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Microbial flora of the Croatian traditionally fermented sausage

Mikrobielle Flora von traditionell hergestellten kroatischen Rohwürsten

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Summary

Microbial and physicochemical changes during the ripening of the Croatian traditionally fermented sausages was studied. Three batches of sausages were produced under controlled conditions according to the standard procedure. Lactic acid bacteria constituted the dominant microbial flora (7–8 $\log_{10}$ cfu g^{-1} in the final product), while coagulase-negative cocci were found in small numbers (2–3 $\log_{10}$ cfu g^{-1}) just in the second phase of ripening (between 14 and 28 days). An important finding was the high number of yeasts (4–5 $\log_{10}$ cfu g^{-1}), which have an important role in the evolution of sensory features of fermented sausages. During the fermentation phase, the pH values rapidly decreased (from initially 6.15 to 5.20 on day 7), resulting in a progressive elimination of the population of enterobacteria and pathogenic staphylococci present at the beginning of the fermentation process. Among the lactic acid bacteria isolated, the predominant genus was *Lactobacillus*, with prevalence of *Lactobacillus plantarum*. The collected isolates of coagulase negative cocci were determined as *Staphylococcus (S.) xylosus*, *S. capitis*, *S. carnosus* and *S. saprophyticus*. The sensory evaluation of sausages showed some deficiencies due to the prolongation of the regular ripening time.

Keywords: fermented sausages, *Lactobacillus* spp., *Staphylococcus* spp., yeasts, pH values, sensory evaluation

Zusammenfassung

In vorliegender Arbeit wurden mikrobiologische und physikalisch-chemische Veränderungen während der Reifung von traditionell hergestellten kroatischen Rohwürsten untersucht. Dabei wurden in drei Durchgängen Würste nach dem Standardverfahren unter kontrollierten Bedingungen hergestellt. Bei der mikrobiologischen Untersuchung ergab sich, daß Milchsäurenbakterien dominierten (7–8 $\log_{10}$ cfu g^{-1} im Endprodukt), während Koagulase-negative Kokken erst in der zweiten Phase der Reifung (zwischen 14 und 28 Tagen) in kleinen Mengen (2–3 $\log_{10}$ cfu g^{-1}) nachgewiesen wurden. Als wichtig ist auch der Nachweis von Hefen in relativ hohen Keimzahlen (4–5 $\log_{10}$ cfu g^{-1}) herauszustellen, da diese eine wichtige Rolle in der Entwicklung der sensorischen Eigenschaften von Rohwürsten spielen. Während der Fermentationsphase fiel der pH-Wert rasch ab (von anfangs 6,15 auf 5,20 am 7. Tag der Reifung), was zu einer progressiven Eliminierung von Enterobakterien und pathogenen Staphylokokken führte. Unter den Milchsäurenbakterien stellte *Lactobacillus* das am häufigsten nachgewiesene Genus dar, wobei hier *Lactobacillus plantarum* dominierte. Bei den Koagulase-negativen Staphylokkoken wurden vier Arten isoliert: *Staphylococcus* (*S.*) *xylosus*, *S. capitis*, *S. carnosus* und *S. saprophyticus*. Bei der organoleptischen Beurteilung der Würste zeigten sich einige Mängel, was einer experimentell bedingten Verlängerung der Reifung im Vergleich zum üblichen Verfahren zugeschrieben wurde.

Schlüsselwörter: Rohwürste, *Lactobacillus* spp., *Staphylococcus* spp., Hefen, pH-Werte, sensorische Untersuchung

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Introduction

Traditional production of fermented sausages represents an important factor of national identity in Croatia. Excellent acceptance of these meat products resulted in industrial scale production, i.e. under controlled conditions, during the entire year in order to ensure the permanent supply of the market with products of equable quality. In the northwest part of Croatia, traditional fermented sausages are industrially manufactured from pork, beef and pork back fat with the addition of nitrite salt and a specific spice mixture (ground black pepper, minced red pepper, garlic), filled in natural swine casings and ripened at lower temperatures.

Fermentation is an ancient method of preservation and prolongation of shelf life of meat. During fermentation, ripening and drying of fermented sausages many complex microbial, biochemical and physicochemical processes take place, influencing the quality and safety of final products (Hadžiosmanović et al., 2005). Prevailing physicochemical changes are manifested in lowering pH, increasing salt content and decreasing water activity, which all have a great impact on the composition of the microbial flora. Many studies have shown that lactic acid bacteria (LAB) and coagulase negative cocci (CNC) are the most active microorganisms in the filling of fermented sausages; first in the acidification process, second in denitrification, lipolysis and proteolysis (Lücke, 2000; Hammes and Hertel, 1998). In addition, some types of fermented sausages are characterised by the presence of yeasts, moulds or enterococci (Samelis et al., 1998; Baldini et al., 2000).

The most commonly isolated LAB in fermented sausages are lactobacilli, namely *Lactobacillus* (*L.*) *sakei*, *L. plantarum* and *L. curvatus* (Hammes, 1990). Within the family *Micrococcaceae*, the most frequently found species in the same substrate belong to the genus *Staphylococcus* (Papamanoli et al., 2002; Mauriello et al., 2004). Good adaptation and technological acceptability of the selected strains of LAB and CNC are crucial factors for their use as meat starter cultures (Hugas and Monfort, 1997). The aim of the present study was to investigate the microbial ecology of raw materials, additives and fermented sausages, as well as microbiological and physicochemical changes during the manufacturing process. In addition, the composition of LAB and CNC in the fermented sausages was evaluated by means of biochemical tests.

Materials and methods

Fermented sausage technology and sampling schedule

Sausages were produced in meat industry following the standard operating procedure. In the production of the filling were used lean pork (60 %) and beef (10 %), pork back fat (24 %), salt with 0.5 % $NaNO_2$ (2.5 %), sugar (0.5 %) and a mixture of ground black pepper, minced red pepper and garlic (3 %). Frozen meat and fat (-15 °C) were pre-tempered to -2 °C before use. After grounding to 12 mm in diameter, the other ingredients were added separately. Meat and fat were further cut to 2 mm in diameter under continuous mixing and homogenisation of the mixture. The mixture was filled into porcine casings (32–34 mm in diameter) under vacuum. After stuffing, sausages were allowed to equilibrate at room temperature (12 hours at 20 °C and 95 % relative humidity), then cold smoked for 48 hours at the same temperature and 85–90 % relative humidity (RH). Finally, sausages were kept in a fermentation chamber till day 28. During ripening, temperature and relative humidity were gradually reduced to 16–18 °C and 75 % RH, respectively.

Three batches were produced for experimental purposes. For microbiological analyses, 3 samples were taken from each batch on day 0, 2, 4, 7, 14 and 28. Samples were transported to the laboratory at +4 °C and kept at the same temperature up to analysis.

Microbiological, physicochemical and sensory analysis

Prior to preparation of sausages, all ingredients were subjected to microbiological and physicochemical analysis. In raw materials, additives and sausages the following microbiological parameters were monitored: total viable count, LAB, CNC, yeasts and moulds, aerobic spore formers, sulphite-reducing clostridia, *Pseudomonas* spp., enterobacteria, enterococci, *Staphylococcus* (*S.*) *aureus* and presence of *Salmonella* spp. and *Listeria monocytogenes*.

For microbiological analysis, 25 grams of test sample were taken under aseptic conditions, diluted in 225 ml of salt peptone water and homogenised for 2 minutes (Stomacher 400 Circulator, Seward, UK). After serial dilution, appropriate dilution samples (1 or 0.1 ml) were poured or spread on agar plates. Total viable count was determined on Plate Count Agar (PCA, BioMérieux, Marcy l'Etoile, France) at +30 °C for 72 hours, LAB count on MRS agar (BioMérieux) overlaid with 5 ml of the same medium at +30 °C for 48–72 hours, CNC on Manitol Salt Agar (MSA, BioMérieux) at +30 °C for 48 hours, enterobacteria-*Escherichia* (*E.*) *coli* on Coli-ID agar (BioMérieux) at +37 °C for 48 hours, enterococci on Kanamycin Aesculin azide agar (Merck, Darmstadt, Germany) at +37 °C for 48 hours, *S. aureus* on Baird-Parker agar (BP, Merck) at +37 °C for 48 hours, using Bactident Coagulase (Merck) and Slidex Staph Plus agglutination test (BioMérieux) for confirmation, yeasts and moulds on oxytetracycline-glucose yeasts extract agar (Oxoid, Basingstoke, Hampshire, England) at +25 °C for 72–120 hours, *Pseudomonas* spp. on cetrimide agar (BioMérieux) at +25 °C for 48 hours, aerobic spore formers using PCA (Merck) at +30 °C for 48 hours after heating at +80 °C for 10 min, sulphite-reducing clostridia on Sulphite Polymyxin Sulphadiazine agar (SPS, Merck) anaerobically at +37 °C for 72 hours, respectively. For the isolation of *Salmonella*, 25 g of test sample were homogenised in 225 ml of peptone water, incubated at +37 °C for 16 hours, then 0.1 ml of pre-enrichment was transferred into 10 ml Rappaport-Vassiliadis broth (RV, Merck) and incubated at +42 °C for 24 hours. After the incubation, the culture was streaked on BPLS and XLD (Merck) and incubated at +37 °C for 24 and 48 hours, respectively. Suspicious colonies were serologically tested (Imunološki zavod, Zagreb) and biochemically determined by means of the API20E diagnostic system (BioMérieux). For isolation of *Listeria monocytogenes*, 25 g of test sample were homogenised in Half-Fraser broth and incubated at +30 °C for 24 hours. Then, 0.1 ml of pre-enriched culture was transferred into Fraser broth and incubated at +37 °C for 48 hours. After the incubation period, the culture was streaked on Oxford agar (BioMérieux). Suspicious colonies were biochemically evaluated using API Listeria kit (BioMérieux).

For the pH measurement, 10 g of test samples (raw materials, sausages) were diluted in 90 ml of distilled water. After homogenisation, the pH values were deter-

mined with a digital pH-meter (WTW, Weilheim, Germany). Besides pH, water content, salt and nitrites were also monitored during the ripening of sausages according to the AOAC (2002) methodology.

A panel of 10 persons was created in order to evaluate the sensory properties of the fermented sausages (Comi et al., 2005). The panel was constituted of tested staff of the Department of Hygiene and Technology of Animal Foodstuffs, Veterinary Faculty, University of Zagreb, with lasting experience in sensorial evaluation of foodstuffs of animal origin. Each parameter in sensorial analysis was evaluated by means of a grading scale from 1 to 10 points. Prior to tasting, color, cut surface, coherence between fat and muscle tissue and smell (as well as off odor and appearance) were evaluated. The products were tasted and graded for rancidity, fat quality, acidity, juiciness, tenderness and overall flavour. In addition, sausages were graded for aftertaste 10 minutes after tasting. Finally, the panellists graded their overall impression on the sensorial evaluation. After the evaluation, mean values were calculated for each parameter.

Characterisation of indigenous microbial flora

In total, 150 LAB (gram-positive, catalase negative bacilli or cocci-bacilli) isolates were collected during all phases of sausage production in all three batches included in the study. Isolates were grown in MRS broth at +30 °C for 24 hours, sub-cultivated and purified on MRS agar plates and stored in MRS broth with 30 % glycerol at -20 °C. Sugar fermentation patterns were tested using API 50 CHL diagnostic test (BioMérieux). Identification was performed by the computer program APILAB Plus.

In total, 72 isolates of CNC were collected during the second part of ripening in two batches of sausages. Isolates were grown in BHI broth (BioMérieux) at +37 °C for 24 hours, sub-cultivated and purified on BHI agar plates and stored in BHI broth with 30 % glycerol at -20 °C. Identification was performed by API Staph system (BioMérieux).

Results and discussion

Microbiological, physicochemical and sensory analysis

The results of microbiological analyses of raw materials, additives and sausages are shown in Tables 1 and 2. Total viable count in pork was within the permissible limits according to the Croatian microbiology standards for cut meat (10^6 g^{-1}). On the other hand, in beef and pork back fat the total viable count exceeded the set standard. In the stuffing of investigated sausages the total viable count was 6–7 $\log_{10}$ cfu g^{-1} during ripening with a slight decrease towards the end of the process. During the fermentation and ripening phase, lactic acid bacteria exceeded the total count of bacteria. In batch 1, the initial LAB count was low (4 $\log_{10}$ cfu g^{-1}), and in batches 2 and 3 no bacteria were isolated on MRS. In pork and beef the LAB count was also very low (3 $\log_{10}$ cfu g^{-1} and 2 $\log_{10}$ cfu g^{-1}, respectively). The population of LAB in sausages from batch 1 increased from 4 $\log_{10}$ cfu g^{-1} to 7 $\log_{10}$ cfu g^{-1} on day 7 and finally reached 8 $\log_{10}$ cfu g^{-1} in the final product. In batch 2, LAB increased from 3 $\log_{10}$ cfu g^{-1} on day 2 to 7 $\log_{10}$ cfu g^{-1} on day 7 and stayed constant till the end of ripening. In batch 3, LAB increased from 3 $\log_{10}$ cfu g^{-1} on day 2 to 7 $\log_{10}$ cfu g^{-1}

TABLE 1: *Microbial counts* ($\log_{10}$ cfu g^{-1} ± SD) of raw materials and additives.*

Microorganisms	Pork	Beef	Pork back fat	Sugar	Mixed spices	Garlic	Casings
Total viable count							
Batch 1	5.93 ± 0.12	6.08 ± 0.23	6.40 ± 0.17	5.83 ± 0.11	6.66 ± 0.19	NT	4.40 ± 0.22
Batch 2	5.08 ± 0.42	7.08 ± 0.09	6.63 ± 0.22	5.77 ± 0.24	6.35 ± 0.21	NT	5.28 ± 0.27
Batch 3	5.15 ± 0.21	5.30 ± 0.11	6.11 ± 0.08	5.72 ± 0.43	6.12 ± 0.34	NT	5.18 ± 0.35
Lactic acid bacteria							
Batch 1	3.08 ± 0.15	2.40 ± 0.24	/	/	NT	NT	/
Batch 2	/	1.30 ± 0.16	/	/	NT	NT	/
Batch 3	/	/	/	/	NT	NT	/
Enterobacteria-*E. coli*							
Batch 1	/	/	/	/	NT	NT	/
Batch 2	/	< 2	/	/	NT	NT	/
Batch 3	/	/	/	/	NT	NT	/
Pseudomonads							
Batch 1	1.48 ± 0.21	1.85 ± 0.43	/	/	NT	NT	/
Batch 2	/	/	/	/	NT	NT	/
Batch 3	/	/	/	/	NT	NT	/
Aerobic spore-formers							
Batch 1	/	/	/	1.85 ± 0.12	2.70 ± 0.01	NT	/
Batch 2	/	/	/	/	2.50 ± 0.08	NT	/
Batch 3	/	/	/	/	2.06 ± 0.04	NT	/
Yeasts							
Batch 1	3.00 ± 0.12	/	3.70 ± 0.31	/	5.40 ± 0.12	/	2.85 ± 0.40
Batch 2	4.36 ± 0.25	4.60 ± 0.18	4.13 ± 0.26	/	/	/	2.48 ± 0.23
Batch 3	3.99 ± 0.10	3.00 ± 0.22	4.18 ± 0.17	/	/	/	2.85 ± 0.17
S. aureus							
(all batches)	< 2	< 2	< 2	/	/	/	< 2

* Each number is the mean of three sausage samples taken from the same batch.
NT: not tested
/ : beneath detection limit (1 $\log_{10}$ cfu g^{-1})
All samples were free of coagulase-negative cocci, enterococci, sulphite-reducing clostridia, *L. monocytogenes* and *Salmonella* spp.

TABLE 2: *Microbial counts* (log_{10} cfu g^{-1} ± SD) of the naturally fermented sausages.*

Microorganisms	Day 0	Day 2	Day 4	Day 7	Day 14	Day 28
Total viable count						
Batch 1	7.11 ± 0.02	6.11 ± 0.11	7.26 ± 0.13	5.83 ± 0.02	6.08 ± 0.50	6.41 ± 0.15
Batch 2	7.28 ± 0.12	7.15 ± 0.21	6.32 ± 0.05	8.08 ± 0.12	7.80 ± 0.47	6.74 ± 0.24
Batch 3	5.51 ± 0.14	7.56 ± 0.13	6.26 ± 0.21	6.18 ± 0.16	6.93 ± 0.21	6.81 ± 0.34
Lactic acid bacteria						
Batch 1	4.08 ± 0.02	4.13 ± 0.01	6.34 ± 0.25	7.53 ± 0.03	7.72 ± 0.22	8.08 ± 0.04
Batch 2	/	3.15 ± 0.15	5.32 ± 0.21	7.62 ± 0.12	7.79 ± 0.60	7.72 ± 0.21
Batch 3	/	3.76 ± 0.74	5.79 ± 0.41	6.91 ± 0.14	6.71 ± 0.15	6.92 ± 0.19
Coagulase-negative cocci						
Batch 1	/	/	/	/	2.64 ± 0.14	/
Batch 2	/	/	/	/	/	3.61 ± 0.02
Batch 3	/	/	/	/	/	/
Enterobacteria-*E. coli*						
Batch 1	< 2	< 2	/	/	/	/
Batch 2	< 2	< 2	< 2	/	/	/
Batch 3	/	< 2	/	/	/	/
Yeasts						
Batch 1	4.28 ± 0.02	4.41 ± 0.01	4.56 ± 0.03	4.34 ± 0.01	3.51 ± 0.04	/
Batch 2	4.51 ± 0.01	5.69 ± 0.09	5.83 ± 0.21	5.88 ± 0.05	4.11 ± 0.06	/
Batch 3	4.86 ± 0.05	5.32 ± 0.10	5.67 ± 0.19	4.62 ± 0.07	4.23 ± 0.07	/
S. aureus						
(all batches)	< 2	< 2	< 2	/	/	/

* Each number is the mean of three sausage samples taken from the same batch.
/ : beneath detection limit (1 log_{10} cfu g^{-1})
All samples were free of enterococci, *Pseudomonas* spp., aerobic sporo-formers, sulphite-reducing clostridia, *L. monocytogenes* and *Salmonella* spp.

(day 28). In spite of the fact that the initial population of LAB in our investigation was low in batch 1 and LAB were not isolated in the batches 2 and 3 (day 0), lactic acid bacteria became the dominant microbial flora during the ripening process.

Yeasts were detected in high numbers (4–5 log_{10} cfu g^{-1}) during ripening, which was probably due to the pork, beef and pork back fat used, where the counts amounted to 3 log_{10} cfu g^{-1} and higher, as well as to the mixed spices and natural casings (Tab. 1). Yeasts play an important role in generation of sensory characteristics of fermented sausages and other meat products due to lipolytic and proteolytic activity (Hammes and Hertel, 1998). Pseudomonads were isolated from pork and beef used as raw materials in batch 1, but no *Pseudomonas* was detected in sausages during fermentation. The enterobacteria – *E. coli* population was lower than 2 log_{10} cfu g^{-1} after stuffing and towards day 7 these microorganisms were progressively eliminated. The proper progress of fermentation resulted in elimination of the initially present gram-negative bacteria that are most sensitive to acidic environment (Adams and Nicolaides, 1997). The number of aerobic spore forming bacteria (< 2 log_{10} cfu g^{-1} in sugar and 2 log_{10} cfu g^{-1} in spices) was also very low. Coagulase-negative cocci were not found in pork, beef and pork back fat (Tab. 1) and their number in sausages during fermentation was also low; they were isolated only in the second part of ripening in batch 1 (> 2 log_{10} cfu g^{-1}) and batch 2 (> 3 log_{10} cfu g^{-1}). This observation reflects their poor competitiveness in the presence of LAB and other influences during fermentation. Coagulase-negative cocci possess a notable proteolytic and lipolytic activity and have important impact on sensory features of sausages (Hadžiosmanović et al., 1979). Some observations regarding sensory characteristics of the investigated sausages could be attributed to the small number and poor activity of the CNC population. Enterococci were not found in raw material and sausages during the entire production cycle. Some studies showed high numbers of enterococci in different kinds of sausages (Drosinos et al., 2005; Comi et al., 2005). Our results confirm the observation of Hugas et al. (2003) that presence and number of enterococci in fermented sausages are in correlation with the hygienic quality of the raw materials used. Moreover, enterococci counts could differ between batches of the same sausage as well. *S. aureus* was detected in pork, beef and pork back fat, but its count did not exceed 2 log_{10} cfu g^{-1} after stuffing, and on day 7 of fermentation no staphylococci were detected. According to some studies, *S. aureus* is fermentation sensitive (Adams and Nicolaides, 1997), but it can also survive under certain conditions. Sulphite-reducing clostridia were not detected in the raw material used and all samples were free of *Salmonella* spp. and *Listeria monocytogenes*.

The initial pH values of sausages were 6.00 and higher (Tab. 3). Beef and pork back fat had initial pH values higher than 6.00 in all batches, too, and only the pork used in batch 1 had a pH value of 5.90. The pH values in all batches of sausages decreased rapidly within 7 days of fermentation, and after that slightly increased towards the end of ripening. The lower pH values in batch 1 are in agreement with the higher initial number of LAB observed in this batch. In batch 2, the pH value decreased from an initial 6.44, but on day 2 and day 4 was still > 6.00. The pH value was 5.23 on day 7 and finally 5.35 at the end of fermentation. In batch 3, the observed pH values were lower than in batch 1. From the second day of fermentation, the population of LAB increased in all batches, and at the same time pH values showed a decrease. Nitrite content was very low, detected just in traces in all batches. Salt concentration

TABLE 3: *Changes in physicochemical characteristics (mean value ± SD) during fermentation and ripening of sausages.*

Parameter	Day 0	Day 2	Day 4	Day 7	Day 14	Day 28
pH						
Batch 1	6.00 ± 0.01	5.95 ± 0.03	5.67 ± 0.01	5.12 ± 0.06	5.30 ± 0.05	5.27 ± 0.01
Batch 2	6.44 ± 0.02	6.25 ± 0.05	6.12 ± 0.01	5.23 ± 0.02	5.30 ± 0.01	5.35 ± 0.05
Batch 3	6.00 ± 0.00	5.80 ± 0.01	5.72 ± 0.04	5.27 ± 0.02	5.08 ± 0.03	5.52 ± 0.04
NaCl (%)						
Batch 1	1.56	1.64	2.32	2.32	2.32	2.36
Batch 2	1.56	1.59	1.16	2.02	2.32	2.40
Batch 3	1.40	1.38	1.60	1.82	2.02	2.12
Nitrite (mg/kg)						
Batch 1	in traces	in traces	in traces	in traces	in traces	in traces
Batch 2	in traces	in traces	in traces	in traces	in traces	in traces
Batch 3	in traces	in traces	in traces	in traces	in traces	in traces
Nitrate (mg/kg)						
Batch 1	in traces	in traces	in traces	in traces	in traces	in traces
Batch 2	in traces	in traces	in traces	in traces	in traces	in traces
Batch 3	in traces	in traces	in traces	in traces	in traces	in traces
Moisture (%)						
Batch 1	58.8	59.6	48.4	32.4	22.4	15.2
Batch 2	51.6	50.8	42.0	32.8	23.6	12.8
Batch 3	59.6	58.8	56.0	50.4	22.4	13.2

increased and moisture content decreased due to sausage desiccation (Tab. 3). Moisture content was quite high both in the raw material (70–75 %) and sausages at the beginning, but it was extremely low at the end of production (below 20 %). This result is associated with the duration of fermentation in our investigation, which was prolonged for 7 days for the purpose of monitoring all parameters during the study. This also had serious repercussions on the sensory evaluation of finished sausages.

Generally, all batches showed acceptable color, coherence, flavour without off-odour, low rancidity, since the grades for these parameters were above the acceptable level (5.0). On the other hand, slightly lower marks were noticed for cut surface, fat quality, acidity and aftertaste, which were attributed to the too high content of fat tissue in the mixture. There were serious deviations related to tenderness and juiciness, which were evaluated below the acceptable level (Fig. 1), and this had repercussions on the overall impression. We assume that these values are in connection with the extended time of ripening, while the sensory evaluation had better scores on day 21. This type of sausages is usually ready for consumption after 21 days in the commercial production. As the fermentation and ripening of sausages had to be 28 days (agreement in the project), the results of sensory evaluation showed some characteristics of inferior value.

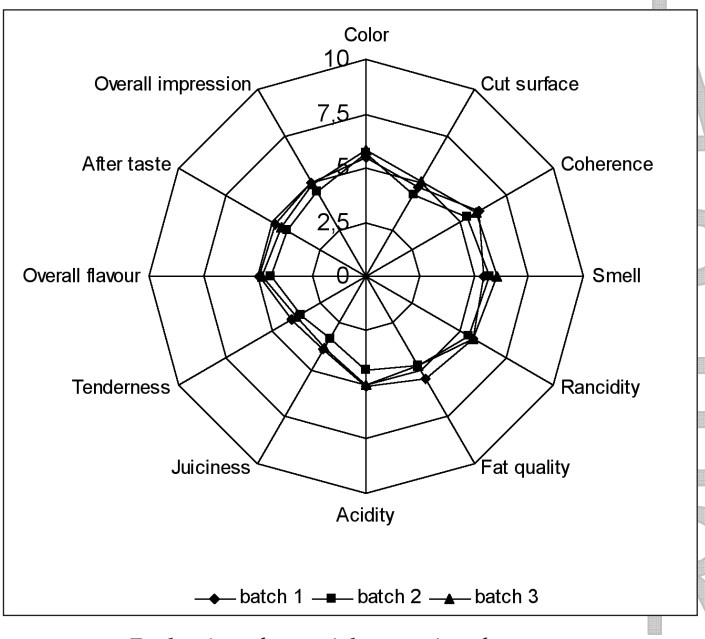

FIGURE 1: *Evaluation of sensorial properties of sausages (grade scale 1–10).*

Characterisation of indigenous microbial flora

As is shown in Table 2, CNC (72 isolates) were isolated only from the batches 1 and 2. According to API Staph identification system, all isolates belonged to the genus *Staphylococcus* with the following 4 strains almost equally represented: *S. xylosus* (29.2 %), *S. capitis* (25.0 %), *S. carnosus* (25.0 %), *S. saprophyticus* (20.8 %) (Tab. 4). Many other studies confirmed the presence of the mentioned staphylococcus species in different kinds of fermented sausages (Coppola et al., 1997; Papamanoli et al., 2002; Garcia-Varona et al., 2000). Similar to lactobacilli, CNC are commercially used as starter cultures in the production of fermented sausages, particularly *S. xylosus* and *S. carnosus* (Olesen et al., 2004). The importance of staphylococci and micrococci in fermented sausages was intensively investigated with regard to their effect on sensory properties of final products and their suitability as starter cultures (Hammes and Hertel, 1998). Our investigation showed a poor competitiveness of the *Micrococcaceae* population during fermentation and ripening of sausages, which probably affected the sensory characteristics of final products.

The results of the determination of LAB (Tab. 5) show the domination of lactobacilli (88.7 %). The majority of the lactobacilli isolated from the batches belonged to the species *L. plantarum* (39.3 %) and *L. brevis* (20.7 %), followed by *L. curvatus* (18.0 %), *L. pentosus* (6.7 %) and *L. fermentum* (4.0 %). Among lactobacilli, in batch 1 the predominant strain was *L. plantarum* (44 %), followed by *L. brevis* (30 %), *L. pentosus* (8 %) and *L. curvatus* (4 %). *L. plantarum* was also isolated most frequently in batches 2 (38 %) and 3 (36 %), followed by *L. curvatus* (30 % and 20 %, respectively), *L. brevis* (18 % in batch 2 and 14 % in batch 3), and *L. pentosus* (6 % in each batch).

TABLE 4: *Biochemical characterisation of staphylococci isolated from naturally fermented sausages.*

No. of isolates at each stage of process (days)						No. (%) of isolates	Identification (API Staph)
0	2	4	7	14	28		
Batch 1 (34 isolates)							
0	0	0	0	10	0	10 (29.4)	*S. xylosus*
0	0	0	0	8	0	8 (23.5)	*S. capitis*
0	0	0	0	9	0	9 (26.5)	*S. carnosus*
0	0	0	0	7	0	7 (20.6)	*S. saprophyticus*
Batch 2 (38 isolates)							
0	0	0	0	0	11	11 (22.0)	*S. xylosus*
0	0	0	0	0	10	10 (20.0)	*S. capitis*
0	0	0	0	0	9	9 (18.0)	*S. carnosus*
0	0	0	0	0	8	8 (16.0)	*S. saprophyticus*
Batch 3 (0 isolates)							
0	0	0	0	0	0	0	
All batches (72 isolates)							
0	0	0	0	10	11	21 (29.2)	*S. xylosus*
0	0	0	0	8	10	18 (25.0)	*S. capitis*
0	0	0	0	9	9	18 (25.0)	*S. carnosus*
0	0	0	0	7	8	15 (20.8)	*S. saprophyticus*

TABLE 5: *Biochemical characterisation of lactic acid bacteria isolated from naturally fermented sausages.*

No. of isolates at each stage of process (days)						No. (%) of isolates	Identification (API 50 CHL)
0	2	4	7	14	28		
Batch 1 (50 isolates)							
4	2	4	3	5	4	22 (44.0)	*L. plantarum*
0	0	0	0	0	2	2 (4.0)	*L. curvatus*
0	0	1	0	1	2	4 (8.0)	*L. pentosus*
4	1	5	2	2	1	15 (30.0)	*L. brevis*
2	1	0	0	0	0	3 (6.0)	*Lc. lactis* subsp. *lactis*
1	0	2	1	0	0	4 (8.0)	*Ln. mesenteroides* subsp. *mesenteroides*
Batch 2 (50 isolates)							
0	2	1	5	4	7	19 (38.0)	*L. plantarum*
0	3	4	3	2	3	15 (30.0)	*L. curvatus*
0	0	0	1	1	1	3 (6.0)	*L. pentosus*
0	1	5	0	1	2	9 (18.0)	*L. brevis*
0	0	0	2	2	0	4 (8.0)	*Ln. mesenteroides* subsp. *mesenteroides*
Batch 3 (50 isolates)							
0	1	2	1	3	11	18 (36.0)	*L. plantarum*
0	0	0	1	0	2	3 (6.0)	*L. pentosus*
0	1	2	2	1	4	10 (20.0)	*L. curvatus*
0	1	0	2	1	2	6 (12.0)	*L. fermentum*
0	1	2	2	1	1	7 (14.0)	*L. brevis*
0	1	0	0	4	0	5 (10.0)	*Ln. mesenteroides* subsp. *mesenteroides*
0	0	0	0	1	0	1 (2.0)	*P. pentosaceus*
All Batches (150 isolates)							
4	5	7	9	12	22	59 (39.3)	*L. plantarum*
0	4	6	5	3	9	27 (18.0)	*L. curvatus*
0	0	1	2	2	5	10 (6.7)	*L. pentosus*
0	1	0	2	1	2	6 (4.0)	*L. fermentum*
4	3	12	4	4	4	31 (20.7)	*L. brevis*
1	1	2	3	6	0	13 (8.6)	*Ln. mesenteroides* subsp. *mesenteroides*
2	1	0	0	0	0	3 (2.0)	*Lc. lactis* subsp. *lactis*
0	0	0	0	1	0	1 (0.7)	*P. pentosaceus*

L. fermentum was isolated only in batch 3 (12 %). Besides lactobacilli, the following lactic acid bacteria were isolated: *Leuconostoc* (*Ln.*) *mesenteroides* subsp. *mesenteroides* (8.6 %), *Lactococcus* (*Lc.*) *lactis* subsp. *lactis* (2.0 %) and *Pediococcus* (*P.*) *pentosaceus* (0.7 %). *Ln. mesenteroides* subsp. *mesenteroides* was determined in each batch (8 % in batch 1 and 2, and 10 % in batch 3), *Lc. lactis* subsp. *lactis* only in batch 1 (6 %), while *P. pentosaceus* (0.7 %) was isolated only in batch 3.

The majority of investigations of different kinds of European traditional fermented sausages confirmed the predominance of lactobacilli, mostly *L. sakei*, *L. curvatus* and *L. plantarum* (Gasparik-Reichardt et al., 2005). Differences in affirmation of certain strains among various sausages depend on many intrinsic and extrinsic parameters, e.g. climate, adaptation to meat substrate, sausage composition, ripening conditions and technology applied. The predominant species in the Croatian traditionally fermented sausage was *L. plantarum*, which is a regular constituent of the microbial flora of Mediterranean fermented sausages (Drosinos et al., 2005). Besides technologically desirable homo-fermentative lactobacilli, the presence of hetero-fermentative LAB in fermented sausages was also confirmed (Gasparik-Reichardt et al., 2005). In our experiment, hetero-fermentative *L. brevis* and *Ln. mesenteroides* subsp. *mesenteroides* (20.7 % and 8.6 %, respectively) were isolated. The high concentration of carbon dioxide, as a product of hetero-fermentative microbial flora activity, is desirable in some foods (milk products), but not in fermented sausages. Elimination of that deficiency could be possible with the use of well-adapted and technologically acceptable lactic acid bacteria in the form of starter cultures.

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