Microbial characterization of horse meat dry sausage

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scientific paper

Summary

The aim of this study was to evaluate microbiological changes of traditionally produced horse meat sausages depending on maturing phases and season, to determine lactic acid bacteria and to research their inhibitory potential towards L. monocytogenes. Production season influenced significantly on total viable count, lactic acid bacteria, coagulase-negative cocci, enterococci and yeasts in final product (p<0.05). Lactic acid bacteria represented the most numerous microbial population, and significant number of yeasts and coagulase-negative cocci was also determined. Lactobacillus plantarum was determined as the dominant species of lactic acid bacteria population (56 %), while Weisella confusa (26 %), Lactobacillus fermentum (6 %), Lactobacillus pentosus (6 %), Lactococcus lactis subsp. lactis (2 %), Lactobacillus delbrueckii subsp. delbrueckii (2 %) and Weisella viridescens (2 %) were also isolated. Lactobacilli showed the strongest inhibitory effect towards L. monocytogenes in vitro. The results of this research may contribute to better understanding of specific issues of horse meat fermented sausages production compared to sausages produced from other sorts of meat and they can be used for sausage production standardization.

***Key words**: horse, dry sausage, lactic acid bacteria, inhibition*

Introduction

Fermented sausages, as commercially most valuable meat products, are produced from the best parts of meat of different animal species. Horse meat, according to the chemical composition and processing value, is a qualitative basic ingredient for such a production, and produced fermented sausages are products of special quality and value (Feiner, 2006). Horse meat fermented sausages production is not industrialized, which additionally contributes to their value due to the complexity of production and influence of multiple factors in forming a recognizable product. During the process of maturing of fermented sausages, a complex microbiological, physical-chemical and biochemical processes are being developed giving the product final sensorial characteristics. These processes are influenced by numerous factors, hygienic quality of meat and production process being an important one because of the influence on initial microbiota composition (Hutkins, 2006). Beside that, an important role also belongs to sort of meat used for the production due to its water content, protein content, percentage of fat tissue, amount of glycogen and other characteristics influencing sensory properties of final product (color, toughness, juiciness etc.) (Incze,1998). Changes during maturation are beneficial for the development of specific microbiota which contributes with its sacharolytic, proteolytic, lipolytic and microbial activity to progress of the same changes.

The aim of this paper was to evaluate microbiological changes during maturation of naturally fermented horse meat sausages, depending on maturation phases and the season, to determine lactic acid bacteria involved and to research their inhibitory potential towards *Listeria monocytogenes*.

Material and methods

Sausage production and sampling

Horse meat sausages were produced in a private small meat processing enterprise according to the standard production procedure during April, September and November. Sausages are made of horse meat (75%), pork firm fat tissue (25%) and added ingredients (salt, black ground pepper, red minced pepper, garlic, nitrite salt). After filling in the natural casing and draining on the sticks, cold smoking followed for 3 days and then ripening in the fermentation chamber for 33 days. Sampling was done every time with three sausages sampled on the day 0, 14, 28 and 36. At the beginning of production process sampling was done also for the raw material (horse meat and pork fat tissue). The samples were transported to the laboratory in a portable refrigerator (+4 °C). All samples were analyzed for microbiological analyses in triplicate.

Table 1 **Methodology of microbiological analyses**

Parameter	Growth medium	Incubation
Aerobic mesophilic bacteria	Plate Count Agar (PCA)	30 °C / 72 hours
Lactic acid bacteria	De Man Ragosa Sharpe agar (MRS)	30 °C / 72 hours
Coagulase-negative cocci	Manitol Salt Agar (MSA)	30 °C / 48 hours
S. aureus	Baird-Parker agar (BP)	37 °C / 24-48 hours
Enterobacteria	Violet red bile glucose agar (VRBG)	37 °C / 24 hours
E. coli	Coli ID	37 °C / 24 hours
Yeasts and moulds	Oxytetracycline yeast agar with tetracycline (OGY)	25 °C / 3-5 days
Enterococci	Kanamycin esculin agar (KEA)	37 °C / 48 hours
Pseudomonas spp.	Cetrimide-fucidin-cephaloridine (*CFC*) agar	25 °C / 48 hours
Sulphite reducing clostridia	Sulphyte Polymixine Suphadiazine agar (SPS)	37 °C / 5 days
Salmonella spp.	Buffered peptonic water Rappaport-Vasiliadis broth Muller-Kauffman tetratyonate/novobiocin broth Brilliant fenol lactose sugar agar (BPLS) XLD	37 °C / 16 hours 42 °C / 24 hours 37 °C / 48 hours 37 °C / 24 hours 37 °C / 48 hours
L. monocytogenes	Half-Fraser broth Fraser broth Palcam RAPID'*L. mono*	30 °C / 24-48 hours 37 °C / 24 hours 37 °C / 24 hours 37 °C / 24 -48 hours

Table 2 **Aerobic mesophilic bacteria count (log_{10}CFU/g) during the ripening of horse meat sausage**

Parameter	Series (Month)	Days 0. X	± SD	14. X	± SD	28. X	± SD	36. X	± SD
Aerobic mesophilic bacteria (log_{10}CFU/g)	April	7.04^{a}	± 0.04	8.61^{a}	± 0.04	8.26^{a}	± 0.12	7.58^{a}	± 0.12
	September	7.33^{a}	± 0.05	8.57^{a}	± 0.47	8.80^{ab}	± 0.06	8.72^{a}	± 0.08
	November	5.95^{a}	± 0.04	8.27^{a}	± 0.04	8.35^{b}	± 0.04	8.60^{a}	± 0.05

^{a,b} within the same column, values marked with the same letter are statistically significantly different (p<0.05)

Table 3 **Lactic acid bacteria count (log_{10}CFU/g) during the ripening of horse meat sausage**

Parameter	Series (Month)	Days 0. X	± SD	14. X	± SD	28. X	± SD	36. X	± SD
Lactic acid bacteria (log_{10}CFU/g)	April	4.07^{a}	± 0.04	8.57^{a}	± 0.06	8.00^{a}	± 0.09	7.01^{a}	± 0.07
	September	4.20^{a}	± 0.04	8.87^{a}	± 0.07	8.68^{a}	± 0.05	7.02^{b}	± 0.07
	November	4.29^{a}	± 0.04	7.87^{a}	± 0.04	8.35^{a}	± 0.04	8.60^{ab}	± 0.05

^{a,b} within the same column, values marked with the same letter are statistically significantly different (p<0.05)

Microbiological analyses

Microbiological analyses of raw material and sausages during maturation were done to determine the number of aerobic mesophilic bacteria, lactic acid bacteria, coagulase-negative cocci, *Staphylococcus aureus*, enterobacteria, *Escherichia coli*, yeasts and moulds, enterococci, *Pseudomonas* spp., sulphite reducing clostridia, and presence of *L. monocytogenes* and *Salmonella* spp. Methodology of microbiological analyses are presented in Table 1.

Determination of lactic acid bacteria and their antimicrobial activity

A selection of 75 colonies of lactic acid bacteria was done for further determination. Colonies were Gram stained and then tested for catalase activity. In the procedure of determination of antimicrobial activity of isolate, only gram-positive, catalase negative bacilli and coccobacilli were taken into account (n=50). Isolates were multiplied in MRS broth (Merck) for 24-48 hours on 30°C for biochemical determination. Culture was then plated on MRS agar and incubated on 30°C for 48-72 hours. After that, one isolated colony was taken and plated on the surface of MRS agar and then incubated again on 30°C for 24 hours. Further procedure was done according to directions of API system producer. Anti-

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microbial effect was tested on bacteria *L. monocytogenes* NCTC 10527 using agar spot and agar diffusion test (Zdolec et al., 2009). As a positive control in the test a *Leuconostoc mesenteroides* E131 strain was used (Drosinos et al., 2006) which synthesizes bacteriocin mesenterocin Y. As a negative control strain *Lactobacillus brevis* ATCC B287 was used.

Statistical data analysis

Collected data were processed statistically by using program Statistica 8 (StatSoft, 2008). Basic statistical data processing was followed by checking normal distribution with Kolmogorov-Smirnov test (K-S test). One-way analysis of variance (one way ANOVA) was used for determination of significance of differences during sausage maturing for every season, and also Tukey HSD test for post-hoc analysis.

Results

The results of microbiological analyses during sausage maturation are presented in Tables 2-6. Total viable count significantly increased till the 14th day of maturation in all series of sausages (Table 2). Bacterial count in the sausages produced in September and November increased continuously till the end of maturing, while in the first series of sausages (April) a decreased number of bacteria for 1 log was determined after 14th day of maturing. Statistically significant differences (P<0.05) between series can be noticed when considering the number of bacteria in relation to the season of production.

Lactic acid bacteria count increased in all series till the 14th day of maturation (p<0.01). In the first two series (April and September) the number of lactic acid bacteria decreased afterwards to the end of maturation (1.5 and 1.8 log respectively), while in the third series (in November) the population was in constant increase. Significant differences in LAB counts were found in relation to season during the whole ripening period (p<0.05), except among the final products from April and September (Table 3). As shown in table 4, the population of coagulase-negative staphylococci was stabile during maturation in all series (>4 $\log_{10}$ CFU/g). Statistically observed, their final number significantly decreased in series produced in April and September, while in November the number increased (p<0.01). Significant differences are observed (p<0.05) in their number in relation to season of production according to days of maturation. The final number of coagulase-negative cocci in April was significantly lower than in sausages produced in September and November. Population of enterococci, despite relatively high initial count, decreased towards the end of production process in sausages produced during April and November (for approximately 1 log; p<0.01). During sausage maturation from the second series (in September) the number of enterococci increased till the 28th day and then slightly decreased towards the end of maturation, so that the number of enterococci stayed stabile, being actually increased in relation to the beginning of maturation process (p<0.01). The number of enterococci in sausages produced in September stayed statistically significantly higher (p<0.05) in the second phase of maturation in relation to the other two series of sausages. The number of enterococci in that period of maturation did not statistically differ in sausages from April and November (Table 5). Population of yeasts and moulds decreased during sausage maturation independently of the season. Table 6 shows that the level of decreased number and number of yeasts and moulds in final product was depending on initial population (p<0.01). When comparing the number of yeasts and moulds depending on season, it is noticed that in the second phase of maturation the number of yeasts and moulds was statistically lower in sausages produced in April, while their number was not statistically different when comparing the sausages produced in September and November (p>0.05). *S. aureus* was not determined during sausage maturation produced in April and November, while during September there was a small number registered (2.18 $\log_{10}$ CFU/g), being below detection level (<2 log) in further maturation. Sulphite - reducing clostridia were present till the 14th day in the first series (April), and on day 0 in the September series. The following LAB strains were determined (n=50) from horse meat sausages with API 50 CHL test: *Lactobacillus plantarum* (n=28; 56 %), *Weisella confusa* (n=13; 26 %), *Lactobacillus fermentum* (n=3; 6 %), *Lactobacillus pentosus* (n=3; 6 %), *Lactococcus lactis* subsp. *lactis* (n=1; 2 %), *Lactobacillus delbrueckii* subsp. *delbrueckii* (n=1; 2 %), *Weisella viridescens* (n=1; 2 %). The results of investigation of inhibitory effect of isolate towards *L. monocytogenes* showed that all strains of *L. plantarum* suppress the growth in agar spot test. Inhibition was also determined with action of other lactobacill strains, like *L. fermentum*, *L. pentosus* and *L. delbrueckii* subsp. *delbrueckii*. On the other hand, inhibition was not recorded using *W. confusa*, *Lc. lactis* subsp. *lactis* and *W. viridescens*. Further on, there was no inhibition recorded with neutralization of supernatants for any of the tested strains.

Table 4 **Number of coagulase - negative cocci ($\log_{10}$CFU/g) during horse meat sausage maturation**

Parameter	Series (Month)	Days											
		0.			14.			28.			36.		
		X	±	SD	X	±	SD	X	±	SD	X	±	SD
Coagulase-negative cocci ($\log_{10}$CFU/g)	April	4.58^{a}	±	0.06	4.55^{a}	±	0.08	4.30^{a}	±	0.11	3.77^{a}	±	0.07
	September	4.90^{a}	±	0.05	4.30^{ab}	±	0.05	3.78^{a}	±	0.05	4.68^{a}	±	0.04
	November	4.43^{a}	±	0.05	4.50^{b}	±	0.08	4.39^{a}	±	0.04	4.88^{a}	±	0.08

^{a,b} within the same column, values marked with the same letter are statistically significantly different (p<0.05)

Table 5 **Number of enterococci ($\log_{10}$CFU/g) during horse meat sausage maturation**

Parameter	Series (Month)	Days											
		0.			14.			28.			36.		
		X	±	SD	X	±	SD	X	±	SD	X	±	SD
Enterococci ($\log_{10}$CFU/g)	April	4.60^{a}	±	0.07	4.02^{a}	±	0.18	3.69^{a}	±	0.08	3.51^{a}	±	0.04
	September	4.51^{a}	±	0.07	4.98^{a}	±	0.10	5.01^{ab}	±	0.10	4.84^{ab}	±	0.04
	November	4.27^{a}	±	0.09	4.48^{a}	±	0.05	3.75^{b}	±	0.09	3.48^{b}	±	0.06

^{a,b} within the same column, values marked with the same letter are statistically significantly different (p<0.05)

Table 6 **Number of yeasts and moulds ($\log_{10}$CFU/g) during horse meat sausage maturation**

Parameter	Series (Month)	Days											
		0.			14.			28.			36.		
		X	±	SD	X	±	SD	X	±	SD	X	±	SD
Yeasts and moulds ($\log_{10}$CFU/g)	April	4.65^{a}	±	0.06	4.35^{a}	±	0.09	4.42^{ab}	±	0.02	4.17^{ab}	±	0.11
	September	5.61^{a}	±	0.04	5.84^{a}	±	0.03	5.50^{a}	±	0.05	5.44^{a}	±	0.06
	November	6.26^{a}	±	0.11	5.67^{a}	±	0.05	5.50^{b}	±	0.06	5.46^{b}	±	0.07

^{a,b} within the same column, values marked with the same letter are statistically significantly different (p<0.05)

Discussion

The process of maturation and physical-chemical changes in fermented sausages is suitable for multiplication of certain groups of microorganisms, with halofilic, osmofilic and acidofilic characteristics (Hutkins, 2006). These are primarily lactic acid bacteria as the most numerous/most active representatives, then coagulase-negative cocci (micrococci, staphylococci), and yeasts and moulds (Huerta et al., 2004; Kozačinski et al., 2008; Cocolin et al., 2009). During maturation we determined multiple increase of lactic acid bacteria population (from 4 $\log_{10}$ CFU/g in the beginning to 8 $\log_{10}$ CFU/g), that was expected and in correspondence with data from professional literature regarding the dynamics of their growth in fermented sausages from other sorts of meat (Fontana et al., 2005; Kozačinski et al., 2006; Drosinos et al., 2007; Zdolec et al., 2008; Alagić et al., 2009). Proteolytic and lipolytic changes in raw material are influenced, beside tissue enzymes, by staphylococci, micrococci, yeast and mould activity (Metaxopoulos et al., 2001; Ferreira et al., 2007). Table 6 shows a stabile population of yeasts which did not change much during sausage maturation, and also a stabile number of coagulase – negative cocci (Table 4), which tended to grow in the second phase of maturation (except in April). The composition and dynamics of the population of these micro-organisms are similar to the results of investigation of fermented sausages of other authors (Ferreira et al., 2007; Zdolec et al., 2008). As already mentioned, maybe the most controversial group of micro-organisms in fermented sausages represent enterococci, since they express beneficial and non beneficial characteristics in the context of quality and safety of these products (Franz et al., 2003; Barbosa et al., 2009; Jofré et al., 2009). Our investigation showed significant differences in their number depending on the season of sausage production, which can be more or less correlated with the microbiological quality of raw material, but also with the hygiene of sausage production. Hugas et al. (2003) found that the number of enterococci in fermented sausages can vary a lot, depending on the sort of sausages and the season of production, and even emphasize the possibility of differences in the number of enterococci in sausages of the same production batch. The differences in the number of enterococci in our samples support the findings of other authors which determined either decrease or stagnation of their number during maturation (Urso et al., 2006; Zdolec et al., 2008). The results of our investigation show that horse meat fermented sausages are microbiologically safe in the sense of number of pathogenic bacteria. That is confirmed by the results of other authors who studied microbiota of regular sausage production (Drosinos et al., 2005; Kozačinski et al., 2006), or sausages inoculated with pathogens such as *L. monocytogenes* (Johnson et al., 1998; Zdolec et al., 2007ab). However, the results of investigation of Encinas et al. (1999), Colak et al. (2007) and other authors point out the possibility of presence of *L. monocytogenes* in final products (sudžuk, chorizo). Determination of LAB fermentation profiles showed the domination of *L. plantarum* which is often found in fermented sausages (Drosinos et al., 2005; Kozačinski et al., 2006), but the majority of authors state that the most numerous and adaptable lactobacilli in that substrate are *L. sakei* and *L. curvatus* (Hammes, 1990; Rantsiou et al., 2005). However, the results of procedures of determination depend very much on the

methodology applied (Quere et al., 1997; Vermeiren et al., 2004). So the results gained by using biochemical test API 50 CHL should be carefully explained because the test does not cover *L. sakei* profile, which is, as it is stressed, the most important and the most numerous lactobacillus in meat fermentation. In that sense, it could be assumed that also isolates *L. fermentum* belong to *L. sakei/L. curvatus* group, as commented by Vermeiren et al. (2004). Beside *L. plantarum*, other lactobacilli species were determined in a small percentage, which corresponds to literature data (Parente et al., 2001; Gasparik-Reichardt et al., 2005; Drosinos et al., 2005). The results of investigation of inhibitory action of isolates towards *L. monocytogenes* confirm the well-known inhibitory potential of lactobacilli (Zdolec et al., 2007c; Zdolec et al., 2009). Further characterization of the dominant *L. plantarum* should be done in order to select most appropriate strains for practical applications in food or animal models (Marcinčák et al., 2009; 2010).

Conclusion

During horse meat sausage maturation lactic acid bacteria were determined as the most numerous microbial population, as well as a significant number of yeasts and coagulase–negative cocci. The season of production significantly influenced on the total number of bacteria, the number of lactic acid bacteria, coagulase – negative cocci, enterococci and yeasts in final product (P<0.05). The dominant strain in lactic acid bacteria group was *L. plantarum*. Among isolated LAB, lactobacilli strains had the strongest anti-listerial effect in the laboratory conditions.

Acknowledgements

This research was conducted as part of a project of the Ministry of Science, Education and Sport of the Republic of Croatia (053-0531854-1853). We thank to Zvonko Šanjek, who enabled the pilot production of sausages in his facility.

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Mikrobiologische Charakterisierung der Dauerwürste aus Pferdefleisch

Zusammenfassung

Das Ziel dieser Arbeit war, mikrobiologische Änderungen in Dauerwürsten aus Pferdefleisch in Bezug auf die Reifephasen und Herstellungssaison zu untersuchen, die Bakterien der Milchsäure zu determinieren und ihre inhibitorische (verhindernde) Leistungsfähigkeit gegen Bakterie listeria monocytogenes zu überprüfen. Die Herstellungssaison hatte einen bedeutenden Einfluss auf die gesamte Bakterienzahl, Bakterienzahl der Milchsäure, Koagulation der negativen Kokken, Enterokokken und Hefe im Fertigprodukt (p<0,05). Die Bakterien der Milchsäure waren die zahlreichste Mikrobenpopulation in der Füllung, nebst bedeutender Zahl von Hefe und Koagulation der negativen Kokken. Die häufigst isolierte Bakterienart war lactobacillus plantarum (56 %), weisella confusa (26 %), lactobacillus fermentum (6 %), lactobacillus pentosus (6 %), lactococcus lactis subsp. lactis (2 %), lactobacillus delbrueckii subsp. delbrueckii (2 %) und weisella viridescens (2 %). Die stärkste inhibitorische Wirkung gegen l. monocytogens in vitro zeigten die Isolaten von Lactobacillen. Die erzielten Resultate können einer besseren Verständigung der Spezifität der fermentiereten Würste aus Pferdefleisch in Bezug auf andere Fleischsorten im Verfahren der Herstellungsstandardisation dienen.

Schlüsselwörter: *Pferde, Dauerwürste, Bakterien der Milchsäure, Inhibition (Hemmung)*

Situazione microbiologica delle salsicce di lunga durata fatte da carne di cavallo

Somario

Questo lavoro voleva indagare i cambiamenti morfologici nelle salsicce di lunga durata fatte da carne di cavallo, a seconda delle fasi di maturazione e della stagione di produzione, ma voleva anche determinare i batteri dell'acido lattico ed esaminare la loro potenziale inibitoria nei confronti del battero Listeria monocytogenes. La stagione di produzione ha fortemente influito sul numero totale di batteri, il numero di batteri dell'acido lattico e la coagulasi di cocchi negativi, gli enterococchi ed i lieviti nel prodotto finale (p<,0,05). I batteri dell'acido lattico erano la più numerosa popolazione in ripieno, ma c'era presente anche un numero di lieviti e la coagulasi di cocchi negativi. La più spesso isolata specie di batteri di acido lattico era il Lactobacillus plantarum (56 %), e poi la Weisella confusa (26 %), il Lactobacillus fermentum (6 %), il Lactobacillus pentosus (6 %), il Lactococcus lactis subsp. lactis (2 %), il Lactobacillus delbrueckii subsp. delbrueckii (2 %) e la Weisella viridescens (2 %). Gli isolati di lattobacili hanno rivelato la più forte azione inibitoria verso il L. monocytogenes in vitro. I risultati ottenuti potrebbero essere utilizzati per una migliore comprensione del fatto che la fermentazione di salsicce fatte da carne di cavallo è specifica per quanto riguarda gli altri tipi di carne, ma potrebbero anche essere utili nel processo stesso della standardizzazione di produzione.

Parole chiave: *cavalli, salsicce di lunga durata, batteri dell'acido lattico, inibizione*

Received: July 15, 2011
Accepted: September 24, 2011