic reactions, and creates the biochemical conditions to attain the new sensory properties of the ripe products through modification of the raw materials [12]. Currently, the use of starters as functional flora is gaining importance; the designed starter cultures have properties in addition to those of the more classic type, helping to optimize the sausage fermentation process and to produce safer and healthier products [13].

The aim of this study was to examine the effect of starter culture (*L. sakei, Staphylococcus carnosus* and *S. xylosus*) on growth of inoculated *Y. enterocolitica* during fermentation.

2. Materials and Methods

2.1 Bacterial Strain and Preparation of Inoculum

Y. enterocolitica subsp. *enterocolitica* ATCC® 9610™ was used for the inoculation studies. The bioserotype of *Y. enterocolitica* strain was biotype 1 serotype 0:8. A pure culture of *Y. enterocolitica* strain was grown in brain heart infusion (BHI) broth (Merck, Germany) at 30 °C for 24 h to stationary phase. At stationary phase, *Y. enterocolitica* reached a density of approximately 10^8 CFU/mL in the broth. The dilution was used immediately for the inoculation of the ground pork meat samples.

2.2 Meat Preparation

Ground meat was obtained from the local slaughterhouse and prepared in meat laboratory at the Faculty of Veterinary Medicine, University in Belgrade, Serbia. Meat was minced in the sterile grinder (meat grinder TB-300E, Thunderbird Food Machinery, USA). Sausages were prepared according to the specifications of a local manufacturer, by mixing and coarsely mincing pork meat (35%), beef meat (23%) and fat (20%). During mixing, nitrite salt (3%, resulting in 175 ppm sodium nitrite), glucose (1%) and white pepper (0.2%) were added. A commercial starter culture Biostart Sprint ("RAPS GMBH", A-5162 Obertrum, Austria) was used to prepare sausages. This product contains *L. sakei, S. carnosus* and *S. xylosus*, and starter cultures were added and the batter was mixed with gloved hands for 5 min. Starter culture was added 20 g/200 kg of meat.

After mixing, the meat was divided into four equal portions of 10 kg each. The first portion was control group (CI group) without addition of *Y. enterocolitica* and starter culture. The second portion (CII group) was inoculated starter, the third portion (EI group) was inoculated with *Y. enterocolitica*, and the fourth portion (EII group) was inoculated *Y. enterocolitica* and starter culture. Each of sausage batters was mixed for 5 min after inoculation and then stuffed into cellulose casings (Viscofan Cellulose Products, Navarra, Spain) to make links 55 mm in diameter and 150 mm in length.

2.3 Fermentation, Drying and Sampling Sausage

The sausage links were placed in the chamber for squeezing (10 °C, 18 h), then smoked in the smoking chamber (Mauting, UKM 2007E, Czech Republic) for 78 h (20-23 °C). Drying was completed in a Travaglini drying chamber (Milano, Italy) at 17 °C

(75% relative humidity) for 35 d. Sampling was performed by randomly selecting two links of each sausage preparation at the 0, 3rd, 6th, 9th, 15th, 25th, and 35th day of drying.

2.4 Analysis of Sausage

Triplicate samples were recovered and analyzed for chemical and microbiological parameters, pH and water activity. The samples were examined immediately at the time of sampling, except for the analysis of microbiological parameters. These samples were frozen until analysis at a later occasion.

The pH was assayed directly in 3 mL of a sub-sample of BHI-broth or on 10 g of sausage homogenized in 40 mL distilled water using a PCH2401-8 electrode in combination with a meterlab PHM120 pH-meter (Radiometer Analytical SAs, France). The 1:4 dilution was used for practical reasons and the bias introduced is assumed to be small. The dilution may increase the measured pH. The effect of using a 1:4 instead of a 1:1 dilution has been evaluated for shrimps, salmon, minced beef, hard cheese, mayonnaise/vegetable spread and pickled herring and was found to be between 0.01 to 0.12 pH units [14].

The water activity was measured using an aqualab water activity meter series 3 TE (Decagon Devices Inc., USA) on approximately 10 g sausage according to the instructions of the manufacturer.

The water content of the sausages was determined on 5 g sausage samples by drying to constant weight at 105 °C in ceramic bowls.

Ash contents were determined on raw patties following the methods described by Horwitz and Latimer [15]. Protein content was measured with a LECO FP-2000 nitrogen determinator (Leco Corporation, St. Joseph, MI, USA) and fat content was evaluated according to Bligh and Dyer [16].

From each sample, 25 g of sausage was transferred to the stomacher bag (Sampling, Stomacher 400 classic bags, VICOR), then 225 mL maximum recovery diluent (MRD) (Merck, Germany) was added

and content was homogenized for 1 min with a stomacher blender (Stomacher 400 circulator, Seward, UK). For enumeration of LAB, 1 mL of the appropriate 10-fold serial dilution was inoculated into MRS agar (Man, Rogosa and Sharpe, Oxoid, UK, Germany). The MRS plates were incubated at 30 °C for 72 h. The selective medium was used for enumeration of *Y. enterocolitica*, i.e., cefsulodin-irgasan-novobiocin agar (CIN; Yersinia Selective Agar Base CM0653, Oxoid, UK) and *Yersinia* selective supplement (SR0109, Oxoid, UK), and incubated at 30 °C for 24 h. LAB and *Y. enterocolitica* counts were determined on 0, 3rd, 6th, 9th, 15th, 25th and 35th day of drying. 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). 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 [17].

2.5 Statistical Analysis

Statistical analysis of the results were elaborated using software GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA and Microsoft Office Excel 2007. All parameters were represented by descriptive statistical parameters (mean ± standard deviation). One-factor analysis of variance (ANOVA) and post Tukey test were used for testing differences between overall average *Y. enterocolitica* number, *Lactobacillus* spp. and Enterobacteriaceae in all sausage of pork meat during the storage time. All analyses were done in triplicate.

3. Results and Discussion

3.1 Chemical Character of Sausage during the Fermentation

The final moisture content of the fermented sausage was obtained from 29.45% ± 0.20% (EII group) to 29.96% ± 0.19% (CI group). The lipid content was

between 39.51% ± 0.18% (CI group) and 39.92% ± 0.23% (EII group). The protein content in fermented sausage was between 25.33% ± 0.14% (CI group) and 25.38% ± 0.17% (CII group). Group CI had the lowest content of ash (5.19% ± 0.11%) (data not shown). No significant differences in chemical composition between CI, CII, EI and EII group. In the composition of the muscular tissue of adult animals, the proteins (about 18%) come in the second place, after water. These components are important because of their regenerative and growing function in the body [18]. Sausages' minimal protein required by the standars is to be 15% of the finished product weight [18]. The results in the paper showed that in all cases, total protein content of the analyzed samples overcomes the maximum legal limit, constituting a balance in consumers' diet, with excellence in nutritional quality.

The initial pH value of the pork was 6.14 ± 0.05 in all samples (day 0) and decreased during the storage period. Significant differences ($P < 0.01$; $P < 0.05$) were found between the control group (CI and CII group) and experimental groups (EI and EII group) during all samples days, except for at beginning day (Table 1).

Lactobacillus ssp. propagates lactic acid and antagonistic substances that inhibited the growth of spoilage and pathogenic bacteria and also contributed to color formation [12]. Also, in this study the lactic acid produced by LAB reduced the pH, and pH values were lower in sausage with starter cultures.

During the fermentation period, water activity decreased from 0.98 ± 0.0006 (day 0) to 0.93 ± 0.0010 (day 35) in CII and EII group. Also, water activity decreased in CI and EI group (Fig. 1). In this study, no

Table 1   pH value at the storage period in control and experimental groups.

Time (days)	pH value (Mean ± SD)			
	CI	CII	EI	EII
0	6.14 ± 0.05	6.14 ± 0.05	6.14 ± 0.05	6.14 ± 0.05
3rd	5.63[Aa] ± 0.03	5.87[Ab] ± 0.15	5.72[b] ± 0.03	5.78[a] ± 0.03
6th	5.57 ± 0.03	5.53[a] ± 0.03	5.59 ± 0.03	5.55[a] ± 0.03
9th	5.39 ± 0.04	5.32[A] ± 0.05	5.46[A] ± 0.08	5.36 ± 0.06
15th	5.19[ABC] ± 0.03	4.97[A] ± 0.12	5.00[B] ± 0.05	4.98[C] ± 0.04
25th	5.10[a] ± 0.06	5.03 ± 0.12	5.01 ± 0.05	5.00[a] ± 0.04
35th	5.09[a] ± 0.06	5.03 ± 0.12	5.05 ± 0.05	4.98[a] ± 0.04

Means with same uppercase letter within a row are significantly different at $P < 0.01$ and with same lowercase significantly different at $P < 0.05$.
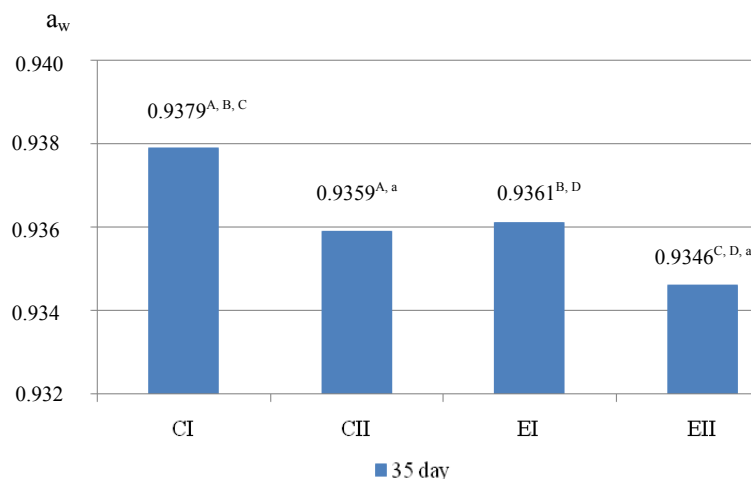

Fig. 1   Water activity in fermented sausages at day 35 of fermentation period.
Means with same capital letter are significantly different at $P < 0.01$ and with same lowercase significantly different at $P < 0.05$.

significant differences ($P > 0.05$) in water activity were observed among the control group and experimental group. These results are similar to results studied by Ceylan and Fung [7]. After 12 d, it was about 0.90, a level that allows most bacteria to survive. Leistner et al. [19] presented the minimum a_w values needed for microorganisms reproduction, such as 0.97 for *Pseudomonas*, 0.95 for *Salmonella*, 0.93 for *Lactobacillus* and *Streptococcus*, 0.90 for *Micrococcus* and *Pedicoccus*.

The final pH of the sausages was 4.98 (EII group) to 5.09 (CI group), typical of low acidity sausages, and this was the result of the classical trend of microbial growth in the fermented sausages, where LAB is increasing in numbers at the very beginning of the fermentations, producing acids and decrease in the pH, followed in the phases of maturation by the activity of micro/staphylococci that are able to neutralize the acids produced. Water activity (a_w) showed a constant decrease during the maturation, and protein, fat, inorganic matter and NaCl content increased during the ripening because of the effect of dehydration.

Comi et al. [20] presented that in all the fermentations followed, the values of pH were about 5.60-5.70 in the final product. The a_w decreased gradually during ripening, reaching values of 0.91-0.92, with a relative humidity (RH) of 40%-46%. The final value of the protein content in all three fermentations was around 20%, while the final fat content was 35%, 27% and 32% for the first, second and third fermentation, respectively. Carbohydrates were not detected anymore after the 7th day of maturation. The inorganic matter reached values of 4.0-4.3 at the end of maturation, and the sodium chloride added at the beginning of the production to the value of 2.5%, increased to 3.3%. The final values for NO_3 and NO_2 were about 10 ppm and 8 ppm, respectively.

3.2 *Y. enterocolitica* in Sausage during the Fermentation

The minced meat was examined before inoculation and it was free of *Y. enterocolitica*. So, in control group *Y. enterocolitica* was not determined. In EI group, initial number of *Y. enterocolitica* was 5.82 ± 0.15 log CFU/g. During the storage, number of *Y. enterocolitica* significantly decreased from 5.82 ± 0.15 log CFU/g to 5.63 ± 0.46 log CFU/g (15th day). After 15th day of storage, *Y. enterocolitica* was not determined in the experimental group (EI) (Table 2). Number of *Y. enterocolitica* decreased during the storage time from 6.16 ± 0.04 log CFU/g to 4.40 ± 0.06 log CFU/g in EI group. Compared with EI group on 15th day of storage, number of *Y. enterocolitica* was numerically lower in EII group. Significant differences ($P < 0.01$) were found between EI and EII group during 0, 3rd, 9th and 15th days of storage (Table 2). After 15th day, *Y. enterocolitica* was both not determined in EI and EII group.

Table 2   Levels of *Y. enterocolitica* in experimental group.

Time (days)	*Y. enterocolitica* level (log CFU/g) Mean ± SD	
	EI	EII
0	5.82[A] ± 0.15	6.16[A] ± 0.04
3rd	5.02[A] ± 0.07	4.64[A] ± 0.10
6th	6.34 ± 0.15	6.47 ± 1.54
9th	5.07[A] ± 0.01	4.53[A] ± 0.01
15th	5.63[A] ± 0.46	4.40[A] ± 0.06
25th	ND	ND
35th	ND	ND

Means with same superscript letter within a row are significantly different at $P < 0.01$. ND: not determined.

3.3 Effect of Using Starter Culture on Chemical Character and Microbial Changes in Sausage during the Fermentation

Few studies have been investigated to inactivate *Y. enterocolitica* in fermented sausage, but the importance of starter cultures and pH value of inactivation has been reported. In Turkish, dry fermented sausage produced using starter culture and inoculated with about 5 log CFU/g *Y. enterocolitica* levels were below detection after 3 d of fermentation, during which pH was reduced from 6.3 to 4.7. In sausage without starter culture, levels of *Y. enterocolitica* were still detectible after 12 d of drying [7]. To eliminate similar levels of *Y. enterocolitica* in fermented sausage within 8 d, the addition of 80-120 mg nitrite/kg was required [21]. *Y. enterocolitica* persisted during 35 d trial, if less than 50 mg nitrite/kg was added [21].

Number of *Lactobacillus* spp. increased from 5.09 ± 0.02 log CFU/g (day 0) to 8.44 ± 0.02 log CFU/g (days 35) in the control group. Significant differences in the number of *Lactobacillus* spp. during the storage were found between CI, CII, EI and EII group ($P < 0.01$) (Table 3).

LAB have been used for thousands of years as starter cultures in the production of fermented foods (e.g., cheese, yoghurt, fermented sausages). The fermentative metabolism causes a desired change in texture, flavour, etc., of the product. In addition, the metabolism has a preservative effect due to the production of antimicrobial substances, such as hydrogen peroxide, organic acids, bacteriocins, etc. [22, 23]. In the production of fermented sausages, fermentation of sugars to lactic acid by homofermentative LAB causes the pH to fall to 4.6-5.3. Together with a reduced water activity ($a_w < 0.95$), microbial stability of the product is ensured [24]. Because of the latter preserving property, there is an increasing interest in the possible use of LAB as protective cultures to control pathogenic bacteria, e.g., *Y. Enterocolitica*, *E. coli*, *L. monocytogenes* [25]. In a spontaneously fermented European sausage, facultative homofermentative lactobacilli constitute the predominant flora, *L. sakei* and *L. curvatus* generally dominate the fermentation process [26].

With a commercial scale fermentation process, starter cultures of *L. sake* and *P. acidilactici* in sausage completely eliminated *Y. enterocolitica* after 4 d of fermentation. A quick reduction of the pH levels of the lactic acid produced was the most important parameter in the inhibition of *Y. enterocolitica* [27]. Present of salt and nitrite also can reduce the number of *Y. enterocolitica* in the fermented sausage. In this study, *Y. enterocolitica* was inhibited after 15 d of storage. Number of Enterobacteriaceae decreased in the control group, and after 21 d, they were not determined by the control group. Significant differences in number of Enterobacteriaceae during storage were found between the control group and experimental group ($P < 0.01$; $P < 0.05$) (Table 4).

Table 3 Levels of *Lactobacillus* spp. in strorage period in control and experimental group.

| Time (days) | *Lactobacillus* spp. level (log CFU/g) Mean ± SD | | | |
	CI	CII	EI	EII
0	5.09[AB] ± 0.02	6.23[ACD] ± 0.08	5.20[CE] ± 0.09	6.66[BDE] ± 0.08
3rd	5.09[ABC] ± 0.02	8.28[ADa] ± 0.38	6.64[BDE] ± 0.01	8.63[CaE] ± 0.01
6th	7.38[ABC] ± 0.84	8.34[A] ± 0.09	8.55[B] ± 0.09	8.30[C] ± 0.09
9th	8.37[A] ± 0.01	7.33[A] ± 0.94	7.97 ± 0.09	8.08 ± 0.18
15th	9.34[A] ± 0.04	8.00[ABC] ± 0.04	9.11[B] ± 0.09	9.15[C] ± 0.47
25th	8.33[AB] ± 0.06	8.97[ACD] ± 0.13	8.56[BCE] ± 0.48	8.26[DE] ± 0.33
35th	8.44[ABC] ± 0.02	8.09[ADE] ± 0.04	7.94[BDa] ± 0.01	7.99[CEa] ± 0.01

Means with same capital letter within a row are significantly different at $P < 0.01$, and with same lowercase within a row are significantly different at $P < 0.05$.

Table 4 Levels of Enterobacteriaceae in strorage period in control and experimental group.

Time (days)	Enterobacteriaceae levels (log CFU/g) Mean ± SD			
	CI	CII	EI	EII
0	$4.64^a \pm 0.32$	$4.50^A \pm 0.05$	$5.29^{aA} \pm 0.06$	4.80 ± 0.53
3rd	3.84 ± 0.01	4.41 ± 0.03	5.46 ± 0.03	4.63 ± 0.08
6th	$5.28^{AB} \pm 0.09$	$4.20^{ACD} \pm 0.10$	$5.20^{CE} \pm 0.03$	$6.63^{BDE} \pm 0.01$
9th	$5.34^{AB} \pm 0.007$	$5.30^{CD} \pm 0.007$	$5.90^{ACE} \pm 0.11$	$6.40^{BDE} \pm 0.12$
15th	4.01 ± 0.04	3.85 ± 0.02	4.56 ± 0.11	2.41 ± 0.12
25th	ND	ND	ND	ND
35th	ND	ND	ND	ND

Within a row, means with same capital letter are significantly different at $P < 0.01$, and with same lowercase significantly different at $P < 0.05$.

Inactivation of pathogens during maturation is, in addition to the control of growth and initial pathogen level, a crucial step in the safe production of fermented sausage. The present results indicate that the reduction of *Y. enterocolitica* levels in fermented sausage may be satisfactory when starter cultures (LAB) added to fermented sausage.

The value of pH, in the range 4.2-6.0 is limiting factor affecting the persistence and competitiveness of the starter culture over the entire fermentation process [13]. *L. sakei* can grow at 4 °C, in the presence of 6.5% NaCl and at pH 4.2 [28]. At 15 °C and in the presence of 2% NaCl, this meat-borne LAB shows growth rates, which allow production of 0.55 generations/h [28]. Its psychrotrophic character and salt tolerance may be due to its ability to efficiently accumulate osmo- and cryoprotective solutes, such as betaine and carnitine, and to its cold stress response; *L. sakei* has more putative cold-stress genes than any other lactobacilli [29].

Models of Little et al. [29] taking not only pH and temperature into consideration, but also the amount of lactic acid concentration [30] as well as the undissociated lactic concentration, and microbiological interactions with starter cultures have also been presented [31, 25].

Raccach and Henningsen [32] found that LAB controlled the growth of *Y. enterocolitica* O:3 and O:8 in meat. At 27 °C, a growth of *Y. enterocolitica* O:3 and O:8 after 12 h of incubation was reduced of 3.6 log CFU/g and 2.0 log CFU/g by sodium chloride (3%)

and sodium nitrite (0.0156%), respectively. The lactic acid produced by LAB was a major factor. In the nature fermentation, the major inhibition factors that reduced the number of *Y. enterocolitica* were sodium chloride, nitrite and lactic acid.

The microbial changes during sausage ripening showed very good growth of mesophilic LAB in all samples, corresponding to a pH decrease. As known, the final period of dry sausages ripening is characterized by the colonization on the sausages surface of abundant moulds, which oxidise lactate with consequent increase of pH [33]. *S. carnosus* and *S. xylosus* are the most recommended staphylococcal starter culture for dry sausage production in Europe [34]. Most *S. xylosus* strains showed technological properties that would make them eligible as starter cultures for fermented meat products [10].

4. Conclusions

Fermentation process using starter cultures, e.g., *L. sakei*, *S. carnosus* and *S. xylosus*, low pH value and low water activity were effective in inactivation of *Y. enterocolitica* in fermented sausage. Lactic acid was the major factor in the elimination of *Y. enterocolitica*. Screening of starter culture can improve safety and quality of fermented sausage, free from pathogenic bacteria.

Acknowledgments

Animal Origin and the Control Measures from Farm to Consumer" (31034; project leader professor Sava Buncic), the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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