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Comparison of Sensory Properties, Shelf-Life and Microbiological Safety of Industrial Sausages Produced with Autochthonous and Commercial Starter Cultures

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Summary

The aim of this research is to use isolated and characterized autochthonous functional starter cultures from traditional Croatian dry sausages and to evaluate their capacity for industrial production of five sausages (Čajna sausage, Zimska sausage, Bečka sausage, Srijemska sausage and Slavonski kulen). These defined autochthonous functional starter cultures (combination of *Lactobacillus* and *Staphylococcus* strains) were used to produce five different industrial sausages which were compared by a panel. The viability of introduced autochthonous *Lactobacillus* and *Staphylococcus* strains and their effect on the final product characteristics, namely microbiological, physicochemical and sensory properties were monitored. The obtained results indicate that autochthonous starter cultures survived industrial production of sausages and can be used for production of sausages under controlled conditions. Autochthonous starter cultures obtained better results in the organoleptic evaluation, microbial safety and prolonged shelf-life in comparison with commercial starter cultures.

Key words: autochthonous starter cultures, sausage production, sensory properties, lactic acid bacteria, staphylococci

Introduction

Naturally fermented sausages are traditional Mediterranean meat products with a large diversity within different regions. Their manufacture can be divided into three stages. In the sausage preparation period, raw materials and ingredients are comminuted and blended, and then the mixture is stuffed into casings. In the fermentation period, lactic acid bacteria are responsible for lactic acid production and pH decrease (*1*). In the subsequent ripening period, the sausage is further dried and develops final texture and flavour. Although the utilization of commercially defined starter cultures is standardized in the industrial production, many manufacturers still produce naturally fermented sausages since they possess a typical sensory quality attributed to the specific composition and metabolic activity of the autochthonous microflora, which is in this case combined with the activity of endogenous meat enzymes. Moreover, commercial starter cultures are often outgrown by the autochtho-

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nous microflora so the final sensory properties of the product are not necessarily the result of their activity. Hence, there is a great need for defined autochthonous functional starter cultures featuring growth and fermentation capabilities which will result in industrial production of sausages with traditional sensory properties. The main challenge in the selection of starter cultures is to improve food safety, and also to preserve the typical sensory quality of traditional sausages (*1,2*). The most promising microorganisms for starter cultures are those selected from autochthonous microflora since they are well adapted to the meat environment and to the specific manufacturing process and are capable of dominating the microbiota of the product due to their specific metabolic capabilities. An increasing number of studies have focused on the isolation and identification of autochthonous functional starter cultures, with the aim of developing new functional probiotic meat products, which will be recognized and labelled as autochthonous due to the influence of climate and vegetation of the region where they are produced (*1,3,4*). The use of commercial starter cultures in the industrial production of sausages results in the loss of typical regional characteristics due to the replacement of the complex native microflora with a defined starter culture. Hence, an application of the autochthonous starter cultures might guarantee the improved quality and safety of this product by preserving typical sensory quality of the traditional fermented product and inhibiting the growth of undesirable microorganisms while simultaneously preserving the characteristics that define the identity of traditional sausages (*1,5*). Starter cultures have to be selected to suit the conditions used by different industries, such as ripening time, temperature, relative humidity, pH, NaCl, and the type of raw meat and other ingredients (*6*).

Therefore, the aim of this study is implementation of well-defined functional autochthonous starter cultures (*1*) for the industrial production of five different sausages (Čajna sausage, Bečka sausage, Srijemska sausage, Zimska sausage and Slavonski kulen) to obtain authentic aroma that cannot be achieved with standard commercial starter culture. The primary objective of this study is to evaluate whether the autochthonous starter cultures survive controlled conditions (temperature, relative humidity) for industrial production of fermented meat products, since the production of most indigenous and traditional products is not standardized and it is not carried out under controlled conditions. Effects of the added autochthonous functional starter cultures on microbiological, physicochemical and sensory properties and shelf-life of five sausages were also monitored.

Material and Methods

Microorganisms

Bacterial strains *Lactobacillus plantarum* 1K and *Staphylococcus carnosus* 4K1 originally isolated from traditional Croatian fermented sausages were characterised as functional (*1,7–12*) and used as starter cultures for industrial sausage production. Bacterial cultures were obtained from the collection of microorganisms of the Laboratory for General Microbiology and Food Microbiology, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia.

Lactobacillus plantarum 1K and *Staphylococcus carnosus* 4K1 were stored at –70 °C in de Man-Rogosa-Sharpe (MRS) broth (Difco™, Detroit, MI, USA) and nutrition broth (Biolife, Milano, Italy) with 30 % (by volume) glycerol, respectively. The strains were activated in the same corresponding broths and maintained at 4 °C.

Identification of autochthonous starter cultures

Matrix-assisted laser desorption-ionization time-of--flight mass spectrometry (MALDI-TOF MS) was used for re-identification of the used autochthonous starter cultures because previous identification of these strains was done only with biochemical API test. For MALDI--TOF MS analysis, samples were prepared using the procedure recommended by the manufacturer (Bruker Daltonik, Bremen, Germany). Measurements were made using a Microflex LT™ instrument (Bruker Daltonik). For the identification, the peaks from the generated mass spectra were compared with reference spectra of the integrated database using MALDI Biotyper Software package. Official identification of the two strains was done by AFLP DNA fingerprinting and *dnaJ* sequence analysis from BCCM™/LMG Identification Service (Ghent, Belgium).

Preparation of wet biomass

The LAB were grown in MRS broth at 30 °C for 48 h and staphylococci in nutrition broth at 37 °C for 48 h. The bacterial cells were harvested under aseptic conditions by centrifugation (6000×*g*, 10 min) at room temperature, washed three times in saline water (0.5 %) and resuspended in sterile saline water. The total viable count (TVC) was performed by the standard dilution method on MRS (Difco™) and nutrition (Biolife) agar after incubation at 37 °C for 48 h. The final number of 10^{11} viable bacterial cells of *L. plantarum* 1K per g of wet biomass, and final number of 10^7 viable bacterial cells of *S. carnosus* 4K1 per g of wet biomass were obtained.

Commercial starter culture

Commercial starter cultures BITEC LS-25 ($1.5 \cdot 10^{12}$ per 100 g) containing dry biomass of *Lactobacillus sakei* and *Staphylococcus carnosus* were obtained from Frutarom, Savory Solutions GmbH, Korntal-Münchingen, Germany.

Sausage manufacture

Five types of fermented sausages: Čajna, Zimska, Bečka, Srijemska and Slavonski kulen were prepared with commercial starter cultures in a local meat factory according to industrial practices, and used as control. Samples of the same types of fermented sausages were produced with autochthonous starter cultures, containing *L. plantarum* 1K and *S. carnosus* 4K1 strains, and inoculated into the meat mixture of sausages as wet inocula.

A 400-kg batch of each type of sausage (Čajna, Zimska, Bečka, Srijemska and Slavonski kulen) consisted of lean pork meat, fat pork meat, beef, nitrate salt, additives and spices according to the manufacturer's specification (unknown in details). Frozen fat, beef and pork meat were ground at 0 °C in a cutter. This was then

mixed with the rest of the ingredients and inoculated with final count of *L. plantarum* 1K in the range from $3.83 \cdot 10^7$ to $5.24 \cdot 10^8$ cells per g and *S. carnosus* 4K1 from about 10^3 to 10^5 cells per g. The prepared cooled mixtures (–2 to –3 °C) for Čajna, Srijemska, Bečka and Zimska were stuffed into collagen casings 380 mm long, with the inner diameter of 35 mm for Čajna and Srijemska sausages, and 50 mm for Bečka and Zimska. Each type of sausage was put on a trolley and left in cold (below 8 °C) to rest for 12–15 h. The ripening process of Zimska and Bečka sausages proceeded as follows: the first stage of straining was done at 24 °C and 85 % relative humidity (RH) until the temperature in the middle of the sausages was reduced to 19–20 °C. The sausages were placed in a drying chamber until the casing was completely dry. Then, the sausages were smoked, at the end of the smoking process, the temperature was progressively decreased from 18 to 16 °C, and the humidity increased to 88 %. Sausages were then left to ripen for 15 days with the control of humidity, temperature and pH (the parameters are known to the manufacturer) and the mass reduction, which was between 35 and 40 % depending on the type of sausage, was the indicator of the end of ripening. The stages of straining, smoking and ripening of Čajna and Srijemska sausages were the same as of Zimska and Bečka, but lower mass reduction of 28 or 30 % indicated the end of the ripening. The final products were vacuumed, declared, labelled, packaged and stored in a chilling chamber in the dark.

Manufacture of Slavonski kulen is different from other sausage manufacturing. During the stuffing of cooled mixture (–1 °C), casings should be filled 10 % over the diameter, in order to obtain a better final product. After stuffing into collagen casings with inner diameter of 50 mm, the kulen was put on a trolley and left overnight to rest at 8 to 14 °C. Straining stage was done at 22 to 23 °C and 85 % RH until the temperature in the middle of the sausage was reduced to 21 °C. Kulen was placed in a drying chamber at the RH of 83–88 % (it can be reduced to 80–85 % to speed up the drying process), and temperature of 20–22 °C. After drying, the kulen was smoked for about 40 h at RH between 83 and 88 % and temperatures between 18 and 20 °C. In the ripening stage, humidity and temperature progressively decreased so that the casing would not dry up and also to prevent sudden drop in pH. The final product was vacuum-packed, labelled and maintained at 8 °C in the dark chilling chamber until distribution.

Microbial analysis

Classical microbiological analyses (Table 1) were used for determination of microbial population from raw material at the end of ripening process (30 days) and at the end of 90 and 180 days of shelf-life in five different fermented sausages. Isolation of microbial populations was carried out on samples of sausages produced in 2011 in a local meat industry according to the Regulation on microbiological criteria for foodstuffs (*13*) and the following parameters were analyzed: Enterobacteriaceae, *Staphylococcus aureus* and sulphite-reducing clostridia, as well as yeast and moulds in 1 g of sample, and *Salmonella* sp. and *Listeria monocytogenes* in 25 g of sample.

A mass of 10 g of samples of each sausage was homogenized in 90 mL of sterile 0.5 % NaCl solution and serially diluted before plating on selective media (Table 1). The plates were incubated under aerobic conditions at 37 °C for 48 h for bacteria, at 25 °C for 48–72 h for yeast and 25 °C for 5 days for moulds. The microbial growth was determined using traditional plate counting (CFU/g).

Viability of lactobacilli and staphylococci in sausages during fermentation and shelf-life

Classical microbiological methods were used for the count of lactobacilli/staphylococci during fermentation (0, 15 and 30 days) and during shelf-life (90 and 180 days). The identity and viability of applied autochthonous starter cultures in the final product (90 and 180 days of shelf-life) were determined using API 50 CH and API Staph identification kits (bioMérieux, Marcy l'Etoile, France) and then confirmed by RAPD PCR analysis (only after 180 days).

Randomly taken colonies from MRS (60 colonies) and Baird Parker agar (60 colonies) from each of the five sausages were identified using API 50 CH and API Staph identification kits (bioMérieux) and then confirmed by RAPD analysis (only after 180 days).

For RAPD analysis, genomic DNA was isolated from 1.5 mL of overnight cultures using a method described previously (*1,3,8*), which is a modification of the salting-out procedure described by Miller (*14*).

Table 1. Classical microbiological methods for isolation and identification of microorganisms

Microorganisms	Method	Nutrient media	Incubation condition	
			Temperature	Time
			°C	h
Salmonella sp.	HRN ISO 6579	RP broth, XLD (Biolife)	37	24–48
Enterobacteriaceae	HRN ISO 5552	VRBG (Biolife)	37	24
Staphylococcus aureus	HRN ISO 6888-1	BP (Merck)	37	48
Sulphite-reducing clostridia	HRN ISO 15213	SPS (Merck)	37	72
Listeria monocytogenes	HRN ISO 11290-1	Palcam agar (Merck)	37	24
Lactic acid bacteria	HRN ISO 13721	MRS agar (Biolife)	30	48–72
Yeasts	HRN ISO 13681	Sabouraud agar (Biolife)	25	48–72
Moulds	HRN ISO 7954	YGCA (Biolife)	25	120

Physicochemical analysis

Sausages for physicochemical analysis were taken at the end of ripening process (30 days). The samples were cut into small pieces and homogenized in a household blender. The pH value was determined according to the ISO method 2917:2000 using WTW Microprocessor pH--meter (WTW, Weilheim, Germany) (*15*).

Water activity (a_w) was measured using AquaLab LITE (ser. no. AL1514; Decagon Devices Inc., Pullman, Washington, DC, USA). Determination of moisture, total protein, total fat and collagen was performed according to the AOAC Official Method 2007.04 (*16*) with a FoodScan™ device (FOSS Analytic, Hillerod, Denmark), which uses the near-infrared spectrophotometer system. Three independent measurements were made on each sample. Average and standard deviations were calculated.

Sensory analaysis

Samples were evaluated at the end of ripening by seven trained panellists selected from staff members of the Faculty of Food Technology and Biotechnology, Zagreb, Croatia. These panellists had completed a training course in sensory analysis, which includes different issues: sensory vocabulary, senses, sensory properties and techniques such as recognition, description, ranking and scaling of stimuli, discriminative test and sensory profiles according to the international standards (*17*).

The sensory analysis was carried out at the sensory laboratory located at the Department of Food Quality Control and Nutrition at the Faculty of Food Technology and Biotechnology, Zagreb, Croatia, with all requirements according to the international standard (*17*). All analyses were performed at the same time of the day, between 10 a.m. and 12 p.m. In total, ten sessions were conducted (two per day, with an hour break between servings to reduce fatigue) and 30 samples were examined, three in each session. Two panel replications were carried out on each sample.

Three slices (2 mm thick) of each sample, cut from the middle of the samples with a slicing machine, served at room temperature on white plastic dishes and coded with three-digit number, were served to the panelists. Water and unsalted bread were provided to clean the palate between samples.

The 20-point scoring system with weighted factors was used to describe the sensory quality of the sausages. The appearance, slice surface appearance, texture, odour and taste of the sausages were assessed using a 5-point scale in which samples were given scores from 1 (very poor) to 5 (excellent). Scores of each attribute were multiplied with specified weighted factor (appearance×0.2, slice surface appearance×0.8, texture×0.6, odour×0.8 and taste×1.6) and the overall quality was expressed as the sum of these five products. The used method was a combination of scoring system used in Croatia for sensory evaluation of dry fermented sausages (*18*) and similar works done by other authors (*19*,*20*).

Statistical analysis

Statistical analysis was carried out using the statistical package STATISTICA v. 7.1 for Windows 10.0 (Stat--Soft, Inc, Tulsa, OK, USA). Averaged data of the effects of the used starter type and type of sausages on the panellists were assessed by Analysis of Variance (ANOVA) with two factors. Student´s *t*-test was used to determine whether there were differences in each sensory attribute between the batches produced with different starter cultures. Differences were considered significant at $p<0.05$.

Results and Discussion

In a previous work we isolated, identified and characterized two autochthonous functional starter cultures (*Lactobacillus plantarum* 1K and *Staphylococcus xylosus* 4K1) from traditional Croatian sausages (Slavonski kulen), which showed desirable technological and functional characteristics for starter cultures (*1*). Therefore, the aim of this paper was to examine the capacity of autochthonous functional strains *L. plantarum* 1K and *S. xylosus* 4K1 as starter cultures for the production of five different industrial sausages, in order to obtain superior quality products with traditional taste and longer shelf-life. Before the application of these autochthonous functional starter cultures in industrial production of sausages, we wanted to confirm our previous identification using biochemical API tests (*1*). MALDI-TOF MS confirmed the identification of *L. plantarum* 1K strain (score 2.443), but *Staphylococcus xylosus* 4K1 was not identified. These samples were identified as *Staphylococcus carnosus* with score 2.383. Official identification of our two strains, *L. plantarum* and *S. carnosus*, was also confirmed by BCCM™/LMG Identification Service (Figs. 1 and 2).

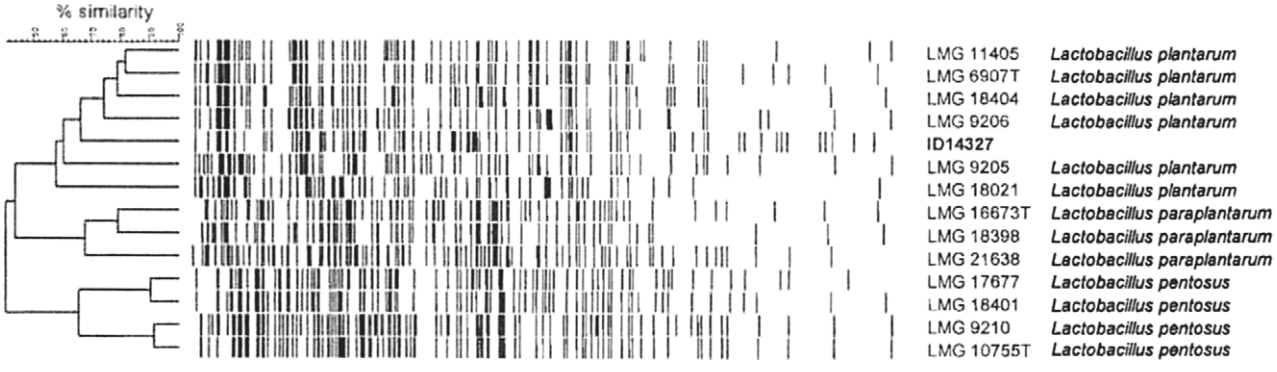

Fig. 1. A dendrogram based on BioNumerics v. 5.1 software-generated AFLP™ DNA fingerprints (Applied Maths NV, Sint-Martens-Latem, Belgium). Based on the results of the AFLP™ DNA fingerprinting, strain indicated with ID14327 (in the text *L. plantarum* 1K) was identified as *Lactobacillus plantarum*

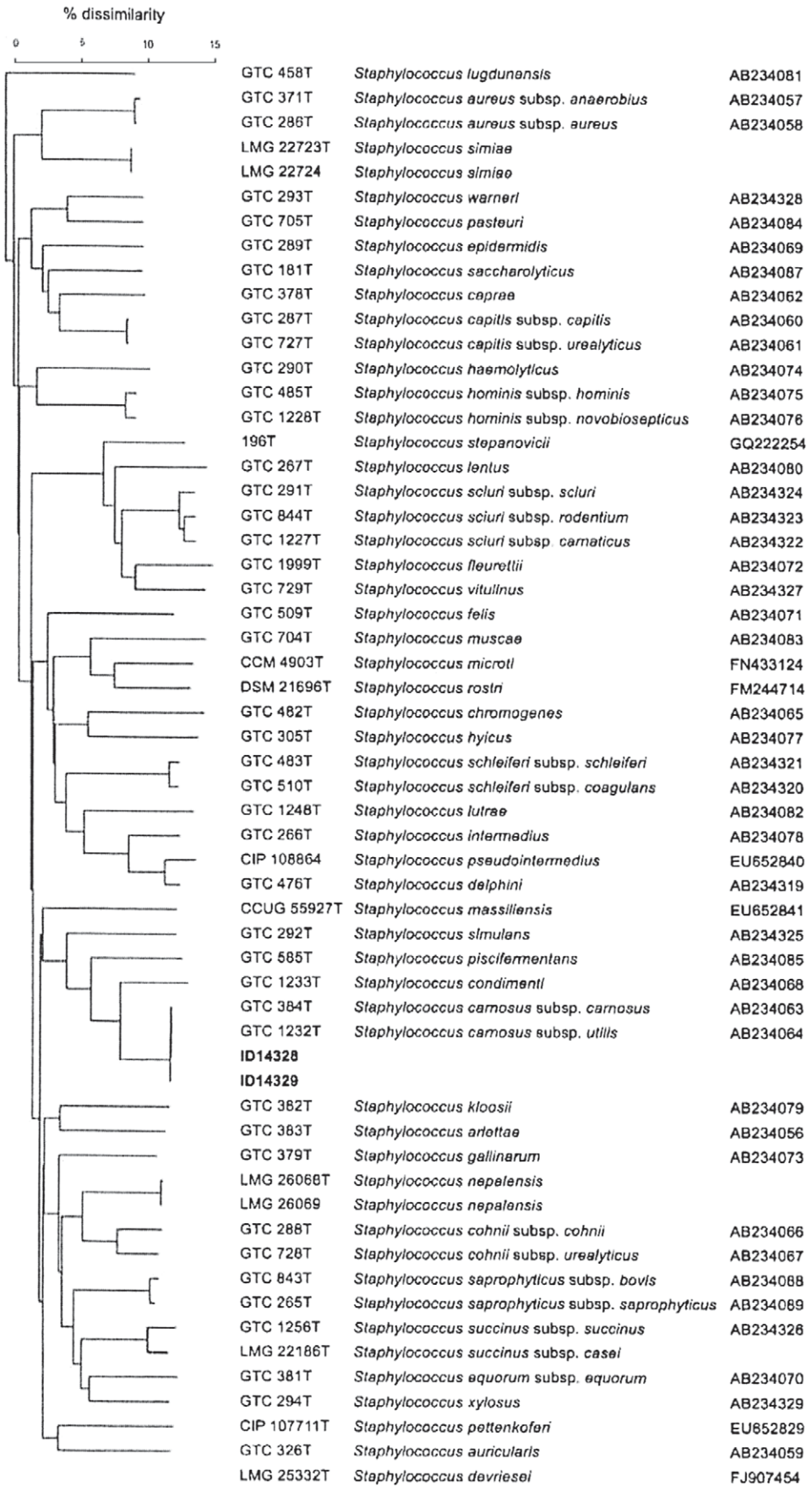

Fig. 2. Neighbour-joining tree based on *dnaJ* sequences. Based on the results of the *dnaJ* sequence analysis and phylogenetic study, strain indicated with ID14329 (in paper *S. carnosus* 4K1) was identified as *S. carnosus*

Then, 400 kg of prepared mixtures for each type of sausages were inoculated with both confirmed strains. Microbiological analyses were performed during both ripening and storage (raw materials were microbiologically safe; data not shown). They were designed to define safety aspect of the product and to follow the fermentation process carried out by the autochthonous functional starter cultures. The results of the microbiological analysis of the raw materials underlined the good hygienic quality of the meat, spices and natural casings used in

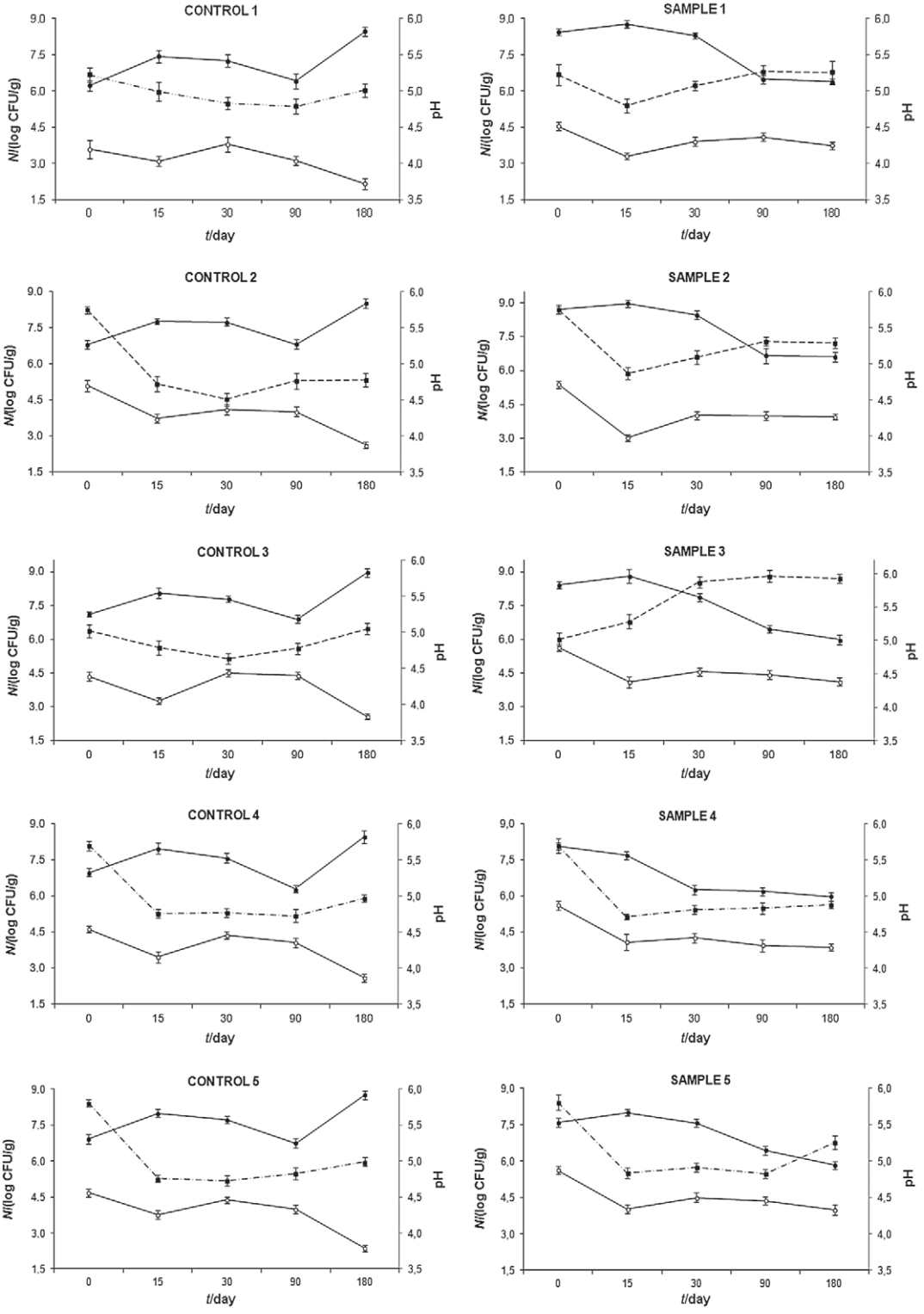

Fig. 3. Evolution of lactobacilli, staphylococci and pH during fermentation (15 days), ripening (30 days) and storage (90 and 180 days) of fermented sausages (average±SD; *N*=3). Control=sausages with comercial starter culture. Sample=sausages with autochthonous starter culture: 1=Bečka sausage, 2=Slavonski kulen, 3=Zimska sausage, 4=Čajna sausage, 5=Srijemska sausage
–●– *Lactobacillus* –○– *Staphylococcus* –■– pH

the production of all five types of sausages. Fig. 3 shows that the number of lactobacilli and staphylococci revealed statistically significant differences between the control and autochthonous starter culture at the beginning of ripening (15 days). Counts of autochthonous starter cultures in MRS agar were around $3.83 \cdot 10^7$–$5.24 \cdot 10^8$ CFU/g in all types of sausages, and in controls $1.7 \cdot 10^6$–10^7 CFU/g. The counts of autochthonous starter culture batches reached maximum levels in the range from $5.05 \cdot 10^7$ to 10^9 CFU/g after 15 days, but, in contrast, they remained at 10^7 CFU/g in control (Fig. 3). The impact of staphylococci on sausages was low, potentially represented by the inoculated strain, which remained below 10^5 CFU/g. At the beginning of fermentations, LAB were increasing in numbers, producing acids and causing decrease in the pH, followed in the phases of maturation by the activity of staphylococci, which were able to neutralize the produced acids (Fig. 3). The pH was typical of low acidity sausages because after 15 days it started to increase, reaching a final value in a range from 4.82 to 5.88. In the production of traditional sausages, the fermentation profile must have a short lag phase in order to ensure the growth of the added starter culture, and not of the unwanted bacteria. The acidification profile must be rather flat, not going below pH=4.8–5.0 at any time (Fig. 3). This will ensure that staphylococci maintain their activity over a longer period of time; foremost their nitrate reductase and flavour-forming activities. The principal role of LAB is to acidify the sausage, although they may also show proteolytic and lipolytic activities. The pH should reach 4.6–5.1, a value close to the isoelectric point of myofibrillar proteins (*21*). It is known that there are several positive technological aspects of acidification: the inhibition of pathogenic and altering microflora, faster drying and improved texture through the denaturation and coagulation of proteins, the activation of muscle proteases and reddening through the formation of nitric oxide and nitrosylmyoglobin (*22*). However, excessive acidification, as in the case of control sausages (Fig. 3), may alter the aroma and taste, and even lead to colour defects due to inhibition of the coagulase-negative staphylococci (CNS) (Fig. 3), the ideal being a balance between acidity, reddening and flavour. Synergy between different starters is important and their association should improve the metabolic activities that each one presents individually. LAB activity must permit adequate growth of CNS, especially at the beginning of the ripening stage. However, activity of starter cultures in dry-cured sausage may vary with the ingredients and additives (origin of the meat and fat, casing, water, salt, fermentable sugars, spices, colourings, flavourings, nitrate, nitrite) and processing (pretreatment of the meat, mincing, mixing, stuffing and drying).

At the end of the ripening process (30 days) the number of lactobacilli in our samples was in the range from 10^6 to 10^8 CFU/g, while in control samples the number of lactobacilli was about 10^7 CFU/g. The counts of staphylococci in our samples and in control group were about 10^4 CFU/g. After 30 days of ripening, statistically significant differences were observed in chemical composition, pH value and a_w in all five types of sausages regarding the manner of production (with autochthonous starter cultures or with commercial starter cultures). No significant differences were observed only in the proteins in Čajna sausage, in the pH value of Čajna and Bečka sausages, and in the a_w of Zimska sausage (Table 2). The values of a_w between 0.877 and 0.898 in all five types of sausages are normal for this type of meat products (*23,24*). The decrease in pH values (Table 2) in sausages may partly be explained by the absence of enterobacteria, as has been observed by other authors (*23,24*). All five types of sausages produced with autochthonous starter cultures as well as their controls produced with commercial starter cultures showed the same aspects of safety during the ripening process (30 days) and during three months of shelf-life. *L. monocytogenes*, *Salmonella* ssp., *S. aureus*, *E. coli* or *Enterobacteriaceae* were not detected in the sausages during three months (results not shown). Three months is the final storage period for these industrial sausages produced with commercial starter cultures, after that their food quality standard is questionable.

After six months of storage, yeasts, moulds and a high number of lactobacilli were detected in control sausages, which resulted in pH in the range of 4.78–5.05 and low number of staphylococci, about 10^2 CFU/g (Table 3). However, after six months, in the samples of fermented sausages moulds were not detected, and the number of lactobacilli was slightly lower (10^6 CFU/g) although the number of staphylococi showed a slight increase (10^3 to 10^4 CFU/g) in comparison with the results of control samples (Table 3, Fig. 3). Therefore, we can conclude that the applied autochthonous starter cultures have better adapt-

Table 2. Chemical composition, pH and a_w of five different types of sausages at the end of ripening (30 days)

Type of sausages	Type of starter culture	*w*(moisture)/%		*w*(protein)/%		*w*(fat)/%	
		x±σ	p-value	x±σ	p-value	x±σ	p-value
Bečka	commercial	32.1±0.2	$3.09 \cdot 10^{-4}$	26.24±0.03	$2.13 \cdot 10^{-3}$	36.75±0.04	$5.03 \cdot 10^{-5}$
	autochthonous	20.71±0.01		27.50±0.05		43.81±0.03	
Slavonski kulen	commercial	31.1±0.1	$5.40 \cdot 10^{-5}$	23.66±0.03	$5.67 \cdot 10^{-5}$	41.86±0.02	$6.32 \cdot 10^{-5}$
	autochthonous	17.22±0.02		27.86±0.01		46.65±0.01	
Zimska	commercial	32.9±0.2	$2.24 \cdot 10^{-4}$	26.04±0.03	$1.74 \cdot 10^{-4}$	37.55±0.04	$3.69 \cdot 10^{-5}$
	autochthonous	19.51±0.01		28.58±0.01		45.78±0.03	
Čajna	commercial	35.2±0.2	$1.32 \cdot 10^{-3}$	26.93±0.05	$1.61 \cdot 10^{-1}$	37.05±0.05	$1.43 \cdot 10^{-4}$
	autochthonous	29.68±0.02		26.89±0.02		42.10±0.02	
Srijemska	commercial	34.4±0.4	$2.54 \cdot 10^{-3}$	26.02±0.03	$1.06 \cdot 10^{-5}$	31.80±0.04	$2.01 \cdot 10^{-5}$
	autochthonous	27.51±0.01		24.72±0.03		42.31±0.02	

Table 2. – continued

Type of sausages	Type of starter culture	w(collagen)/%		pH		a_w	
		x±σ	p-value	x±σ	p-value	x±σ	p-value
Bečka	commercial	1.82±0.00	$1.16\cdot10^{-4}$	4.83±0.02	$9.29\cdot10^{-2}$	0.90±0.00	$4.09\cdot10^{-2}$
	autochthonous	2.75±0.01		5.07±0.06		0.88±0.01	
Slavonski kulen	commercial	2.73±0.00		4.57±0.03	$7.84\cdot10^{-4}$	0.90±0.00	$4.83\cdot10^{-2}$
	autochthonous	3.39±0.01		5.09±0.1		0.88±0.21	
Zimska	commercial	1.15±0.00	$2.86\cdot10^{-5}$	4.64±0.03	$5.71\cdot10^{-6}$	0.90±0.01	$5.83\cdot10^{-2}$
	autochthonous	3.02±0.01		5.88±0.05		0.88±0.01	
Čajna	commercial	2.43±0.00		4.76±0.02	$7.43\cdot10^{-2}$	0.90±0.00	$2.37\cdot10^{-2}$
	autochthonous	2.52±0.00		4.82±0.06		0.88±0.01	
Srijemska	commercial	2.69±0.00	$1.78\cdot10^{-4}$	4.79±0.01	$3.70\cdot10^{-2}$	0.89±0.01	$4.83\cdot10^{-2}$
	autochthonous	2.31±0.00		4.92±0.07		0.88±0.01	

ed to the meat environment and to the specific manufacturing process compared to the commercial starter cultures and they prolonged the shelf-life of the final product for three more months (Fig. 3, Table 3). In order to monitor the viability and presence of all applied bacterial strains in the produced sausages, classical microbiological method on selective agars was used. More than one hundred colonies obtained after incubation on different agar media (60 colonies from MRS and 60 colonies from Baird Parker agar) were subjected to biochemical analysis using API 50CH and API Staph identification kits. Two bacterial species were identified: *Lactobacillus plantarum* with 99.9 % and *Staphylococcus carnosus* with 98.7 % similarity with the type species from the database of the API systems. In addition to physiological (API) tests, to avoid possible misclassification, final identification of the two bacterial species was confirmed by a genotypic method, RAPD-PCR (Fig. 4). The RAPD-PCR patterns of the isolates from five different sausages produced with autochthonous starter cultures showed identical patterns to our referent strains *L. plantarum* 1K and *Staphylococcus carnosus* 4K1, which indicated that these isolates belonged to the same strains (Fig. 4). These results showed that the used autochthonous starter cultures survived industrial production of sausages. As the aim of this work was to produce five different Croatian sausages (Slavonski kulen, Zimska sausage, Čajna sausage, Srijemska sausage and Bečka sausage) with the addition of the well defined autochthonous functional cultures (*1*) under controlled conditions, but with aroma characteristics typical

Table 3. The values of pH, a_w and microbiological parameters (range of values) of five fermented sausages after 6 months of storage

Parameter	Statistical parameters	Control		Samples
pH	x±σ	4.96±0.1		5.32±0.38
	range	4.78–5.05		4.88–5.93
	p-value		0.072	
a_w	x±σ	0.81±0.01		0.81±0.04
	range	0.79–0.82		0.8–0.85
	p-value		0.925	
N(*Staphylococcus* spp.)/(log CFU/g)	x±σ	2.46±0.20		3.94±0.14
	range	2.15–2.63		3.73–4.12
	p-value		$1.04\cdot10^{-6}$	
N(LAB)/(log CFU/g)	x±σ	8.64±0.21		6.16±0.32
	range	8.47–8.95		5.83–6.61
	p-value		$5.5\cdot10^{-7}$	
N(yeast)/(log CFU/g)	x±σ	4.19±0.38		2.80±0.43
	range	3.73–4.76		2.32–3.29
	p-value		$6.3\cdot10^{-4}$	
N(moulds)/(log CFU/g)	x±σ	2.21±0.83		n.d.
	range	1.20–3.24		n.d.
N(Enterobacteria)/(log CFU/g)		n.d.		n.d.

n.d.=not determined
Control=sausages with commercial starter culture
Sample=sausages with autochthonous starter culture

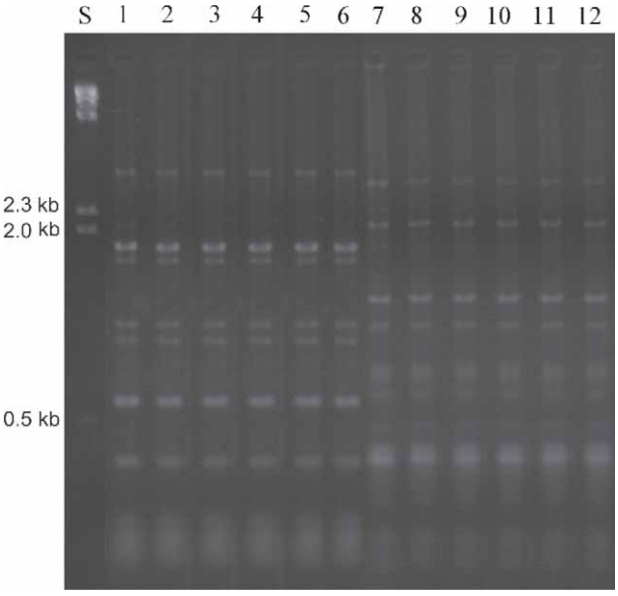

Fig. 4. RAPD fingerprints of lactic acid bacteria and staphylococci from final products after 180 days. 1=*Lactobacillus plantarum* 1K; 2–6=*Lactobacillus* isolates from final products (Slavonski kulen, Čajna sausage, Zimska sausage, Bečka sausage and Srijemska sausage, respectively); 7=*Staphylococcus carnosus* 4K1; 8–12=*Staphylococcus* isolates from final products (Slavonski kulen, Čajna sausage, Zimska sausage, Bečka sausage and Srijemska sausage, respectively); S=molecular mass size standard

of traditional Croatian sausages, the sensory properties were also investigated. Considered sensory attributes of the produced sausages were: outer appearance, colour and inner appearance, texture, odour and taste. The aim of the sensory evaluation was to determine whether the use of autochthonous starter cultures affected the sensory quality in comparison with the same sausages produced with commercial starter cultures (Table 4). The results of this research indicate that two autochthonous functional strains (*L. plantarum* 1K and *S. carnosus* 4K1) can be successfully used as autochthonous functional starter cultures for the production of sausages with specific sensory characteristics, typical of traditional Croatian sausages (*9,12,25*). All these sausages produced with autochthonous starter cultures received higher scores for almost all sensory attributes than control sausages produced with commercial starter cultures, although statistically no significant differences ($p>0.05$) were observed between the mean values of each sensory attribute. Only the total mean scores of Srijemska sausage were characterized by significant difference ($p<0.05$) in relation to the starter cultures and the type of sausages (Table 4). This can be explained by the fact that the autochthonous starter cultures used in this study, although they belong to the species commonly regarded as responsible for sausage fermentations, possess specific physiological, technological and sensory characteristics that make these traditional Croatian sausages with geographical origin unique. The use of autochthonous starter cultures can improve the slice surface appearance and odour of Bečka sausage, the taste of Zimska sausage, Srijemska sausage and Slavonski kulen and slice surface appearance and texture of Čajna sausage and thus the overall sensory quality of the investigated products, but not in a statistically significant manner. Only the taste of Srijemska sausage produced with autochthonous starter cultures was statistically significantly different from those produced with commercial starter cultures. Isolation and characterization of essential microbes in mixed cultures from region-specific brand products is of great importance. Implementation of this culture in industrial production should improve the quality and uniformity of the final product as well as food safety by preserving typical sensory quality of the traditional fermented product and inhibiting the growth of undesirable microorganisms. Detailed

Table 4. Mean scores obtained for each sensory attribute in five different Croatian sausages produced with commercial and autochthonous starter cultures

Croatian sausages	Type of starter culture	Sensory attributes					
		Appearance	Slice surface appearance	Texture	Odour	Taste	Total mean score±σ
Bečka	commercial	1.0±0.0	3.3±0.3	2.7±0.3	3.6±0.4	7.7±0.6	18.4±0.5
	autochthonous	1.0±0.0	3.5±0.4	2.7±0.3	3.9±0.3	7.7±0.7	18.8±0.9
	p-value		0.6		0.3		0.4
Zimska	commercial	1.0±0.0	3.7±0.4	2.6±0.3	3.9±0.3	6.9±0.8	18.1±0.6
	autochthonous	1.0±0.0	3.6±0.4	2.6±0.3	3.9±0.3	7.5±0.8	18.5±0.8
	p-value		0.6			0.3	0.4
Čajna	commercial	1.0±0.0	3.5±0.4	2.7±0.3	4.0±0.0	8.0±0.0	19.2±0.7
	autochthonous	1.0±0.0	3.9±0.3	2.9±0.2	4.0±0.0	8.0±0.0	19.8±0.4
	p-value		0.1	0.3			0.1
Srijemska	commercial	1.0±0.0	3.6±0.4	2.7±0.3	4.0±0.0	6.9±0.8	18.2±0.4
	autochthonous	1.0±0.0	3.2±0.0	2.6±0.3	4.0±0.0	8.0±0.0	18.8±0.3
	p-value		0.1	0.6		0.0	0.0

knowledge about microbiological population that is responsible for ripening process, tehnological parameters and resulting sensory properties is needed. Variations in overall quality, especially organoleptic characteristics of the product, represents a huge problem that remains to be solved before the sausages investigated in this research meet new market opportunities. Therefore, besides isolation and characterization of dominant microorganisms, technological and functional characteristics of specific microbial population have to be defined in order to be implemented in the production by small to medium enterprises. In this way the product will meet all regulations, as needed on a broad market, and it will remain autochthonous, safe and recognizable.

Conclusions

In conclusion, the microbiological, physicochemical and sensory aspects as well as shelf-life of the five Croatian sausages produced with autochthonous functional starter cultures studied in this paper are slightly different from the same sausages produced with commercial starter cultures. However, their organoleptic profile, characterized by an acceptable acidity and low rancidity, is distinctive for these traditional sausages. Further studies will be carried out to detail phenotypic, genotypic and physiological characterization of the isolated strains of staphylococci and LAB from different traditional Croatian meat products with additional purpose of creating a Croatian bank of autochthonous functional starter cultures specific for industrial production of traditional Croatian fermented meat products.

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