The use of *Pediococcus pentosaceus*, *Staphylococcus carnosus* and *Staphylococcus xylosus* starter cultures in the production of kulen

Nežak[1], J., N. Zdolec[2], S. Vidaček[3], N. Marušić[3], H. Medić[3]

znanstveni rad

Summary

During the process of kulen production, the micro-climatic, physical and chemical and microbiological changes in the samples were monitored. The results obtained indicate the justification of using starter cultures, which bring the pH value down to the required levels, as shown by the final sensory assessment. The use of starter cultures led to the domination of technologically and hygienically justified microflora, which contribute to improving quality, safety in production and the hygienic course of the process of fermentation of kulen. This study points out the advantages and possible disadvantages of producing kulen with starter cultures, in comparison to the traditional production.

Key words: *kulen, physical, chemical, microbiological and microclimatic changes, starter cultures.*

Introduction

Kulen is an original domestic meat product, whose origins and development are linked to the agricultural economy of Slavonia. In terms of nutritional value, it is a highly valuable meat product with a large proportion of protein, and a relatively small quantity of water, which is a rich source of valuable proteins and calories (Petričević et al., 2010).

The presence of microorganisms in meat, as an unavoidable factor in the production of fermented sausages, has prompted many researchers to study the action and composition of native micro flora on the fermentation process, which was the basis for the development of starter cultures (Kozačinski et al., 2008; Zdolec et al., 2009; Frece et al., 2010; Babić et al., 2011; Alagić et al., 2011).

Starter cultures, by their biochemical activity, cause changes to the pH of the meat, speed up the development of colour, consistency, flavour, aroma, and in general bring about the formation of the characteristic qualities of the product. Alongside their desired effect, some microbe cultures may cause the product to spoil; it is also possible to find microorganisms which may be harmful for the health of consumers. The traditional production of kulen is based on the fermentation of the filling by the naturally present microflora or modulations with products of good quality (Petričević et al., 2010).

In starter cultures aimed at the production of rapidly fermented sausages in the USA, lactic acid bacteria of the *Lactobacillus* and *Pediococcus* strains are predominant. Their use shortens the production process. Due to the high temperatures of fermentation (30- 45˚C) the taste of the product becomes very acid (Buckenhüskes, 1994; Weber, 1996; Čavlek, 1997; Medić et al., 2009; Feiner, 2006). In Europe, bacteria from the strains *Lactobacillus, Pediococcus, Micrococcus* and *Staphylococcus* are used for the same purpose. During the process of fermentation, which takes place at significantly lower temperatures, there is a gradual fall

1 dr .sc. Jadranko Nežak, PIK Vrbovec d.d., Zagrebačka 148, 0340 Vrbovec, Hrvatska

2 dr. sc. Nevijo Zdolec, Veterinarski fakultet Sveučilišta u Zagrebu, Zavod za higijenu i tehnologiju namirnica animalnog podrijetla, Heinzelova 55, 10000 Zagreb, Hrvatska

3 dr.sc. Sanja Vidaček, docent; Nives Marušić, dipl. ing.; dr. sc. Helga Medić, izvanredni profesor Prehrambeno-biotehnološki fakultet Sveučilišta u Zagrebu, Zavod za prehrambeno-tehnološko inženjerstvo, Pierottijeva 6, 10 000 Zagreb, Hrvatska; e-mail hmedic@pbf.hr,

Table 1. **Changes in mass of kulen samples with or without starter cultures during the processing**

PRODUCTION IN DAYS	WITHOUT STARTER CULTURES			WITH STARTER CULTURES		
	MASS	CHANGES TO MASS		MASS	CHANGES TO MASS	
	(g)	(g)	%	(g)	(g)	%
1	2190	-	-	2200	-	-
2	2104	86	3.9	2132	68	3.09
3	2026	164	7.48	2064	136	6.18
4	1978	212	9.68	2022	178	8.09
5	1934	256	11.68	1984	216	9.81
6	1907	283	12.88	1962	238	10.78
7	1885	305	13.9	1941	259	11.77
8	1868	322	14.70	1920	280	12.72
9	1844	346	15.79	1898	302	12.72
10	1829	361	16.47	1887	313	14.22
11	1814	376	17.16	1876	324	14.70
12	1779	411	18.73	1866	334	15.18
24	1638	551	25.20	1748	452	20.54
40	1528	662	30.20	1555	645	29.30
60	1366	824	37.60	1425	775	35.20
90	1305	885	40.40	1324	876	39.81
120	1265	925	42.23	-	-	-

Table 2. **Changes to diameter of kulen samples with or without starter cultures during the processing**

PRODUCTION IN DAYS	WITHOUT STARTER CULTURES			WITH STARTER CULTURES		
	DIAMETER	CHANGES TO DIAMETER		DIAMETER	CHANGES TO DIAMETER	
	(mm)	(mm)	%	(mm)	(mm)	%
1	126.00	-	-	118.00	-	-
2	121.00	5.00	3.96	116.00	2.00	1.69
3	120.70	5.30	4.20	115.00	3.00	2.54
4	120.50	5.50	4.36	113.50	4.50	3.81
5	120.00	6.00	4.76	113.00	5.00	4.23
6	119.70	6.30	5.00	112.50	5.50	4.66
7	119.30	6.70	5.31	112.00	6.00	5.08
8	119.00	7.00	5.55	111.50	6.50	5.50
9	118.50	7.50	5.95	111.00	7.00	5.93
10	118.20	7.80	6.19	110.70	7.30	6.18
11	118.00	8.00	6.34	110.50	7.50	6.35
12	117.50	8.50	6.74	110.30	7.70	6.52
24	115.20	10.80	8.57	108.40	9.60	8.13
40	112.80	13.20	10.47	106.80	11.20	9.49
60	109.80	16.20	12.85	103.60	14.40	12.20
90	107.90	18.10	14.36	101.80	16.20	13.72
120	107.20	18.80	14.92	-	-	-

in pH values, which contributes to the stability of the production and the creation of products of better quality. The use of starter cultures ensures their dominant growth in relation to contaminating microflora which may be present in fresh meat, which guarantees the hygienic safety of the product (Zdolec et al., 2008; Bonomo et al., 2011). Over the recent years, *P. pentosaceus* bacteria have been used as starter cultures in the production of long-life sausages, since they have a lower optimal temperature than *P. acidilactici* (Raccach, 1981; Feiner, 2006), so they produce lactic acids much slower and at lower fermentation temperatures, from 15 to 27˚C. Bacterial strains from the *Pediococcus* genus are also used successfully in the production of fermented meat products because they have not shown any undesirable characteristics which could have a negative effect on the quality of the product (Medić et al., 2009; Vatanyoopaisarn et al., 2011).

Although starter cultures from the genus *Pediococcus* were introduced primarily because of their fermentation characteristics, in time their ability to inhibit undesirable microorganisms (Daly et al., 1973; Yuksegdag and Aslim, 2010) was also seen to be one of their desirable characteristics. Bacterial strains from the *Pediococcus* genus proved to be very effective in inhibiting undesirable microorganisms including *Staphylococcus aureus*, *Clostridium botulinum*, and types from the genus *Salmonella*, *Escherichia* and *Bacillus*, and mould. The inhibitive action depends on the type of bacteria and genus, the incubation temperature and the concentration and speed of creation of lactic acid (Raccach, 1981; Smith and Palumbo, 1981; Vatanyoopaisarn et al., 2011).

Among the problems which characterize the contemporary production of kulen which are of particular interest for this type of product in this country, it is necessary to point out the need to study the process of conversion of the raw filling into a mature product. Therefore the knowledge of the factors which af-

fect the course of the osmotic diffusion processes which take place in the filling of the kulen is crucial. In literature, of course, there is a great deal of information about the character and course of these processes (Feiner, 2006; Toldrá, 2008). However their weakness is that they are not completely applicable to the production conditions in this country. For this reason, we have examined the effect of the addition of starter cultures on the course of the osmotic and diffusion processes in the filling and the characteristics of the product, in relation to the traditional production without the addition of starter cultures.

Material and Methods

For kulen production, pork meat and pork fatty tissue obtained by boning out previously cooled pork half- carcasses are used. The meat of most breeds of physiologically mature pigs is suitable, especially cross Duroc Landrace and Yorkshire pigs, rich in intramuscular fat which is vital for the course of the lypolitic process without which the development of the distinctive flavour and aroma of kulen would not take place.

Boned pork meat from the thigh and back is obtained by removing as completely as possible the layers of fatty and connective tissue and the lymph nodes and blood vessels. The firm fatty tissue suitable for kulen production is obtained from the anatomic location of the neck, chin and back. Meat prepared in this way is hung to drain and frozen at -3˚C for 24-36 hours, whereby it loses 7 – 9 % of meat juices, so that it contains 52 – 54% of water before mincing. We freeze the fatty tissue to -6 ˚C. In the preparation of kulen we also use nitrite salt ("Derma" Varaždin), glucose ("Wiberg" Austria), lactose ("Wiberg" Austria), a microorganism starter culture (Biobak K. "Wiberg" Austria), seasoning (sweet and hot ground paprika, garlic) and pork casing (appendix).

Table 3. **Changes to pH and a_w values in kulen samples during the processing**

PRODUCTION		pH VALUES		a_w
		MIDDLE	EDGE	
DAYS	PHASE OF PRODUCTION	Without starter/ with starter	Without starter/ with starter	Without starter/ with starter
0	**FILLING**	5.62/5.62	5.62/5.62	0.961/0.961
1	CONDITIONING	5.74/5.74	5.74/5.74	0.959/0.960
2	**PRE-FERMENTATION AND SMOKING**	5.50/5.27	5.53/5.30	0.959/0.960
3		5.00/4.94	5.09/5.02	0.958/0.959
4		4.90/4.88	4.93/4.90	0.956/0.957
5		4.92/4.89	4.95/4.92	0.953/0.955
6		4.93/4.90	4.98/4.93	0.950/0.953
7		4.95/4.92	5.00/4.96	0.948/0.951
8		4.98/4.93	5.08/4.98	0.940/0.949
9		5.02/4.95	5.10/4.99	0.938/0.947
10		5.05/4.96	5.15/5.00	0.937/0.944
11		5.09/4.98	5.20/5.03	0.933/0.940
12		5.12/5.00	5.28/5.05	0.930/0.936
24	**FERMENTATION**	5.28/5.10	5.40/5.19	0.916/0.922
40		5.62/5.18	5.72/5.31	0.900/0.908
60		5.82/5.52	5.90/5.65	0.881/0.880
90		5.98/5.79	6.14/6.02	0.862/0.869
120		6.16/-	6.26/-	0.848/-
180/150	**STORAGE**	6.28/5.90	6.41/6.26	-

Table 4. **Chemical composition of kulen filling produced without the addition of starter cultures during the processing**

PRODUCTION		SAMPLES WITHOUT STARTER			
DAYS	PRODUCTION PHASE	WATER %	PROTEINS %	FAT %	SALT %
0	FILLING	55. 54	16. 30	23. 50	2. 10
12	AFTER SMOKING	38. 20	18. 02	38. 55	2. 68
60	MID FERMENTATION	32. 40	21. 44	40. 60	2. 86
120	END OF FERMENTATION	28. 40	24. 20	42. 10	3. 07

For the filling, the basic frozen raw materials were used, at a temperature of – 2 ºC and taken for machine mincing by a German Treif mincer, with 12 mm cutters and 20 % to 8 mm. The meat having been minced is then placed in an Italian VELATI blender, which can blend 100 kg of frozen filling. The hard fatty tissue is ground at - 6 ºC in a KRAMER-GRÄBE cutter (Germany 200 lit.) to 5-6 mm. The fatty tissue ground in this way is placed in the blender mentioned, with the addition of seasoning, additives and sugar with or without the starter culture and the rest, and it is mixed under reduced pressure until the mixture becomes even and sticky. The temperature of the filling at the end of blending is from 0 to – 2 ºC. The filling prepared in this way is taken by cart to the filler, made by the German manufacturer VEMAG H.P.15, which works under low pressure. The technological process of preparing the filling was the same, but separate, with or without the starter cultures.

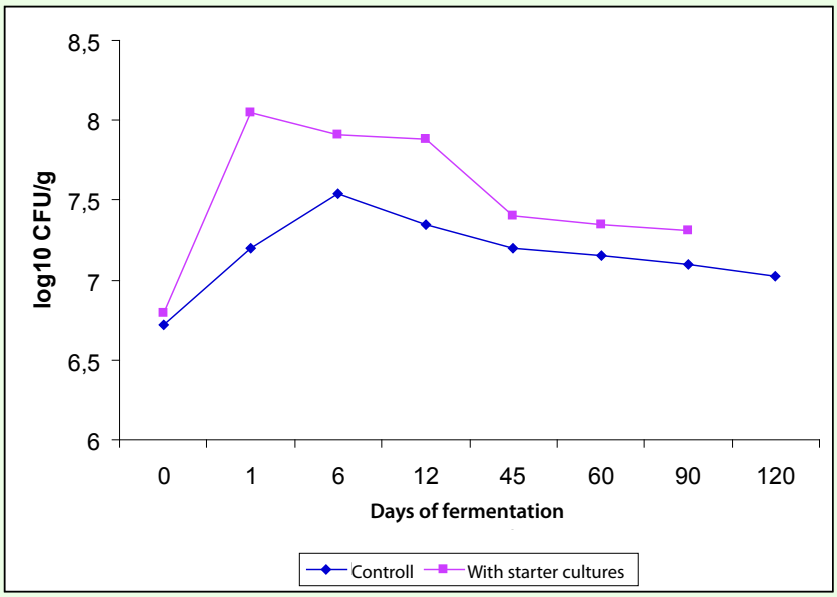

Figure 1. **Total number of bacteria in kulen samples with and without the addition of starter cultures**

Table 5. **Chemical composition of kulen filling made with starter culture Biobak K. during the processing**

PRODUCTION		SAMPLES WITH BIOBAK K.			
DAYS	PRODUCTION PHASE	WATER %	PROTEIN %	FAT %	SALT %
0	FILLING	55. 54	16. 30	23. 50	2. 10
12	AFTER SMOKING	39. 80	17. 90	38. 80	2. 64
60	MID FERMENTATION	33. 50	20. 80	40. 34	2. 82
120	END OF FERMENTATION	30. 20	23. 20	41. 70	2. 96

Table 6. **Sensory evaluation of kulen with and without the addition of starter cultures after processing and storage**

Sensory evaluation	Mean values	
	Sample	
	Without starter cultures	With starter culture Biobak K.
After processing	3.82	4.80
After storage	3.70	4.46

Mixed starter cultures are used as follows: *Pediococcus pentosaceus*: > 6 x 10^9/g, *Staphylococcus carnosus*: > 5 x 10^{10}/g and *Staphylococcus xylosus*: > 7 x 10^{10}/g. The starter cultures are used as lyophilized cultures packed in air tight aluminium foil, kept at a temperature lower than -18˚C. The quantity of starter is 50g, which is appropriate for 100 kg of product.

The pig appendix casing was salted, soaked in warm water, and then well drained so no water remained at all. After filling by hand and tying, forming a bundle, the kulen is hung on sticks and placed on the cart. In this phase of the process samples were taken and marked as needed for physical, chemical and microbiological testing, which went through the further stages of production in the course regular production. The carts with kulen were transferred to a special conditioning room in order to remove the condensed water from the surface of the casing and to equalize the temperature of the filling with the room temperature (17-19 ºC).

In this cycle it is important to reach the required temperature as quickly as possible for the growth and development of the desired microorganisms (20 - 22 ºC). After the temperature has been equalized, that is, when the desired temperature is reached in all parts of the product, smoking begins at intervals over a period of 12 days. In this period it is important to maintain the permeability of the casing, for the best possible loss of moisture from the inside. This is attained by preventing sudden drying of the surface of the casing, and gradual and cold smoking up to 22 ºC, so that during the process of pre-fermentation permeability is not lost through baking or closure by layers of pitch from the smoke on the surface.

Kulen is kept in this chamber for 12 days, where it loses 14-18 % of its initial weight at a temperature of 12 – 22 ˚C and relative humidity of 76 – 92 %. After this, the product is moved to a fermentation chamber where it remains until the end of the process. The relative humidity in the chamber is from 76-80 %, and the temperature between 12 – 14 ˚C. Kulen remains there until the end of the process, which lasts between 100 and 150 days. In both chambers, the microclimatic conditions are monitored throughout the process.

At the end of the process the samples are stored in a chamber at a temperature between 10–15 ˚C. Samples needed for testing were taken at each phase of the process and after storage of 60 days.

The following physical tests were undertaken:

- The temperature in the kulen filling was measured until it was equal to the environment using a digital device "testo" 926 or "testo" H1, (digital thermometer with testo 926 probe)

- pH values of the kulen filling were measured (by digital pH meter D 810 "Fuchs messetechnik")
- measurement of kulen filling a_w values (cryometer AWK – 10 Nagy)
- changes in mass (digital scales: "Vage" Zagreb)
- changes in diameter (moveable markers)
- microclimatic conditions in the rooms (temperature, relative humidity, air circulation, ventilation).

The microclimatic conditions in the production rooms were monitored using measuring instruments for the chambers, and a microprocessor in a chamber cupboard, where all data are recorded graphically and parameters set for air circulation, ventilation, relative humidity and temperature, and the portable instruments mentioned earlier.

Fat, protein and ash contents were estimated according to methods recommended by the AOAC (AOAC, 1999). Moisture content and sodium chloride were determined in the *biceps femoris* according to AOAC methods (AOAC, 1984).

In the kulen samples the total bacteria count was monitored according to HRN ISO 4833.

Sensory assessment of samples of the fermented sausages was conducted according to the modified DLG method (Anon., 1993). The method includes determining four characteristic attributes of the product: external appearance, colour, structure, and appearance when sliced; consistency, aroma and taste. All four attributes were given a score from 1-5 in that each attribute was given an importance factor according to its importance, as follows: external appearance – 1; colour, structure and appearance of slice – 3; consistency – 2 and aroma and taste – 4. Assessment was performed by trained panel.

Results and discussion

The temperature and relative humidity of the air in the chambers affect the pace of water loss from the product but also other changes during the production process. The results obtained indicate that the changes in temperature and relative humidity are related to the course of the production process. The work of the chambers, or the microclimate conditions in them, must be set to ensure slow and even drying, and to monitor the gradual reduction of a_w values in the filling.

During the conditioning phase the goal is to equalize the temperature of the cold filling with the given temperature in the chamber, and to remove condensed water from the surface of kulen and dry it. In this phase of the process, the air temperature was at about 18˚C and relative humidity about 77%, with rapid and continuous air circulation. The phase of pre-fermentation and smoking takes place as a rule at the highest relative air humidity and temperature levels of those we used in this study. During drying and fermentation the temperature is constant and between 12 and 14˚C, and relative air humidity is gradually reduced from the initial 92% to 76-80% at the end of the process. The relatively small quantity of kulen produced in industrial conditions, and the variety of production processes make it impossible to compare data from literature or use experience gained. However, the pace of changes in microclimate conditions in the case of our test is in line with data found in literature, although in the production of some types of fermented products slightly higher temperatures are often used (Bartolović, 1974; Savić and Tadić, 1992; Jessen, 1995; Gonzazes and Diaz, 2002; Comi et al., 2005).

By tracing the changes to the diameter and loss of weight of the kulen from Tables 1. and 2., it may be seen that the pace of change is quickest at the beginning of the process, that is, during the conditioning and pre-fermentation phase, when the product, depending on the means of fermentation applied, loses about 2 % daily, which is also seen in the diameter of the kulen. In the phase of fermentation and drying the changes decrease gradually, on average amount to about 0.6% towards the end of fermentation and significantly less. The changes to the mass and diameter depend on the pace of drying, which must be adjusted and aligned to the gradual reduction of water activity in the filling. Working in this way, it is possible to prevent the edges of the kulen from drying out, which would then prevent the further normal course of the process, and affect the quality and pace of fermentation and drying (Jessen, 1995; Čavlek, 2001; Ulmer et al., 2006). Depending on the initial diameter of the sample, the differences in changes in mass and sample diameter are clear, and in the initial phases of the process were much more visible, and less in the finished product. These differences can be linked to the pace of loss of mass of kulen. The differences in mass and diameter of kulen are related to the use of different additives. The changes in samples produced with the starter culture Biobak K are more expressed, whilst the changes in the samples made without the addition of starter cultures, that is with only native microflora present, are less expressed, which is also indicated in data from literature (Incze, 1991; Feiner, 2006).

In the initial phases of the production process there is a significant fall in the pH value (Table 3.), which is expressed the most between the fifth and seventh day of production, depending on whether kulen was

made with or without the addition of starter culture, that is in the phase of pre-fermentation and smoking, and it then begins to rise gradually. In the samples made with the starter culture Biobak K, the pH values were somewhat lower in the initial phase of the process and in the finished product, whilst the pH values in kulen made without the addition of starter cultures were somewhat higher throughout the process. The tests showed that the intensity of the pH changes are significantly affected by the presence of added sugars, and starter cultures.

The changes in water activity ($a_{w)}$ values depend on the pace of drying. The values moved from the initial 0.961 to 0.848 at the end of the process, that is on day 120, in the samples made without the addition of starter cultures, whilst the a_w values in samples made with the starter culture Biobak K moved from the initial 0.961 to 0.869 on day 90. We may conclude that a_w values are significantly affected by the pace of drying, as well as the presence of added or native flora.

The loss of mass as the result of dehydration is reflected in the changes of the initial chemical indicators. The results show that the most intensive changes to the proportion of water occur in the initial phases of the process during conditioning and pre- fermentation, and in the further course of the process the pace of changes decreases. The movements of other tested parameters also relate to these changes, as their relative share rises during the production process. A comparison of the results obtained from the samples made without the addition of starter cultures and with the starter culture Biobak K, shows that the samples made without the addition of starter cultures have a slightly smaller proportion of water, and a larger proportion of proteins, salt and fat. However, this change in the quantitative relationship between these chemical compounds, which basically depends on the gradual separation of water from the filling, cannot be seen only as an increase in the non-evaporating compounds (protein, fats and salt) at the expense of water, which evaporates. It is a more complex process which is caused not only by the chemical diversity of the filling and the influence of microclimate factors, but also the influences on the drying process of the pressure and firmness of placing the filling into the casing, the quality and method of processing the particles in the filling, the status of the myofibril and sarcoplasmic proteins, the quality and quality of processing of the casing etc.

The changes to the total number of microorganisms in the kulen filling (Figure 1.) are partially in accordance with data from the literature (Savić and Tadić, 1992). The discrepancies between the groups of samples should be linked to the addition of starter cultures, the type and quantity of added sugar, and the samples with only native flora. The total number of microaerofilic lactic acid bacteria is greater in the samples with the starter culture Biobak K than in the samples without starter cultures added. The results indicate the visible sudden development of microflora in the initial phases of the fermentation process, where higher temperatures and more water activity are present.

The samples produced with and without the addition of starter cultures were assessed at the end of drying and fermentation, that is on the ninetieth and one-hundred and twentieth day, and after sixty days of storage.

The results obtained by the modified DLG method (Anon., 1993) give individually assessed samples a varying degree of approval from the testers. On the basis of the results obtained, there is a clear significant difference in the results obtained between the samples made without the addition of starter culture and the samples made with the starter culture Biobak K. Samples made with the starter culture Biobak K were given higher scores by panel, and therefore a higher mean final score, in comparison to the samples made without the addition of starter cultures. The samples made without the additional starter cultures had a prominent, undesirable bitter taste, with visible discolouration, and the filling was not compact. The sensory testing after storage of the samples with the starter culture showed a fall in the sensory scores. This indicates that the period of distribution and sales may be shortened.

Conclusion

The results of the testing undertaken, regarding the quality, economics, continuity and stability of production, demonstrate the justification of using microorganism starter cultures in the production of kulen in controlled conditions, that is throughout the entire year, as shown by the sensory characteristics of the samples. However, the production of kulen in controlled conditions, with dynamic changes in parameters, without the addition of starter cultures, that is by means of native flora, is almost impossible, since in the early phase of fermentation the edges of the kulen may dry out and the fermentation time is extended due to the insufficient homogeneity of the filling. In some samples the filling separates and becomes discoloured, which has a negative effect on the sensory characteristics of the product.

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Received: February, 15, 2011
Accepted: March, 24, 2011

U broju 1/2011. časopisa MESO nenamjerno smo objavili pogrešku vezano za proizvodnju robne marke „SAMOBORSKA SALAMA". Jedina tvrtka koja je pod tim nazivom i u jasno definiranoj ambalaži smije proizvoditi i na tržište plasirati je IGO- MAT d.o.o.