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Impact of salt reduction on the number of microorganisms and a sensory analysis for Kranjska sausages during their shelf-life

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Avrelija Cencič passed away on 14.12.2012.

ABSTRACT

Salt is an important ingredient in the production of meat product. Any reduction of salt requires a special treatment. This study was conducted to evaluate the effect of salt reduction on the growth of microorganisms in Kranjska sausages during their shell-life and to carry out a sensory assessment. The 18 lots of sausages were prepared under salt-reduced (1.6%) and control (2.3%) salt concentrations, directly on the production line. A total of 85 sausages were analysed and the data were used for the comparisons of groups (ANOVA) and to detect the significant variables (polynomial models) influenced on the total number of microorganisms (TNMs). The significant differences were determined between the lots (representing the microbiological status of the stuffing), between the salt-reduced samples and control samples, and between the different humidity levels. The correlations and significant relationships were determined between the TNMs and the lots, the salt concentrations, and the relative humidity. The polynomial models were to general to be used for the prediction. For sensory analysis implemented on 40th day 18 sausages were assessed. The reduction of salt resulted in lower scores in the sensory evaluation. The less-salted sausages contained more microorganisms.

Key words: Kranjska sausage, reduction of salt, sensory evaluation, models, relative humidity

INTRODUCTION

Table salt is one of the oldest food preservatives. It was probably already used in prehistoric times. For decades, the main question has been what is the healthy daily amount of salt in our diet and, in particular, what is its impact on human health? The WHO and FAO recommend 5 g of salt (or 2 g of sodium) per person as the maximum daily intake (WHO, 2003). Regarding the WHO recommendations, reformulations to reduce the amount of salt have been made in bread, meat and in some dairy and convenience foods (Kloss et al. 2015).

A large intake of salt has proven to be harmful to human health. Table salt is an important nutritional risk factor for hypertension and a resulting stroke, as well as other cardiovascular diseases (Doyle and Glass 2010). Slovenia is among four countries where the inhabitants have a blood pressure that is too high. Slovenians are also among the European consumers with a very high daily intake of salt. Therefore, Slovenia adopted a National Action Plan to reduce the salt in the diets of people in Slovenia for the period 2010–2020 (Kloss et al. 2015).

Salt is essential for human life, and the meat products are one of the important sources of salt. It influences the texture, the flavour and the colour of meat products, and consequently the microbiological stability and the shelf-life of meat products.

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The impact of salt on texture, flavour and colour

A salty taste is only detected if the taste receptors react with the salt in the solution (Desmond 2007, Doyle and Glass 2010). In certain food types NaCl could be replaced by alternative salts and other additives: KCl, a mixture of phosphates and polyphosphates, glycine and potassium lactate, different proteins, nitrates, nitrites, magnesium sulphate, spices, and L-lysine hydrochloride (Desmond 2006). Salt also affects the pigment of the meat. By accelerating the oxidation of myoglobin in meat myoglobin, an undesirable grey colour begins to form in the muscle tissue. With the selection of the appropriate technological procedures (Kilcast 2007) the quantity of the required salt could be reduced. However, the reduction in the amount of salt had a significant impact on the colour and the flavour of the sausage (Aaslyng 2014).

The impact of salt on microbiological stability and shelf-life

Water in an appropriate form is essential for the growth of microorganisms in meat products. From ancient times, the preservation of food has therefore focused on controlling the moisture in the food. The current hypotheses about binding water in the meat are based on the electrostatic, osmotic and capillary forces, upgrading recently on the water-holding hypothesis: surface force, electric double-layer force, hydrophobic interaction, and repulsive entropic forces. The influence of salt on the ability of minced meat and fat to bind water is very complex and depends on the involved structures of many proteins, i.e., the myosin family, actin, actomyosin, titin and many enzymes and their ability to interact (Puolanne and Halonen 2010, Soladoye et al. 2015).

Salt is also dissolved in the water contained in the meat, and in this way the salt concentration and the osmotic pressure in the meat juice is increased. This increased osmotic pressure inhibits the life processes in bacteria, their growth and reproduction to the point where they can no longer have a detrimental impact on the meat. Part of the free water is bound to salt; therefore, the activity of the water (a_w) is decreased (Desmond 2007, Doyle and Glass 2010).

Salt reduction causes problems with food safety because the microbiological stability of meat products is worsened and the shelf-life is shortened. Before reducing the amount of salt it is necessary to optimize recipes with respect to the replacement of salt with the appropriate equally-acting substances. The most common foodborne pathogens in meat and meat products are bacteria of the genera *Salmonella, Campylobacter*, *Listeria*, *Escherichia, Staphylococcus, Clostridium*, *Vibrio*and*Bacillus*(Desmond 2006, Desmond 2007, Doyle and Glass 2010) and analysis of determination of the types of micro-organisms are necessary (Hwang 2009, Pereira 2011, Giaouris 2014, Sharedeh 2015).

Therefore, the predictions of the growth rates for harmful microorganisms in meat products during their shelf-life have been driven mainly from the point of view of the microbiological safety of the food. Food safety and the development of growth models were initiated by governments, for example, the USDA Pathogen Modelling Program (U.S. Department of Agriculture), Combase (Institute of Food Research from UK, USDA, and Food Safety Center from Australia), and Seafood spoilage and Safety Predictor (Technical University of Denmark) (Doyle and Glass 2010, Hwang 2013). These models are based on real data, such as meat-product recipes (i.e., type of meats, salt, seasonings, nitrite content and more), food-processing characteristics, storage conditions, etc. Growth models were developed for different foodborne pathogens such as *Listeria monocytogenes*, *Salmonella spp.*, *Yersinia unterocolitica*, *Escherichia coli*. Some models are based on only one effect, i.e., the storage temperature or additional effects, i.e., temperature, pH, NaCl concentration, activity of the water, modified atmosphere of packed food (Mejlholm et al. 2010).

The aims of this study were to statistically evaluate the impact of salt reduction on the number of microorganisms and on a sensory analysis on the 40th day after production for Kranjska sausages during their shelf-life. A total of 85 sausages were analysed according to the selected experimental design. The variability of the TNM during the shelf-life was expressed with the intra-assay coefficient of variability. The statistical analysis was designed to reveal the differences between two groups of sausages, i.e., the salt-reduced (1.6% salt concentration) and control samples (2.3% salt concentration), by using ANOVA and taking into account the total number of microorganisms, the stuffing (lots), the salt concentrations, and the humidity on the morning of the production day. And secondly, to determine the influence/importance of the stuffing (lot), selected sampling days, salt concentrations, morning humidity on the day of the production, and the influence of the daily production environment on the number of microorganisms on the basis of polynomial models. The sensory analysis was performed on 18 sausages to find out if salt reduction had an influence on the colour, odour and taste of the sausages on the 40th day after production.

MATERIALS AND METHODS

Production of Kranjska sausages and technological specificities

Kranjska sausage is a pasteurized, semi-durable sausage made from coarsely minced pork meat category I (percentage of fat up to 10%), solid fat, also category I, and selected spices. First, 400 kg of pork meat was cut in the meat grinder. Then, 150 kg of this pork was salted with 2.80 kg of 0.5% nitrite salt. After 24 hours the stuffing for the sausages was prepared by mixing 150 kg of pork meat, bacon, selected spices (garlic, pepper), a specific quantity of 0.5% nitrite salt and a specific amount of coarsely minced pork meat, so that the total weight of the stuffing was 400 kg. A total of 50 kg of well-homogenized mass was then taken, tagged and filled into a pork intestine with a diameter of 32 cm. The target concentration was from 1.5% to 1.7% (Ministry of health 2010). To the remaining amount of stuffing we added a specified amount of salt to reach the target concentration

of 2.33% and mixed it well. The stuffing was then filled in thin a pork intestine and closed with a short wooden stick in pairs with coupled ends (Figure 1). The sausages were then smoked hot and pasteurized to the target central temperature of 70°C. This procedure guarantees that no pathogen microorganism harmful to human health remained in the sausages. The sausages were then cooled to the target core temperature of 6°C, packed in a controlled atmosphere, and stored at a temperature of 6°C.

Fig. 1: Kranjska sausage.

The production day for the sausages is expressed as the "lot". For example, lot 11215 means the year 2011, while 215 means the day of the year when the sausage was produced. In this case, the production day was the 3rd of August 2011.

Kranjska sausage is eaten warm, after a brief overheating in hot water to obtain a very specific taste and a high culinary quality. The surface of the sausage is reddish brown (see Figure 1), with a mild odour of smoke; the meat is pinkish red in the cross-section, and the bacon is white and insoluble. The texture is taut, crispy and juicy. The aroma is full and typical for a desalinated, specifically seasoned and smoked pork.

Analytical determination of the NaCl in the sausages after a thermal and a cooling process

The actual salt content in Kranjska sausages was analytically determined after the thermal processing at 70°C and after the cooling of the product to 6°C using the Mohr method. The determination of concentrations of NaCl is based on the titration of chlorides by $AgNO_3$ (ISO 1841-2:1996).

Determination of the total number of microorganisms (TNMs)

The analysis was performed according to ISO 4833:2003E. This method is appropriate for a determination of the TNM for aerobic mesophilic bacteria in food and feed, in this case in 20g of the sausage, by counting the number of grown colonies on a solid medium after aerobic incubation at 30°C. Pathogens were not expected due to the thermal and cooling process. Therefore, the species of microorganisms and pathogens were not analysed. The TNMs were counted on days 1, 5, 10, 15, 20, 25, 30, 35 and 40.

We were not able to determine the TNM of the raw pork meat before its chopping in the meat grinder because it consisted of many small meat parts. The first measurement of the TNM was meaningful on the 1st day after the sausage was prepared, heated to 70°C and cooled to 6°C.

Sensory evaluation on the 40th day after production

The sensory evaluation took place on the 40th day after production. Three trained evaluators performed the sensory evaluation. The evaluators assessed the reduced-salt samples and the samples with the standard salt content.

Before the assessment, the samples of sausages were heated and served on white plates. The evaluators assessed the colour of the sausage cross-section, together with the colour of the exterior of the sausage, the odour and the taste of the sausage. The maximal score was 33. The sensory assessment criteria are shown in Table 1.

Table 1: Sensory assessment criteria.

Sensory assessment criteria	Score	Description
Section colour	3	Typical colour, without grey areas
	0	Atypical colour of the section and with expressed grey areas
Odour	3	Pleasant, typical and harmonious
	0	Non-harmonic, atypical and with the emergence of a rancidity, sour or musty
Taste	5	Pleasant, typical and harmonious
	0	Non-harmonic, an insignificant, or an unpleasant taste, as a result of excessive smoke, a bitter taste or flavour of rancidity, as a result of low-quality raw materials.

Sampling

The production of sausages was carried out under constant conditions. The technological process enshured the homogeneity of ingredients in the stuffing. No more than one lot was prepared per day.

From 400 kg of the stuffing (one lot) 1666 sausages are usually prepared. In this case before the final salting, 50 kg of stuffing were taken for the preparation of 208 less-salted sausages. The rest of the stuffing was additionally salted and 1458 sausages were then prepared with the controlled concentration of the salt. 18 lots were included with the following lots numbers 11215, 11216, 11217, 11220, 11221, 11222, 11223, 11224, 11228, 11229, 11235, 11236, 11237, 11238, 11240, 11264, 11265, and 11266.

The study was conducting and sampling was implemented according to the selected experimental design in three parts:

1. TNM counting on 40th day after production for 18 lots for two salt concentrations

In this case 36 sausages were randomly taken as samples for the analysis of each lot and for both salt concentrations. The data from the samples were divided into two sets for the statistical analysis because the last three lots were prepared one moth later then the others. Therefore, Set A consisted of the data for 30 samples/sausages for 15 lots: 11215, 11216, 11217, 11220, 11221, 11222, 11223, 11224, 11228, 11229, 11235, 11236, 11237, 11238, and 11240. Set B consisted of data for 36 samples: set A and lots 11264, 11265, and 11266.

2. TNM counting on 9 days: 1st, 5th, 10th, 15th, 20th, 25th, 30th, 35th, and 40th for three lots 11264, 11265, 11266 and two salt concentrations.

In this case 54 sausages were randomly picked as the representative for 3 lots, for two salt concentrations and for 9 sampling days.The TNM values on the 40th days of 6 samples for lots 11264, 11265, and 11266 and for two salt concentrations were used also in the first part of the study. One replication of the TNM counting on the 30th day was made for the less-salted sausages. The two salt-reduced sausages on the 30th day were taken from lot 11264. Set C consisted of analysed data for 55 sausages, also taking into account the replication.

Set D consisted of data for all 85 analysed sausages.

3. Additionally, 18 sausages were randomly selected for lots 11264, 11265, 11266 for the implementation of the sensory analysis, i.e., 3 assessors x 3 lots x 2 salt concentrations.

The sausages prepared and used in the present study were in line with the Commission Regulation for the microbiological criteria for meat products (Commission Regulation 2005) and Guidelines for microbiological safety, which are intended for the final consumer (Rupel et al. 2009). This meant that all samples were free of pathogenic microorganisms due to the selected technological procedure.

Table 2: The study variables.

The independent variables

Sign	Name	Type of variable	The range or the scale points
X1	The stuffing for the sausages(the lot)	Numeric- discrete value	18 lots with short names and actual numbers in bracket L1(11215), L2(11216), L3(11217), L4(11220), L5(11221), L6(11222), L7(11223), L8(11224), L9(11228), L10(11229), L11(11235), L12(11236), L13(11237), L14(11238), L15(11240), L16(11264), L17(11265), L18(11266)
X2	The sampling days after the sausages' production	Numeric- discrete value	9 sampling days 1, 5, 10, 15, 20, 25, 30, 35, 40 day
X3	The NaCl concentration	Numeric -continuous value	2 concentrations area – reduced from 1.43% to 1.65% marked as R and control from 2.09% to 2.50% marked as C

The independent and confounding variables

X4	The relative humidity on the morning on the day of the production	Numeric- continuous value	62% to 88 %
X5	The day of the week when production took place	Numeric - discrete value	6 days Monday, Tuesday, Wednesday, Thursday, Friday and Sunday or 1, 2, 3, 4, 5, 7

The dependent variable

Sign	Name	Type of the variable	The measured range
Y	The total number of microorganisms (TNM)	Numeric - continuous value	1000–39000

Statistical analysis

On the basis of the collected data and analysed values the variables for the statistical analysis were selected. Table 2 shows the selected variables. The independent variables were the lot (X1), the sampling days (X2), the NaCl concentration (X3), the confounding/independent variables for the relative morning humidity (X4) and the day of the week expressing the daily production conditions (X5) and one dependent variable TNM (Y). X1 represented the impossible-to-define microbiological status of the stuffing for the sausages (small parts of meat from different origins) at the beginning of the production. X4 was the relative humidity in the morning (%) on the day of the production (ARSO 2011). X4 was recorded as a hidden independent variable. Therefore, the levels were not planned in advance. X5 was recorded as the potential hidden variable, which represented the influence of the daily working environment, i.e., the personnel. The dependent variable TNM (Y) was the total number of microorganisms.

The statistical analysis included:

1. Data visualizations

Scatter plots were used for the visualization of the data from set B and set C.

2. Tests for normality

The Shapiro-Wilk and Kolmogorov-Smirnov tests were implemented for testing the normality of the data for sets A, B, C and D, with the purpose of selecting parametric or non-parametric methods for the statistical analysis.

3. Parametric and non-parametric tests for the comparisons of the salt-reduced and control samples.

For the analysis of set B, Friedman's two-way non-parametric analysis of variance (ANOVA) and the non-parametric Kruskal-Wallis test were used. For the analysis of set C the analysis of variance for normally distributed data was calculated.

4. Calculation of correlations between two selected variables and expression of the importance of selected independent variables according to a selected dependent variable

The Pearson product-moment and Spearman rank-order correlations were calculated to determine the correlations between the variables. The regression analyses were carried out to confirm the relationships between selected variables and their significance on the basis of statistically significant models (Freund and Littell 1991).

The linear regression was used to test the relationship between the number of microorganisms TNM (as the dependent variable) and the selected independent variables (Table 2). The general equation for the linear polynomial models with the intercept was:

$$Log_{10}Y = intercept + Ai*X1 + Bi*X2 + Ci*X3 + Di*X4 + Ei*X5 \quad /1/$$

or without the intercept:

$$Log_{10}Y = Ai*X1 + Bi*X2 + Ci*X3 + Di*X4 + Ei*X5 \quad /2/$$

The criteria for the evaluation of the models were: the F and p values of the model, the Error, the Correlation coefficient, the RMS (%) and the Shapiro-Wilk and the Kolmogorov-Smirnov normality tests for checking the normal or non-normal distributions of the residuals. The equation for RMS(%) was as follows:

$$RMS(\%)=SQRT(Error/n)/(Ymax-Ymin)*100 \quad /3/$$

The "Error" was the square root of the difference between the measured Y and the calculated Y. "n" was the number of samples. Ymax was the maximum value of the measured Y and Ymin was the minimum value.

A variance inflation factor (VIF) and a condition index (C.I.) are tests for detecting the multi-collinearity to exclude possible hidden dependencies between selected independent variables in the polynomial models. The multi-collinearity existed if one variable represented a linear combination of the other variables (Montgomery and Peck 1989, Freund and Littell 1986).

The correct metric with which to analyse the selected variables was not apparent. The Box-Cox power transformation of the dependent variable and the standardization of independent variables were performed to improve the fit of the polynomials to the data (Box-Cox 1964, Winsberg and Ramsay 1980, SAS/Stat 2008). The standard procedure PROC TRANSREG (SAS 9.4) was carried out. The optimal transformation for Y was determined on the basis of changing the λ list from -2 to 2 by 0.05 and it was the decimal logarithm $log_{10}Y$. For the independent variables the spline transformations (spline is a piecewise polynomial of degree k with function values and the first k – 1 derivatives) with a certain number of knots were the most appropriate. The knots were the number of discontinuous areas within the spline. A larger number of knots increased the fit of the spline function to the data. The starting number of knots in the case of the spline transformations was the number of levels of the independent variables. We increased the number of knots up to 10 to bend the curve more closely to the data.

5. Variability determination

The repeatability and variability of the data were expressed on the basis of one replicated TNM counting and an intra-assay coefficient of variation (intra CV) for the set C. The intra CV was also calculated to avoid misleading conclusions on the basis of only one TNM replicated counting.

6. Parametric and non parametric tests for the sensorial analysis

This was obtained on the basis of the parametric and non-parametric tests of the differences between the control and the salt-reduced samples' scores for the odour, the colour of the section, the outside colour, and the taste. The parametric tests (the paired t-test, two-independent samples t-test) and non-parametric tests (Wilcoxon Signed-rank test, Wilcoxon-Mann Whitney test) were calculated. The paired T test or Wilcoxon signed-rank test were used for a comparison with the independent tests because the same stuffing (one lot) was used for the preparation of the salt-reduced sausages and after the addition of the salt also for the control sausages. The normality test Shapiro-Wilk for checking the distributions of the differences between the samples was also employed.

A statistical analysis was calculated with the program SAS 9.4 for Windows.

RESULTS AND DISCUSSION

The results of the first part of the study, set A,B: detecting of the variables affecting the TNM on the basis of group

Table 3: The p-values under the null hypothesis of normality of Shapiro-Wilk and Kolmogorov-Smirnov tests are displayed.

		A set	B set	C set	D set
Variable	Test for normality	p Value	p Value	p Value	p Value
Y	Shapiro-Wilk	< 0.0001	< 0.0001	0.0003	< 0.0001
Y	Kolmogorov-Smirnov	< 0.0100	< 0.0100	< 0.0100	< 0.0100
logY	Shapiro-Wilk	0.0070	0.0033	0.0493	0.0004
logY	Kolmogorov-Smirnov	<0.0116	0.0114	>0.1500	0.0394

Table 4: Friedman's two-way non-parametric analysis of variance (ANOVA) and non-parametric Kruskal-Wallis test are displayed for set B.

Friedman's two-way nonparametric analysis of variance
4.A Model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	18	3667.611111	203.756173	24.76	<.0001
Error	17	139.888889	8.228758		
Corrected Total	35	3807.500000			

R-Square	CoeffVar	Root MSE	RlogY Mean
0.963260	15.50584	2.868581	18.50000

4.B Significance of the variables in the model

Source	DF	Anova SS	Mean Square	F Value	Pr > F
x1	17	3034.250000	178.485294	21.69	<.0001
x3	1	633.361111	633.361111	76.97	<.0001

4.C Fisher's least-significant difference test (pairwise t tests): comparisons significant at the 0.05 level are indicated by *.**

Comparisons significant at the 0.05 level are indicated by ***.				
x3 Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
R - C	8.3889	6.3716	10.4062	***

comparisons and polynomial models.

The TNM for 18 lots changed from 1,000 to 39,000. The TNM values of the controlled samples ranged from 1,000 to 23,000 and from 1,000 to 39,000 for the salt-reduced samples. The reduced-salt samples had an approximately 3-times higher TNM than the control samples. The exception was sample taken from lot L11, where it was assumed that the TNM was very low in comparison to the other samples. The salt content changed in the case of salt-reduced samples from 1.43 to 1.65 and from 2.09 to 2.50 in the case of controlled samples. The morning humidity during the implementation of the study was from 72 % up to 88%. It should also be noted that the relative humidity was higher in the case of samples with higher TNMs. The most often day of the week of the production of the sausages was Wednesday (5 times). Table 2 presents the study variables: types, ranges or their scale points, units if there are any. Figure 2 displays the described experimental set-up. Figure 2 shows the difference in the TNMs between the control and the salt-reduced samples for all lots on the 40th day.

Testing for normality (see Table 3) did not confirm the normal distribution for sets A (30 samples and 15 lots) and B (36 samples and 18 lots); therefore, the non-parametric analysis of variance was used. The results of the analysis of variance are displayed in Table 4. The model (Table 4.A) represented the relation between dependent TNM ($Rlog_{10}Y$), which was first ranked, and two independent variables lot (X1) and salt concentration (X3). These results indicated that the overall model is statistically significant, F= 24.76, p-values was less than 0.0001, and accounted for a significant portion of the variables X1 and X3. The R-squared was 0.96326, and indicated that model accounted for 96% of the variations of X1 and X3. The variables X1 and X3 (Table 4.B) are also statistically significant (p-values were less than 0.0001).

4.D Fisher's least-significant difference test (pairwise t tests): comparisons significant at the 0.05 level are indicated by *.**

x1 Comparison	
L17 - L9, L3, *L5, L1, L12, L13, L4, L8, L14, L7, L10, L11*	***
L6- L3, *L5, L1, L12, L13, L4, L8, L14, L7, L10, L11*	***
L16 - L3, *L5, L1, L12, L13, L4, L8, L14, L7, L10, L11*	***
L18 - *L5, L1, L12, L13, L4, L8, L14, L7, L10, L11*	***
L15 - *L5, L1, L12, L13, L4, L8, L14, L7, L10, L11*	***
L2- L12, L13, L4, L8, L14, L7, L10, L11	***
L9- L17, L14, L7, L10, L11	***
L3- L17, L6, L16, L11	***
L5- **L17, L6, L16, L18, L15**, L11	***
L1- **L17, L6, L16, L18, L15**	***
L12 - **L17, L6, L16, L18, L15**, L2	***
L13 - **L17, L6, L16, L18, L15**, L2	***
L4- **L17, L6, L16, L18, L15**, L2	***
L8- **L17, L6, L16, L18, L15,**L2	***
L14 - **L17, L6, L16, L18, L15**, L2, L9	***
L7- **L17, L6, L16, L18, L15**, L2, L9	***
L10 - **L17, L6, L16, L18, L15**, L2, L9	***
L11 - **L17, L6, L16, L18, L15**, L2, L9, L3, L5	***

Comparison of the rank means between the salt-reduced (R) and control (C) samples showed significant difference at the 0.05 (Table 4.C).

Comparisons of the rank means between lots showed significant ones at the 0.05 and they are listed in Table 4.D. Depending on the significant comparisons, the lots could be classified into three groups. In the first group, the lots had TNM less than 10,000. The first group lots were L5, L1, L12, L13, L4, L8, L14, L7, L10, and L11. In the second group, the lots had, TNM less than or equal to 10,000, and less than 20,000. There were lots L2, L9 and L3. In the third group, had a TNM equal to or greater than 20,000. The third group lots were L6, L15, L16, L17, L18. The classifications of the lots are shown in Figure 2 using the two bold black horizontal lines. Each member of the first group was significantly different from each of the third group of lots and vice versa. L2, L9 and L3 were, with regard to their means, not significantly different to particular members of the first or to the third group and therefore were classified into second group. Such significant differences between the lots based on the TNM were not expected. This was the main reason that the humidity (x4) was included in the calculations. It was important variable, which influenced on TNM.

Nonparametric Kruskal-Wallis tests (Table 4.E) indicated the same as nonparametric ANOVA, i.e., that there were the significant differences between the lots (X1) (p-value=0.0462) and the salt-reduced and control samples

Non-parametric tests
4.E Non-parametric tests

Test	Variable	Chi-Square	Pr> Chi-Square
Kruskal-Wallis	X1	27.8920	0.0462
Kruskal-Wallis	X3	5.8221	0.0158

X3 (p-value=0.0158).

Findings for sets A on the basis of models A.A. and A.B and for set B on the basis of models B.A and B.B (Table 7):

The intercept was statistically not significant in models A.A and B.A. The correlation coefficient was much higher in the model A.B and B.B without the intercept, the RMS values were approximately 20 %. The explanation was that the majority of the lots had a low TNM at the beginning, but still not zero (see Figure 2).

TNM ($\log_{10}$Y) was inversely proportional to the salt content (X3).

The relation between TNM ($\log_{10}$Y) and lot (X1): X1 was not statistically significant for the models A.A, A.B and B.A, but it was significant in model B.B. Figure 2 shows that lots L16, L17 and L18 had higher TNMs in comparison to the other lots and set B had these lots included. Therefore X1 became statistically significant.

TNM ($\log_{10}$Y) was proportional with the relative humidity X4 (%) and it was statistically significant. In the future the relative humidity should be permanently controlled during the production of meat products.

The relation between TNM ($\log_{10}$Y) and the day in the week (X5) was not statistically significant .

The models A.A, A.B, B.A and B.B were used for prediction of TNM for lots L16, L17 and L18, but calculated TNM values were too low. The models were too general for prediction, but they determined relationships between selected variables.

The results of the second part of the study, set C, D: determination of variables influencing on TNM on the basis of comparison of groups, expressing the variability of the TNM.

The data for set C were normally distributed (see Table 3) for $\log_{10}$Y (the p value for Kolmogorov-Smirnov test was 0.15).

For lots L16, L17 and L18, the TNMs were never bellow 1,000 on the first day after production. The average TNM was 1,833 on the first day for the 2.37% salt concentration and 4,100 for the 1.61% concentration. The relative humidity during the production days of lots L16, L17 and L18 was quite high, i.e., over 80%.

Figure 3 shows the TNMs of the samples on days 1, 5, 10, 15, 20, 25, 30, 35, and 40. The higher TNMs are for the salt-reduced samples (dashed lines), and the lower TNMs for the control samples (solid lines). On average, the significant change was between the 30th and 35th day, after which it was slowly increasing, decreasing or staying the same: 3 samples out of 6 had a lower TNM, 1 had the same TNM, and 2 samples had a higher TNM than on the 35th day. In the case of the previously mentioned samples of lot L11 the number of microorganisms drastically decreased before the 40th day after the production. The TNMs on the 20th and 25th days after the production for lot L16 were greater in comparison

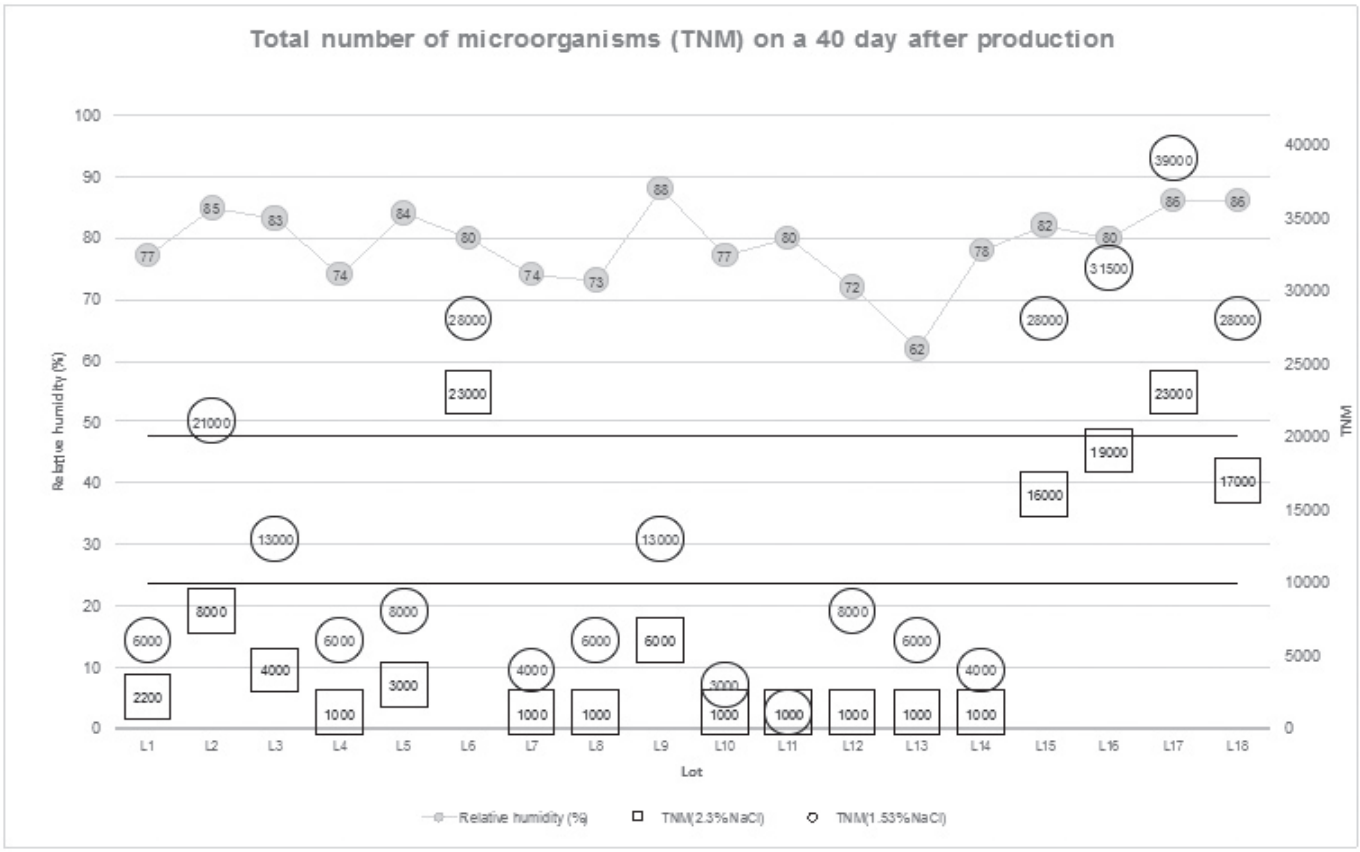

Fig. 2: Total number of microorganisms (TNMs) on the 40th day after production for 18 lots with relative humidity in % on the day of the production (numbers at the top of the dashed horizontal lines) for the control samples with average salinity 2.32% (circles with TNM values) and the salt-reduced samples with average salinity 1.55% (squares with TNM values).

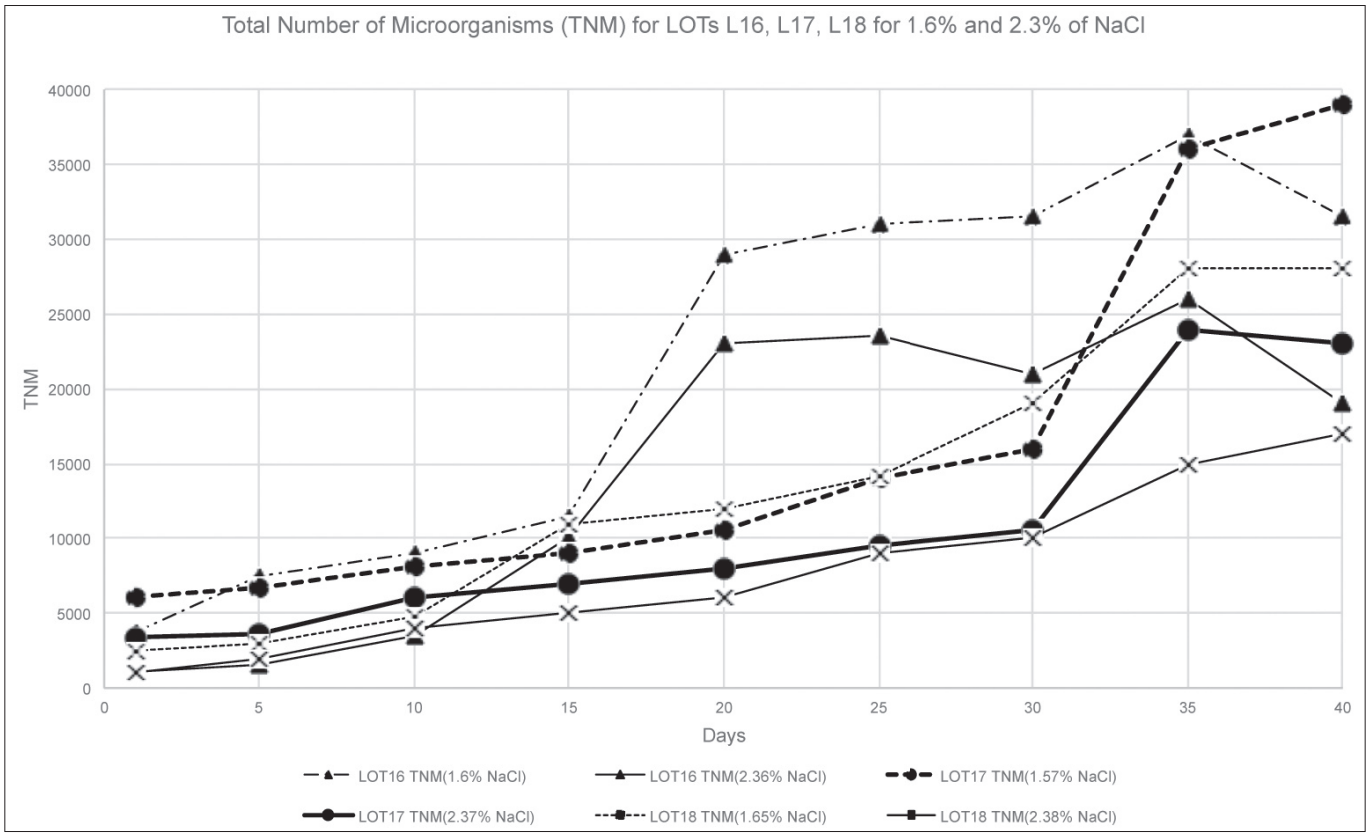

Fig. 3: Total number of microorganisms (TNMs) for lots L16 (lines with triangles), L17 (lines with circles) and L18 (lines with squares) on the 1st, 5th, 10th, 15th, 20th, 25th, 30th, 35th and 40th day after the production. The solid lines present the TNMs for the control samples. The dashed lines present the TNMs for the salt-reduced samples.

Table 5: The analysis of variance for Set C is presented.

5.A Model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	12	7.93937633	0.66161469	40.34	<.0001
Error	41	0.67236900	0.01639924		
Corrected Total	53	8.61174533			

R-Square	Coeff Var	Root MSE	logY Mean
0.921924	3.207547	0.128060	3.992444

5.B Significance of the variables in the model

Source	DF	Anova SS	Mean Square	F Value	Pr > F
x1	2	0.41303633	0.20651817	12.59	<.0001
x2	8	6.55682700	0.81960338	49.98	<.0001
x3	1	0.74483267	0.74483267	45.42	<.0001
x4	1	0.22468033	0.22468033	13.70	0.0006

5.C Fisher's least-significant difference test (pairwise t tests): comparisons significant at the 0.05 level are indicated by ***.

Comparisons significant at the 0.05 level are indicated by ***.				
x1 Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
L16–L17	0.06450	-0.03930	0.16830	
L16–L18	0.20917	0.10537	0.31296	***
L17–L18	0.14467	0.04087	0.24846	***

5.D Fisher's least-significant difference test (pairwise t tests): comparisons significant at the 0.05 level are indicated by ***.

Comparisons significant at the 0.05 level are indicated by ***.				
x3 Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
R - C	0.23489	0.16450	0.30527	***

5.E Fisher's least-significant difference test (pairwise t tests): comparisons significant at the 0.05 level are indicated by ***.

Comparisons significant at the 0.05 level are indicated by ***.				
x4 Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
H1 - H2	0.13683	0.06218	0.21149	***

H1 = 80% humidity, H2 = 86% humidity

to lots L17 and L18.

Findings for Set C

The results of the analysis of variance are presented in Table 5. The model (Table 5.A) represented the relation between dependent TNM ($\log_{10}$Y) and the four independent variables lot (X1), the sampling day (X2), the salt concentration (X3) and humidity (X4). These results indicated that the overall model was statistically significant, F= 40.34, p-values were less than 0.0001, and accounted for a significant proportion of variables X1 – X4. The R-squared was 0.921924 and indicated that the model accounted for 92% of the variations of X1-X4. The variables X1 – X4 (Table 5.B) were also statistically

Table 6: Spearman rank-order and Pearson product-moment correlations, the p-value under the null hypothesis of zero correlations for the pairs of analysis variables, one independent variable X_i and the dependent variable Y, are displayed for sets A, B, C, and D.

Set	Variables	r	Prob>|r|	Correlation
A	X3	-0.32	0.0846	Pearson
(30 samples)	X4	0.39	0.0333	Pearson
	X1	-0.16	0.3942	Spearman
	X5	0.17	0.3567	Spearman
B	X3	-0.26	0.1222	Pearson
(36 samples)	X4	0.49	0.0024	Pearson
	X1	0.27	0.1061	Spearman
	X5	0.22	0.1960	Spearman
C	X3	-0.29	0.0293	Pearson
(55 samples)	X4	-0.25	0.0684	Pearson
	X1	-0.24	0.0806	Spearman
	X2	0.86	0.0001	Spearman
	X5	-0.24	0.0806	Spearman
D	X3	-0.26	0.0158	Pearson
(85 samples)	X4	0.21	0.0598	Pearson
	X1	0.21	0.0552	Spearman
	X2	0.10	0.3449	Spearman
	X5	0.03	0.7770	Spearman

significant (p-values were less than 0.0001 for X1, X2, X3 and less than 0.0006 for X4). A comparison of the means between the lots showed significant difference at the 0.05 (Table 5.C) between L16 and L18 and between L17 and L18, but not between L16 and L17. Comparison of the means between the salt-reduced (R) and the control (C) samples showed significance at the 0.05 (Table 5.D). A comparison of the means between the morning humidity on the day of the production (H1 = 80% humidity, H2 = 86% humidity) also showed a significant difference at the 0.05 (Table 5.D).

During the shelf-life the sausages were kept under the controlled conditions. One repetition of counting TNM was done for lot L16, the salt-reduced sample and for sampling on 30th day after production. The TNM of the first one was 31,000 and for the second one it was 10,500. The mean value was 20,800 and the standard deviation was+/-14,849. The coefficient of variation (CV) was 70%. CV was extremely high, but CV often shows misleading values. Therefore, the intra CV was calculated to describe a variability within a population of sausages during the shelf-life in the lots L16, L17, and L18.The calculated intraCV was 36.78% for the set C (including the repetition).The intra CV for the salt-reduced samples in set C was 35.66% and for the control salt samples it was 43.24%. The control salt samples had higher variability than the salt-reduced samples. The values showed high variability. The reason was that TNM did not depend only on the salt concentration but on the humidity, lot, some uncontrolled contaminations, micro-flora of a particular sausage during the shelf life and some other reasons. In this case Figure 3 shows the variability of TNMs in the sausages during the shelf-life when the sausages were cooled to 6^{o}C. Nevertheless, to the intra CV, the analysis of variance showed the significant differences between the salt-reduced and control sausages according to the TNM and the salt concentrations, lots and morning humidity (Table 5).

Pearson and Spearman correlations between variables were calculated. They are presented in Table 6. The correlation between the humidity (X4) and TNM ($\log_{10}$Y) were significant, with a p-value of less than 0.03 (set A) and 0.00 (set B). This indicated strong, positive relationship between these two variables. The relationship was on the limit in the case of set D, p-value was 0.0598. Set C showed the oposite correlation between X4 and Y. In the case of set C only two values of humidity appeared, 80% and 86%, which is not enough for a determination of the correlations. A significant p-value was also between salt concentration (X3) and TNM ($\log_{10}$Y) for set C and D. For set A it was near the limit (p-value was 0.0846) and for set B it was not significant.The relationship between days of shelf-life (X2) and TNM ($\log_{10}$Y) was significant only in set C, where the p-value was 0.0001. The relationship between the day of the production (X5) and TNM ($\log_{10}$Y) was not significant for all sets.

Table 7: Models' results for A.A, A.B, B.A, B.B, D.A and D.B showing independent variables affecting the dependent variable TNM. The logarithmic values of TNM ($log_{10}Y$) were taken into the calculations. The variance inflation factor (VIF) and condition index (C.I.) are showing that there is no collinearity between variables. The complex transformations of the independent variables were used for a calculation of models A.C, B.C and D.C with the purpose to decrease RMS(%) and increase the correlation coefficient values just to confirm the relationship between selected independent variables and the dependent variable and their importance on the basis of their statistical significance.

Set	Model	F value	p value Prob > F	Error	Correlation coefficient	RMS (%)	Shapiro-Wilk* p Value	Kolmogorov-Smirnov* p Value
A	A.A	6.36	0.0011	3.56129	0.4250	21.65	0.7605	>0.1500
A	A.B	659.58	<.0001	3.91450	0.9887	22.70	0.9381	>0.1500
A	A.C	6.20	0.0155	1.53837	0.8048	14.23	0.7104	>0.1500
B	B.A	9.75	<.0001	4.56247	0.4999	22.37	0.7977	>0.1500
B	B.B	876.27	<.0001	4.68143	0.9898	22.66	0.9197	>0.1500
B	B.C	11.72	0.0003	1.56702	0.8884	13.11	0.6948	>0.1500
D	D.A	19.72	<.0001	8.20458	0.5270	19.53	0.1614	>0.1500
D	D.C	6.64	<.0001	6.51144	0.7928	17.40	0.4287	>0.1500

· *Tests for normality of residuals

Model A.A				
Parameter Estimates				
Variable	Parameter Estimate	p value Prob > |t|	VIF	C.I.
Intercept	3.90588	0.1279	0	1
X1	-0.00949	0.3086	1.21	6.12
X3	-0.58188	0.0028	1.01	12.82
X4	0.03394	0.0074	1.11	30.78
X5	0.09629	0.0572	1.10	100.37

Model A.B				
Parameter Estimates				
Variable	Parameter Estimate	p value Prob > |t|	VIF	C.I.
X1	0.00383	0.2864	127.22	1
X3	-0.54865	0.0050	24.49	5.57
X4	0.04512	<.0001	109.45	12.15
X5	0.08151	0.1062	6.96	29.18

Model B.A				
Parameter Estimates				
Variable	Parameter Estimate	p value Prob > |t|	VIF	C.I.
Intercept	-1.03484	0.3756	0	1
X1	0.00941	0.0308	1.11	6.59
X3	-0.52914	0.0029	1.01	12.98
X4	0.04303	0.0003	1.04	33.11
X5	0.06037	0.2044	1.07	48.61

Model B.B				
Parameter Estimates				
Variable	Parameter Estimate	p value Prob > |t|	VIF	C.I.
X1	0.00693	0.0324	128.86	1
X3	-0.55775	0.0014	24.38	6.01
X4	0.03792	0.0001	118.58	12.25
X5	0.06231	0.1885	7.97	29.89

Model D.A				
Parameter Estimates				
Variable	Parameter Estimate	p value Prob > |t|	VIF	C.I.
Intercept	-2.53089	0.0026	0	1
X1	0.01635	<.0001	2.03	5.31
X2	0.02367	<.0001	1.77	9.53
X3	-0.40268	<.0001	1.01	14.68
X4	0.03098	0.0002	1.39	50.73
X5	-0.02814	0.3950	1.13	66.87

Set C in general did not show the same correlations as sets A, B and D; therefore, the linear regression was not calculated. The calculated correlation coefficients justified the use of the linear regression for the sets A, B and D to determine the relationships between variables on the basis of the models in more detail.

Table 7 shows the parameters of the calculated linear polynoms. RMS (%) values were in all cases approximately 20%; nevertheless, the models were highly significant (p values Prob >F were lower than 0.05). A decimal logarithmic transformation of the dependent variable was used. By using

Table 8: The results of parametric and non-parametric comparison tests of sensory analysis including the colour of the section and the exterior colour, the odour and the taste on the 40th day from three evaluators for the lots 11264, 11265 and 11266 are presented. ColourR, OdourR, and TasteR means the salt-reduced samples. ColourC, OdourC, and TasteC means the control samples.

	ColourR-ColourC	ColourR-ColourC	OdourR-OdourC	OdourR-OdourC	TasteR-TasteC	TasteR-TasteC
Normality test for differences	Statistic W	P Value Pr <W	Statistic W	P Value Pr <W	Statistic W	P Value Pr <W
Shapiro-Wilk	0.833482	0.0489	0.85671	0.0883	0.751938	0.0057
Parametric test	T value	p Value Pr > |t|	T value	p Value Pr > |t|	T value	p Value Pr > |t|
Paired t-test	-4.24	0.0028	-0.60	0.5632	-3.77	0.0054
Non-parametric test	Statistic S	P Value Pr >= |S|	Statistic S	P Value Pr >= |S|	Statistic S	P Value Pr >= |S|
Wilcoxon Signed-Rank test (Paired test)	-14	0.0156	-4	0.6875	-19.5	0.0234
Parametric test	t Value	Pr > |t|	t Value	Pr > |t|	t Value	Pr > |t|
Two independent samples t-test	4.02	0.0010	0.71	0.4867	2.05	0.0570
Non-parametric test	Z	Two-Sided Pr > |Z|	Z	Two-Sided Pr > |Z|	Z	Two-Sided Pr > |Z|
Wilcoxon-Mann-Whitney test (two independent samples)	-2.8815	0.0040	-0.7867	0.4314	-1.6784	0.0933

additional transformations of the independent variables (spline polynoms) the RMS decreased to 14.23 % (A.C) 13.11% (B.C).

The normality tests (Shapiro-Wilk, Kolmogorov-Smirnov) of the distributions of residuals for models were not significant; the distributions of residuals were not significantly different from a normal distribution.

The multi-collinearity diagnostic parameters, the variance inflation factor (VIF) and the condition index (C.I.), for each term in the model were calculated. If VIFs exceed 10 and C.I. 100, than the multicollinearity could be indicated. The C.I. values were smaller than 100 and therefore we could conclude that the diagnostic parameters did not show the existence of multi-collinearity.

Findings for Set D on the basis of linear regression

Set D had 84 samples and one repetition. We confirmed all the already-determined correlations. Statistically significant were X1, X2, X3 and X4, and had a strong influence on TNM.

The result of the third part of the study: significance of the influence of salt reducing on taste, odour and colour of the sausages

Table 8 shows the sensory evaluation of the Kranjska sausages from lots L16, L17 and L18 on the 40th day after production. The normality test confirmed the normal distribution of differences for odour (p=0.0883). The p value for the colour was on the limit (p=0.0489) and for taste, a normal distribution was not confirmed. Q-Q plots were not exceptionally non-normal; even the Shapiro-Wilk test assumes a normal distribution only for difference OdourR-OdourC. Because the number of samples was rather small we calculated all test for all differences.

The difference between the colours (colour of the section and the external colour) of the two products with a different salt content was statistically confirmed. All tests gave p value smaller then 0.05. All tests confirmed no difference between the samples for the odour. In all cases p value was higher then 0.05. This meant that a reduced salt content did not affect the odour of the sausage. The paired tests confirmed the difference in taste between the salt-reduced and control sausages and the independent tests not. The paired t-test is usually not sensitive to deviations from normality, i.e., these deviations must be really abnormal; this was not the case in our study. There were no abnormal deviations. But because the evaluators gave higher ranks to sausages with higher salt concentration, it is obvious that the paired tests were more realistic. The p-value was 0.0054 for the paired t-test and 0.0234 for the Wilcoxon signed-rank test.

CONCLUSIONS

The conclusion of the study was that salt is an important preservative that affects the microbiological stability of the Kranjska sausage and a sensory analysis. All the lots of the sausages with a reduced salt content had an increased content of TNMs.

The important variables during the production of the sausages, besides the salt concentration, were the relative humidity and the lot. The relative humidity should in future be permanently monitored during production. The lot was representing the microbiological status of the meat particles at the beginning of the production for the sausages. The origin of the meat particles was monitored and it will be also in the future, because the suppliers or the microbiological status of the meat particles could be changed.

The variability of TNM during the shelf-life was expressed with the intra CV and was lower for the salt-reduced sausages and higher for the control salt concentration. It was obvious that TNM did not depend only on the salt concentration, but also on some other variables, for example, humidity and lot. There was also the possibility of the development of the microflora inside the sausages during the shell-life showing higher TNMs, but in this case more samples have to be analysed and the type of microorganisms should be determined.

The developed polynomial models determined and confirmed the correlations and strong influences between TNM and the variables salt concentration, lots and humidity. Polynomial models were too general and not precise enough to be also used for the prediction, but they were useful for a determination of the relations between variables, i.e., TNM was increasing with humidity and decreasing with higher salt concentration.

The sensory assessment showed that the salt reduction had no statistically significant effect on the odour of the sausage. However, there was a statistically significant impact on the colour and the flavour of the sausages.

According to the results of our study, we demonstrated that salt is a preservative that plays an important role in the production of sausages; it has an impact on both the flavour and the colour of the product and on the microbiological stability and shelf-life.

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Vpliv zniževanja soli na vsebnost skupnega število mikroorganizmov in senzorično analizo v kranjskih klobasah v času njihovega roka uporabnosti

IZVLEČEK

Sol je pomembna sestavina v proizvodnji mesnih izdelkov in vsako zniževanje vsebnosti soli zahteva posebno obravnavo. Ta študija je bila izvedena z namenom ugotavljanja vpliva zmanjševanja soli na rast mikroorganizmov v kranjskih klobasah in na senzorično oceno v času njihovega roka trajanja. 18 lotov klobas je bilo pripravljenih z zmanjšano količino (1,6%) in z kontrolno količino (2,3%) soli neposredno na proizvodni liniji. Analiziranih je bilo 85 klobas. Podatki so bili uporabljeni za primerjave skupin (ANOVA) in za odkrivanje značilnih spremenljivk (polinomski modeli), ki vplivajo na skupno število mikroorganizmov (SŠM). Signifikantne razlike so bile določene med loti Kranjskih klobas (ki predstavljajo mikrobiološko stanje nadeva), med vzorci klobas z zmanjšano količino in kontrolno količino soli in med različnimi stopnjami zračne vlage. Korelacije in signifikantna razmerja so bila določena med SŠMs in loti, koncentracijo soli v klobasi in relativno vlažnostjo. Polinomi so bili preveč splošni, da bi se lahko uporabljali za napovedovanje. Senzorična analiza je bila izvedena na 40. dan. Ocenili smo 18 klobas. Pri ocenjevanju je zmanjšanje soli prineslo klobasam nižje ocene. Manj slane klobase so vsebovale več mikroorganizmov.

Ključne besede: Kranjska klobasa, zmanjševanje soli, senzorično ocenjevanje, modeli, relativna vlažnost