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# The effects of immunocastration on male pig yield parameters and meat quality

*Jelena Janjic[1], Jelena Ivanović Ciric[1], Jelena Aleksic[1], Nataša Glamoclija[1], Marija Starcevic[1], Anita Radovanovic[1], Milan Z. Baltic[1]*

*A b s t r a c t: The aim of this study was to evaluate the effects of immunocastration on pork meat and carcass quality, compared to meat from surgically castrated males and entire males. Ninety (Duroc x Pietrain) x (Landrace x Yorkshire) crossbred pigs were assigned to three experimental groups: surgically castrated males (barrows; castrated up to the seventh day of age), entire males (males), and vaccinated males (immunocastrates). Carcass and meat quality characteristics such as weight of hot and chilled carcass, meat yield, chilling loss and chemical parameters were examined. Surgically castrated pigs had significantly lower ($p<0.01$; $p<0.05$) weight before slaughter, than males and immunocastrates, and also lower ($p<0.01$) warm carcass weight than males. The average carcass meatiness of castrates was significantly lower ($p<0.01$) than the average meatiness of males and immunocastrates. Chilling loss of barrows was significantly lower ($p<0.01$) than chilling loss of males or immunocastrates. It was also found that the chilling loss of immunocastrates was significantly lower ($p<0.05$) than chilling loss of males. According to the results obtained, it can be concluded that immunocastration could be a good alternative to surgical castration considering meat and carcass quality characteristics.*

***Keywords:*** *immunocastration, yield, quality, pork.*

## Introduction

The continual growth of the human population world-wide requires innovative solutions in order to significantly increase food production, in particular for foods of animal origin. The production of high quality pork meat has been an ultimate goal of the pig industry for many decades (*Dokmanovic et al.,* 2014; *Dokmanovic et al.,* 2015). One of the options to increase pork production is *via* fattening uncastrated male pigs, which are known to produce more meaty carcasses than surgically castrated males (barrows) and young female pigs (gilts). However, not castrating male pigs can cause the occurrence of the meat defect known as boar taint, due to the presence of androstenone or skatole in adipose tissue. Negative consumer perception of meat from entire male pigs has been reported by many authors, not only in fresh pork but also in processed products such as bacon and dry cured ham (*Matthews et al.,* 2000; *Font-i-Furnols et al.,* 2008; *Font-i-Furnols et al.,* 2012).

Surgical castration of male pigs at an early age is carried out in most countries to prevent boar taint, increase intramuscular and subcutaneous fat content for certain quality products and prevent aggressive behavior. Males and barrows have been shown to differ in carcass and meat quality traits (*Lundstrom et al.,* 2009). However, consumers concerns for animal welfare are increasing pressure on the pig industry to abandon surgical castration (*Fàbrega et al.,* 2010).

Immunological castration of pigs is an attractive alternative to surgical castration, and nowadays, is increasingly used in many countries to reduce boar taint and improve pork quality. Moreover, immunocastrated pigs showed reduced sexual and aggressive behavior compared to entire male pigs, thus improving animal welfare (*Zamaratskaia and Krøyer Rasmussen*, 2015). A vaccine for the immunocastration of male pigs (Improvac®, against GnRH) to avoid boar taint has been recently accepted for use in the European Union (*European Medicines Agency*, 2013). While vaccination has been shown to be effective against boar taint, performances can differ between entire males and males vaccinated against GnRH (*Aluwé et al.,* 2015). Many scientists have examined the effects of immunocastration on meat quality parameters (*Zamaratskaia et al.,* 2008; *Pauly et al.,* 2009; *Gispert et al.,* 2010; *Aleksic*, 2012). In fact, immunocastration is likely to result in

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carcasses which do not diverge a lot from the normal meatiness of boar carcasses (*Aleksic*, 2012).

The aim of this study was to evaluate the effect of immunocastration on meat quality by comparing three groups of pigs: surgically castrated males, entire males and immunocastrated males, slaughtered at the same age.

## Materials and Methods

The study was conducted using three groups of 30 pigs each. The groups comprised surgically castrated males (castrated at up to seven days old), entire males, and immunocastrated males. All pigs were descendants of a one single boar (a crossbred of Duroc and Pietrain) and sows of the same line (crossbreds of Landrace and Yorkshire). At eight weeks of age they were transferred to the experimental barn, individually housed and fed a commercial diet *ad libitum*. At that time, the first vaccination against GnRH (2 ml of Improvac® vaccine per animal, Pfizer Animal Health) was applied by a veterinarian. Thereafter, pigs were assigned to three treatment groups: surgically castrated males (n=30, weight 18.26±2.19 kg), entire male boars (n=30, weight 18.76±2.86 kg), and immunocastrated males (n=30, weight 18.54±2.33 kg). The second vaccination of immunocastrated males (2 ml of vaccine) was performed at 5 weeks prior to slaughter. At the end of the study (178 days), surgically castrated males weighed 102.50±9.55 kg, male boars 111.40±6.22 kg, and immunocastrated males 107.70±7.92 kg.

After fattening and transport to the slaughterhouse, pigs were slaughtered and carcass processing was performed in the same way for all animals. Meat yield parameters were determined after slaughtering, processing and cooling. For chemical analyses, ten samples of meat (*m. longissimus dorsi pars lumbalis*) were taken from each group of pigs.

Pigs were weighed before slaughter after they were unloaded from the transport vehicle, on a walk-through balance located in the corridor (measurement accuracy was ±0.5 kg). The weight of hot or chilled carcasses was measured on a balance with accuracy of ±0.1 kg. Dressing percentage was calculated from the weight of live animals and warm carcass weight. Meat yield was expressed in percentages and kilograms, and was determined according to local Regulation on the quality of pigs for slaughter (*Serbia, SFRJ,* 2/85, 12/85, 24/86). According to this Regulation, meat yield is determined by the sum of the thickness of loin fat and backfat in millimeters, and warm carcass weight is expressed in kilograms. Based on these measures, meat yield was determined from the tabular values in kilograms or as a percentage. Chilling loss was determined based on the difference between warm carcass weight and weight of carcass after 24 h cooling, and was expressed as a percentage. For chemical analyses, standard AOAC methods (*AOAC*, 1990) were used.

### *Statistical analysis*

Statistical analysis of the results was elaborated using software GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All parameters were described by mean and standard deviation (SD). The significance of differences between mean values of two groups was measured using the t-test. To test the significance of differences among three or more groups, ANOVA and Tukey's test were used. Significance levels of 0.01 and 0.05 were applied.

## Results and Discussion

Vaccination very strongly reduced the size of testes in immunocastrates compared to the male boars (Figure 1). Numerous studies showed reduction of testes (16% to over 60%) in immunocastrates when compared to male boars (*Metz et al.,* 2002; *Jaros et al.,* 2005; *Zamaratskaia et al.,* 2008), as found in this study. In addition to macroscopic changes, this (Figure 2) and other studies (*Jaros et al.,* 2005; *Gökdal et al.,* 2010) showed histological differences between the testes of immunocastrates and entire male boars.

Surgically castrated pigs had significantly lower ($p<0.01$; $p<0.05$) weight before slaughter than males and immunocastrates, and also lower ($p<0.01$) warm carcass weight than males (Figure 3). Live weight in all groups increased as the day of fattening period progressed (data not shown), although the entire males seemed to be superior to the other groups (surgically castrated and immunocastrates). Gonadal steroids play a critical role in animal growth and development (*Ribeiro et al.,* 2004). Also, *Ribeiro et al.* (2004) reported that residual levels of testosterone secreted in immunocastrates have anabolic effects that, possibly, are sufficient to sustain a high rate of growth and development. In other studies (*Skrlep et al.* 2010; 2012), barrows were heavier than boars, while in our study, male boars were heavier than barrows ($p<0.01$). Several studies have pointed to a higher body weight in barrows and immunocastrates

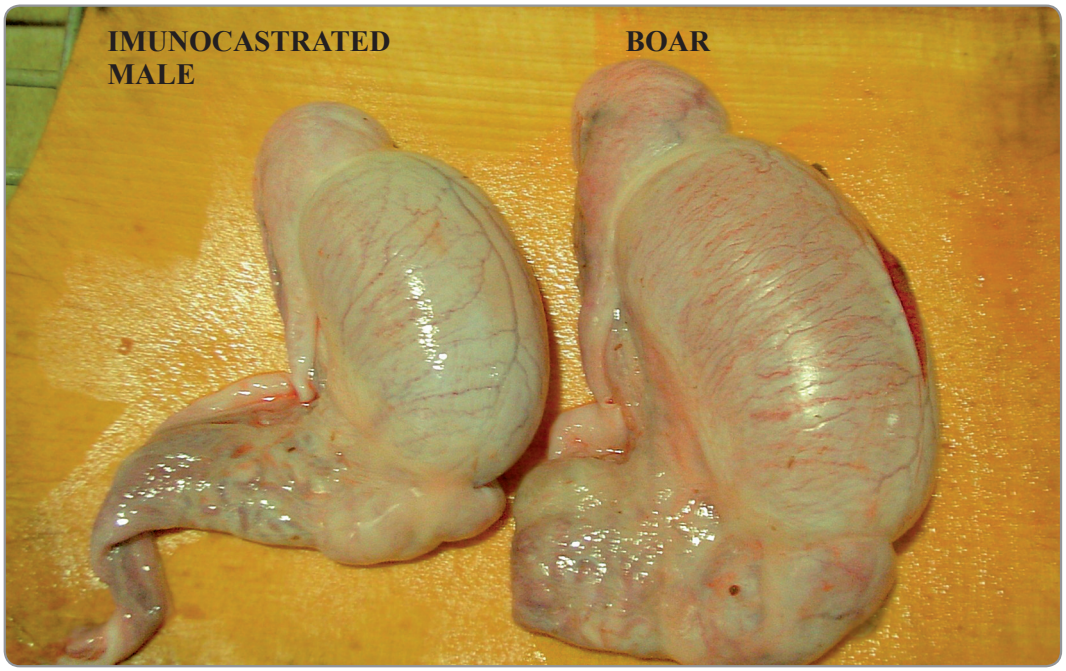



**Figure 1.** Representative image of testes belonging to an immunocastrated male and an entire male boar.

compared to entire male boars (*Dunshea et al.,* 2001; *Oliver et al.,* 2003; *Gispert et al*., 2010). In the study of *Fuchs et al.* (2009), there were no significant differences between immunocastrates and barrows in average body weight and weight of carcasses after slaughter. In our study there were no significant differences between the average warm carcass weight of immunocastrates and barrows, or between immunocastrates and entire males (Figure 3), which is consistent with the results of *Fuchs et al*. (2009).

Chilling losses of barrows, entire males and immunocastrates are shown in Figure 4. Chilling loss of barrows was significantly lower ($p<0.01$)

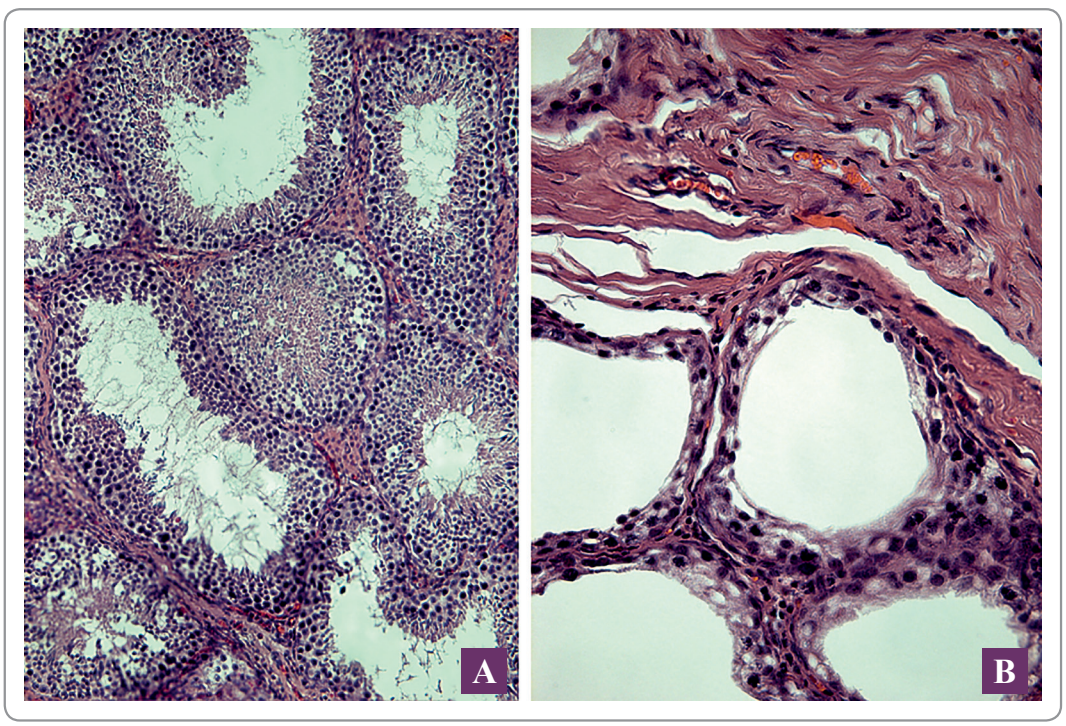
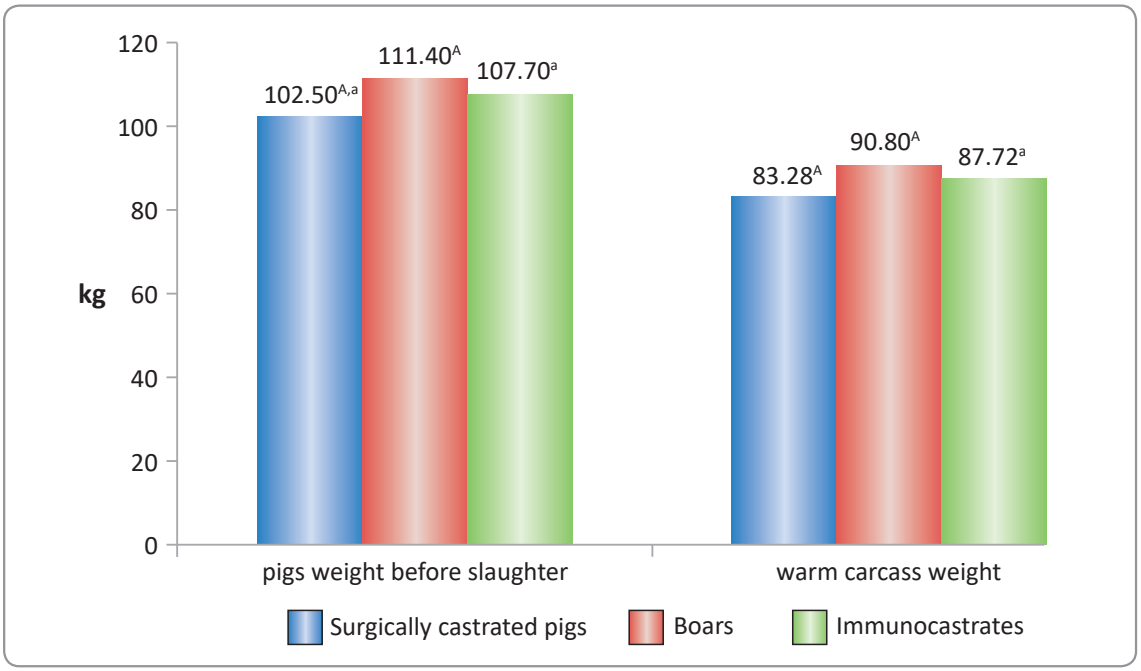



**Figure 2.** Typical testicular histology of an entire male boar (A) and an immunocastrated male (B).



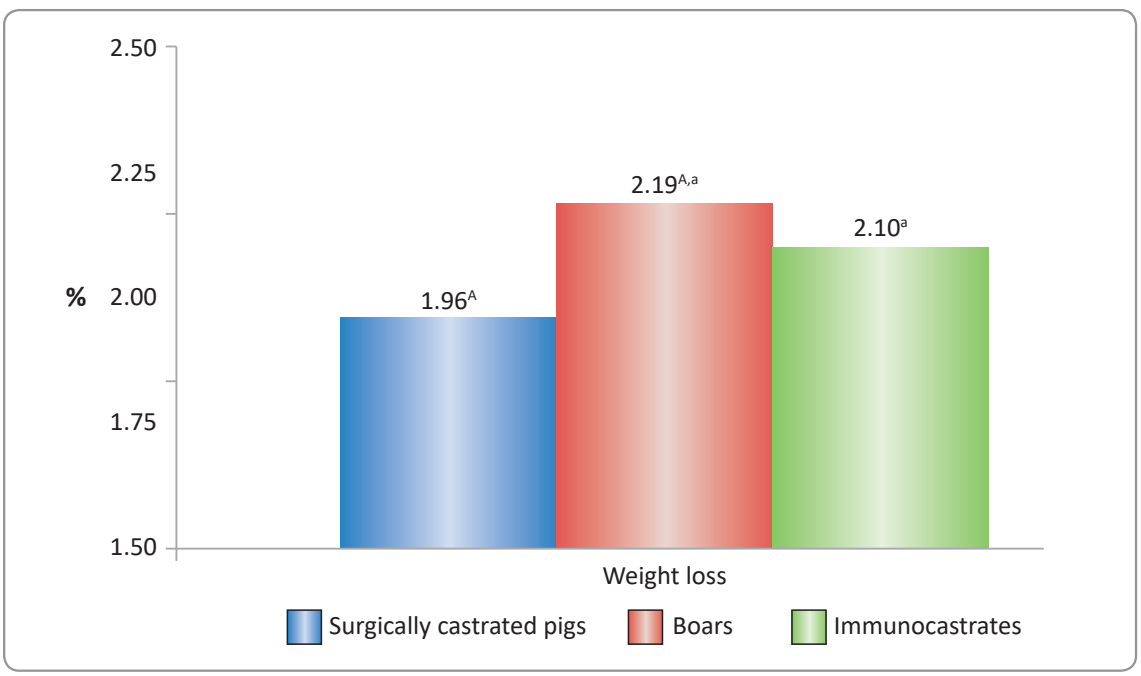

**Legend:** Means with a common superscript letter are significantly different: $^{A}$ – $p<0.01$; $^{a}$ – $p<0.05$.

**Figure 3.** Pig weight before slaughter and warm carcass weight ($\bar{X}$).

than chilling loss of males or immunocastrates. It was also found that the chilling loss of immunocastrates was significantly lower ($p<0.05$) than chilling loss of entire males.

Carcass yields for the three pig groups was calculated on the basis of chilled carcass weights and ranged from 79.06±2.03% to 79.77±2.30%. There were no significant differences between the average carcass yields of the three pig groups (Figure 5).

Numerous factors, such as breed, type, genotype, nutrition, weight and age at slaughter affect the quality of live and slaughtered pigs, and




**Legend:** Means with a common superscript letter are significantly different $^{A}$ - $p<0.01$; $^{a}$ – $p<0.05$.

**Figure 4.** Chilling loss ($\bar{X}$) of barrows, males and immunocastrates.

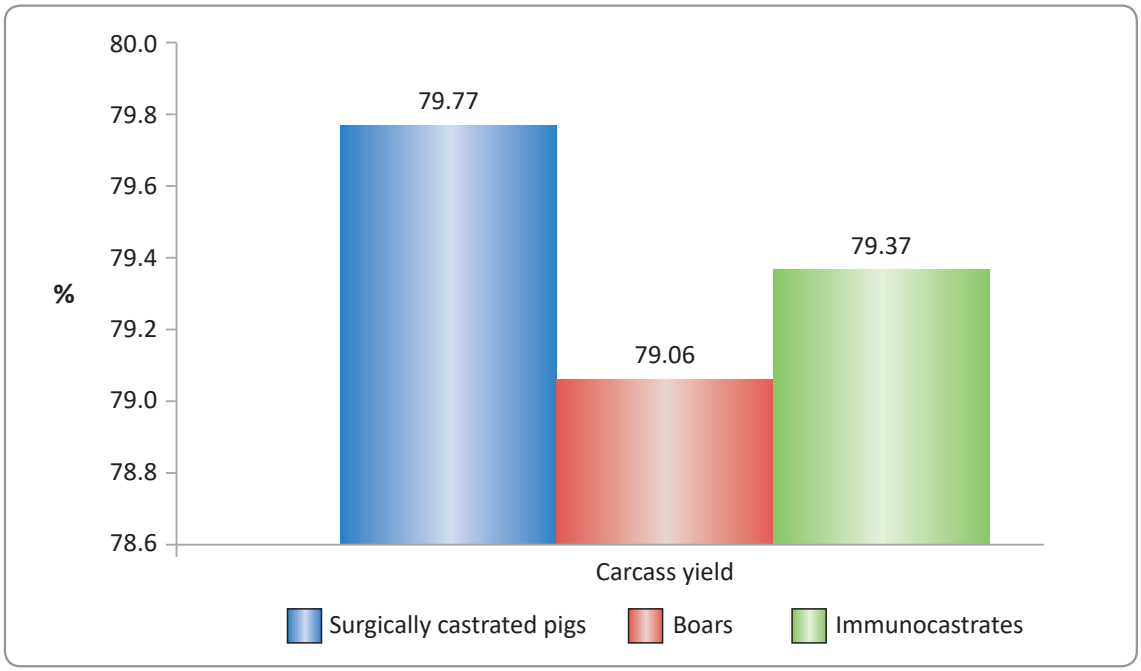
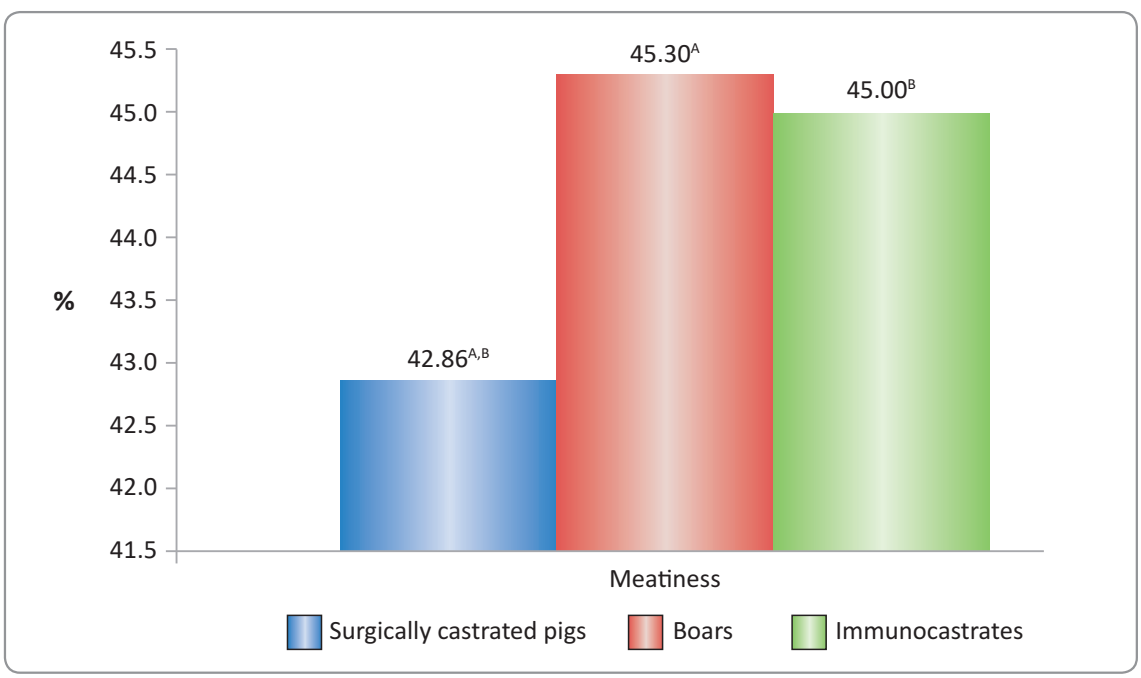



**Figure 5.** Carcass yield ($\overline{X}$) of the three pig categories.

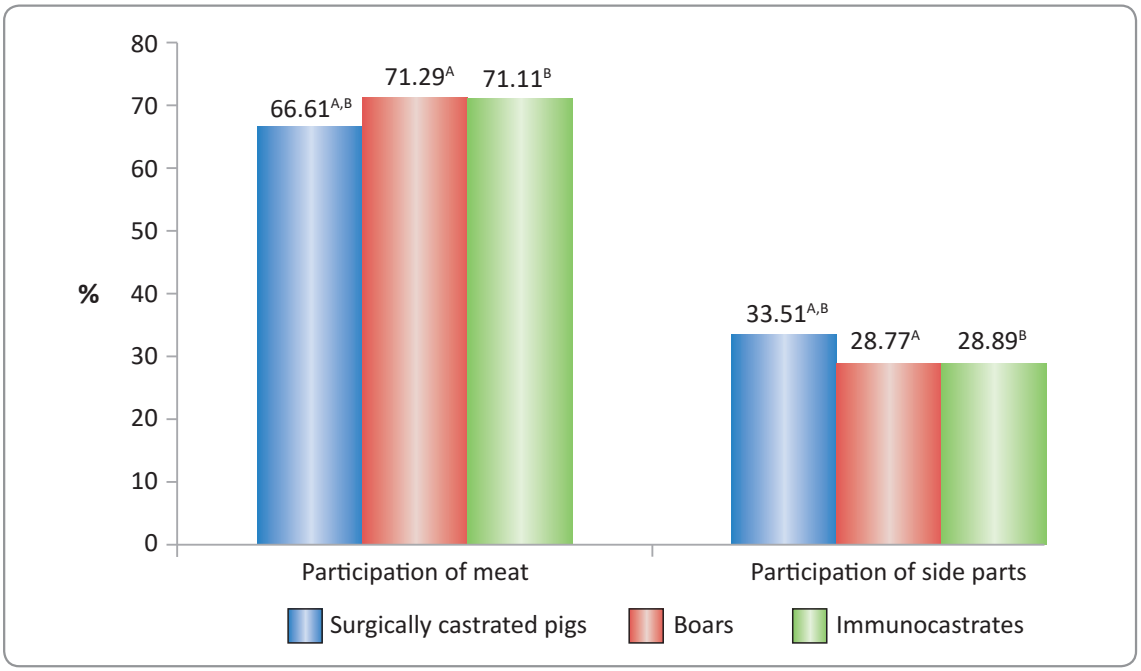
characteristics of the meat and fat. In fattening pigs kept in groups and fed with concentrated feed *ad libitum*, *Sencic et al.* (2005) indicated that increased body weight results in increased yield, although this also depended on the pig type. In fatty type pigs, carcass yield is about 82.6%, in half fat types it is around 81.5%, and in fleshy type pigs, carcass yield is around 80.9% (*Jaros et al*., 2005). Most often, carcass yield varies from 78% to 82% (*Jaros et al*., 2005).

Meatiness of carcasses, expressed as a percentage, is shown in Figure 6. The average carcass meatiness of surgical castrates (42.86±1.12%) was significantly lower ($p<0.01$) than the average meatiness




**Legend:** Means with a common superscript letter are significantly different $^{A, B}$ – $p<0.01$.

**Figure 6.** Meatiness of carcasses ($\overline{X}$) from the three pig categories.




**Legend:** Means with a common superscript letter are significantly different $^{A, B}$ – $p<0.01$.

**Figure 7.** Percentage of meat and side parts in leg ($\bar{X}$) from the three pig categories.

of males (45.30±0.77%) and the average meatiness of immunocastrates (45.00±0.64%).

Elimination of puberty and production of carcasses without boar taint are the main reasons to apply Improvac vaccine (*Bonneau et al.,* 1994; *Dunshea et al.,* 2001; *Metz et al.,* 2002; *Turkstra et al.,* 2002; *Jaroš et al.,* 2005). The effects of this castration method are reflected in greater meatiness of immunocastrate carcasses, compared to those of surgical castrates. The study of *Jaros et al.* (2005) found that meat quality and proportion of muscle tissue are significantly better in immunocastrates compared to castrates. This is of special importance to producers, who thereby achieve better economic effects, as well as for the meat industry, which is always interested in more meaty carcasses.

Leg is the most valuable part of the pig carcass according to meat quality, and the amount of meat. The average percentages of meat and side parts of leg (knuckle, skin with subcutaneous fat tissue, bone) of barrows, entire males and immunocastrates obtained during processing are shown in Figure 7. The average percentages of meat in the leg from males and immunocastrates were significantly higher ($p<0.01$) compared to the average meat percentage from surgical castrates. Also, the average percentage of the side parts from barrows was significantly higher ($p<0.01$) than the side parts from males and immunocastrates.

There were no differences in the average weight of leg between the immunocastrate and males, which is in accordance with other results (*Fuchs et al.,* 2009; *Pauly et al.,* 2009; *Bonneau et al.,* 1994). In the study of *Skrlep et al.* (2010), immunocastrates had a higher proportion of muscle tissue in leg compared to castrates, while there was no different in the proportion of muscle tissue in legs of males and immunocastrates.

**Table 1.** Percentage of side parts in total leg weight of the three pig categories.

| Pig category | Knuckle | Bones | Skin with subcutaneous fat tissue |
|---|---|---|---|
| Surgically castrated pigs | $12.34^{A}$±0.36 | $5.73^{A}$±0.32 | $15.32^{A,B}$±2.34 |
| Entire males | $11.92^{B}$±0.64 | $5.70^{B}$±0.30 | $11.09^{A}$±2.24 |
| Immunocastrates | $13.29^{A,B}$±0.78 | $6.36^{A,B}$±0.46 | $9.25^{B}$±2.28 |

**Legend:** Data are mean ± standard deviation. Within a column, means with a common superscript letter are significantly different $^{A, B}$ – $p<0.01$.

**Table 2.** Chemical composition (%) of meat from the three pig categories.

| Pig category | Water | Proteins | Lipids | Ash |
|---|---|---|---|---|
| Surgically castrated pigs | $72.61^{A}\pm0.46$ | $21.83^{A}\pm0.27$ | $4.27^{A,B}\pm0.19$ | $1.40\pm0.02$ |
| Entire males | $73.54^{A,a}\pm0.41$ | $22.52^{A,B}\pm0.33$ | $2.59^{A,C}\pm0.20$ | $1.40\pm0.01$ |
| Immunocastrates | $73.04^{a}\pm0.32$ | $22.05^{B}\pm0.15$ | $3.67^{B,C}\pm0.25$ | $1.41\pm0.02$ |

**Legend:** Data are mean ± standard deviation. Within a column, means with a common superscript letter are significantly different $^{A,B,C}$ – $p<0.01$; $^{a}$ – $p<0.05$.

The percentage of side parts (knuckle, bones, skin with subcutaneous fatty tissue) in the total leg weight of barrows, males and immunocastrates are shown in Table 1. The percentages of knuckles and bones in the total leg weight of immunocastrates were significantly higher ($p<0.01$) then percentages of these parts in the total leg weight of barrows and males. In contrast, the percentage of skin with subcutaneous fat tissue was significantly higher ($p<0.01$) in the total leg weight of surgically castrated pigs than it was in males and immunocastrates.

Entire male pig carcasses have a higher proportion of bones, while barrows have a lower proportion of bones and consequently produce fewer losses due to deboning. Entire males had a higher proportion of muscle tissue, and among other groups (castrates, immunocastrates) there were no differences (*Cruz-Bustillo et al.,* 1989; *Judge et al*., 1990). Additionally, a higher proportion of leg, loin and shoulder was found in entire males than in immunocastrates, and in immunocastrates compared to castrates (*Cruz-Bustillio et al.,* 1989; *Judge et al*., 1990). The carcasses of males have about 5% more muscle tissue compared to castrates (*Cruz-Bustillio et al.,* 1989; *Judge et al*., 1990).

Chemical parameters of meat quality were studied in *m. longissimus dorsi pars lumbalis* of our three pig types (Table 2). Average water content in the meat from entire males was significantly higher ($p<0.01$) than the average water content in barrow meat. We also found that the average water content in immunocastrate meat was significantly lower ($p<0.05$) than the average water content in meat from entire males. There were no significant differences between the average water content in meat from barrows and immunocastrates. Ash content in the meat of males and surgical castrates was identical, and that in meat from immunocastrates was slightly higher; however, there were no statistically significant differences among the groups (Table 2). These results are similar to those reported by *Gokdal et al.* (2010).

Differences between the average fat content in meat of barrows, males and immunocastrates were significant. The main effects of immunocastration included a reduction of intramuscular fat in proportion to surgical castrates, but not to the average level we determined in meat from entire males ($p<0.01$; Table 2). It is expected that these differences would adversely affect the tenderness and juiciness of meat. In contrast, the force required for meat cutting is reduced, which should be beneficial for tenderness. Also, characteristics such as intramuscular fat, meat color and chilling loss were better for immunocastrates compared to castrates (*Hennessy et al.,* 2000).

An integral evaluation, incorporating the results of production (*Fàbrega et al.,* 2010), meat and carcass quality and sensory characteristics (*Font-i-Furnols et al.,* 2008; *Font-i-Furnols et al.,* 2012), suggests that vaccination with Improvac for boar taint control will provide a good alternative to surgical castration.

## Conclusion

Regarding our results we can conclude that immunocastration, from the point of view of meat and carcass quality, could be a good alternative to surgical castration.

**Conflict of Interest.** The authors declare that they have no conflicts of interest.

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# Effect of genotype on physico-chemical characteristics of rabbit meat

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*A b s t r a c t: Basic chemical composition (water, protein, fat, ash), water holding capacity and cooking loss of rabbit meat (Oryctolagus cuniculus) were investigated. The meat originated from three different genotypes (New Zealand White, Californian and crossbred animals of these two breeds). Animals up to 30 days old fed exclusively on their mother's milk and then received commercial feed ad libitum until they were 90 days old. Before slaughter, rabbits had an average live weight of 2038.17 g. Muscles from the hind legs from 21 animals (7 of each genotype) were taken for examination at 48 h post mortem. Chemical composition was determined according to standard methods. Water holding capacity was determined by the Grau-Hamm filter paper press method. Cooking losses were measured by dipping 10 g meat pieces into boiling water for 10 minutes, as well as by roasting chops in an electric oven at 180°C to an internal temperature of 78 °C. On average, rabbit meat contained 74.49% water, 21.79% protein, 2.78% fat, 1.24% ash and its energy value was 494.79 kJ 100g$^{-1}$. Genotype had no significant effect on chemical composition or water holding capacity of meat. The content of free water in the meat amounted, on average, to 52.28% and bound water content was 22.21%. Cooking loss was significantly ($P<0.05$) lower in meat from crossbreds. During boiling, the meat, on average, lost 32.73% of its weight, compared with a 38.11% loss during roasting.*

***Keywords:*** *rabbit meat, chemical composition, water holding capacity, cooking loss.*

## Introduction

Rabbit meat production is based on pure breeds (selected for meat production) and their crossbreds. New Zealand White (NZW) and Californian (CAL) are the most popular breeds in commercial production (*Ozimba and Lukefahr*, 1991; *Shemeis and Abdallah*, 1998). Rabbit meat is appreciated due to its high nutritional and dietetic properties: it is lean, contains highly unsaturated lipids (60% of total fatty acids are unsaturated), is rich in proteins (20–21%) and has amino acids of high biological value, while it is poor in cholesterol and sodium and rich in potassium, phosphorus and magnesium (*Dalle Zotte*, 2000). That is why the rabbit meat is more easily digested compared to other kinds of meat (beef, lamb or pork) and is recommended for consumption, e.g. for persons with cardiovascular illnesses (*Pogány Simonová et al*., 2010). It is a recommended food for elderly, hypertensive or diabetic patients. The nutritive value is on a par with fish meat (*Para et al*., 2015). Rabbit meat is one of the best white lean meats available on the market, very tender and juicy. There is no religious taboo or social stigma regarding the consumption of this meat (*Nistor et al*., 2013).

World rabbit meat production amounted to 1.56 million tonnes in 2014. The leading world producer of rabbit meat is China with 762,627 t year$^{-1}$, while, in Europe, the main producer is Italy (268,980 t), followed by Spain (63,790 t), France (53,292 t), Czech Republic (38,602 t) and Germany (34,253 t year$^{-1}$). Unfortunately, for most Balkan countries we have not managed to find data, except for Greece (6,799 t), Bulgaria (6,629 t) and Romania (143 t year$^{-1}$) (FAOSTAT, 2014).

Chemical composition, water holding capacity (WHC) and cooking loss are the part of physico-chemical characteristics of meat, according to which its quality is estimated. Depending on genotype, age, sex, diet, region of the carcass, rabbit meat contains 65.93 to 77.34% water, 19.43 to

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24.40% protein, 0.90 to 4.10% fat and 0.99 to 2.08% ash (*Panic et al*., 1986; *Mohamed*, 1989; *Metzger et al*., 2003; *Metzger et al*., 2006; *Omojola and Adesehinwa*, 2006; *Skandro et al.,* 2008; *Rafay et al*., 2008; *Baiomy and Hassanien*, 2011; *Bivolarski et al*., 2011).

Water in meat can be free, loosely bound or tightly bound. Free water is extracted from the meat by gravity, loosely bound using a force, and tightly bound by drying (*Karan-Djurdjic and Peric*, 1966). The ability of meat to retain its own water and to bind added water is one of the most important technological properties of meat. The WHC of meat includes the ability of meat to retain its own water when applying force (pressing, centrifugation, chopping or warming), as well as to bind added water. Depending on the method applied and other factors (genotype, diet, rabbit age, part of the carcass, time *post mortem* etc.), WHC varies widely from 15.42% to 57.16% (*Omojola and Adesehinwa*, 2006; *Rafay et al*., 2008; *Bivolarski et al.,* 2011; *Suradi and Yurmiaty*, 2011).

The losses during meat cooking depend on the same factors that affect WHC. Various procedures are applied to determine the cooking loss (boiling, roasting, different temperatures and duration of treatment), which varies from 30.22 to 39.15% (*Hernández et al*., 1998; *Dal Bosko et al*., 2001; *Yalçın et al.,* 2006; *Omojola*, 2007). The diversity of rabbit breeds offers the opportunity to increase the efficiency of meat production by commercial crossbreds. The aim of this research was to examine the effect of genotype on chemical composition, WHC and cooking loss of NZW and CAL rabbits and crossbreds between them.

## Materials and methods

Meat from three genotypes of rabbit (*Oryctolagus cuniculus*): NZW, CAL and crossbreds between these two breeds, with an average live weight of 1794.4, 1706.2 and 2613.9 g, respectively, were examined. Young rabbits up to 30 days old fed exclusively on their mother's milk and then recieved a commercial feed *ad libitum* until they were 90 days old, when they were slaughtered. Chemical composition and WHC were determined using minced and homogenized muscles from the hind legs, 48 h *post mortem*. Seven samples from each genotype, making a total of 21 samples, were investigated.

*Chemical composition and energy value*

Chemical composition was determined using minced, homogenized meat according to standard methods (*AOAC, 2005*). The water content was determined by drying the meat in an oven at 105°C according to AOAC 950.46. Total proteins (Nx6.25) were determined using the Kjeldahl method according to AOAC 928.08. Crude fat content was measured according to AOAC 991.36 and ash content according to AOAC 920.153.

The energy value of the meat was calculated by multiplying the determined percentage of fat by 37.7 kJ and the percentage of proteins by 17.9 kJ. The sum of these two obtained values is reported as the energy value of the meat.

*Water holding capacity (WHC)*

WHC was determined by the pressing method as described by Grau and Hamm (*1953*), using a hydraulic press (Johann Stiel Maschinenbau, Germany). On a previously desiccated filter-paper (Whatman no° 1), 300±3 mg of meat was weighed and pressed between two plexiglass plates and a load of 1000 kg was applied for 5 min. Mean values of two replications were used for analysis. The area of the extruded meat juice (wet area) was measured by planimeter (Reiss-precision, BR 3005, Germany) and expressed in $cm^2$. The content of free (or loosely bound) water, expressed in mg, was calculated as follows:

$$\text{mg } H_2O = \frac{\text{Wet area } (cm^2)}{0.\ 0948} - 8.0 \qquad \text{(eq. 1)}$$

The percentage of free (or loosely bound) water in the meat and in the total water was calculated as follows:

$$\text{Free water (\%) in meat} = \frac{\text{mg free water}}{300 \text{ mg}} \times 100 \qquad \text{(eq. 2)}$$

$$\text{Free water (\%) in total water} = \frac{\text{mg free water}}{\text{total water (mg)}} \times 100 \qquad \text{(eq. 3)}$$

$$\text{Total water (mg) in 300 mg meat} = \frac{\text{\% water} \times 300}{100} \qquad \text{(eq. 4)}$$

The percentage of bound water in meat = % of total water minus % of free water in meat. The percentage of bound water in total water = 100 minus % of free water in total water.

*Cooking losses*

Cooking loss during boiling meat was determined by dipping cubic pieces of thigh muscle, weighing 10 g, into boiling water for 10 minutes. The boiling weight loss was calculated by the difference in weight of a meat cube before and after boiling, expressed as a percentage of its initial weight.

Cooking loss during roasting meat was determined using samples of chops, weighing about 30 g, placed into an open porcelain dish and roasted in an electric oven at 180±3°C, until the core temperature was 78°C. The chops were cooled to room temperature and weighed again. The roasting weight loss was calculated by the difference in weight of a chop before and after roasting, expressed as a percentage of its initial weight.

*Statistical analysis*

Statistical evaluation of the results was performed by analysis of variance (ANOVA). The differences between the mean values of the groups were tested using Tukey's test. The results are given as means±standard deviation.

## Results and discussion

The chemical composition of hind legs muscles was not significantly different (P>0.05) between genotypes (Table 1). No significant differences between the chemical composition of meat of NZW and CAL rabbits were found in another study (*Baiomy and Hassanien*, 2011). The established water content in our NZW rabbit meat

**Table 1.** Chemical composition and energy value of rabbit meat (mean±standard deviation), n=7

| Traits | NZW | CAL | NZW x CAL |
|---|---|---|---|
| Water, % | 74.60±4.61 | 74.85±3.71 | 74.02±1.69 |
| Proteins, % | 21.76±1.23 | 21.59±1.31 | 22.01±1.27 |
| Fats, % | 2.88±0.29 | 2.62±0.42 | 2.84±0.15 |
| Ash, % | 1.23±0.09 | 1.37±0.06 | 1.12±0.03 |
| Energy value, kJ $100g^{-1}$ | 498.08±27.32 | 485.23±34.15 | 501.05±31.75 |

**Legend:** NZW – New Zealand White rabbit; CAL – Californian rabbit; NZW × CAL – crossbred New Zealand White × Californian rabbit

**Table 2.** Water holding capacity of rabbit meat (mean±standard deviation), n=7

| Traits | NZW | CAL | NZW x CAL |
|---|---|---|---|
| Total water in meat (%) | 74.60±4.61 | 74.85±3.71 | 74.02±1.69 |
| Total water in 300 mg meat (mg) | 223.80±24.15 | 224.55±25.16 | 222.06±24.82 |
| Free or loosely bound water: | | | |
| $cm^2$ | 15.71±2.87 | 15.31±2.48 | 15.86±1.89 |
| mg | 157.72±21.15 | 153.50±21.25 | 159.30±19.78 |
| Free or loosely bound water (%) in: | | | |
| meat | 52.57±5.43 | 51.17±5.22 | 53.10±4.85 |
| total water | 70.47±7.72 | 68.36±7.69 | 71.73±6.67 |
| Bound water (%) in: | | | |
| meat | 22.03±2.34 | 23.68±2.79 | 20.92±2.12 |
| total water | 29.53±3.25 | 31.64±3.37 | 28.27±2.96 |

**Legend:** NZW – New Zealand White rabbit; CAL – Californian rabbit; NZW × CAL – crossbred New Zealand White × Californian rabbit

**Table 3.** Cooking loss (%) of rabbit meat (mean±standard deviation), n=7

| | NZW | CAL | NZW x CAL |
|---|---|---|---|
| Boiled meat | 35.48±1.85[a] | 33.46±1.39[a] | 29.25±3.25[b] |
| Roasted meat | 39.80±1.34[a] | 39.51±1.31[a] | 35.02±3.05[b] |

**Legend:** NZW – New Zealand White rabbit; CAL – Californian rabbit; NZW × CAL – crossbred New Zealand White × Californian rabbit; [a, b] – Values with different letters within a row are significantly different (P<0.05)

(74.60%) was close to the results of other studies on the same breed: *Skandro et al.* (2008) (74.39 to 74.93%), *Rafay et al*. (2008) (74.84%), *Metzger et al.* (2003) (73.9 to 75.0%). However, lower water content (65.93 to 71.42%, 70.2%, 71.5%, respectively) (*Omojola and Adesehinwa*, 2006; *Baiomy and Hassanien*, 2011; *Chrenek et al.,* 2012) as well as higher ones (77.34%) (*Mohamed*, 1989) have also been reported. The mean protein level we determined in NZW rabbit muscle (21.76%) was similar to the findings of *Mohamed* (1989) (21.55%), *Metzger et al.* (2003) (21.3 to 21.5%), *Skandro et al.* (2008) (21.79 to 22.02%) and *Chrenek et al.* (2012) (21.12%). *Omojola and Adesehinwa* (2006), *Baiomy and Hassanien* (2011) found lower protein (19.43 to 21.05% and 20.3%, respectively), while *Rafay et al.* (2008) found a higher protein level (22.12%). The fat content determined in NZW rabbit muscle (2.88%) is in agreement one previous result (2.32%; *Rafay et al.,* 2008), and lower than the 3.35% elsewhere reported (*Chrenek et al.,* 2012). Depending on the dressing methods, fat content was 1.49 to 3.58% (g $100g^{-1}$) (*Omojola and Adesehinwa*, 2006), and depending on the age of weaning and muscle type, it was 2.20 to 3.61% (*Bivolarski et al*., 2011). Depending on housing for the rabbits, the amount of fats in the hind legs ranged from 2.48 to 3.36% (*Metzger et al.,* 2003). It seems that there are large variations in fat content. The ash content we determined (in NZW; 1.23%) was close to the published results of *Metzger et al.* (2003) (1.29 to 1.31%), *Skandro et al.* (2008) (1.17 to 1.26%), *Bivolarski et al*. (2011) (1.08 to 1.26%), and lower than that reported by *Mohamed* (1989) (1.63%). The water content (74.85%) in the hind leg muscles of CAL was close to the result published for CAL (73.80%; *Panic et al.,* 1986), and higher than the 69.6% reported elsewhere (*Baiomy and Hassanien*, 2011). The percentage of proteins we determined in CAL rabbit muscle (21.59%) agrees with the published value of 21.87% (*Panic et al.,* 1986), but is higher than 20.4% (*Baiomy and Hassanien*, 2011). The fat content of our CAL rabbit muscle (2.62%) was lower than 3.21% (*Panić et al.,* 1986) and particularly lower than 8.11% (*Baiomy and Hassanien*, 2011). The percentage of ash (1.37%) was higher than 1.07% (*Baiomy and Hassanien*, 2011). Regarding the chemical composition of meat from crossbred (NZW x CAL) rabbits, the water content (74.02%) was higher, and of percentage of proteins (22.01%) lower than the values (71.79 to 72.32% and 23.22 to 24.11%, respectively) obtained previously for the same crossbreds (*Marongiu et al.,* 2008). In Hyla hybrid and other hybrid rabbits, water content was from 73.2% to 74.12%, proteins from 22.2 to 22.7%, fats 1.85 to 3.4% and ash 1.06 to 1.3% (*Nizza and Moniello*, 2000; *Dal Bosco et al*., 2001).

Calculated energy values for the three genotypes (485.23 to 501.05 kJ $100g^{-1}$) were higher than those reported in the literature (415 to 458 kJ $100g^{-1}$) (*Rafay et al.,* 2008; *Pogány Simonová et al*., 2010; *Chrenek et al.,* 2012).

No significant differences (P>0.05) were found between WHC of the three genotypes (Table 2). In the literature, WHC varies. The reasons for this include numerous methodology differences, different calculation of survey data, the great heterogeneity in the terminology and expression of results, and the use of different pressures when using the method of Grau and Hamm (*1953*). At different pressure, different amounts of water are extruded from meat. Using a pressure of 1 kg, 2.25 kg and free mechanical force, 22.67%, 26.83% and 42.73% free water were obtained, respectively (*Pla and Apolinar*, 2000). Therefore, *Hofmann* (1971; 1977) points out that extruded water is a function of pressure. Over the years, a wide range of conditions have been reported for meat sample evaluation. They range from forces of 0.01 to 44 kN, sample sizes of 0.3 to 1.5 g, temperatures of 4 to 23 °C and compression times from 1 to 20 minutes. In addition, different filter papers have been used. As a consequence, it is difficult to propose a standard procedure for measuring WHC by the press method because too many variations are present in published studies, so results between studies are not comparable (*Petracci and Baéza*, 2007).

Losses during boiling and roasting the meat were lower (P<0.05) in the crossbreds than in pure breeds (Table 3). The differences may be due to the different rabbit weights at slaughter. *Pla et al*. (1998) found that cooking losses were higher in lighter rabbits. Similar losses to those we measured were established by other authors (*Hernández et al.,* 1998; *Pla et al*., 1998; *Yalçin et al*., 2006). Lower cooking losses were obtained in hybrid rabbits (31.5% by boiling, 30.22% by roasting) (*Dal Bosko et al.,* 2001), probably due to the thermal treatment used. Our boiling weight losses of meat corresponded to data obtained by other researchers (*Omojola and Adesehinwa*, 2006; *Omojola*, 2007; *Yurmiati et al.,* 2010). However, it is worth noting that rabbit meat is, generally, not eaten boiled (*Dal Bosko et al.,* 2001).

## Conclusion

Rabbit genotype showed no significant effect on basic chemical composition or WHC of rabbit hind leg muscles. Meat from crossbred rabbits underwent significantly (P<0.05) lower cooking loss, compared to the pure breeds. Cooking losses were higher during roasting than during boiling meat. Rabbit meat contained a high percentage of proteins and low amounts of fat, so it can be considered as a dietary product or healthful food.

**Conflict of Interest.** The authors declare that they have no conflicts of interest.

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# Saturated fatty acids and total fat daily intake through consumption of processed meat products

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*A b s t r a c t: Processed meat/fish products (pâtés, cooked chicken sausages, canned chopped meat and dry fermented sausages) were evaluated for their contribution to the total daily intake of saturated fats and total fats, in relation to Serbian and European regulations. Estimations of saturated fat daily intakes indicated that fish pâté and chicken cooked sausages would provide similar amounts to the recommended daily limit of saturated fat (limited to 10% of total fat intake), while the calculated contributions from pork and beef meat products were much greater (pâtés 20–53%; canned chopped meat 22–23%; dry fermented sausages 65–81%). The ratio of n-6/n-3, as an indicator of lipid quality, was ≤4 in fish pâtés and was considerably higher in turkey, ham and chicken pâté (17.5, 21.9 and 46.9 respectively), cooked chicken sausages (8.55–14.98), canned chopped meat (13.28–16.07) and dry fermented sausages (22.12–26.78). The results obtained could be of importance for the establishment of tables for nutritional value of products. It was confirmed that a regular intake of saturated fat and total fat via consumption of processed meat products, in particular processed pork products, was likely to be high in the Serbian general population. We speculate that this is, in turn, is likely to increase the potential risk for development of coronary heart disease (CHD). The increased awareness of the meat industry regarding the importance of the fat content/quality in processed meat products and its impact on health, optimization of the product specifications (replacement of SFAs with unsaturated fats), health promotion activities by public health authorities, as well as better education of consumers about beneficial nutrition habits (e.g. Mediterranean diet) should reduce the rate of CHD.*

***Key words:*** *processed meat products, total fats, saturated fats, fatty acids.*

## Introduction

In Western countries, the percentage of fat in the diet is high and a consequence is that the excess fat from consumed food is regularly deposited in adipose tissue (*Wood et al.,* 2008). Lipids in blood are transported by means of lipoproteins, which have a very important role in lipid metabolism. Higher levels of triglycerides in the blood usually increase the level of low density lipoprotein (LDL). LDL cholesterol, high density lipoprotein (HDL) cholesterol and triacyl-glycerol levels in blood are directly linked to quantity and quality of fat in the diet (*Vandendriessche*, 2008). The various saturated fatty acids (SFAs) differ in their effects on the blood lipoprotein profile, e.g. lauric (12:0), myristic (14:0) and palmitic (16:0) acids can raise blood total and LDL cholesterol concentrations, while stearic acid (18:0) has no effect (*EFSA*, 2010). Therefore, a healthy diet should include different types of fat, but in small amounts. Linoleic acid (18:2*n*-6) is the most important *n*-6 fatty acid of plant origin. In humans, it can lower levels of total and LDL cholesterol and, to some extent, HDL cholesterol, which is obviously an undesirable effect (*Lada & Rudel*, 2003). The most important *n*-3 fatty acids are eicosapentaenoic acid (20:5*n*-3, EPA), docosahexaenoic acid (22:6*n*-3, DHA) and α-linolenic acid (18:3*n*-3, ALA). ALA can be introduced into processed meats by changing the product specification through the introduction of ALA-rich vegetable oil instead of animal fat. However, it has been demonstrated that the increased consumption of *n*-3 from ALA has no beneficial effect on health (*Wang et al.*, 2006; *Fretts et al.,* 2014).

Desirable total fat intake in the diet, according to most experts, should amount to only 25–30% of the total daily energy intake (*EFSA*, 2010), provided that 10–15% of the daily energy intake comprises monounsaturated fatty acids (MUFAs), which have a neutral/favourable effect on development of coronary heart disease (CHD). In the UK, the recommended fat intake should be reduced to less than 30% of the total energy intake (*Department of Health*, 1994), while up to 10% of energy intake in

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the form of SFAs would enable the body to completely fulfil its essential metabolic functions, at the same time diminishing the risk of CHD (*Wood et al.,* 2008; *Micha & Mozaffarian*, 2010). Furthermore, although dietary guidelines often recommend reduction in SFA consumption, such guidelines often do not highlight any specific nutrient as preferable for replacing SFA in the diet (*U.S. Department of Agriculture and U.S. Department of Health and Human Services,* 2010).

Many authors have tried to develop meat products with a lower content of SFAs, *trans* fatty acids and/or cholesterol by trying to find fat replacers which preserve the sensory qualities and shelf life of the products and guarantee their acceptance by consumers (*Muguerza et al.,* 2002; *Sampaio et al.,* 2004). A variety of fat replacers and loads were evaluated to reduce the fat added to meat products; however, the ideal fat/load combination has not yet been identified. Sunflower, maize, peanut, tea seed, coconut, palm, soy, and olive oils as well as fish oil have been evaluated in numerous studies (*Muguerza et al.,* 2002; *Valencia et al.,* 2007; *Guillevic et al.,* 2009; *Rahimi et al.,* 2011). The use of vegetable oils can be compared with the use of soft fats, which result in products characterized by their poor appearance, difficulty to cut, and greater tendency to oxidize than hard fats (*Gandemer*, 2002). This tendency of products containing vegetable oils instead of lard to oxidize is due to the greater unsaturated fatty acid content; it has to be considered that shelf life reductions mainly result from increased rancidity and decolouration, which significantly affect the sensory acceptability of products. In many studies, even if total SFAs are decreased, such products still had very high total fat and especially saturated fat daily intake values (*Estévez et al.,* 2006; *D'Arrigo et al.,* 2004; *Terassa et al.,* 2016; *Xiong et al.,* 2016).

Observational studies have found that the Serbian population eats more fat than is recommended (*Gajic & Gudelj*, 2003). In the Province of Vojvodina and its rural areas, the consumption rate of animal fats and animal foods was the largest, and, at the same time, vegetable fat consumption was the lowest in the country. The SFA content in diets was low in all regions in Serbia, except in Vojvodina (*Gajic & Gudelj*, 2003). The greater part of the Serbian population consumes meat every day, and among them, more than 50% of children from 7 to 18 years of age follow this meat consumption pattern (*Sarcevic et al.,* 2013). Data from the Institute of Public Health of Serbia indicate that cardiovascular diseases were a leading cause of death in Serbia in 2014 (*Institute of Public Health of Serbia "Dr Milan Jovanovic Batut"*, 2014).

The aim of this study was to investigate: a) the fatty acid composition of processed meat products, and b) the daily SFA and total fat intake, as calculated by estimated consumption of processed meat/fish products (pâtés, cooked chicken sausages, canned chopped meat and raw, dry fermented sausages) present in the Serbian market. To our knowledge, there are no similar studies in Serbia nor in the Western Balkan countries, which share the same principles and technology in manufacturing these products.

## Materials and Methods

### *Samples*

Meat/fish products were purchased from meat companies from Serbia with production portfolios reflecting the typical processed meat products consumed in Serbia, as follows: twelve different flavours of meat or fish pâtés with net weights ranging from 60 g to 90 g, six cooked chicken sausages with net weights from 300 g to 370 g and four canned chopped meats with net weights of 150 g. Meat pâtés mainly consisted of about 30% mechanically separated poultry/turkey meat (chicken and turkey pâtés only), 30% soups/liquid and 30% of fat/oil, salt and spices. Refined sunflower oil was commonly used in the production process, and the microbiological safety of these products was achieved by commercial sterilization procedures (e.g. 118°C 25 $min^{-1}$; 2.5 $bar^{-1}$). Pâtés made of pork liver are traditional products in the Serbian meat industry that have been taken as a reference by the fish industry for the development of similar products elaborated with different fish species, including salmon and tuna.

Cooked chicken sausages were made from meat paste of different meat categories, fat tissue, and additives. The microbiological safety of these products was achieved by commercial pasteurization procedures (e.g. 74°C for 60–90 min – depending on the sausage diameter). Canned chopped meats were derived from at least 45–60% beef or pork meat, back fat, as well as salt, spices, and additives. The microbiological safety of these products was achieved by commercial sterilization procedures (e.g. 121°C 30 $min^{-1}$; 2.5 bar).

Twelve raw, dry fermented sausages typical of those products on sale in the Serbian market were also examined. These types of fermented sausages are typical of those traditionally manufactured in Serbia (Province of Vojvodina) and neighbouring countries, Croatia (Provinces of Slavonija and Baranja) and Hungary. Sausage production usually starts in winter when ambient temperatures are

relatively low (e.g. <10 ºC). The meat batters intended for stuffing normally comprise 70/30 chilled lean pork and pork back fat, plus the main additives including red hot paprika powder, salt, raw garlic etc. Sausages are subjected to cold smoking, fermentation and ripening, whereby a specific colour, smell and taste are formed. After the end of the ripening process, sausages were packed in packaging that preserves freshness and juiciness of the product and also prolongs its shelf life.

*Fatty acid analysis by capillary gas chromatography*

The total fat content was determined according to ISO standard method (*ISO,* 1973). Total lipids for fatty acid determination were extracted from meat products by accelerated solvent extraction (ASE 200, Dionex, Sunnyvale, CA) according to the method of *Spiric et al.* (2010). Fatty acid methyl esters (FAMEs) in the extracted lipids were prepared by transesterification using 0.25 M trimethylsulfonium hydroxide (TMSH) in methanol (*ISO,* 2000). FAMEs were determined by gas-liquid chromatography (GLC, Shimadzu 2010) equipped with flame ionization detector and capillary HP-88 column (length 100m, i.d. 0.25 mm, film thickness 0.20 μm). Injector and detector temperature were 250 ºC and 280 ºC, respectively. Nitrogen was used as the carrier gas at flow rate of 1.33 mL $min^{-1}$. The injector split ratio was set at 1:50. To achieve complete separation of the examined compounds, a programmed column oven temperature starting at 125 ºC and ending at 230 ºC was applied. Total analysis time was 50.5 min. The chromatographic peaks in the samples were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, USA). Each sample was analysed in duplicate. Results were expressed as mass of fatty acid (g) in 100 g of fatty acids.

*Calculation of daily intake total fat and saturated fats*

Percentages of total fat and saturated fat derived from heat treated ready-to-eat pâtés and raw, dry fermented ready-to-eat meat products were calculated in relation to the reference intake of 2,000 kcal. The rules on labelling and advertising in Serbia (*Serbia*, 2013) and in the U.S. (*U.S. Department of Agriculture and U.S. Department of Health and Human Services*, 2010) recommend an intake of total fats of 70 g $d^{-1}$, and saturated fats of 20 g $d^{-1}$. These values are informative for consumers in interpreting nutritional values of food products. Total fat daily intake and saturated fat intake was calculated by dividing total fat and saturated fat content expressed in 100 g of product by 70 g and 20 g, respectively.

*Statistical analysis*

Data obtained for the fatty acid compositions were subjected to analysis of variance (ANOVA) with the Tukey-Kramer HSD test for the comparisons of means at the 5% level of significance. Statistical analysis was performed using SAS Institute Inc. JMP 10 software.

## Results and Discussion

*Pâtés*

The average fatty acid compositions of the 12 pâtés are presented in Table 1.

Pâtés were generally characterized by low amounts of SFAs. However, significant differences ($P<0.05$) were found for the total SFA content of the pâtés. The smallest level of SFAs was found in the tuna pâté (9.52%) and the highest in the ham pâté (39.72%) The total MUFA content of the pâtés was significantly different ($P<0.05$), with oleic acid (C18:1n-9) being the most common MUFA. The amount of MUFAs in pâtés was the highest in fish pâtés (56.58% and 57.06%, tuna and salmon, respectively), while in turkey, chicken, pork liver and ham pâtés, levels of MUFAs were lower and ranged from 35.30% to 46.89%. The most common *n*-6 PUFA was linoleic acid. It was present in higher levels in turkey and chicken liver pâtés (Table 1). The most common *n*-3 PUFA was ALA, which was more abundant in fish pâtés (Table 1), while it occurred in lower amounts in turkey, chicken liver, pork liver and ham pâtés. Generally, significantly higher contents of EPA and DHA ($P<0.05$) were found in salmon pâté than in tuna pâté. However, in fish pâtés in our study, particularly in salmon pâté, the levels of EPA and DHA were higher than in commercial and experimental fish pâtés in the study of *Aquerreta et al.* (2002).

Polyunsaturated fatty acid to SFA ratios (P/S), as one of the quality parameters of lipid foods, were far greater than 0.4 in all pâtés (2.01–3.43), with the exception of pork pâtés and ham pâté, where these ratios were relatively close to the recommended ratio (0.38 and 0.33, respectively). The ratio of *n*-6/*n*-3 was ≤4 only in fish pâtés, while this ratio was considerably higher in pork liver pâté (15.49), turkey pâté (17.53), ham pâté (21.86) and chicken

**Table 1.** Fatty acid composition (%) of pâtés

| Podnaslov | Tuna pâté n=2 | Salmon pâté n=2 | Turkey pâté n=2 | Chicken liver pâtén=2 | Pork liver pâtén=2 | Ham pâté n=2 |
|---|---|---|---|---|---|---|
| C14:0 | 0.36 ± 0.03 $^{c}$ | 1.60 ± 0.01 $^{a}$ | 0.40 ± 0.20 $^{c}$ | 0.24 ± 0.03 $^{c}$ | 1.26 ± 0.04 $^{b}$ | 1.34 ± 0.15 $^{ab}$ |
| C15:0 | 0.05 ± 0.01 $^{bc}$ | 0.12 ± 0.01 $^{a}$ | 0.03 ± 0.01 $^{c}$ | 0.03 ± 0.01 $^{c}$ | 0.05 ± 0.01 $^{bc}$ | 0.08 ± 0.03 $^{b}$ |
| C16:0 | 5.92 ± 0.13 $^{d}$ | 8.83 ± 0.18 $^{c}$ | 12.38 ± 1.24 $^{b}$ | 13.78 ± 0.16 $^{b}$ | 24.81 ± 0.48 $^{a}$ | 25.70 ± 0.50 $^{a}$ |
| C16:1 | 0.63 ± 0.63 $^{d}$ | 2.18 ± 0.10 $^{c}$ | 1.42 ± 0.30 $^{c}$ | 1.49 ± 0.08 $^{bc}$ | 1.85 ± 0.01 $^{ab}$ | 2.06 ± 0.05 $^{a}$ |
| C17:0 | 0.10 ± 0.01 $^{b}$ | nd | 0.07 ± 0.02 $^{b}$ | 0.08 ± 0.01 $^{b}$ | 0.25 ± 0.01 $^{a}$ | 0.33 ± 0.07 $^{a}$ |
| C18:0 | 2.16 ± 0.01 $^{d}$ | 2.52 ± 0.05 $^{d}$ | 4.18 ± 0.53 $^{c}$ | 4.64 ± 0.23 $^{c}$ | 12.81 ± 0.27 $^{a}$ | 11.91 ± 0.28 $^{b}$ |
| C18:1*n*-9 | 53.95 ± 0.36 $^{a}$ | 50.30 ± 0.81 $^{b}$ | 34.07 ± 1.97 $^{d}$ | 33.55 ± 0.45 $^{d}$ | 42.16 ± 0.39 $^{c}$ | 43.86 ± 1.14 $^{c}$ |
| C18:2*n*-6 | 25.82 ± 0.21 $^{b}$ | 14.88 ± 0.21 $^{c}$ | 43.76 ± 2.82$^{a}$ | 43.58 ± 0.64 $^{a}$ | 13.61 ± 0.19 $^{c}$ | 11.75 ± 1.35 $^{c}$ |
| C18:3*n*-3 | 5.03 ± 0.20 $^{a}$ | 4.73 ± 0.09 $^{b}$ | 0.53 ± 0.03 $^{d}$ | 0.94 ± 0.03 $^{c}$ | 0.74 ± 0.02 $^{cd}$ | 0.49 ± 0.10 $^{d}$ |
| C18:3*n*-6 | nd | nd ± | nd | 0.12 ± 0.02 | nd | nd |
| C20:0 | 0.46 ± 0.02 $^{a}$ | 0.39 ± 0.01 $^{b}$ | 0.17 ± 0.01 $^{c}$ | 0.17 ± 0.01 $^{c}$ | 0.22 ± 0.01 $^{c}$ | 0.20 ± 0.05 $^{c}$ |
| C20:1 | 2.00 ± 0.05 $^{b}$ | 4.58 ± 0.11 $^{a}$ | 0.27 ± 0.07 $^{d}$ | 0.25 ± 0.02 $^{d}$ | 0.93 ± 0.01 $^{c}$ | 0.96 ± 0.16 $^{c}$ |
| C22:0 | 0.32 ± 0.01 $^{a}$ | 0.19 ± 0.01 | nd | 0.32 ± 0.01 $^{a}$ | nd | nd |
| C20:2 | 0.17 ± 0.02 $^{b}$ | 0.71 ± 0.15 $^{a}$ | 0.10 ± 0.05 $^{b}$ | 0.16 ± 0.03 $^{b}$ | 0.56 ± 0.03 $^{a}$ | 0.57 ± 0.01 $^{a}$ |
| C20:3*n*-6 | nd | nd | nd | 0.28 ± 0.02 $^{a}$ | nd | nd |
| C20:3*n*-3 | 0.27 ± 0.01 $^{b}$ | 2.31 ± 0.07 $^{a}$ | nd | nd | 0.09 ± 0.01 $^{c}$ | 0.08 ± 0.01 $^{c}$ |
| C20:4*n*-6 | 1.23 ± 0.01 $^{b}$ | 1.77 ± 0.02 $^{a}$ | 0.15 ± 0.01 $^{e}$ | 0.23 ± 0.02 $^{d}$ | 0.28 ± 0.02 $^{c}$ | 0.32 ± 0.01 $^{c}$ |
| C20:5*n*-3 | nd | 1.34 ± 0.15 $^{a}$ | nd | nd | nd | nd |
| C22:5*n*-3 | 0.25 ± 0.04 $^{b}$ | 0.98 ± 0.09 $^{a}$ | nd | nd | 0.06 ± 0.02 $^{b}$ | nd |
| C22:6*n*-3 | 0.77 ± 0.22 $^{b}$ | 2.35 ± 0.24 $^{a}$ | nd | nd | nd | nd |
| C24:0 | 0.13 ± 0.01 $^{a}$ | nd | 0.12 ± 0.01 $^{a}$ | 0.10 ± 0.01 $^{b}$ | nd | nd |
| SFA | 9.52 ± 0.14 $^{d}$ | 13.66 ± 0.25 $^{c}$ | 17.56 ± 2.19 $^{b}$ | 19.38 ± 0.10 $^{b}$ | 39.60 ± 0.61 $^{a}$ | 39.72 ± 0.23 $^{a}$ |
| MUFA | 56.58 ± 0.36 $^{a}$ | 57.06 ± 0.80 $^{a}$ | 35.76 ± 2.34 $^{c}$ | 35.30 ± 0.56 $^{c}$ | 44.95 ± 0.40 $^{b}$ | 46.89 ± 1.36 $^{b}$ |
| PUFA | 32.65 ± 0.22 $^{b}$ | 27.51 ± 1.06 $^{b}$ | 46.52 ± 4.53 $^{a}$ | 45.07 ± 0.69 $^{a}$ | 15.16 ± 0.19 $^{c}$ | 13.04 ± 1.57 $^{c}$ |
| P/S | 3.43 ± 0.03 $^{a}$ | 2.01 ± 0.11 $^{b}$ | 2.69 ± 0.60 $^{b}$ | 2.32 ± 0.04 $^{b}$ | 0.38 ± 0.01 $^{c}$ | 0.33 ± 0.04 $^{c}$ |
| *n*-6 | 26.13 ± 0.05 $^{b}$ | 15.78 ± 0.55 $^{c}$ | 44.01 ± 2.63 $^{a}$ | 44.13 ± 0.66 $^{a}$ | 14.24 ± 0.16 $^{c}$ | 12.47 ± 1.48 $^{c}$ |
| *n*-3 | 6.52 ± 0.27 $^{b}$ | 11.72 ± 0.50 $^{a}$ | 2.51 ± 1.90 $^{c}$ | 0.94 ± 0.03 $^{c}$ | 0.92 ± 0.03 $^{c}$ | 0.57 ± 0.09 $^{c}$ |
| *n*-6/*n*-3 | 4.01 ± 0.17 | 1.35 ± 0.01 $^{b}$ | 17.53 ± 2.86 $^{a}$ | 46.98 ± 0.79 $^{a}$ | 15.49 ± 0.32 $^{ab}$ | 21.86 ± 1.04 $^{ab}$ |

**Legend:** *n, number of samples; results are represented as mean ± SD; nd = not detected. Values in the same row with the same letter are not significantly different (P≥0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S = polyunsaturated/saturated fatty acids

liver pâté (46.98). In other studies, the *n*-6/*n*-3 ratio was higher in commercial tuna (38.2) and salmon (9.3–11.3) pâtés (*Aquerreta et al.,* 2002; *Echarte et al.,* 2004). However, it is possible to produce pork pâtés with healthier *n*-6/*n*-3 ratios (<4) by using linseed oil with α-tocopherol addition for lipid stability, without producing negative effects on the textural or sensory properties (*D`Arrigo et al.,* 2004).

*Cooked sausages and canned chopped meat*

The FA compositions of different chicken cooked sausages and chopped canned meats are presented in Table 2.

Chicken cooked sausages were generally characterized by lower amounts of SFAs (26.07–31.92%) than canned chopped meat (41.43–43.12%), and high

**Table 2.** Fatty acid composition (%) of chicken cooked sausages and chopped canned meats

| Fatty acids | Chicken sausage n=2 | Mini chicken sausage n=2 | Junior chicken sausage n=2 | Pork luncheon meat n=2 | Beef luncheon meat n=2 |
|---|---|---|---|---|---|
| C14:0 | 0.34 ± 0.01 $^{e}$ | 0.62 ± 0.01 $^{d}$ | 0.79 ± 0.01 $^{c}$ | 1.68 ± 0.06 $^{b}$ | 2.22 ± 0.03 $^{a}$ |
| C15:0 | 0.07 ± 0.01 $^{b}$ | 0.08 ± 0.01 $^{b}$ | 0.08 ± 0.01 $^{b}$ | 0.20 ± 0.01 $^{b}$ | 0.30 ± 0.03 $^{a}$ |
| C16:0 | 19.84 ± 0.24 $^{c}$ | 21.21 ± 0.41 $^{c}$ | 23.53 ± 0.19 $^{b}$ | 25.82 ± 0.23 $^{a}$ | 26.78 ± 0.89 $^{a}$ |
| C16:1 | 3.14 ± 0.01 $^{c}$ | 3.27 ± 0.10 $^{c}$ | 3.75 ± 0.01 $^{b}$ | 3.45 ± 0.01 $^{d}$ | 4.26 ± 0.01 $^{a}$ |
| C17:0 | 0.10 ± 0.01 $^{c}$ | 0.14 ± 0.01 $^{bc}$ | 0.15 ± 0.01 $^{bc}$ | 0.32 ± 0.04 $^{b}$ | 0.70 ± 0.10 $^{a}$ |
| C18:0 | 5.62 ± 0.01 $^{d}$ | 6.48 ± 0.11 $^{cd}$ | 7.37 ± 0.04 $^{c}$ | 13.10 ± 0.60 $^{b}$ | 14.52 ± 0.01 $^{a}$ |
| C18:1*n*-9 | 41.12 ± 0.09 $^{b}$ | 39.72 ± 0.56 $^{b}$ | 39.69 ± 0.02 $^{b}$ | 44.10 ± 0.41 $^{a}$ | 45.49 1.39 $^{a}$ |
| C18:2*n*-6 | 24.74 ± 0.07 $^{a}$ | 23.58 ± 0.81 | 21.45 ± 0.05 $^{b}$ | 9.21 ± 0.94 $^{c}$ | 5.93 ± 0.01 $^{d}$ |
| C18:3*n*-6 | 0.14 ± 0.03 $^{a}$ | 0.11 ± 0.01 $^{b}$ | nd | nd | nd |
| C18:3*n*-3 | 2.92 ± 0.01 $^{a}$ | 2.54 ± 0.17 $^{a}$ | 1.28 ± 0.01 $^{b}$ | 0.58 ± 0.12 $^{c}$ | 0.45 ± 0.06 $^{c}$ |
| C20:0 | 0.11 ± 0.02 $^{ab}$ | 0.11 ± 0.01 $^{ab}$ | nd | 0.14 ± 0.04 $^{a}$ | 0.09 ± 0.01 $^{ab}$ |
| C20:1 | 0.73 ± 0.01 $^{a}$ | 0.78 ± 0.04 $^{a}$ | 0.57 ± 0.01 $^{b}$ | 0.80 ± 0.03 $^{a}$ | 0.27 ± 0.03 $^{c}$ |
| C20:2 | 0.21 ± 0.01 $^{bc}$ | 0.23 ± 0.06 $^{bc}$ | 0.34 ± 0.01 $^{ab}$ | 0.43 ± 0.05 $^{a}$ | 0.12 ± 0.03 $^{c}$ |
| C20:3*n*-6 | 0.39 ± 0.01 $^{a}$ | 0.34 ± 0.01 | 0.23 ± 0.01 $^{b}$ | 0.17 ± 0.03 $^{b}$ | 0.17 ± 0.01 $^{b}$ |
| C20:3*n*-3 | nd | nd | nd | nd | nd |
| C20:4*n*-6 | 0.32 ± 0.01 $^{ab}$ | 0.50 ± 0.15 $^{a}$ | 0.58 ± 0.01 $^{a}$ | 0.27 ± 0.01 $^{ab}$ | 0.17 ± 0.06 $^{b}$ |
| C20:5*n*-3 | nd | nd | 0.19 ± 0.02 $^{a}$ | nd | nd |
| C22:5*n*-3 | 0.06 ± 0.01 | 0.11 ± 0.02 $^{a}$ | nd | nd | nd |
| SFA | 26.07 ± 0.20 $^{d}$ | 28.63 ± 0.54 $^{d}$ | 31.92 ± 0.23 $^{c}$ | 41.43 ± 0.52 $^{b}$ | 43.12 ± 1.31 $^{a}$ |
| MUFA | 44.98 ± 0.09 $^{b}$ | 43.77 ± 0.71 $^{b}$ | 44.01 ± 0.03 $^{b}$ | 48.06 ± 0.46 $^{ab}$ | 50.60 ± 2.24 $^{a}$ |
| PUFA | 28.44 ± 0.11 $^{a}$ | 26.91 ± 1.07 $^{a}$ | 23.49 ± 0.03 $^{b}$ | 10.24 ± 0.99 $^{c}$ | 6.73 ± 0.01 $^{d}$ |
| P/S | 1.09 ± 0.01 $^{a}$ | 0.94 ± 0.05$^{b}$ | 0.74 ± 0.01 $^{c}$ | 0.25 ± 0.03 $^{d}$ | 0.16 ± 0.01 $^{e}$ |
| *n*-6 | 25.47 ± 0.11 $^{a}$ | 24.25 ± 0.86 $^{a}$ | 22.02 ± 0.19 $^{b}$ | 9.64 ± 0.85 $^{c}$ | 6.24 ± 0.01 $^{d}$ |
| *n*-3 | 2.98 ± 0.01 $^{a}$ | 2.66 ± 0.21 $^{a}$ | 1.47 ± 0.02 $^{b}$ | 0.60 ± 0.15 $^{c}$ | 0.47 ± 0.03 $^{c}$ |
| *n*-6/*n*-3 | 8.55 ± 0.01 $^{b}$ | 9.12 ± 0.40 $^{b}$ | 14.98 ± 0.08 $^{ab}$ | 16.07 ± 3.75 $^{a}$ | 13.28 ± 1.02 $^{b}$ |

**Legend:** *n, number of samples; results are represented as mean ± SD; nd = not detected. Values in the same row with the same letter are not significantly different (P≥0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S = polyunsaturated/saturated fatty acids.

MUFA contents (44.01–44.98%), with oleic acid being the most common. The most common *n*-6 PUFA in chicken cooked sausages was linoleic acid, ranging from 21.45% to 24.74%, while it was present in lower levels in canned chopped meat (5.93–9.21%). Among the *n*-3 PUFAs, the most common was ALA in cooked sausages and canned chopped meat. The P/S ratio in cooked chicken sausages was higher than 0.4, ranging from 0.74 to 1.09. In canned chopped meat, it was lower than the recommended value (0.16–0.25). The FA profiles and *n*-6/*n*-3 ratios (8.55–14.98) observed in the current study were similar to those of a control group of chicken frankfurters (*Jeun-Horng et al.,* 2002). The *n*-6/*n*-3 ratios in pork luncheon meat and beef luncheon meat in the current study were 16.07 and 13.28, respectively. *Guillevic et al.* (2009) showed it was possible to cook and manufacture typical French cooked meats enriched in n-3 PUFA with *n*-6/*n*-3ratios below 4 and without deleterious effects on global consumer appreciation.

*Fermented sausages*

The fatty acid composition of dry fermented sausages is presented in Table 3.

Small variations in the composition of fatty acids were detected in the raw, dry fermented sausages, although they were mainly characterized by similar concentrations of SFAs, ranging from 36.07% to 37.10% and a high concentration of MUFAs, which ranged from 48.10% to 49.78%, with oleic acid as the most predominant. Among the *n*-6 PUFAs, linoleic acid was the most common,

**Table 3.** Fatty acid composition (%) of dry fermented sausages

| Fatty acids | Čajna sausage n=3 | Kulen n=3 | Budimska sausage n=3 | Sremska n=3 |
|---|---|---|---|---|
| C14:0 | 0.98 ± 0.09 | 0.93 ± 0.07 | 1.04 ± 0.07 | 0.95 ± 0.06 |
| C15:0 | 0.05 ± 0.01 | 0.03 ± 0.02 | 0.07 ± 0.01 | 0.02 ± 0.03 |
| C16:0 | 23.65 ± 1.65 | 22.82 ± 1.22 | 23.21 ± 0.27 | 23.18 ± 1.20 |
| C16:1 | 2.21 ± 0.38 | 1.94 ± 0.17 | 2.04 ± 0.07 | 1.91 ± 0.24 |
| C17:0 | 0.36 ± 0.02 | 0.30 ± 0.07 | 0.34 ± 0.01 | 0.44 ± 0.11 |
| C18:0 | 11.47 ± 1.05 | 12.02 ± 0.98 | 11.08 ± 0.32 | 12.30 ± 0.80 |
| C18:1*n*-9 | 44.8 ± 0.39 | 46.87 ± 2.64 | 45.06 ± 2.74 | 46.91 ± 0.02 |
| C18:2*n*-6 | 13.2 ± 3.58 | 12.1 ± 2.11 | 13.90 ± 2.35 | 11.44 ± 1.04 |
| C18:3*n*-3 | 0.48 ± 0.11 | 0.45 ± 0.15 | 0.53 ± 0.17 | 0.35 ± 0.12 |
| C20:0 | 0.20 ± 0.11 | 0.19 ± 0.01 | 0.18 ± 0.07 | 0.19 ± 0.08 |
| C20:1 | 1.10 ± 0.20 | 0.98 ± 0.07 | 1.01 ± 0.04 | 0.89 ± 0.10 |
| C20:2 | 0.69 ± 0.14 | 0.59 ± 0.12 | 0.64 ± 0.03 | 0.56 ± 0.08 |
| C20:3*n*-6 | 0.39 ± 0.14 | 0.34 ± 0.14 | 0.28 ± 0.06 | 0.31 ± 0.20 |
| C20:3*n*-3 | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.02 | 0.06 ± 0.01 |
| C20:4*n*-6 | 0.29 0.01 | 0.32 ± 0.10 | 0.32 ± 0.05 | 0.39 ± 0.01 |
| SFA | 36.73 ± 2.94 | 36.3 ± 1.25 | 36.07 ± 0.40 | 37.10 ± 0.47 |
| MUFA | 48.11 ± 0.98 | 49.78 ± 2.46 | 48.10 ± 2.80 | 49.71 ± 0.36 |
| PUFA | 14.86 ± 3.92 | 13.61 ± 2.57 | 15.49 ± 2.57 | 12.78 ± 0.83 |
| P/S | 0.40 ± 0.14 | 0.37 ± 0.07 | 0.43 ± 0.07 | 0.34 ± 0.03 |
| *n*-6 | 14.28 ± 3.86 | 13.07 ± 2.41 | 14.82 ± 2.31 | 12.32 ± 0.76 |
| *n*-3 | 0.58 ± 0.06 | 0.54 ± 0.18 | 0.67 ± 0.26 | 0.46 ± 0.07 |
| *n*-6/*n*-3 | 24.62 ± 3.97 | 24.20 ± 6.00 | 22.12 ± 7.78 | 26.78 ± 2.48 |

**Legend:** *n, number of samples; results are represented as mean ± SD; nd = not detected. Values in the same row without a letter are not significantly different (P≥0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S = polyunsaturated/saturated fatty acids.

ranging from 11.44% to 13.90%. Among the *n*-3 PUFAs, the most common was ALA, which ranged from 0.35% to 0.53%. Dry fermented sausages enriched in ALA could be successfully elaborated with an adequate technological process by the incorporation of a gelled emulsion prepared with linseed oil (*Alejandre et al.,* 2016). Final acceptable products in that study had a lower fat content and very favourable *n*-6/*n*-3 ratio. The reformulation process did not cause oxidation problems, and no perceptible differences were reported for taste and juiciness as compared to a traditional product. This composition of fatty acids is typical for pork meat used as a raw material for the production of these sausages (*Wood et al.,* 2008). The FA profiles observed in the current study were similar to those of fermented sausages from a study by *Campos et al.* (2013). P/S ratios in the current study, as one of the quality parameters of food lipids in the fermented sausages, ranged from 0.34 to 0.43. However, these P/S ratios were close to the recommended ratio (0.4) (*Simopoulos*, 2002; *Wood et al.,* 2008).

The *n*-6/*n*-3 ratios in all dry fermented sausages were above the recommended levels of 4:1 (*Simopoulos*, 2002) and ranged from 22.12 (Budimska sausage) to 26.78 (Sremska sausage); these findings are in accordance with previously published data (*Saicic et al.,* 2010). The *n*-6/*n*-3 ratios were higher in our study than in the similar studies (*Campos et al.,* 2013; *Utrilla et al.,* 2014; *Valencia et al.,* 2007). In the study of *Pelser et al.* (2007), up to 20% of pork backfat was substituted with flaxseed oil or canola oil. The addition of flaxseed oil and canola oil progressively increased the

P/S ratio and decreased the *n*-6/*n*-3 ratio, leading to values closer to those considered optimal.

More scientific knowledge and new technologies will be necessary to fit the growing requirements of the market (*Vandendriessche*, 2008). If industry does not find an answer for the problem of fat in meat products, but without producing major differences in taste and flavour, substitutes for these products with other foods will be more likely. Quality will be judged by the consumer as sensorial quality first.

*Nutritional value of processed meat products*

The estimated percentage of daily fat intake for pâté is presented in Table 4.

Consumption of 100 g of fish pâtés per day equated to from 19% to 21% of total recommended fat intake, while turkey, chicken liver, pork liver and ham pâtés contained, on average ≥30% of fats. Only consumption of fish pâtés and chicken cooked sausages was estimated to result in the recommended daily intake of 10% saturated fats (6–12%), while consumption of the other types of pâtés and canned chopped meat was estimated to result in saturated fat intakes much greater than 10% (20–53%) (*Department of Health*, 1994; *Wood et al.,* 2008). However, no reduction of evaluated total fat and saturated fat intake was obtained in another study (*D`Arrigo et al.,* 2004) of pork liver pâté (intake of saturated fat was 67%, while it was estimated as 53% in our study, Table 4). In the study of *Terassa et al.* (2016), sunflower oil instead of pork back fat was applied to reduce fat content in chicken liver pâtés, but the reduction of the fat content was followed by decreasing oleic acid and increasing SFA levels. The evaluated intake of total fat was 44% and saturated fat intake was 20% higher than in our study (Table 4). The results obtained by *Xiong et al.* (2016) indicate that healthier chicken liver pâtés can be formulated with sunflower and canola oil combinations substituting 30–40% of the pork backfat. Even if a reduction of total fat intake was not obtained, the evaluated saturated fat intake ranged from 46% to 63% (*Xiong et al.,* 2016). However, these intakes were still higher than in our study (Table 4, estimated saturated fat intake was 20%).

In cooked, ready-to-eat meat products, estimated saturated fat daily intake was 7–12% (Table 5). However, reduction of evaluated total fat in chicken frankfurter fed with supplemental fish oil in the study of *Jeun-Horng et al.* (2002) was about 26%, and saturated fat intake was 30% which was higher than in our study (Table 5). There was a significantly lower oxidative stability of chops derived from pigs fed a linseed diet compared to pigs fed a control diet (*Guillevic et al.,* 2009), but also, higher evaluated total fat and saturated fat daily intakes via cooked sausages (about 27% and 40%, respectively) than in our study of pork products (Table 5). *Juárez et al.* (2009) showed that pork products can be modified to provide a significant increase in functional lipids, which can have positive influences on health. However, evaluated total fat was about 28% in control and about 30% in modified pork products, and saturated fat intake was about 35%, which was higher than in our study (about 22%, Table 5).

Consumption of 100 g of fermented dry sausages per day resulted in an estimated total fat intake ranging from 50% to 63% of the recommended daily fat intake, and a daily intake of saturated fats ranging from 65% to 81% of the recommended amount (Table 5). However, in *Campos et al.* (2013), the obtained fat intake via fermented sausages was reduced to 32%, with a saturated fat daily intake of 44.8%. These intakes were lower than the daily intakes calculated in our study (Table 5). *Campos et al.* (2013) made venison salchichon using 25% pork meat and 75% lean venison that ensured a pleasant texture, odour, flavour and appearance for the

**Table 4.** Percentage of total fat and saturated fat derived from heat treated, ready-to-eat pâtés in relation to the reference intake of 2,000 kcal per day

| Daily intake | Tuna pâté | Salmon pâté | Turkey pâté | Chicken liver pâté | Pork liver pâté | Ham pâté |
|---|---|---|---|---|---|---|
| Total fat, g 100 $g^{-1}$ of product | 13.00 | 14.68 | 21.58 | 20.67 | 26.79 | 21.09 |
| Total fat intake,% | 19 | 21 | 31 | 30 | 38 | 30 |
| Saturated fat, g 100 $g^{-1}$ of product | 1.24 | 2.01 | 3.79 | 3.74 | 10.61 | 8.38 |
| Saturated fat intake,% | 6 | 10 | 38 | 20 | 53 | 42 |

**Table 5.** Percentage of total fat and saturated fat derived from heat treated and dry fermented ready-to-eat meat products in relation to the reference intake of 2,000 kcal per day

| Daily intake | Chicken sausage | Mini chicken sausage | Junior Chicken sausage | Pork luncheon meat | Beef luncheon meat | Čajna sausage | Kulen | Budimska sausage | Sremska sausage |
|---|---|---|---|---|---|---|---|---|---|
| Total fat, g 100 $g^{-1}$ of product | 8.95 | 4.77 | 4.40 | 11.23 | 10.50 | 44.11 | 42.53 | 37.67 | 34.79 |
| Total fat intake,% | 13 | 7 | 6 | 16 | 15 | 63 | 61 | 54 | 50 |
| Saturated fat, g 100 $g^{-1}$ of product | 2.33 | 1.37 | 1.40 | 4.40 | 4.53 | 17.12 | 16.02 | 13.59 | 12.91 |
| Saturated fat intake,% | 12 | 7 | 7 | 22 | 23 | 81 | 77 | 68 | 65 |

consumer. *Valencia et al.* (2007) showed that algae oil can be used as a functional ingredient in dry fermented sausages in a limited amount. The obtained products had good sensorial quality, showed better *n*-6/*n*-3 ratios than traditional sausages, and supplied a relevant amount of DHA. However, even though their oxidation stability should be guaranteed with the use of antioxidants and storage under vacuum conditions, such products did not result in a reduced evaluated total fat intake (this was about 47%) or saturated fat intake (about 63%), which were lower than the intakes we estimated for our pork products (Table 5). Dry fermented sausages resulted in reduced total fat intake, down from 44% to 37.8% and reduced saturated fat intake, down from 52.2% to 42.3% (*Alejandre et al.,* 2016), which were also lower intakes than estimated in our study (Table 5). Although sensory and physical analyses showed that sausage formulations with encapsulated oils and control sausage were comparable, the new formula sausages were better liked by the sensory panel in the study of *Pelser et al.* (2007). However, the new sausages also resulted in higher evaluated total fat daily intake (up from 58% to 56%) and evaluated saturated fat daily intake (up from 68% to 81%), which were similar values to those calculated in our study (*Pelser et al.,* 2007) (Table 5).

Given that processed meats in Serbia might be consumed in amounts significantly greater than 100 g per serving, actual fat intakes per serving could be higher. Processed meats in Serbia are eaten not just at the main meal, but can be consumed for breakfast or as a between-meals snack. Given that the tinned pâté products are relatively inexpensive, they are often consumed by pensioners or others on limited budgets. They are also simple to prepare and consume (open and eat), so they are a common food for children. The fish pâtés look more interesting, in that some health benefits might ensue if people could be persuaded to consume these instead of meat pâtés.

Analysis of available data from forty countries indicated that calories from animal foods correlated positively with CHD mortality rates, while calories from vegetable foods correlated negatively. Differences in coronary disease mortality rates worldwide could largely be explained by differences in dietary cholesterol and saturated fat intake (*Odegaard*, *et al.,* 2012). Studies have indicated that healthy diet has an important role in the prevention of hypertension (*McEvoy et al.,* 2012).

## Conclusion

Cholesterol and triglycerides are essential components of the body, which become harmful only when they occur in increased amounts, and they may be important risk factors for the development of atherosclerosis in humans. It was confirmed that a regular intake of saturated fat and total fat via consumption of processed meat products, in particular processed pork products, was likely to be high in the Serbian general population. We speculate that this is, in turn, is likely to increase the potential risk for development of CHD. The works of other authors have shown that it is difficult to make a product that will meet the sensorial criteria and at the same time achieve a reduced daily intake of total fat and especially saturated fat. However, the results obtained should be of importance for the establishment of tables for nutritional value of products. The increased awareness of the meat industry regarding the importance of the fat content/quality in processed meat products and its impact on health, optimization of the product specifications (replacement of SFAs with unsaturated fats), health promotion activities by public health authorities, as well as better education of consumers about beneficial nutrition

habits (e.g. Mediterranean diet) should reduce the rate of CHD. Further research is needed to understand better the optimal combination of unsaturated fatty acids in processed meat products and recommended daily intakes which should maintain and enhance the health status of consumers.

**Conflict of interest.** The authors declare that they have no conflicts of interest.

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# Effect of pork to beef meat ratio on the physicochemical properties of frankfurters

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*A b s t r a c t: This study was conducted to investigate the effects of raw material, pork and beef meat, ratio on the physicochemical characteristics of emulsion-type sausage. Five different frankfurter formulations were calculated as follows: B100 (40% beef meat); B75 (30% beef and 10% pork meat); B50 (20% beef and 20% pork meat); B25 (10% beef and 30% pork meat) and B0 (40% pork meat). Frankfurters made solely from beef meat (B100) showed significantly better emulsion stability than those made with pork meat (B0). The increase in the fat content also decreased gel strength, leading to lower values of hardness, cohesiveness, gumminess and chewiness. The optimal ratios between pork and beef meat to enhance the textural properties of frankfurters were B50 and B75. The lightness values (L*) increased with increasing pork meat content, while the redness values (a*) demonstrated the opposite trend. Protein, fat and total pigments displayed a positive relation, whereas water content exhibited a negative relation with a* values.*

***Key words:*** *Emulsion-type Sausages; Meat Ratio; Composition; Texture; Colour.*

## Introduction

Emulsification technology has been used over several hundred years for the preparation of emulsion-type meat products. Emulsified meat products such as frankfurter sausages are generally consumed in many countries. They tend to be more popular than other processed meat products, because they are convenient and are utilized in a variety of foods (*Allais, 2010*). The wide diversity in physicochemical and sensory characteristics of food emulsions is due to the variety of ingredients and processing conditions. Emulsified meat products, also called meat batters, are complex systems in which fat is emulsified into a viscous fluid mainly composed of solubilized myofibrillar proteins previously extracted from meat from different animal species (*Ugalde-Benítez, 2012*).

One of the most important quality characteristics for processed meat products such as emulsified sausages is emulsion stability between fat and water contents. Fat is one of the most variable raw materials in emulsified meat products, as it plays an important role in the formation of meat emulsions with other ingredients, and is related to flavor intensity, juiciness, and tenderness in sausage products (*Hughes et al. 1997*).

Protein is also an important material for binding both the fat and water constituents in the meat emulsion. For example, soluble myofibrillar proteins are extracted by salt surrounding the fat particle, and they subsequently form the emulsion matrix with water and fat (*Youssef and Barbut, 2010*). Meat emulsion formation includes the activation of most of the proteins present in the muscle by disrupting the sarcolemma to release myosin and actin, which are subsequently solubilized by salts and phosphates. Myofibrillar proteins, with fibrous structures, turn into a viscous fluid during protein activation. This fluid is responsible for fat emulsification and immobilization of added water. Changing fibrous proteins into a viscous fluid is relatively easy with pork and chicken meat, but more difficult with beef and lamb (*Feiner, 2006*). This is because different animal species can present a wide variety of protein characteristics, probably due to interaction effects (*Zorba, 2006*). According to Feiner (2006), meat hardness, as a result of fiber thickness variation among meat type and cuts, is also related to protein solubility variation within the same animal species. Another major component of the meat

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emulsion is water. In the emulsion, water performs a number of functions such as: 1) functioning as a curing solution; 2) regulating the temperature of the batter; 3) saving on production costs; and 4) having an impact on the texture and juiciness of the product (*Ockerman and Basu, 2014*).

Numerous studies regarding the processing technology of emulsion-type sausages have been published, including research into additives, substitutes, chopping temperature, pressure, mixing time, and processing procedure (*Bañón et al. 2008 ; Carballo et al.1995; Colmenero et al.1995; Wang et al.2009* ). However, although the raw material components have a big impact on emulsion-type sausages, very few studies have been conducted to assess the physicochemical characteristics based on the ratio of raw materials. Therefore, the objective of this study was to investigate the effect of the pork to beef meat ratio on physicochemical characteristics of emulsion-type frankfurter sausages, and to determine the optimized ratios of these raw material components for frankfurter production.

## Materials and methods

### *Frankfurter preparation*

As raw material, post-rigor pork and beef meat (mixture of round and shoulder muscles) and fresh back fat were obtained from the slaughterhouse at the Institute for Animal Husbandry (*Belgrade, Serbia*). The meat was trimmed of visible fat and connective tissue. Frankfurters were manufactured in a small meat processing plant at the Institute under commercial processing conditions.

Five different formulations were calculated to yield a 20 kg batch as follows: B100 (40% beef meat); B75 (30% beef and 10% pork meat); B50 (20% beef and 20% pork meat); B25 (10% beef and 30% pork meat) and B0 (40% pork meat). All the formulations also contained: 30% of pork back fat, 30% water (ice), 1.5% nitrite-salt (Prima Commerce, Serbia), 0.3% polyphosphate (Tari K2, BK Giulini GmbH, Germany), 1% soy protein isolate (Supro 548 IP Non-GMO, Solae™) and 0.4% of a ready-to-use frankfurter spice mixture (Prima Commerce, Serbia) (Table 1).

All formulations were produced on the same day and in an identical manner: meat and fat were chopped to 8 mm particle size in a meat grinder (Balint, Serbia) and then mixed with ice, nitrite-salt, soy protein and spices in a meat cutter (Belje, Croatia). The prepared batter was stuffed into 24 mm diameter collagen casings, after which they were hung, smoked and cooked for approximately 2 hours in a smoking/cooking chamber (Belje, Croatia), until the temperature in the central part of the sausages reached 72°C10 $min^{-1}$. The cooked frankfurters were showered in cold water and stored at 5 ± 1°C for 48h before testing.

### *Composition analysis of the frankfurters*

Ten samples from each formulation of frankfurters were used for the composition examination. The casing was removed and the sausages were ground in a mixer (Ultra Turrax T18, IKA, Germany) before all analysis were carried out, in triplicate. The moisture content was determined by drying at 105°C (*ISO 1442, 1997*); protein content by the Kjeldahl method and a multiplication factor of 6.25 (*ISO 937, 1978*); total fat content by the Soxhlet method (*ISO 1443, 1973*), and ash content by mineralization at 550 ± 25°C (*ISO 936, 1998*). The pH value

**Table 1.** Experimental design and composition of frankfurters

| Ingredients (%)[1] | 100B | 75B | 50BP | 75P | 100P |
|---|---|---|---|---|---|
| Beef | 40.0 | 30.0 | 20.0 | 10.0 | – |
| Pork | – | 10.0 | 20.0 | 30.0 | 40.0 |
| Pork back fat | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 |
| Water (ice) | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 |
| Total | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Nitrite-salt | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Polyphosphate | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Soy protein | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Spices | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |

**Legend:** [1] Formulations were calculated to yield a 20 kg batch.

was measured using a pH-meter, Hanna, HI 83141 (Hanna Instruments, USA), equipped with a puncture electrode. The pH meter was calibrated using standard phosphate buffers (*ISO 2917, 1999*).

*Textural profile analyses*

The frankfurters were cut into 15 mm thick slices and from each slice, the 15 mm diameter core was removed to obtain cylindrical samples for textural profile analysis (TPA). The TPA tests were carried out using TA.XT Plus Texture Analyzer (Stable Micro Systems Ltd., UK) with 50 kg load cell. Frankfurter cores were placed upright on a platform (sample height 15 mm), and compressed with a 25 mm diameter cylindrical aluminum probe (P/25). The texture attributes obtained from TPA were: hardness, springiness, cohesiveness gumminess and chewiness according to Pons and Fiszman (*1996*). Test speed was 60 mm $min^{-1}$, and strain was 50%. The TPA tests were performed without rupture of the cores, so rupture force, rupture work of energy and rupture deformation of sausages was obtained from a rupture test, also according to Pons and Fiszman (*1996*), in single compression at 65% strain, with the same test speed and with the same probe. Texture attributes from each type of sausage were obtained from at least eight measurements.

*Pigment content and instrumental colour measurement*

The content of total pigments and nitroso-myoglobin (mg per kg of sausage) were determined on a spectrophotometer (Spekol 1300, Analytic Jena, Germany) at 640 and 540 nm respectively, according to the method described by *Hornsey* (1956). For instrumental colour analyses, each sausage was cut and the colour was measured three times using Chromameter CR-400 (Minolta Co. Ltd, Tokyo, Japan), configured with the following parameters: D65 light source, 0° observer, and 8 mm aperture size and calibrated using a white ceramic tile. Values were given in the colour space CIE, where L* – lightness; a* – redness; b* – yellowness (CIE, 1976). The colour measurements were performed at room temperature (20 ± 2°C). Chroma (C*) and hue angle (h) were calculated using the available software.

*Statistical analysis*

Data entry and decoding were 100% verified. A one-way ANOVA was conducted to compare the results of the different assays, using SPSS Statistics 17.0 (*Chicago, Illinois, USA*) data analysis software. An alpha level of $p<0.05$ was used to determine significance. For the TPA tests, multivariate analysis of variance (MANOVA) was performed, with pork:beef ratio as fixed factor, and textural attributes as dependent variables. Subsequent univariate analyses were also conducted, and in post-hoc analysis, Duncan's test was performed to obtain homogeneous subsets of samples for each texture attribute.

## Results and discussion

*Composition of frankfurters*

The observed pH and composition values (Table 2) show that frankfurters B0 and B25 had somewhat higher fat content and consequently lower moisture content compared to B50, B75 and B100 frankfurters. Protein contents varied from 12.83% (B100) to 11.49% (B0) and were significantly different between all formulations. The variations in pork to beef meat ratios used in frankfurter formulations in our investigation revealed a clear pattern that connects slightly higher pH values, higher protein levels, higher moisture and reduced fat content with higher beef content frankfurters (Table 2).

**Table 2.** Composition and pH values of frankfurters (mean ± standard deviation)

| Sample | pH | Moisture (%) | Fat (%) | Protein (%) | Ash (%) |
|---|---|---|---|---|---|
| B100 | $5.97^{a} \pm 0.02$ | $56.13^{a} \pm 0.06$ | $27.39^{a} \pm 0.91$ | $12.83 \pm 0.03$ | $2.75 \pm 0.01$ |
| B75 | $5.97^{a} \pm 0.02$ | $59.63^{a} \pm 0.06$ | $24.25^{a} \pm 0.17$ | $11.72 \pm 0.02$ | $2.59^{a} \pm 0.01$ |
| B50 | $5.97^{a} \pm 0.01$ | $59.05^{a} \pm 0.17$ | $26.41^{a} \pm 0.40$ | $11.61 \pm 0.01$ | $2.48 \pm 0.01$ |
| B25 | $5.84 \pm 0.01$ | $54.14 \pm 0.08$ | $31.13 \pm 0.16$ | $11.58 \pm 0.04$ | $2.58^{a,b} \pm 0.02$ |
| B0 | $5.88 \pm 0.01$ | $53.61 \pm 0.04$ | $31.73 \pm 0.10$ | $11.49 \pm 0.07$ | $2.52^{b} \pm 0.03$ |

**Legend:** B100 (40% beef meat); B75 (30% beef and 10% pork meat); B50 (20% beef and 20% pork meat); B25 (10% beef and 30% pork meat); B0 (40% pork meat); Means in the same column that have no superscript in common are significantly different ($p<0.05$)

**Table 3.** The effect of pork to beef meat ratio on color and pigments of frankfurters

| | B100 | B75 | B50 | B25 | B0 |
|---|---|---|---|---|---|
| L* | 68.8±0.7 | 69.6±0.3 | 71.9±0.8 | 72.8±0.2 | 75.1±0.6 |
| a* | 9.2$^{a,b}$±0.2 | 9.7±0.3 | 9.0$^{a}$±0.4 | 8.9$^{b}$±0.2 | 8.2±0.3 |
| b* | 12.3±0.2 | 11.9±0.2 | 11.5±0.2 | 11.2±0.3 | 10.8±0.3 |
| h | 15.4$^{a}$±0.2 | 15.3$^{a}$±0.1 | 14.6$^{b}$±0.4 | 14.3$^{b}$±0.2 | 13.6±0.4 |
| C | 53.1$^{b}$±0.7 | 50.9$^{a}$±1.2 | 51.9$^{a,c}$±1.0 | 51.4$^{a}$±1.1 | 52.9$^{b,c}$±0.8 |
| Total pigments (mg/kg) | 84.3±1.2 | 68.9±2.4 | 54.6±1.1 | 51.1±0.9 | 39.6±1.0 |
| Nitroso-myoglobin (mg/kg) | 31.6$^{a}$±1.0 | 25.8$^{b}$±1.0 | 25.5$^{b}$±1.6 | 32.2$^{a}$±1.1 | 25.4$^{b}$±2.0 |
| Conversion rate | 39.6$^{a,b}$±0.5 | 35.2$^{a}$±0.3 | 43.8$^{b}$±2.5 | 60.4$^{c}$±2.0 | 60.2$^{c}$±6.2 |

**Legend:** B100 (40% beef meat); B75 (30% beef and 10% pork meat); B50 (20% beef and 20% pork meat); B25 (10% beef and 30% pork meat); B0 (40% pork meat); Means in the same row that have no superscript in common are significantly different ($p<0.05$)

*Textural profile analyses*

MANOVA was calculated, examining the effect of pork:beef ratio on textural attributes, and a significant effect was found [Wilks' Lambda (32, 138 ) = 0.1 and $p<0.05$]. Subsequent univariate analysis showed that the pork:beef ratio did not have a statistically significant effect only on springiness . Mean values of other texture attributes are presented (Table 3), along with homogeneous subsets for each attribute. The means listed under each subset comprise a set of means that are not significantly different from each other.

Compared to other formulations, the highest hardness values were observed for frankfurters made solely from beef meat (100B) (Table 3). The highest values of cohesiveness, gumminess, and chewiness were each observed for the same frankfurter formulation (100B), which had significantly the greatest protein content. According to Choe et al. (*2013*), emulsion sausages with higher values in cohesiveness, gumminess and chewiness showed significantly greater emulsion stability than those with low values.

Our results also revealed that increasing the fat content also decreased gel strength, leading to lower values of hardness, cohesiveness, gumminess and chewiness. This result was likely due to the lack of emulsifying agents from salt-soluble proteins such as myosin, which would result in poorer quality characteristics in the frankfurters. Similar results were also reported by a number of research groups (*Bañón et al., 2008; Bloukas and Paneras, 1993; Cofrades et al.2000 ; Colmenero et al., 1995*). An excessive fat content causes the emulsion to break down, due to lack of protein content surrounding the fat globules in emulsion formation. *Hughes et al.* (1998) reported that a decrease in fat content significantly reduced cohesiveness and gumminess in Frankfurter sausages. This is in contrast to our result, where we observed that the fat content showed a negative relationship to all textural attributes investigated (Table 3).

*Pigments and colour*

The instrumental colour data (Table 4) show the pork to beef meat ratio had a significant effect on L*, a* and b* measurements of frankfurters. We observed that frankfurters made exclusively from beef meat (B100) had the lowest L* values compared to all other formulations ($p<0.05$). The lightness increased with increasing pork meat content, while the redness values (a*) demonstrated the opposite trend, with the lowest value (8.2) observed in frankfurters made solely from pork meat. Protein, fat and total pigments displayed a positive relation, whereas water content exhibited a negative relation with a* values (Tables 2 and 4 ). Our results are in concurrence with the findings of *Youssef and Barbut* (2011), where the higher levels of protein and lean meat resulted in a significant increase in redness values in the emulsion meat batter. Additionally we have confirmed their assumption that this phenomenon is attributed to a higher myoglobin content. This theory is also supported by the work of *Carballo et al.* (1995), where the dilution of myoglobin through reduced protein content in the formulation led to a lower redness value.

According to *Hughes et al.* (1997), it was previously noted that reducing the fat content resulted in a decrease of lightness and increase of redness of

**Table 4.** Homogeneous subsets for different textural parameters and the effect of pork to beef meat ratio

| a) Hardness | Subset | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| B0 | 2054.8 | | | |
| B25 | 2276. | 2276.7 | | |
| B50 | | 2567.4 | 2567.4 | |
| B75 | | | 2709.6 | |
| B100 | | | | 3608.2 |

| b) Gumminess | Subset | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| B0 | 1599.6 | | | |
| B25 | 1745.3 | 1745.3 | | |
| B50 | | 1992.7 | 1992.7 | |
| B75 | | | 2075.0 | |
| B100 | | | | 2540.3 |

| c) Chewiness | Subset | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| B0 | 1400.9 | | | |
| B25 | 1520.8 | 1520.8 | | |
| B50 | | 1714.9 | 1714.9 | |
| B75 | | | 1833.4 | |
| B100 | | | | 2180.2 |

| d) Cohesiveness | Subset | |
|---|---|---|
| | 1 | 2 |
| B0 | ,7061 | |
| B25 | | ,7659 |
| B50 | | ,7678 |
| B75 | | ,7783 |
| B100 | | ,7816 |

**Legend:** B100 (40% beef meat); B75 (30% beef and 10% pork meat); B50 (20% beef and 20% pork meat); B25 (10% beef and 30% pork meat); B0 (40% pork meat);

frankfurter sausages. In a number of previous studies, it was observed that the colour of emulsion-type products was mostly influenced by fat content (*Bloukas et al.,1997 ; Carballo et al., 1995*). In our research, the fat content increased in the series from B100 to B0, and the lightness of the frankfurters also increased. As the water content in the frankfurters increased in the series from B0 to B100, the yellowness increased (Tables 2 and 4 ).

## Conclusion

We investigated a number of pork:beef meat ratios used in the preparation of emulsion-type frankfurter sausages, and examined their effects on the physicochemical characteristics of the sausages. All of the treatments investigated showed normal pH (5.84–5.97), protein content between 11.49% and 12.83%, fat content between 24.25 % and 31.73%, and moisture in the range of 53.61-59.63%. It was observed that both the fat and protein contents significantly affected the textural profile of the sausages, where the frankfurters containing only beef meat had the highest values for hardness, cohesiveness, gumminess, and chewiness . Frankfurter colour was also influenced by variations in pork:beef meat ratios and fluctuations in protein, fat and total pigment content. Lightness was significantly reduced with increasing water content, while yellowness values were amplified. In conclusion, therefore, increasing the moisture content and reducing the fat and protein contents of the frankfurters resulted in reduced values of a range of physicochemical characteristics. Frankfurters made solely from beef meat showed significantly better emulsion stability than those made with pork meat.

Knowing that consumer studies are crucial for understanding the relation between food properties on the one hand and human preferences and purchasing behaviour on the other, we also conclude that its absence is a thoughtful limitation in our investigation. This exactly why we would suggest an exploratory study of the perceived relationship of price and quality of frankfurters with different pork:beef meat ratios among Serbian consumers should be a subject of future research.

**Conflict of Interest.** The authors declare that they have no conflicts of interest.

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*Review paper*

# The application of high pressure processing in decontamination of dry fermented sausages

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*A b s t r a ct: High pressure processing (HPP) is a non-thermal postprocessing technology for decontamination of foods with minor impact on their nutritional and sensory characteristics. In the meat industry, HPP is mainly used to increase shelf-life and improve the safety of processed and ready-to-eat meat products. HPP can also be considered as a technique for creation of innovative meat products. In the last decade, numerous scientific studies on HPP application to dry fermented sausages have been published. Many factors, including sausage formulation and processing parameters as well as time, temperature and intensity of treatment, can influence the effectiveness of high pressure in processing these types of products. In general, pressure higher than 400 MPa is necessary to achieve efficacious microbial inactivation. HPP treatment has no detrimental effects on sensory qualities of dry fermented sausages or these effects are minor compared to other decontamination technologies. Also, HPP is environmentally friendly, and hence, wider application of this technology in the food industry is expected in the near future.*

***Key words:*** *high pressure processing, dry fermented sausage, meat safety, food quality.*

## Introduction

In the last two decades, increased demand for minimally processed and additive– and preservative-free products has highlighted high pressure processing (HPP) as one of the most prominent recent innovations in the food industry. HPP is a non-thermal post-processing technology, mainly used to increase shelf life and to improve food safety. HPP uses a pressure of ≥100 MPa that is transmitted immediately and uniformly through food products using a liquid transmitter, whilst keeping the freshness and nutritive value of the treated products. On the other hand, some negative impacts have also been seen with application of this technology, including changes of quality parameters such as colour, texture and water holding capacity (*Garriga and Aymerich,* 2009*; Simonin et al.,* 2012*; Rastogi,* 2016).

In the meat industry, HPP is mostly applied to ready-to-eat (RTE) meat products (*Mor-Mur and Saldo*, 2012). The main purpose of HPP technology is inactivation of foodborne pathogens and spoilage microbiota, but it can also be used as a technique for creating innovative meat products (*Campus*, 2010; *Simonin et al.*, 2012).

Dry fermented sausages are mainly considered as microbiologically safe products, the safety assurance of which relies on sufficient anti-pathogen effects of multiple antimicrobial factors during the processes of fermentation and drying. However, in cases of initial contamination of the raw materials with higher levels of pathogenic microorganisms and/or insufficient control due to the antimicrobial factors, the safety of these products can become compromised (*Ducic et al.*, 2014; *Ducic and Markov, 2015*). Therefore, the aim of this review is to present updated knowledge dealing with applications and effects of HPP technology on the safety and quality of dry fermented sausages.

## The main characteristics and application of HPP technology in the meat industry

In order to construct devices that will meet specific microbiological and nutritional food quality requirements, most companies producing HPP equipment directly cooperate with research centres and food producers. In recent years, the problems with production of pumps that can produce sufficient pressure, and concurrently, with production of large-capacity chambers able to withstand large numbers of production cycles, were successfully resolved. Consequently, due to this development of high

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efficiency HPP machines, processing costs have reduced to acceptable levels (*Bermudez and Canovas*, 2011; *Rastogi*, 2016).

The main parts of an HPP system are a pressure vessel, high pressure pump, closure(s) for sealing the vessel, a device for holding the closure in place while the vessel is under pressure, a system for controlling and monitoring the pressure and optionally, the temperature, as well as a system for transferring food product to and from the pressure vessel (*Campus*, 2010). The volume of pressure vessels indented for the food industry is usually between 35 and 320 litres, while the position of pressure vessels can be either vertical or horizontal. The advantages of horizontal layout include easier filling and emptying, as well as simpler and reliable tracking of treated and non-treated products. If high pressure is used for food treatment concurrently with high temperature, the HPP is usually performed in lower volume vessels containing heaters. In such a vessel, temperatures ranging from 20°C to 150°C can be produced in a short time (*Bermudez and Canovas*, 2011).

Due to the compression of matter volume at high pressure, the processing increases food temperature by 5°C to 15°C or about 3°C with each 100 MPa increase, depending on food composition, the rate of pressure increase and the shape and fullness of the vessel. After decompression, the temperature of food returns to the initial temperature. Similarly, pressure reversibly decreases food pH; sometimes a change of more than one unit is manifested, along with reduction of protein stability (*Cheftel*, 1997; *Campus*, 2010; *Mújica-Paz et al*., 2011). Prior to application of HPP on solid food, like fermented dry sausages, the food is vacuum packaged in plastic materials that are able to conform to the treated product's compression of at least 19% of the original volume. Packaging materials are selected on the basis of their integrity and insulating capacity, so they are not distorted by the application of high pressure, and if migration of molecules from packaging into the food product occurs, this must be within the permissible limits (*Juliano et al*., 2010). Modified atmosphere packaging is also used in some circumstances instead of vacuum packaging, but in that case, due to additional gas compression, application of HPP takes longer which, as a consequence, increases the production costs (*Mújica-Paz et al*., 2011).

## Mode of HPP action on microorganisms

The effects of HPP on microorganisms in/on meat and meat products are dependent on many characteristics of microorganism and food product. HPP inactivation of microorganisms is caused by various changes that occur in the cell membrane, the cell wall, ribosomes and enzymes. Damage of cell membrane is the main cause of cell death, due to disturbances of permeability, osmotic pressure and transport systems (*Patterson*, 2005; *Campus*, 2010; *Simonin et al.*, 2012; *FDA*, 2014). Additionally, high pressure directly leads to denaturation and agglomeration of proteins and subsequent inactivation of the enzymes (*Bajovic et al.,* 2012).

Single- or multi-cell parasites are severely affected as a consequence of their complex structure, even at a lower pressure ranging from 200 to 300 MPa (*Yuste et al.,* 2001; *Simonin et al.*, 2012). Moulds and yeasts exhibit moderate HPP resistance, with the exception of certain ascospores of heat resistant moulds (*Neosartorya*, *Talaromyces*, *Byssochlamys*), which are able to withstand pressures higher than 600 MPa (*Chapman et al.,* 2007; *Smelt*, 1998).

The vegetative forms of bacteria are more resistant than moulds and yeasts. Due to the thicker and stronger cell wall, Gram positive bacteria are more resistant than the Gram negatives; also, cocci are more resistant than rods (*Murchie et al*., 2005; *Patterson*, 2005). Bacterial cells in the exponential phase are more sensitive to pressure compared to the cells in the stationary phase (*McClements et al.*, 2001; *Manas and Mackey*, 2004; *Hayman et al.,* 2007). Spores cannot be destroyed by application of high pressure alone, as treatment intensity at usual processing temperatures is inadequate. Furthermore, pressure-assisted thermal processing (PATP) is a method used for food sterilization that combines high pressure (>600 MPa) and temperatures above 60°C. The advantages of PATP include a lower processing temperature and/or shorter exposure of the product to high temperature, compared to conventional sterilization (*Cheftel*, 1995; *Bermúdez-Aguirre and Canovas*, 2011; *Mújica-Paz et al., 2011*; *Simonin et al.*, 2012). Another strategy combines high pressure and temperature (<100°C) – the aim of this treatment is to enable germination of bacterial spores first, so the resultant vegetative bacteria are sensitive to the high pressure (*Rendueles et al.*, 2010). This method is still not used commercially, and the reasons include high variability of spore germination under the HPP, as well as the economic aspects of the process (*Ahn et al.*, 2007; *Wilson et al.*, 2008; *Tores et al.*, 2010).

Viruses exhibit diverse resistance to HPP, but can be inactivated by high pressure. Prions are destroyed only by using extremely high pressure (≥700 MPa) concurrently with high temperature (≥60°C) (*Campus*, 2010). *Heindl et al.* (2008) found that

after application of 800 MPa (5 min, 80°C), infectivity of prions significantly decreased.

The effect of HPP technology on microorganisms additionally depends on the product characteristics, such as water activity ($a_w$), pH, salt content and the composition of the raw material. To achieve appropriate antimicrobial effects on products with lower $a_w$, especially if $a_w$ is lower than 0.9, these products need to be exposed to higher pressure. Microorganisms injured by HPP are usually more sensitive to low $a_w$, so HPP on low $a_w$ products is followed by inhibition of microbial recovery during storage (*Considine et al.,* 2008; *Simonin et al.*, 2012). HPP's antimicrobial effects increase with declining pH (*Garriga and Aymerich*, 2009). However, the composition of more complex food products (i.e. contain proteins, lipids, sugars, vitamins and some cations) reduces the antimicrobial effects of HPP (*Hauben et al*., 1998; *Rubio et al*., 2007; *Considine et al*., 2008; *Mor-Mur and Escriu*, 2009; *Campus*, 2010). Furthermore, HPP works synergistically with, or is supported by, some antimicrobial substances (e.g. bacteriocins, nitrites, organic acids), modified atmosphere packaging (containing $CO_2$ or vacuum packed), as well as by low temperatures during food storage (*Garriga and Aymerich*, 2009; *Jofré et al*., 2010; *Bajovic et al*., 2012; *Rodríguez-Calleja et al*., 2012 ). Considering that, the results of investigating HPP antimicrobial effects in buffers and synthetic media cannot be always extrapolated (*Ananou et al*., 2010; *Campus*, 2010).

## HPP effects on microbiological and sensorial quality of dry fermented sausages

In the meat and meat product industry, pressure of 400–600 MPa for 3 to 7 minutes, mostly at room temperature, is applied for inactivation of pathogens and spoilage microbiota (*Bajovic et al*., 2012). The most important foodborne pathogens associated with dry fermented sausages are nontyphoidal *Salmonella* spp., *Listeria monocytogenes* and pathogenic *Escherichia coli* (*Ducic et al*., 2016). Numerous published studies on HPP effectiveness on microbiological and sensorial quality of dry fermented sausages in production of dry fermented sausages are outlined below.

*Omer et al.* (2010) investigated HPP application on inactivation of enterohaemorrhagic *E. coli* (*E. coli* O103:25), inoculated at a level of 6.8 log CFU $g^{-1}$ in two types of Norwegian fermented dry sausages (morr and salama). Pressure of 600 MPa applied in three cycles lasting for 200 seconds (6 minutes in total) at an initial temperature of 12°C, led to 3 logs reduction of the inoculated pathogen. In an earlier study, *Gill and Ramaswamy* (2008), applying the same pressure (600 MPa) on two types of semi-dry fermented sausages (All Beef and Hungarian salami; $a_w$ 0.927 to 0.968; pH 4.8 to 6.3), found more than 4 logs reduction of *E. coli* O157, while statistical differences in reductions were not found in treatments that lasted for 3, 6 or 9 minutes. Significantly, Gill and Ramaswamy also investigated sausages after four weeks of storage at 15°C. In All Beef salami, the number of *E. coli* O157 increased during storage, while in Hungarian salami, which had lower pH and $a_w$, the pathogen numbers remained at the same level. In both of these studies, high pressure did not cause any significant changes in the treated products sensorial characteristics.

A comprehensive study performed by *Porto-Fett et al*. (2010) investigated effects of high pressure (483 MPa during 1, 2, 3, 4 and 5 minutes at 20°C as well as 600 MPa during 5, 7, 10 and 12 minutes at 20°C) applied to Genoa salami inoculated with several *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium strains. The initial level of each pathogen strain-cocktail was approximately 7 logs per gram of sausage batter. After fermentation and drying, sausages were HPP treated, stored at 4°C and monitored for levels of inoculated pathogens for 28 days. *S.* Typhimurium and *E. coli* O157:H7 were completely eliminated in the majority of the samples. After the application of HPP (600 MPa during 5 minutes), reduction of *L. monocytogenes* ranged from 1.6 to 6 logs, depending on fermentation and drying conditions. In the same conditions of pressurization (600 MPa, 5min), *L. monocytogenes* was under the detection limit (≤ 1 log) in some samples immediately after the treatment, although it was detected during the storage phase. This study confirmed earlier findings that *L. monocytogenes* is more resistant to high pressure than *E. coli* or *Salmonella*, as well as that the direct antimicrobial impact of high pressure decreases with lower $a_w$ of food product.

*Garriga et al*. (2005) examined the effect of 400 MPa at 17°C during 10 minutes on microbiological and sensorial properties of two Spanish mildly fermented sausages (fuet and chorizo). As a result of the applied pressure/time, the authors stated that safety and shelf life were improved, i.e. the number of *Enterobacteriaceae* was reduced, *L. monocytogenes* was unable to grow, while inoculated *S.* Typhimurium was not detected.

*Marcos et al*. (2007) conducted a study using the same type of sausages but without inoculation of pathogens; beside antimicrobial effects, the HPP (400 MPa, 17°C, 10 minutes) effect on sensorial properties was assessed as well. In fuet and

chorizo, HPP reduced the *Enterobacteriaceae* level by 1 and 3.8 log CFU $g^{-1}$, respectively. The number of *Enterococci* decreased by 2 log CFU $g^{-1}$ in chorizo sausages only, confirming that sensitivity of the enterococcal population to HPP is variable and is influenced by the numbers and species composition of each product. By using a texture profile analysis (TPA) method for the HPP-treated sausages, it was found that these products had higher cohesiveness, chewiness and springiness compared with untreated products. Regarding sensorial properties, the only difference noticed by the sensory panel was a slight decrease in colour intensity manifested in HPP-treated sausage. Furthermore, HPP did not cause higher levels of lipid oxidation (measured as thiobarbituric acid reactive substances (TBAR) values).

*Marcos et al*. (2013) investigated the antilisterial effect of combined use of high pressure and antimicrobial packaging on fermented dry sausages that were produced without NaCl and stored for 90 days at 12°C (considered as the worst case scenario of storage conditions in consumers' refrigerators). Sliced products were prepared using accelerated drying (Quick Dry Slice process – QDS®) and then inoculated on the surface with a three-strain cocktail of *L. monocytogenes* (the concentration of was $5x10^5$ CFU $g^{-1}$). Pressure of 600 MPa (5 min, 12°C) did not reduce pathogen levels, and this was, according to the authors, a consequence of low $a_w$ of the treated products ($a_w$ <0.9). High pressure treatment used in conjunction with nisin led to greater *Listeria* reduction (an additional 0.5 logs, so 1.9 logs compared to 1.4 logs) than was obtained by using nisin only.

Similar research that was conducted by *Stollewerk et al*. (2012) pointed out that the QDS process in combination with high pressure (600 MPa, 5 min, 13°C) provides safe fermented dry sausages even after 91 days of storage under refrigeration. This study investigated sausages that, during production, were inoculated with *L. monocytogenes* (30 CFU $g^{-1}$) and *Salmonella* spp. (15 CFU $g^{-1}$); these concentrations of pathogens were chosen to be in line with the level of "common" contamination of fermented dry sausages on the market found in other studies.

*Jofre et al*. (2009) investigated the sensitivity of *L. monocytogenes*, *Salmonella* and *Staphylococcus aureus* strains to high pressure and enterocins A and B in Spanish fermented dry sausages (fuet). Each of these pathogens was inoculated as a strain-cocktail with concentration of 2.7 log CFU per gram of sausage batter. After the drying process, sausages were subjected to 400 MPa pressure (17°C, 10 min) and stored for 20 days at 20°C and thereafter, at 7°C. In HPP-treated fuet, the reduction of *Salmonella* occurred earlier compared to untreated controls, whereby at the end of storage time, the level of this pathogen was < 1 log CFU $g^{-1}$ in each sausage. Adding enterocins disabled *L. monocytogenes* growth, whilst in control sausages, the level of this pathogen increased by 5 log CFU $g^{-1}$ during the production process. Notably, in sausages with no enterocins added and stored at refrigeration temperatures, high pressure led to a long term inhibitory effect; in other words, *L. monocytogenes* reduction of 5 log CFU $g^{-1}$ occurred no sooner than in the last week of storage. The level of *S. aureus* during sausage production increased by about 3 log CFU $g^{-1}$ and remained at that level during the entire storage time.

In a similar study, *Rubio et al.* (2013) found that 600 MPa (5 min, 15°C) applied at the end of fuet's production process (21 days) reduced the number of *Enterobacteriaceae* by at least 0.3 logs (i.e. from 1.3 log CFU $g^{-1}$ to < 1 log CFU $g^{-1}$). Furthermore, by applying HPP, the levels of *L. monocytogenes* and *S. aureus* (each pathogen inoculated at a concentration of 3.5 log CFU $g^{-1}$) were reduced by about 1 log CFU $g^{-1}$. The HPP effect manifested immediately after the treatment or after 7 days of storage at 14°C. On the other hand, the number of useful bacteria (lactic acid bacteria, coagulase negative cocci) did not change significantly after exposure to HPP.

*Rubio et al*. (2007) investigated HPP effects (500 MPa, 5 min, 18°C) on microbiological, sensorial and physicochemical properties of Spanish dry fermented sausages, salchichon, which were produced from the meat and back fat of pigs fed on control diet or with food enriched in oleic or linoleic fatty acids. After the HPP application, sliced sausages were stored for 210 days at 6°C and during that time were assessed on several occasions for instrumental colour measurement, sensorial properties, as well as numbers of the main groups of microbiota. The sensory properties of HPP-treated versus control sausages were similar, as was colour (lightness, redness, yellowness). Immediately after HPP application, the level of aerobic psychrotrophs and intestinal anaerobes was reduced by up to 2 log CFU $g^{-1}$, and the level of lactic acid bacteria was reduced by about 1 log CFU $g^{-1}$. However, during storage, a significant reduction of these microbial groups was observed in control sausages and thus, after 210 days, the difference between HPP treated and untreated sausages was lower than at the beginning of storage. This study also found HPP reduced *Pseudomonas* spp. (by 3.5 log CFU $g^{-1}$) and moulds and yeasts (by >1.5 log CFU $g^{-1}$).

The possibility of processing fermented dry sausages with high pressure applied at the beginning of the production process was investigated in the study conducted by *Marcos et al.* (2005). Sausage batter

was inoculated with various strains of *Salmonella* and *L. monocytogenes*, while the concentrations of both pathogens were roughly $6x10^2$ per gram of stuffing. One day after production (stuffing into casings), sausages were exposed to 300 MPa (17°C, 10 min) and then subjected to the ripening process. As a result of the cumulative effects of high pressure and the ripening process, the number of *Salmonella* was about 1.5 logs lower in HPP-treated sausages than in control (non-HPP treated) sausages, in which the reduction, as a consequence of ripening only, was 0.5 logs. In contrast, *L. monocytogenes* numbers decreased by ~1 log after HPP, but after ripening, numbers increased to ~ 2 logs, while in control sausages at the end, *L. monocytogenes* was at a very low level or had completely disappeared. According to the authors, the reason for this phenomenon was the HPP-induced, temporary decrease in numbers of lactic acid bacteria; this slowed the pH drop and reduced the production of antilisterial factors and consequently enabled recovery of sub-lethally damaged *L. monocytogenes*. Furthermore, in HPP treated sausages, a "whitening" effect of pressure (increase in brightness (*L* *) was observed, and the authors explained this as a consequence of globin denaturation and/or haeme displacement or release.

*Latorre-Moratalla et al*. (2007) also treated sausages before fermentation, but they applied lower pressure (200 MPa, 17°C, 10 min). As a result, *Enterobacteriaceae* growth during fermentation and drying was absent or it was present to a lesser extent compared with control sausages; HPP also led to reduction of some biogenic amines (like putrescine and cadaverine) in treated sausages. This HPP application did not reduce technologically useful microorganisms (*Lactobacillus* spp., coagulase negative cocci); moreover, their numbers were greater (by up to 1 log CFU $g^{-1}$) in finished, HPP-treated sausages. The authors stated that the pH, $a_w$, proteolysis and product colour were not different from the usual values. This study also found that the pressure of 200 MPa did not reduce tyramine levels, although this flaw can be superseded by adding decarboxylase negative starter cultures to the sausage batter.

*Omer et al*. (2015) stated that if trimmings (frozen or chilled) intended for production of morr sausages or salami are exposed to 600 MPa (6 min, 12°C), a better hygienic status of final sausages is achieved. However, the final products, in that case, were of diminished quality regarding colour, smell, taste and texture as well as of lower total sensorial acceptability. Nevertheless, after six weeks of storage, the difference in sensorial properties was less expressed, especially if frozen trimmings were treated. Also, sausages produced from HPP raw meat had lower $a_w$ and weight compared to those made from untreated meat.

The effect of HPP on reduction of biogenic amines in fermented dry sausages was explored by *Ruiz-Capillas et al*. (2007). Sliced chorizo sausage (3 mm thick slices) was exposed to 350 MPa (15min, 20°C) and then stored for 160 days at 2°C. HPP application induced slightly reduced numbers (0.5 to 1 log CFU $g^{-1}$) of aerobic mesophiles and lactic acid bacteria during storage. Additionally, HPP resulted in significantly less tyramine, putrescine and cadaverine, which was explained to be a consequence of lactic acid bacteria reduction.

*Bolumar et al*. (2015) investigated the possibility of producing fermented sausages that would contain lean meat treated by high pressure (600 MPa, 5 min, 20°C) instead of pig back fat. The sensory panel found that the sausages with HPP-treated lean meat added instead of 35% of fatty tissue had better taste, while the smell, texture and colour (instrumentally measured) were not statistically different compared to the control sausage. Furthermore, there was no difference in the extent of lipid oxidation (TBAR value, peroxide value), or in the numbers of aerobic mesophiles or *Enterobacteriaceae.*

The study conducted by *Alfaia et al*. (2015) examined HPP application on Portuguese fermented sausage (chouriço) with different combinations of pressure (202 to 600 MPa) and time (154 to 1800 seconds). It was found that 400 MPa for 154 to 960 seconds reduced spoilage microbiota (*Enterobacteriaceae* and *Pseudomonas* spp.) and fungi to below the limit of detection (<1 log CFU $g^{-1}$), while technologically useful microbiota (lactic acid bacteria, coagulase negative cocci, *Enterococcus*) were reduced by 1 to 1.5 log CFU $g^{-1}$. This treatment also enhanced colour, cohesion and firmness of sausages. Neither of the pressure/time combinations of treatments led to increased lipid oxidation (TBAR values).

## Conclusion

HPP is a non-thermal technology that improves the safety of meat and meat products and extents their shelf life. Pressures of at least 400 MPa successfully inactivate microorganisms. For fermented dry sausages, HPP is applied, primarily, as an additional decontamination step on packaged products. HPP has no detrimental effects on sensorial qualities of dry fermented sausages. Moreover, improved texture through increased cohesiveness, firmness and chewiness is observed, while negative effects on colour are rare and/or less expressed and are mainly

related to applying HPP treatment at the beginning of the process. Due to the development of highly efficient HPP machines, processing costs have been reduced to acceptable levels, and this has led to wider application of this technology in the food industry. As an environmentally friendly and waste-free technology, HPP has a high acceptance among consumers. HPP encourages development of innovative fermented meat products and it is expected to be more widely applied in the future.

**Conflict of interest.** The authors declare that they have no conflicts of interest.

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# Effect of commercial starter cultures on survival of *Yersinia enterocolitica* and microbiological status of Sremska sausages

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*A b s t r a c t: The aim of this study was to determine the survival of Yersinia enterocolitica (biotype 1, serotype O:8), and the microbiological status (lactic acid bacteria and Enterobacteriaceae), water activity and pH values of Sremska sausage (traditional dry-fermented sausage from Northern Serbia) during ripening (18 days). Four different groups of Sremska sausage were manufactured: CI group – control without starter culture; CII group – control with starter culture; EI group – was inoculated with $10^8$ CFU $mL^{-1}$ of Y. enterocolitica ATCC 9610, without starter culture and EII group – was inoculated with $10^8$ CFU $mL^{-1}$ of Y. enterocolitica ATCC 9610 and with starter culture. During ripening, microbiological examination was conducted according to ISO methods, on days 0, 3, 7, 12 and 18. In the inoculated sausages, Y. enterocolitica did not grow after day 12 of the ripening period. The results revealed that the use of starter cultures increased the number of lactic acid bacteria, while completely reducing the Enterobacteriaceae count compared with the Sremska sausage without starter culture. Also, the sausages manufactured with starter culture had lower pH values compared to the sausages without starter culture. In conclusion, the use of starter cultures contributes to improving the microbial safety of Sremska sausage.*

**Key words:** *Yersinia enterocolitica, Sremska sausage, food safety, microbiological status.*

## Introduction

*Yersinia enterocolitica* is a bacterium which belongs to the family *Enterobacteriaceae*, widely found in natural environments (*EFSA Panel on Biological Hazards*, 2014; *Mitrovic*, 2016). This psychrotrophic bacterium has the capability to survive and multiply at low temperatures (*Baltic et al*., 2013; *Ivanovic*, 2014; *Baltic et al*., 2016; *Ivanović et al*., 2016a). Also, *Y. enterocolitica* is zoonotic, causing yersiniosis, a frequently reported bacterial zoonosis in the European Union *(EFSA and ECDC*, 2013). Among the sources, pork is reported as a major reservoir for *Y. enterocolitica*. The bacterium is often present in the oral cavity of pigs especially tonsils, intestinal content, faeces and lymph nodes (*EFSA Panel on Biological Hazards,* 2014).

Sremska sausage is popular dry-fermented sausage in Serbia (and has a protected designation of origin). It is characterized by a specific hot taste, aromatic and spicy flavour, dark red colour and hard consistency (*Stanisic et al*., 2014). Sremska sausage is one of the local fermented cured meat products that, until the mid-1950s, were produced exclusively on farms. According to the available data, production of Sremska sausage began in the middle of the 18th century. There are assumptions that the forerunner of today's Sremska sausage was the spicy and smoked Lucanica that Roman soldiers carried in their backpacks for encouragement before, and invigorating refreshment after battles (*Stevanovic et al*., 2016). It is well known that the typical characteristics of fermented sausages are generated by chemical, biochemical, physical and microbiological changes occurring during fermentation, ageing and drying. Also, fermentation of raw materials improves the safety, shelf life and acceptability of food and it has a long tradition *(Ivanovic et al*., 2015a; *Domínguez et al*., 2016). There are several lactic acid bacteria, mainly *Lactobacillus sakei* in Europe and *Pediococcus acidilactici* in the USA (*Leroy et al*., 2006), and some staphylococcal

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species, almost exclusively *Staphylococcus xylosus* and *Staphylococcus carnosus*, developed as commercial starters for the manufacture of dry sausages (*Corbiere Morot-Bizot et al*., 2007). The addition of starter cultures has become common in the manufacture of several types of fermented meat products in order to ensure safety, thus reducing the risk of pathogenic and spoilage bacteria, as well as to contribute to colour and flavour development and extend shelf-life (*Lorenzo et al*, 2012; *Essid and Hassouna*, 2013; *Ciuciu Simion et al*., 2014; *Mitrovic*, 2016).

The practice of utilising a short maturation period and storage of fermented sausage at refrigeration temperatures may result in unsatisfactory reductions of pathogens (*Escherichia coli, Listeria monocytogenes and Yersinia enterocolitica*) if present. Thus, inclusion of a maturation period above refrigeration temperatures and using starter culture may increase the safety of fermented sausages (*Lindqvist and Lindblad*, 2009).

In Serbia, there is limited study on *Y. enterocolitica* and the bacterium is not routinely isolated. Therefore, the aim of this study was to evaluate effect of starter cultures on survival of *Y. enterocolitica* and microbiological status of Sremska sausages.

## Materials and methods

### *Sausage production and sampling procedures*

Four different batches of Sremska sausage were manufactured according to traditional techniques, two of them without starter culture (CI and EI) and the other two batches (CII and EII) with addition of commercial starter culture, Biostart Sprint (RAPS GmbH, Obertrum, Austria) at the level defined by the manufacturer in each case (20 g per 200 kg of meat). This product contains *Lactobacillus sakei, Staphylococcus carnosus* and *Staphylococcus xylosus*, and starter cultures were added and the batter was mixed with gloved hands for 5 min. The EI and EII sausages were inoculated with *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610 (http://www.atcc.org), biotype 1, serotype O:8. The preparation of inoculum was according to *Ivanovic et al.* (2015b).

Sausage formulation comprised minced pork meat (35%), minced beef meat (23%), minced fat (20%), nitrite salt (2.3%, resulting in 175 ppm sodium nitrite), glucose (1%) and white pepper (0.2%). The mix was maintained at 4°C for 24 h and then stuffed into natural casings with a diameter of 34 mm. The sausages were fermented for 2 days at 20°C and 80–85% relative humidity and then transferred into a drying-ripening chamber where they were kept for 18 more days at 17°C and 75–80% relative humidity. Sampling was performed by randomly selecting two links of each sausage on days 0, 3, 7, 12 and 18 of ripening.

### *Chemical composition, pH and $a_w$ values*

ISO recommended standards were employed to determine moisture (*ISO*, 1997), fat (*ISO,* 1992), protein (*ISO*, 1992b) and ash (*ISO*, 1999). The pH values were measured with a pH meter, TESTO 205 (Lenzkirch, Germany). The water activity ($a_w$) was measured using an aqualab water activity meter series 3 TE (Decagon Devices Inc., USA) on approximately 10 g of sausage according to the manufacturer's instructions.

### *Microbiological analyses*

For microbiological analysis, 10 g of Sremska sausage was aseptically weighted into a sterile plastic bag, previously removing and discarding the outer plastic. Subsequently, samples were homogenized with 90 mL of a sterile solution of 0.1% (w/v) peptone water (Oxoid, Unipath, Basingtoke, UK), containing 0.85% NaCl and 1% Tween 80 as emulsifier, for 2 min at 20–25°C in a stomacher blender (Stomacher 400 Circulator, Seward, UK), thus making a 1/10 dilution. Serial 10-fold dilutions were prepared by mixing 1 mL of the previous dilution with 9 mL of 0.1% sterile peptone water. For enumeration of lactic acid bacteria, 1 mL of the appropriate 10-fold serial dilution was inoculated into Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK). The MRS plates were incubated at 30°C for 72 h, and all colonies were counted to enumerate lactic acid bacteria (*ISO*, 1998). Selective medium was used for enumeration of *Y. enterocolitica* (CIN – Cefsulodin-Irgasan-Novobiocin: *Yersinia* selective agar base CM0653 and *Yersinia* selective supplement SR0109, Oxoid) and was incubated at 30°C for 24 h (*ISO*, 2003). For an enumeration of *Enterobacteriaceae*, 1 mL of the appropriate 10-fold serial dilution was inoculated into violet red bile glucose agar (VRBG, Merck, Germany) (*ISO*, 2009). The VRBG plates were incubated at 30°C for 24 h. All purple colonies due to rapid fermentation of glucose surrounded by purple haloes of precipitated bile salts were counted (*Fredriksson-Ahomaa et al.*, 2001). Lactic acid bacteria, *Y. enterocolitica* and *Enterobacteriaceae* counts were determined on days 0, 3, 7, 12 and 18 of ripening. After incubation, plates with 30–300 colonies were counted. The microbiological data were transformed into logarithms of the number of colony forming units (log CFU $g^{-1}$).

*Statistical Analysis*

A total of 80 sausages (ten sausages for each batch, four batches, two replicates) were analysed for the different parameters. Statistical analysis of the results was elaborated using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA; http://www.graphpad.com) and Microsoft Office Excel 2013 (Microsoft Corporation, Los Angeles, CA). The parameters were described by means and standard error of means (SEM) (*Y. enterocolitica* counts were described by means and standard deviation). One-way ANOVA with Tukey's *post hoc* test was performed to assess the significance of differences among control and experimental groups. Values of $P<0.05$ and $P<0.01$ were considered significant.

## Results and discussion

*Chemical composition and pH values*

Values corresponding to moisture, protein, fat and ash at the beginning of the study are summarised in Figure 1.

The effect of starter cultures on chemical composition (moisture, fat, protein and ash) and pH values during the ripening period is presented in Table 1. At the beginning of the study, moisture, fat, protein and ash content was 55.36%, 25.71%, 15.45% and 3.54%, respectively (data not shown). The moisture content decreased during storage. There were no significant differences in protein values between the different sausage types. Our results are in agreement with those obtained in another study, which also did not find significant differences in protein values between different sausages (*Lorenzo et al.*, 2014). However, in the current study, moisture, fat and protein content showed significant ($P<0.01$) differences between treatments. *Dalmis and Soyer* (2008) also noticed that inoculated sausages had significantly lower moisture content compared to control sausages, without starter culture. In contrast, *Essid and Hassouna* (2013) found that the addition of *S. xylosus* and *Lactobacillus plantarum* starter culture did not affect the loss of sausage moisture. *Santa et al.* (2014) also found significant differences between moisture content at the end of ripening of Italian sausages. In that case, sausages inoculated with starter cultures had lower moisture and higher fat and ash values than control and experimental sausages without starter culture. *Domínguez et al.* (2016) found significant differences between protein content at the end of ripening of dry-cured foal sausage, unlike our findings.

The pH decreased sharply from initial mean value of 6.14 (data not shown) to reach, after 18 days of ripening, pH 5.32, 5.22, 5.30 and 5.27 for CI, CII, EI and EII sausages, respectively. So, the inoculation of the starter cultures resulted in greater acidification during the 18 days of production. The pH of CI sausages was significantly ($P<0.001$) higher than those inoculated with starter cultures (Table 1). Other authors also reported similar results in dry-fermented sausages (*Lorenzo et al.*, 2014). These authors observed that all sausages inoculated with starter culture reached a lower pH than control sausages. The pH fall could be related to an accumulation of

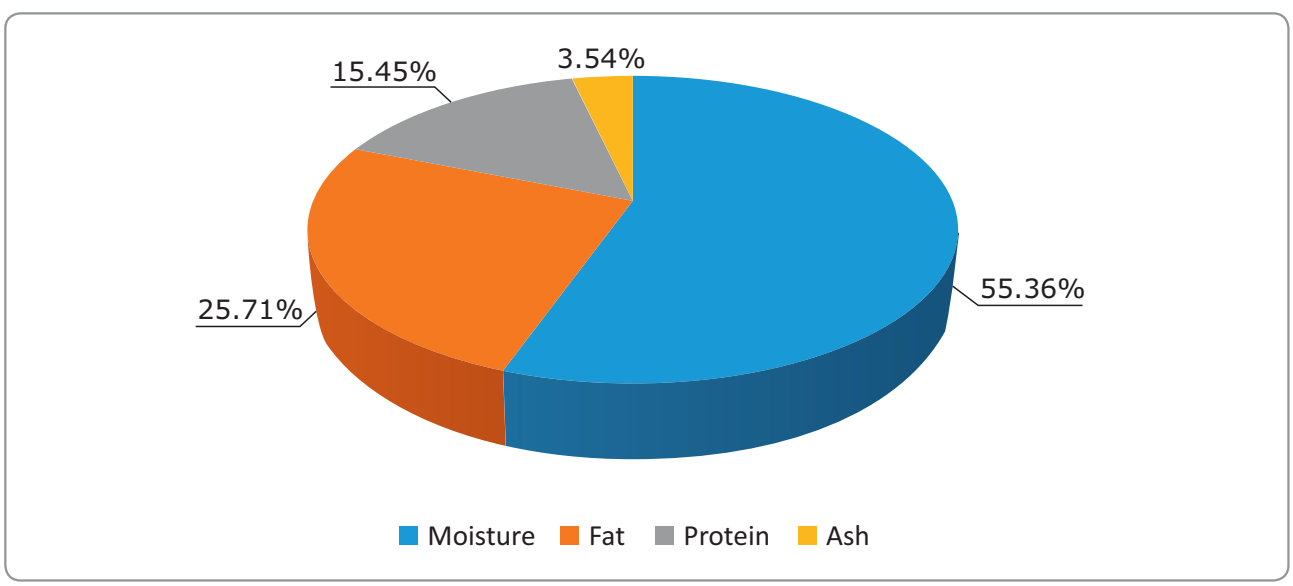


**Figure 1.** Chemical composition of the Sremska sausages at the beginning of the study

**Table 1.** Effect of commercial starter cultures on chemical composition (g 100 $g^{-1}$), pH and $a_w$ values (at the end of ripening period)

| Parameter | Sausage group | | | | SEM | *P* value |
|---|---|---|---|---|---|---|
| | CI | CII | EI | EII | | |
| | 18. day | | | | | |
| Moisture | $29.07^{A}$ | $28.19^{B}$ | $29.34^{A}$ | $28.48^{B}$ | 0.09 | < 0.0001 |
| Protein | 25.49 | 25.67 | 25.42 | 25.63 | 0.05 | 0.18 (ns) |
| Fat | $40.26^{a}$ | $40.87^{a}$ | $40.03^{b}$ | $40.63^{b}$ | 0.12 | 0.03 |
| Ash | $5.17^{A}$ | $5.28^{B}$ | $5.22^{A}$ | $5.27^{C}$ | 0.01 | 0.001 |
| pH values | $5.32^{A}$ | $5.22^{B}$ | $5.30^{C}$ | $5.27^{BC}$ | 0.009 | 0.0004 |
| $a_w$ values | 0.911 | 0.910 | 0.921 | 0.914 | 0.02 | ns |

**Legend:** Within a row, means with a different letter are significantly different: $^{A–C}P<0.01$; $^{a–b}P<0.05$; ns – not significant.
Groups: CI – control without starter culture; CII – control with starter culture; EI –was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610 and with starter culture.

organic acids, mainly lactic, present in this type of sausage as a result of carbohydrate breakdown during fermentation (*Zhao et al*., 2011; *Lorenzo et al*., 2014). In contrast, *Ciuciu Simion et al.* (2014) did not observe differences in pH values between inoculated and non-inoculated Romanian Dacia sausage. However, it must be taken into account that the influence of starter cultures on pH values depends on the microorganisms present in the starter culture, and it is difficult compare the results between studies that used different starter cultures. In our study, $a_w$ decreased throughout ripening in the four types of sausage studied. However, statistical analysis did not show significant differences ($P>0.05$) between the sausages inoculated with starter culture and the control sausages at the end of the study. Our result is in disagreement with those reported by *Kaban and Kaya* (2009), who observed that sausages with starter culture displayed lower $a_w$ values in comparison to those of the control.

*Microbial counts*

Results concerning *Y. enterocolitica* levels detected during ripening of Sremska sausages produced with or without starter culture are reported in Table 2. Statistical analysis showed significant differences ($P<0.01$) between EI and EII sausages on *Y. enterocolitica* counts (on days 3 and 7 of ripening). The differences in *Y. enterocolitica* counts at the end of the process could be related to the addition of starter cultures. This outcome is in agreement with those reported by other authors (*Ivanovic et al*., 2015b; *Mitrovic*, 2016), who found the use of selective starter to produce sausages significantly affects *Y. enterocolitica* levels. Initial counts of *Y. enterocolitica* were 6.17 log CFU $g^{-1}$ in our EI and EII sausages. On day 7 of ripening, counts of *Y. enterocolitica* decreased to 4.30 and 4.99 log CFU $g^{-1}$ for EI and EII sausages, respectively. At the end of the ripening period, *Y. enterocolitica* counts were not detected. The decrease in *Y. enterocolitica* counts during the 18 days of ripening for the inoculated sausages suggests the poor competitiveness of *Y. enterocolitica* due to the intensive growth of lactic acid bacteria and the associated decrease of pH, as reported by other works (*Lindqvist and Lindblad*, 2009; *Ivanovic et al.*, 2015a; *Ivanovic et al.*, 2015b). Most lactic acid bacteria are more tolerant to the antagonistic effects of lactic acid than *Y. enterocolitica*, and they produce large amounts of lactic acid. The lactic acid produced can exercise a negative influence on *Y. enterocolitica* (*Ivanović et al*., 2015b).

The effect of starter cultures on the *Enterobacteriaceae* counts of Sremska sausage is shown in Table 3. Initial counts of *Enterobacteriaceae* were 4.64 log CFU $g^{-1}$ in the control sausages and 5.29 log CFU $g^{-1}$ in the experimental sausages. The number of these bacteria depends mainly on the hygienic quality of the raw materials and the handling conditions during processing (*Ivanovic et al*., 2013). During ripening, this group of bacteria displayed a strong decrease in Sremska sausages inoculated with starter culture (CII sausages from 4.64 log CFU $g^{-1}$ to 2.47 log CFU $g^{-1}$ and EII sausages from 5.29 log CFU $g^{-1}$ to 3.03 log CFU $g^{-1}$). The inhibitory effect exerted by the starter cultures on the pathogenic microbiota was, therefore, evident, especially from day 7 of the ripening period. Our findings are similar to those reported by *Essid and Hassouna*

**Table 2.** *Y. enterocolitica* counts (log CFU $g^{-1}$) during the ripening period of naturally fermented Sremska sausages inoculated with starter culture (groups EI and EII) ($\overline{X}$±SD)

| Day of ripening | EI Sausages | EII Sausages | *P* value |
|---|---|---|---|
| 0 | 6.17±0.34 | 6.17±0.34 | 0.990 (ns) |
| 3 | $4.84^{A}$±0.09 | $4.49^{B}$±0.04 | < 0.0001 |
| 7 | $4.30^{A}$±0.04 | $4.99^{B}$±0.30 | < 0.0001 |
| 12 | nd | nd | |
| 18 | nd | nd | |

**Legend:** Within a row, means with a different letter are significantly different: $^{A–B}P<0.01$; ns – not significant.
Sausage groups: EI – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610 and with starter culture.
nd: Absent from 10 g sample.

(2013) and *Ciuciu Simion et al.* (2014), who observed lower final counts of *Enterobacteriaceae* in control sausages than in sausages inoculated with lactic acid bacteria strains. From the results of the present work, it seems that the inclusion of the starter culture substantially contributes to the decrease of the *Enterobacteriaceae* and *Y. enterocolitica* counts throughout the ripening. Also, a low number of *Enterobacteriaceae* is a very relevant indicator of food safety (*Rubio et al*., 2013; *Ivanovic et al*., 2013, *Janjic et al*., 2015; *Ivanovic et al*., 2016b).

Initial lactic acid bacteria counts in starter culture inoculated sausages (CII and EII, 5.21 log CFU $g^{-1}$ and 6.23 log CFU $g^{-1}$, respectively) were higher than those in the sausages without starter culture (CI and EI, 5.09 log CFU $g^{-1}$ and 6.20 log CFU $g^{-1}$, respectively) (Table 4). The maximum number of lactic acid bacteria was observed on day 12 of ripening period and then a slight decrease was observed and counts reached 9.03 log CFU $g^{-1}$, 9.12 log CFU $g^{-1}$, 9.06 log CFU $g^{-1}$, 9.01 log CFU $g^{-1}$, 9.20 log CFU $g^{-1}$ for CI, CII, EI and EII sausages, respectively, at the end of the ripening period. This slight decrease of lactic acid bacteria during ripening is probably due to the decrease of fermentable carbohydrates (*Lorenzo and Franco*, 2012). Statistical analysis showed significant differences ($P<0.01$) between all groups of Sremska sausages. Nevertheless, *Lorenzo et al*. (2014) did not find significant differences between control and commercial starter cultures (with *Lactobacillus sakei*) groups, while *Rubio et al*. (2013) showed higher lactic acid bacteria counts ($P<0.05$) in control sausages compared to sausages inoculated with starter culture (*L. plantarum* and *Lactobacillus rhamnosus*) .

Due to the good adaptation of lactic acid bacteria to the meat and their faster growth rates during Sremska sausage fermentation, they became the dominant microbe, as expected (*Essid and Hassouna*, 2013; *Zhao et al*., 2011). Lactic acid bacteria belong to the desirable microbiota of fermented sausages (*Ivanovic et al*., 2013, *Mitrovic*, 2016).

**Table 3.** *Enterobacteriaceae* counts (log CFU $g^{-1}$) during the ripening period of naturally fermented Sremska sausages ($\overline{X}$±SEM)

| Day of ripening | Sausage Group | | | | SEM | *P* value |
|---|---|---|---|---|---|---|
| | CI | CII | EI | EII | | |
| 0 | $4.64^{A}$ | $4.64^{A}$ | $5.29^{B}$ | $5.29^{B}$ | 0.050 | < 0.0001 |
| 3 | $4.39^{A}$ | $3.59^{B}$ | $5.00^{C}$ | $4.49^{A}$ | 0.070 | < 0.0001 |
| 7 | $3.01^{A}$ | $2.47^{B}$ | $3.93^{C}$ | $3.03^{A}$ | 0.040 | < 0.0001 |
| 12 | nd | nd | nd | nd | – | – |
| 18 | nd | nd | nd | nd | – | – |

**Legend:** Within a row, means with a differ letter are significantly different: $^{A–C}P<0.01$
Groups: CI – control without starter culture; CII – control with starter culture; EI – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610 and with starter culture.
nd: Absent from 10 g sample

**Table 4.** Lactic acid bacteria counts (log CFU $g^{-1}$) during the ripening period of naturally fermented Sremska sausages ($\overline{X}$±SEM)

| Day of ripening | Sausage Group | | | | SEM | *P* value |
|---|---|---|---|---|---|---|
| | CI | CII | EI | EII | | |
| 0 | $5.09^{A}$ | $5.21^{B}$ | $6.20^{A}$ | $6.23^{C}$ | 0.020 | < 0.0001 |
| 3 | $7.53^{A}$ | $8.40^{B}$ | $8.57^{B}$ | $6.65^{C}$ | 0.050 | < 0.0001 |
| 7 | $8.71^{A}$ | $9.56^{B}$ | $8.06^{C}$ | $8.70^{A}$ | 0.040 | < 0.0001 |
| 12 | $9.25^{A}$ | $9.06^{B}$ | $8.92^{C}$ | $9.30^{B}$ | 0.009 | < 0.0001 |
| 18 | 9.03 | 9.12 | 9.06 | 9.20 | 0.007 | 0.23 (ns) |

Within a row, means with a different letter are significantly different: $^{A–C}P<0.01$; ns – not significant.
Groups: CI – control without starter culture; CII – control with starter culture; EI – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610, without starter culture; EII – was inoculated with $10^8$ CFU $mL^{-1}$ of *Y. enterocolitica* ATCC 9610 and with starter culture.
nd: Absent from 10 g sample

Also, lactic acid bacteria have a positive effect on the hygienic properties of the product, inhibiting pathogenic and spoilage microbiota by acidification or by the production of antimicrobials (*Villani et al*., 2007; *Ivanovic et al*., 2015; *Lindqvist and Lindblad*, 2009).

## Conclusion

In general, the chemical composition parameters of the sausages evaluated in this study were affected by the use of starter cultures. Also, sausage pH seemed to be influenced by starter cultures, since starter culture-inoculated Sremska sausage exhibited a stronger acidification than the control sausage groups during the 18 day ripening period. This acidification, together with the growth of desirable competitive microbiota, might explain the sharp decrease of *Enterobacteriaceae* and *Y. enterocolitica* counts observed in the sausages inoculated with starter culture. The inhibitory effect exerted by the starter cultures on *Y. enterocolitica* is, therefore, evident, especially from day 7 of the ripening period. Therefore, the use of starter culture can improve the microbial food safety of traditional Sremska sausages.

**Conflict of interest.** The authors declare that they have no conflicts of interest.

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# Possibility of partial replacement of sodium chloride with potassium chloride and ammonium chloride in production of meatballs in tomato sauce

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*A b s t r a c t: The aim of this study was to examine the influence of reducing the sodium chloride content in meatballs in tomato sauce. The trial consisted of five groups. In the control group of meatballs and sauce, only sodium chloride was added. In group 1, half of the sodium chloride was replaced with potassium chloride, while in group 2, one third of the sodium chloride was replaced with potassium chloride. In group 3, one third of the sodium chloride was replaced with ammonium chloride, and in group 4, sodium chloride was reduced to half that of the control, and 1 g (0.25%) of ammonium chloride was also added. Partial replacement of sodium chloride with potassium chloride or ammonium chloride affected neither colour acceptability nor consistency of either meatballs or tomato sauce. The intensity of saltiness meatballs from group 4 was significantly lower than in the control and group 3 meatballs (P≤0.05). There was no statistical difference between saltiness acceptability of group 2 meatballs and that of groups 1 and 4 meatballs. All meatballs were acceptable and did not have so bitter a taste as to be sensorily rejected by assessors (P≤0.05). The bitterest sauces were the control and group 3 sauces, and they were significantly different from other groups; from groups 2 and 4 at the P≤0.01 level and from group 1 at the P≤0.05 level. The most acceptable saltiness and taste acceptability was achieved by group 4 meatballs, produced with 0.75% sodium chloride and 0.25% ammonium chloride, while the saltiness acceptability of tomato sauce was not influenced by partial replacement of sodium chloride with other chloride salts.*

***Key words:*** *meatballs, tomato sauce, sodium chloride reduction, potassium chloride, ammonium chloride*

## Introduction

Dietary sodium intake in many cases exceeds recommendations of the World Health Organization (WHO), which has several negative health influences linked mostly to the appearance of essential hypertension and consequential cardiovascular disorders. There are many directives issued from WHO concerning how to reduce sodium intake *via* food and also investigations into sodium reduction in food production where sodium chloride is a main additive. Mostly, the meat industry is the target of these investigations. There are several studies on sodium reduction in cooked sausages, dry fermented sausages and dry meat. However, the meat industry is an important producer of ready-to-eat meals prepared or cooked in advance, with no further cooking or preparation required before being eaten. These ready-to-eat meals have become an important choice for modern consumers, with respect to the fast lifestyle of modern societies.

There are not much literature data on reducing the sodium content in ready-to-eat meals. Many investigations are focused on reducing sodium content in meat products, particularly in those which are not thermally treated (dry fermented sausages, dry ham). Since it is the only salt with a clearly salty taste, in food, sodium chloride cannot be totally replaced with other salts. However, it can be partially replaced, and to this purpose, potassium chloride and less often, other chloride salts are used (*Guàrdia et al.,* 2006*)*. Besides potassium chloride, magnesium and calcium salts and ascorbates are most comonly used as replacers *(Ruusunen and Puolanne,* 2005*)*.

The need to reduce sodium in meat products and generally in food will be an aim of the food industry in the future; fast food chains will also have to address this issue, even if people think the amount

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of salt consumed *via* fast food is not so large (*Moran et al., 2017*). Nonetheless, salt replacers present a difficult problem because of their degradation of desirable sensory characteristics, including texture and, of course, salty taste (*Kamenik et al.,* 2017*; Kang et al.,* 2014).

The aim of this study was to examine the influence of reducing the sodium chloride content in meatballs in tomato sauce, as well as the influence of partial replacement of sodium chloride with potassium chloride or ammonium chloride, with the target of achieving a normal, salty taste with less sodium.

## Materials and Methods

The trial consisted of five groups (Tables 1 and 2). In the control group of meatballs and sauce, only sodium chloride was added. In group 1, half of the sodium chloride was replaced with potassium chloride, while in group 2, one third of the sodium chloride was replaced with potassium chloride. In group 3, one third of the sodium chloride was replaced with ammonium chloride and in group 4, sodium chloride was reduced by one half compared with the control, while 1 g (0.25%) of ammonium chloride was also added.

### *Meatball preparation*

Meatballs were prepared from minced pork leg meat (grind plate 3 mm) purchased from a local market. Meat was well mixed with the ingredients presented in Table 1 to achieve optimal consistency to form into round shapes. Prepared meatballs were briefly fried in a thin layer of sunflower oil.

### *Tomato sauce preparation*

Sauces were prepared from tomato juice (Tomatino classic, Polimark, Serbia) and the ingredients presented in Table 2. A roux was prepared from flour fried in sunflower oil for about 1 minute, and after that, water, tomato juice, salt/salt mixture and sugar were added. The sauces were simmered for 10 minutes.

**Table 1** Composition of meatballs, g

| Group | Minced pork (leg) | Sodium chloride | Potassium chloride | Ammonium chloride | Ground garlic |
|---|---|---|---|---|---|
| Control | 400 | 6.00 | – | – | 2.00 |
| 1 | 400 | 3.00 | 3.00 | – | 2.00 |
| 2 | 400 | 4.00 | 2.00 | – | 2.00 |
| 3 | 400 | 4.00 | – | 2.00 | 2.00 |
| 4 | 400 | 3.00 | – | 1.00 | 2.00 |

**Table 2** Composition of sauce, g

| Group | Tomato juice | Water | Sunflower oil | Flour | Sugar | Sodium chloride | Potassium chloride | Ammonium chloride |
|---|---|---|---|---|---|---|---|---|
| **Control** | 400 | 400 | 6.00 | 6.00 | 6.00 | 6.00 | – | – |
| **1** | 400 | 400 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | – |
| **2** | 400 | 400 | 4.00 | 4.00 | 4.00 | 4.00 | 2.00 | – |
| **3** | 400 | 400 | 4.00 | 4.00 | 4.00 | 4.00 | – | 2.00 |
| **4** | 400 | 400 | 3.00 | 3.00 | 3.00 | 3.00 | – | 1.00 |

*Meal preparation*

Meatballs were cooked in prepared tomato sauce for 45 minutes. Half an hour after cooking, the product was presented to sensory assessors for evaluation.

*Sensory evaluation*

Sensory evaluation was performed by seven assessors (trained by proficiency testing) using numeric scales. Each sensory characteristic was evaluated for both ingredients of complete meal, meatballs and sauce. Colour acceptability, consistency, saltiness acceptability, taste acceptability and overall impression were evaluated with a 1−5 point scale, where 1 was the least acceptable and 5 was the most acceptable. Intensity of saltiness and bitterness of the meatballs and sauces were evaluated with a 1−5 point scale, where 1 was the least and 5 was the most expressed attribute, respectively. Meals (meatballs and sauce) were evaluated using a rank test.

*Statistical evaluation*

The results obtained were statistically evaluated using Microsoft Excel 2010 and are presented as mean±SD. Statistical differences between means of the examined parameters were determined on the level 0.05 and 0.01 by Student´s t-test.

## Results and discussion

The results of sensory evaluation of colour and consistency of meatballs and tomato sauce are presented in Table 3.

Colour and consistency of meatballs from all groups were acceptable, and all assessors evaluated them similarly. Mean values were not statistically different ($P \geq 0.05$). Also, assessors were very consistent in the case of evaluating tomato sauce for these two characteristics. Both colour and consistency of all tomato sauce groups were acceptable and there were no significant differences between means ($P \geq 0.05$).

The intensity of saltiness and bitterness of the meatballs and tomato sauces are shown in Table 4.

Usually, 1.8−2.0% sodium chloride is used in minced meat products. This trial was purposely designed for a maximum salt level of 1.5%, in accordance with results of *Lilic et al*. (2005). Group 4 meatballs had the smallest amount of salt, and this group was evaluated as having the lowest saltiness intensity (Table 4). It was significantly lower than the salt intensities of control and group 3 meatballs ($P \leq 0.05$).

There was no statistical difference between saltiness acceptability of group 2 meatballs and that of groups 1 and 4 meatballs. It was expected that sauce with the largest amount of sodium chloride would be the saltiest, but in fact, group 3 tomato sauce was the saltiest ($P \leq 0.05$). Since one third of the sodium chloride was replaced with ammonium chloride in this group, it is clear that ammonium chloride increased saltiness, particularly in this liquid medium with high water content. Group 3 tomato sauce was significantly more salty compared with the other groups.

Although the evaluations for intensity of bitterness were different, all meatballs were acceptable and did not have so bitter a taste as to be sensorily rejected by the assessors. There was no significant difference in meatball bitterness ($P \geq 0.05$), even where half of the sodium chloride was replaced with potassium chloride (group 1 compared with control). Despite that, the bitterness intensity of tomato

**Table 3 Sensory evaluation of colour acceptability and consistency, mean±SD, n = 7**

| Group | Meatballs | | Tomato sauce | |
|---|---|---|---|---|
| | Colour acceptability | Consistency | Colour acceptability | Consistency |
| **Control** | 4.86±0.35 | 4.57±0.49 | 4.57±0.49 | 4.71±0.45 |
| **1** | 4.71±0.70 | 4.00±0.93 | 4.57±0.73 | 4.57±0.73 |
| **2** | 4.86±0.35 | 4.29±0.88 | 4.29±1.03 | 4.57±0.49 |
| **3** | 4.86±0.35 | 4.71±0.70 | 4.57±0.49 | 4.57±0.73 |
| **4** | 4.86±0.35 | 4.71±0.45 | 4.71±0.45 | 4.71±0.45 |

**Table 4** Sensory evaluation of intensity of saltiness and bitterness, mean±SD, n = 7

| Group | Meatballs | | Tomato sauce | |
|---|---|---|---|---|
| | Intensity of saltiness | Intensity of bitterness | Intensity of saltiness | Intensity of bitterness |
| Control | 3.86±0.64 $^{a}$ | 2.29±1.03 | 3.71±0.70$^{a,q}$ | 4.29±0.45$^{b,y}$ |
| 1 | 3.14±0.99 | 3.43±1.68 | 1.71±0.88$^{y}$ | 3.00±1.07$^{a}$ |
| 2 | 2.86±1.12 | 2.29±1.28 | 3.29±0.88$^{x,q}$ | 3.29±0.45$^{x}$ |
| 3 | 4.14±1.12$^{a}$ | 3.00±1.41 | 4.71±0.70$^{b,z}$ | 4.00±1.31$^{b}$ |
| 4 | 2.71±0.88$^{b}$ | 1.86±0.99 | 2.29±1.16$^{b,q}$ | 2.57±0.90$^{a,x}$ |

**Legend:** $^{(a,b)}$ Values (mean±SD) with different superscript letters are significantly different (P≤0.05);
$^{(x,y;\ q,z)}$ Values (mean±SD) with different superscript letters are significantly different (P≤0.01)

sauces was very differently evaluated. The bitterest sauces were the control and group 3 sauces, which were significantly bitterer than sauce groups 2 and 4 at the level of P≤0.01 and than group 1 sauce at the level of P≤0.05.

Results of sensory evaluation of saltiness and taste acceptability and overall impression of the prepared meals of meatballs in tomato sauce are presented in Table 5.

Group 4 meatballs had the most pleasant taste, which was more acceptable than the taste of groups 1 and 3 meatballs (P≤0.01). However, groups 2 and 4 meatballs were not significantly different in terms of taste than control meatballs (P≥0.05). Although groups 3 and 4 meatballs contained relatively small amounts of sodium chloride, it can be assumed the larger amount of ammonium chloride added intensified the salty taste of sodium chloride, and consequently, made the taste of group 3 meatballs less acceptable compared with group 4 meatballs. The overall impression of the meatballs was significantly different between groups 1 and 4 (P≤0.01) and between the control and groups 3 and 4 (P≤0.01).

The most favourably evaluated sauce, taking into account saltiness and taste acceptability, was group 4 sauce. The taste acceptability of group 3 sauce achieved the worst score, and it was statistically lower than that of groups 2 and 4 sauces (P≤0.05). There were no significant differences between other mean scores (P≥0.05). For overall impression, however, the only differences were between groups 3 and 4 sauces (group 4 sauce achieved a better overall

**Table 5** Sensory evaluation of saltiness and taste acceptability and overall impression, mean±SD, n = 7

| Group | Meatballs | | | Tomato sauce | | |
|---|---|---|---|---|---|---|
| | Saltiness acceptability | Taste acceptability | Overall impression | Saltiness acceptability | Taste acceptability | Overall impression |
| Control | 3.14±1.36$^{a}$ | 3.71±1.48 | 3.29±1.48 $^{a}$ | 2.86±1.12 | 3.14±1.25 | 3.00±1.31 |
| 1 | 3.29±0.70 | 3.57±0.73$^{x}$ | 3.57±0.73$^{x}$ | 3.14±1.36 | 3.57±1.29 | 3.57±1.29 |
| 2 | 3.71±1.03 | 3.71±1.28 | 3.71±1.28 | 2.29±1.16 | 4.29±0.70$^{a}$ | 3.57±1.40 |
| 3 | 2.86±1.46$^{a}$ | 3.43±1.29$^{x}$ | 3.14±1.55 $^{a}$ | 3.14±1.25 | 2.57±1.68$^{b}$ | 2.29±1.39$^{a}$ |
| 4 | 4.57±0.73$^{b}$ | 4.71±0.45$^{y}$ | 4.71±0.45$^{b,y}$ | 3.57±1.18 | 4.14±0.64$^{a}$ | 3.71±1.03$^{b}$ |

**Legend:** $^{(a,b)}$ Values ( mean±SD) with different superscript letters are significantly different (P≤0.05)
$^{(x,y)}$ Values ( mean±SD) with different superscript letters are significantly different (P≤0.01)

impression; P≤0.05), while other scores were quite similar (P≥0.05).

The best-ranked was meal was group 4, and it was significantly better than the rankings achieved by group 1, control and group 3 meals. Five assessors ranked group 4 as the best meal choice, while the second-ranked meal was the group 2 meal.

## Conclusion

Partial replacement of sodium chloride with potassium chloride or ammonium chloride in different amounts affected neither colour acceptability nor consistency of meatballs or tomato sauce.

Intensity of saltiness was a direct result of the amount of sodium chloride added, but also of the ammonium chloride added, so meatballs and tomato sauces with the larger amount of added ammonium chloride were more salty than others.

Bitterness was not influenced by partial replacement of sodium chloride with potassium chloride or ammonium chloride in different amounts in the meatballs, but the tomato sauce produced only with sodium chloride and that with the larger amount of ammonium chloride were more bitter.

The saltiness and taste acceptability of group 4 meatballs were evaluated as the best; these meatballs were produced with 0.75% sodium chloride and 0.25% ammonium chloride. The saltiness of tomato sauce was not influenced by partial replacement of sodium chloride with other chloride salts, but taste acceptability was better in group 2, produced with 1% sodium chloride and 0.5% potassium chloride, and in group 4, produced with 0.75% sodium chloride and 0.25% ammonium chloride.

The overall impression was very similar for all groups of meatballs and tomato sauce. However, group 4 were scored as having the best overall impression.

According to the results obtained, to achieve meatballs in tomato sauce with reduced sodium chloride (and therefore, sodium) content, partial replacement with ammonium chloride is a suitable choice for this product.

**Conflict of Interest.** The authors declare that they have no conflicts of interest.

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# Biopreservation of traditional raw milk cheeses with an emphasis on Serbian artisanal cheeses and their historical production

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*A b s t r a c t: Cheese is one of the oldest food products with preservation based on fermentation, the most common and perhaps the oldest biotechnology. It relies on the biochemical action of lactic acid bacteria (LAB) and is regarded as health-friendly by consumers. Some autochthonous LAB, worldwide and in Serbia, have been characterized as effective producers of antimicrobial compounds such as low-molecular-weight metabolites, hydrogen peroxide, bacteriocins, and bacteriocin-like molecules, and so have demonstrated great potential as food preservatives . The raw milk cheese microbiota, as a good source of novel bacteriocinogenic LAB with high diversity of microbial activity, is key in controlling the microbial load in cheese and achieving diverse sensory characteristics.*

***Keywords:*** *biopreservation, lactic acid bacteria, raw milk cheese.*

## 1. Antimicrobial potential of lactic acid bacteria – an attractive model of biopreservation

The global food industry is continually changing and evolving in order to meet consumers' needs. Consumers want food that is convenient: high-quality, fresh (minimally processed), natural (preservative free), undoubtedly safe and with extended shelf life. Both consumer and food legislative needs call for innovative approaches to preserving food. Therefore, food processors and the scientific community have to explore and implement novel food preservation systems.

Biopreservation is defined as the extension of shelf life and enhanced safety of foods by the use of natural or controlled microbiota and/or antimicrobial compounds (*Schillinger et al.*, 1996; *Stiles*, 1996). Fermentation, the most common and historically-rooted form of biopreservation, relies on the biochemical action of lactic acid bacteria (LAB). Moreover, fermentation, perhaps the oldest biotechnology, has been utilized for millennia (*Ross et al*., 2002) and is regarded as health-friendly by consumers.

The LAB are a heterogeneous group of organisms functionally related by their ability to convert hexoses into lactic acid during homo- or heterofermentative metabolism. Although LAB do not compromise a distinct taxonomic group, they are phylogenetically closely related, with their small genomes and simplified metabolic pathway for carbohydrate fermentation (*Pfeiler and Klaenhammer*, 2007). The ecological distribution of LAB is extensive: they are indigenous to food-related habitats but also associated with the mucosal surfaces of animals (*Makarova et al*., 2006). Molecular studies revealed that considerable genetic adaptation has occurred during the coevolution of LAB with their diverse habitats (*Mayo et al*., 2008). The transition toward a nutrient-rich lifestyle (e.g. milk) to gene loss and metabolic simplification (reduction). Also, LAB adaptation to milk has resulted in acquisition and duplication of genes involved in the metabolism of carbohydrates and amino acid transport, thus ensuring the genetic traits dedicated to efficient exploitation of milk's nutrients (*Makarova et al*., 2006).

The major antimicrobial compounds produced by LAB are organic acids (lactic acid, acetic acid). Rapid acidification is one of the major criteria for selection of LAB starter strains that are utilized in

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the dairy industry. However, it is an important parameter in achieving the microbiological stability of fermented food if we bear in mind that, generally, food safety is guaranteed as soon as the pH value reaches 4.2 or below (*Holzapfel*, 2002). The antimicrobial effect of acids is exerted by interfering with maintenance of cell membrane potential, inhibiting active transport and reducing intracellular pH, thus hindering a variety of metabolic pathways (*Kashket,* 1987; *Lorca and De Valdez*, 2009). Moreover, specific strains of LAB are characterized as effective producers of other antimicrobial compounds such as low-molecular-weight metabolites (reuterin, diacetyl, and fatty acids), hydrogen peroxide, bacteriocins, and bacteriocins-like molecules (*Suskovic et al.*, 2010). Among these, the bacteriocins, proteinaceous compounds with antimicrobial activity against pathogenic and spoilage bacteria, have demonstrated great potential as food preservatives.

## 2. Bacteriocins

Bacteriocins are defined as small, heat-stable, ribosomally synthesized antimicrobial peptides with a narrow or broader spectrum of activity (*Cotter et al.*, 2005). It is reasonable to assume that numerous bacteriocins exist in nature, but industrially important LAB have been mainly exploited as a huge reservoir for bacteriocins, as they have earned the "generally recognized as safe – GRAS" (*FDA*, 1988) status due to their long tradition of safe use in fermented food. Over the years, various schemes have been introduced in order to classify the bacteriocins of Gram-positive bacteria. *Cotter et al.* (2005) suggested a more radical modification of the previous classification schemes where bacteriocins can generally be classified into one of two groups on the basis of whether they undergo post-translational modifications: Class I (modified – lantibiotics) and Class II (unmodified – non-lantibiotics), as opposed to Klaenhammer's (*Klaenhammer*, 1993) four class scheme. The mode of antimicrobial action of bacteriocins differs among classes including membrane permeability, interference with cell wall synthesis, or dependence on a receptor molecule required for binding, inhibition of sugar-uptake system and efflux of intracellular solutes (*Perez et al.,* 2015). It was generally assumed that most bacteriocins were not active against Gram-negative bacteria due the integrity of their lipopolysaccharide outer membrane (*Stevens et al.*, 1991; *Perez et al.,* 2015). In the same studies, the antimicrobial activity was attributed to bacteriocin production as shown by susceptibility of inhibitor substances to degradation by proteolytic enzymes .

Bacteriocins are qualified to be promising tools in the biopreservation due to several characteristics (*Perez et al.,* 2015):

i) Inherent tolerance to thermal stress
ii) Activity over a wide pH range
iii) No adverse effect on quality and flavor
iv) Easy degradation by proteolytic enzymes, which minimizes the development of resistance mechanisms. Occasional resistance is observed, probably due to intrinsic ability of cells to change lipid composition of membrane, but it is unclear if this phenomenon is generated by spontaneous mutation (*Crandall and Montville*, 1998; *Vadyvaloo et al.*, 2002; *Nes and Johnsborg*, 2004).
v) Suitability to bioengineering due to their primary metabolic nature (*Perez et al.,* 2014).

It is noteworthy that very few commercial bacteriocinogenic protective cultures are marketed today owing to the difficulty of developing cultures that are efficient and effective in food systems. *In situ* bacteriocin production is favored as it does not require specific legislative approval, but it does require that the producer strain is well adapted to the food matrix, and capable of active growth and bacteriocin expression. The effectiveness of bacteriocin activity in food is affected by numerous factors: interference with food matrix, enzymatic degradation, and retention of bacteriocin molecule by components of the food system, the antagonistic effect of background microbiota, slow diffusion and insolubility due to inadequate physicochemical parameters and uneven distribution of bacteriocin in heterogeneous food matrix (*Cleveland et al.,* 2001; *Gálvez et al.,* 2007).

Before the introduction of genetic studies, screening for bacteriocins relied on functional assays, in which potential producer organisms were tested for antimicrobial activity against selected indicator organisms. It is not an ideal solution because not only is bacteriocin production plasmid-encoded (which implies instability due to plasmid loss), but it has also been recognized that regulatory mechanisms of bacteriocin synthesis are subject to temperature control (*Diep et al.,* 2000). Therefore, a major obstacle in screening and choosing novel bacteriocin-producing LAB is that their optimal growth temperature can differ from their optimal

temperature for bacteriocin production (*Nes and Johnsborg*, 2004).

Bearing in mind the ubiquity of bacteriocin production, the ecological consideration of this trait has been established, although it is not fully understand what, precisely, that ecological role is (*Riley et al.*, 2003). Subinhibitory concentrations of structurally diverse inhibitor molecules affected transcription of many bacterial genes not necessarily linked to stress response, suggesting that antimicrobial compounds may function as signal molecules in natural habitats when produced at low concentration – a phenomenon previously known as hormesis (*Calabrese and Baldwin*, 2002). So far, it has been suggested that at least some bacteriocins have a dual role, acting as inhibitors at high concentrations but also as signaling compounds at lower concentrations, thus playing a role in mediating microbial population and community interactions (*Fajardo and Martinez*, 2008). Based on comparative genomic analysis of LAB, it has been recognized that molecular systems responsible for bacteriocin production are evolving in response to adaptation, reflecting the long-term existence of LAB in complex, highly competitive microbial communities (*Makarova et al*., 2006). Ecologically speaking, bacteriocin production provides LAB with greater competitiveness.

In recent years, extensive activities of the scientific community have been focused on the antimicrobial properties of LAB (*Leroy and De Vuyst*, 2004; *De Vuyst and Leroy*, 2007; *Suskovic et al*., 2010). Considering consumer aversion to traditional chemical preservatives, which is forcing food processors to reconsider their preservation technology and seek alternative tools, the use of bacteriocins is likely to expand in the future. However, many bacteriocins have not been fully characterized and therefore cannot be extensively exploited in the food industry. To be wholly characterized, bacteriocins have to be tested for a variety of parameters including spectrum of antimicrobial activity, mode of action, sensitivities to heat, pH, proteolytic enzymes, salt and detergents, determination of molecular mass, amino-acid composition and sequence, determination of the genetic basis of the bacteriocin production and secretion (*Todorov*, 2009). Additionally, great effort has to be devoted to fully address the question of efficient *in situ* bacteriocin expression and consequently to optimize the efficiency of bacteriocin production. The heterologous expression of bacteriocins from LAB could be an affordable solution (*Jiménez et al*., 2015), as advances in genome sequencing of LAB favor the genetic manipulation of these bacteria .

## 3. Raw milk cheeses: a bastion against undesirable microorganisms

Recently, there has been a considerable interest in locally-sourced, fresh, organic, natural and sustainable products. One product that ideally embodies these consumer expectations is raw milk cheese. *Paxson* (2008) stated that the raw milk cheese processing could be considered as biotechnology for localism.

Cheese is a living and dynamic ecosystem that involves the growth of complex microbial consortia. The complexity of cheese microbiota is due to abundant bacterial associations and biotic interactions favored by the structure and physicochemical heterogeneity of the cheese matrix. The unique chemosensory profile of cheese, as well as shelf-life, quality and safety are largely determined by composition and evolution of cheese microbiota (*Irlinger and Mounier*, 2009). A recent study (*Bokulich and Mills*, 2013) showed that environmental microbiota originating from the cheese processing facility are capable of establishing in the surface microbiota of wash-rind cheeses and most likely impact the sensory properties of cheese. This established site-specific in-house microbiota with potential to be continuously transferred to successive batches of cheese confirmed the basic ecological principles: “everything is connected to everything else” and “environmental selection.” According to *Marcellino* (2003), processing parameters introduced through the traditional manufacturing of raw milk cheeses are the major driving forces in selection of specific cheese microbiota, far more so than distinct geographical areas with their inherent characteristics (pedioclimatic conditions, pasture). Therefore, *Marcellino* (2003) considered raw milk cheese as a nature-culture hybrid.

There is a general consensus that cheeses are safer than other unfermented dairy products due to the interplay of various abiotic and biotic factors. After comprehensive summary of the epidemiological literature related to raw milk cheese outbreaks, *Donnelly* (2001) stated there is no compelling data indicating that mandatory pasteurization would result in a safer product. Moreover, some microbiologists argued that pasteurization diminishes the principle of competitive exclusion, and provides an environment which supports pathogen growth. According to EFSA-ECDC (*EFSA-ECDC*, 2016), non-compliance with microbiological criteria established for *Listeria monocytogenes* was primarily related to soft and semi-soft cheeses made from pasteurized milk, which implied post-processing contamination. However, there are on-going debates

about the safety of raw milk cheeses, that raise what *Mintz* (2002) recognized as a conundrum of democratic capitalist societies: "how to provide protection of public health on one hand, yet maintain freedom of choice on the other". Moreover, anthropologists and sociologists, especially in the USA, introduced the notion of microbiopolitics as a theoretical frame for understanding debates over the food localism, nutrition, health and safety of raw milk cheeses (*Paxson*, 2008). In Paxson's theory of post-Pasteurian culture, the hyperhygienic Pasteurian claim is that only milk pasteurization makes cheese safe. In contrast, post-Pasteurians, including defenders of traditional cheeses, rely on the complex microbial ecology of cheese as an effective safety strategy. They claim that high diversity of microbial activity coupled with local know-how of the manufacturing process are keys for controlling the microbial load in cheese and achieving diversification of sensory characteristics (*Montel et al*., 2014). *Panari and Pecorari* (1999) stated, with particular reference to traditional processing of Parmigiano-Reggiano: "These processing systems are continually aiming at improvements in quality and only the best technologies persist through the centuries; furthermore, they have accepted corrective practices (as in the modern Hazard Analysis and Critical Control Points [HACCP] protocol) suitable for customers' requirements, including hygienic traits".

Available data from scientific literature and reports from the Center for Disease Control indicate *L. monocytogenes*, verocytotoxin-producing *Escherichia coli* (VTEC), *Staphylococcus aureus*, *Salmonella* and *Campylobacter* as the main microbiological hazards associated with raw milk cheese outbreaks, whereas *Salmonella*, followed by VTEC were the most common etiological agents (*Verraes et al*., 2015). According to EFSA-ECDC **(***EFSA-ECDC*, 2016), out of 592 foodborne outbreaks with strong evidence, cheese was implicated in 1.5% of cases, which was the same prevalence as for the sweets and chocolate category. In the industrialized world, the low prevalence of food-borne outbreaks due to consumption of dairy products, including raw milk cheeses, is achieved by successful implementation of overall management of infectious disease in dairy herds, HACCP implementation, and active surveillance throughout the food chain with adherence to GHP and GMP.

Raw milk cheese processing is an illustrative example of empirical application of hurdle technology. Fermentation as a key biochemical process results in decrease of pH due to acid production, redox potential reduction, and nutrition depletion. The proper development of acidity is the most important process control tool that determines cheese safety. The phases of curd processing and pressing favor syneresis and consequently lead to decrease in water activity ($a_w$). The salting, besides redirecting the biochemical processes, additionally reduces $a_w$. The interplay of these physicochemical parameters establishes a hostile environment for any pathogens possibly introduced to the cheese matrix due to contamination. During the ripening process, the well adapted autochthonous LAB become established in high numbers, owing to their resistance to reduced pH, decreased $a_w$ and high salt concentration. The main advantage of autochthonous LAB compared to other microbial groups is their potential to effectively compete for limited sources of nitrogen (*Sieuwerts et al.*, 2008) due to efficient proteolytic and transport systems. The hurdle effect of natural LAB microbiota on undesirable contaminants seems to be highly variable and is accomplished by competing for nutrients (competitive interactions) and producing inhibitory compounds (organic acids, volatile compounds, $H_2O_2$) and/or antimicrobial compounds (bacteriocins) (*Irlinger and Mounier*, 2009).

## 4. Antimicrobial potential of autochthonous LAB microbiota isolated from raw milk cheeses worldwide

Numerous studies considered raw cheese microbiota as a good source of novel bacteriocinogenic LAB strains (*Franciosi et al.*, 2009; *Dal Bello et al*., 2010; *Ortolani et al.*, 2010). *Moraes et al.* (2012) confirmed that most (93%) of the enterococcal isolates originating from raw milk and cheese in Minas Gerais state, Brazil, harbored at least one lantibiotic or enterocin gene but as the bacteriocinogenic isolates also carried virulence genes, the authors emphasized the need for careful evaluation of their application in food systems.

*Mojsova et al.* (2015) reported that enterococci isolated from Macedonian traditional cheeses showed antimicrobial activity predominantly against *L. monocytogenes*, and among bacteriocinogenic enterococcal isolates, enterocin P, cytolysin and enterocin A were the most frequently detected structural genes. The authors concluded that although screened enterococcal isolates could be of great technological potential as protective cultures in the cheese industry, it is necessary to ensure that potentially applicable isolates are free of virulence factors.

*Enterococcus faecalis* isolates from Italian traditional cheeses were evaluated for their antimicrobial potential against foodborne spoilage and

pathogenic bacteria and against LAB commonly used as starter cultures in dairy fermentation (*Silvetti et al.*, 2014). The analyzed enterococcal isolates inhibited *Bacillus cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *Clostridium sporogenes*, whereas LAB were moderately antagonized. In that study, by applying a molecular approach, only one enterococcal isolate was found to be enterocinogenic, as the structural gene for enterocin AS-48 was the only one amplified. The authors assumed that the antimicrobial properties of other, phenotypically positive isolates could be due to another non-enterocin inhibitory compound.

By applying the spot-on-the-lawn test, *Tulini et al*. (2013) evaluated the antimicrobial potential of *Lactobacillus paraplantarum* FT259, isolated from Brazilian semi-hard artisanal cheese, on a variety of food-related bacteria and LAB. *L. paraplantarum* FT259 inhibited *L. monocytogenes*, *Listeria innocua* and *Lactobacillus sakei* but was not active against Gram-negative bacteria or staphylococci.

*Achemchem et al.* (2005) examined Jben, a soft, farmhouse goat's cheese made in Morocco, as a potential source of LAB bacteriocin producers. Among the isolates, *Enterococcus faecium* F58 was selected for further study according to its broad inhibitory spectrum against *Listeria*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Brochothrix* and *Lactococcus*, although none of the Gram-negative bacteria examined were inhibited. The results obtained concerning the characterization of inhibitory substance(s) produced by *E. faecium* F58 strongly suggested the proteinaceous nature of compound(s), which was named enterocin F58. The high thermostability of enterocin F58 and activity over a wide pH range justified its potential use for biopreservation in food systems.

Out of 2,227 LAB isolated from 27 Peruvian artisanal cheeses, 0.9% showed an inhibitory effect against *L. monocytogenes* CWBI-B2232 (*Gálvez et al*., 2009). As no change in inhibitory activity was observed after acid neutralization and treatment with catalase of the cell-free supernatants (CFS), the authors assumed that the antimicrobial effect of autochthonous LAB was due to bacteriocin-like substances, which was further confirmed by proteolytic digestion of the CFS.

*Milioni et al.* (2005) successfully recovered the anti-staphylococcal *Lactococcus plantarum* LpU4 from an Italian traditional Pecorino cheese. The confirmed the anti-staphylococcal effect of autochthonous LAB strains is a matter of utmost concern considering that 9.9% of all food-related outbreaks reported in the EU in 2015 were caused by staphylococcal toxins and cheese was the most commonly implicated food vehicle in the strong-evidence outbreaks caused by staphylococcal toxins (*EFSA-ECDC*, 2016).

*Cosentino et al.* (2012) reported that bacteriocin-producing strains *of Lactococcus lactis* subsp. *lactis* isolated from traditional Sardinian goat and sheep dairy products, growing in co-culture *with L. monocytogenes*, were able to reduce the *Listeria* counts by approximately 4 log units compared to the control. Comparable results were obtained by *Coelho et al.* (2014), who noticed that bacteriocinogenic *E. faecalis* isolates from a traditional Azorean artisanal cheese (Pico cheese), were able to control growth of *L. monocytogenes* in fresh cheese by decreasing the *Listeria* count by approximately 4 log during the storage period of 7 days.

The anti-listerial effect of bacterial communities in cheese has been frequently observed (*Eppert et al.*, 1997; *Maoz et al.*, 2003; *Mayr et al.*, 2004). Individually, however, the bacteriocinogenic strains isolated from complex anti-listerial microbiota did not show any inhibition of the *Listeria* growth (*Eppert et al.*, 1997), which emphasized the importance of bacterial interaction *in situ* (*Roth et al.*, 2010; *Demarigny and Gerber*, 2014).

*Mezaini et al.* (2009) concluded that *Streptococcus thermophilus* T2, isolated from Algerian traditional cheese, Raib, had antimicrobial potential toward the closely-related Gram-positive bacteria, *L. innocua* and *E. faecalis*, and is a promising candidate to help improve microbiological microbiological safety and increase the shelf life of traditional dairy fermented food.

## 5. Antimicrobial potential of autochthonous LAB microbiota isolated from raw milk cheeses in Serbia

Cheese is one of the oldest food products, and cheese production, dating from 8000 years ago, is a classic method of food preservation (*Savic et al*., 2009). Serbia, as well as other Balkan countries, has a long history in the production of traditional dairy foods, including cheese **(***Golic et al*., 2013). In the Middle Ages, Serbian cheese was manufactured by the Vlach people during summer. Back then, cheese was a rare and expensive food, and so was considered to be food for the nobility. Cheese was not only an important food in Serbian cuisine, but it was also used as a valuable payment mechanism, being exchanged for salt and craft products. Moreover, during the reign of Emperor Dusan in the 1300s, a cheese impost was implemented, which was given as a donation to monasteries (*Marjanovic-Dusanovic*, 2004). Documents from 1417 suggest that Serbia exported

cheese to Dubrovnik, then a well-known trade center. Two main cheese types produced in Serbia were white brined cheeses and much later, hard cheese, the so-called kachkaval (*Pejic*, 1956). According to historical data, the tradition of kachkaval (Tzintzar language the word ″kač″ means cheese) cheese making was introduced by nomads from Greece to locals on Stara Planina Mountain about 100 years ago (*Mijacević and Bulajic*, 2004). Nomadic sheep breeders known as Crnovunci (Blackwool people) started to produce kachkaval by enclosing white cheese in a sheep's stomach, bellows in hot water, then later mixing and salting the cheese. This manufacturing process was passed on to the people of Dojkinci village, who improved it and maintain it today (*Mijacevic and Bulajic*, 2004; *Veljovic et al.*, 2007). Kachkaval was exported to Vienna and Budapest. Zlatar cheese, white brined hard cheese, has been produced traditionally in Serbia for centuries (*Veljovic et al*., 2007). Other brined cheeses, including Sjenica cheese, Svrljig cheese, Golija cheese, Pirot cheese, Sara cheese and Homolj cheese, are traditionally manufactured in households in mountain regions, while Sombor cheese originates in the northern, flat plains of Serbia (*Dozet et al*., 1996; *Mijacevic and Bulajic*, 2008; *Vucic et al*., 2008; *Stevanovic et al.*, 2016).

It is not only a long-lasting manufacturing tradition that makes cheeses from Serbia special. The originality and specificity of the cheeses reflect the presence of natural LAB microbiota originating from raw milk and the specific geographic region **(***Radulovic et al*., 2016). Specificities of microclimate, characteristics of the raw milk and sublethal stresses introduced through the manufacturing process (milk acidification, renneting, whey drainage, salting, and ripening) selectively favor development of LAB with unique phenotypic characteristics **(***Mijacevic et al*., 2005). Their metabolic activity results in end products with specific sensory attributes. Accordingly, autochthonous cheeses might be considered as unique ecological entities (*Licitra*, 1997). Autochthonous, artisanal cheeses in Serbia are produced in households from raw milk (cow's, sheep's and goat's) or milk mixtures, without the use of commercial starter cultures.

Reduction of pH due to production of organic acids (primarily lactic acid) is the main preservation mechanism of LAB. *Veljovic et al.* (2007) and *Nikolic et al.* (2008) showed reasonable acidification autochthonous LAB isolates from Zlatar cheese showed reasonable acidification, able to decrease cheese pH to 4.8 after 5 to 7 h. LAB strains tolerant to high salt concentrations and able to grow at low pH, thus forming the predominant microbiota in cheese, have an important role in the ripening process (*Begovic et al*., 2011). Some LAB isolated from Zlatar cheese produced from sheep's or cow's milk were extremely tolerant to high salt concentrations as well as low pH, meaning they competed well with a variety of bacterial species, including spoilage microorganisms (*Terzic-Vidojevic* et al., 2007; *Veljovic et al*., 2007; *Terzic-Vidojevic et al*., 2013). *Mijacevic et al*. (2003) showed that LAB isolated from kachkaval were resistant to high temperature, low pH and high salt as result of their long-term adaptation to the technological conditions of cheese processing.

In most cases, autochthonous LAB in raw milk cheeses from the Balkan region and Serbia, as part of it, are mesophiles such as *Lactococcus* spp., *Lactobacillus* spp., and *Enterococcus* spp. or thermophiles such as *S. thermophilus* (*Terzic-Vidojevic et al.*, 2007; *Veljovic et al.,* 2007; *Terzic-Vidojevic et al.,* 2009). LAB diversity in raw milk cheeses from Serbia is also reflected in the production of different antimicrobial substances like bacteriocins, hydrogen peroxide, and diacetyl which are antagonistic to a wide bacterial spectrum (*Veljovic et al.,* 2007).

The ability of LAB to produce bacteriocins enables these bacteria to grow competitively in mixed bacterial populations **(***Topisirovic et al*., 2007). A study conducted on 253 samples of Zlatar cheese, production of which is not standardized, and which can be purchased only in local markets or from artisanal producers, reported that 70 *L. lactis* subsp. *lactis*, *E. faecalis* or *Lactobacillus paracasei* subsp. *paracasei* isolates produced proteinaceous, antimicrobial, possibly bacteriocin-like substances **(***Topisirovic et al*., 2007). Comparable results were obtained another study, where 87 of 253 LAB isolates produced antimicrobial proteinaceous compound, possibly bacteriocin-like substances (*Veljovic et al.,* 2007). Enterococci isolated from Zlatar cheese as well as from Pirot kachkaval (an artisanal cheese from Stara Planina Mountain, made from raw cow's milk without starter culture) were reported to be producers of enterocins (*Topisirovic et al.,* 2007; *Terzic-Vidojevic et al.,* 2009). The importance of enterocins lies in their antimicrobial activity against Gram-positive bacteria such as *L. monocytogenes* and *L. innocua*, which was confirmed by *Nikolic et al.* (2008), who isolated enterococcal strains with antilisterial activity from Bukuljac, a homemade goat's cheese. *L. paracasei* subsp. *paracasei* isolated from homemade Sjenica cheese from the Pester Plateau, Serbia, produced bacteriocin BacSJ, a heat-stable proteinaceous bacteriocin, with retained activity after treatment at 100°C for 1 hour at pHs from 2 to 11. All

lactococcal and enterococcal isolates from Vlasina cheese, traditionally made from raw sheep's and goat's milk, were bacteriocin producers, and 53.4% of LAB isolates from white pickled cheeses from rural South Morava and mountainous regions of Eastern Serbia and Golija Mountain were bacteriocin producers active against *S. aureus* **(***Golic et al*., 2013; *Terzic-Vidojevic et al*., 2013). Bacteriocins produced by LAB isolate from Vlasina cheese survived for 30 minutes at 63.5°C (*Terzic-Vidojevic et al*., 2013). Antimicrobial compounds produced by lactococcal and enterococcal isolates from artisanal raw milk cheese produced on Stara Planina Mountain showed activity against *L. lactis* subsp. *lactis* and *L. paracasei* subsp. *paracasei* (*Begovic et al*., 2011).

LAB isolated from Zlatar cheese produced nisin-like substances that inhibited Gram-positive *S. aureus* and *L. monocytogenes*, as well as spore-forming bacteria including *Clostridium* spp. and *Bacillus* spp. Due to the fact that nisin is natural preservative, approved and commercially used in over 50 countries around the world, the safety of Zlatar cheese can be attributed to production of these substances (*Veljovic et al*., 2007).

*Nikolic et al*. (2008) studied the antimicrobial activity of *Lactobacillus* isolates (largely *L. paracasei* subsp. *paracasei*), a producer of the bacteriocin SJ, from Bukuljac cheese,with effect on *Lactobacillus paracasei* subsp. *paracasei*, as well as *Salmonella* Enteritidis.

LAB production of diacetyl is an interesting technological property due to the contribution of this compound to the buttery aroma of fermented cheeses. Moreover, diacetyl is recognized as an antimicrobial substance, although at concentrations which exceed its acceptable sensory level. However, synergistic effects, with diacetyl in combination with other antimicrobial factors, may be an acceptable solution (*Lee and Jin,* 208). Diacetyl inhibits the growth of Gram-negative bacteria by reacting with arginine binding protein, thus affecting arginine utilization (*Jay,* 1982). *Terzic-Vidojevic et al.* (2015) showed that the most of the lactococcal isolates from white pickled and fresh soft cow's milk artisanal cheeses in Serbia and Croatia were producers of diacetyl.

## 6. Conclusion

In conclusion, bacteriocinogenic isolates are common among the autochthonous LAB microbiota of Serbian raw milk cheeses. Further studies are encouraged to evaluate the application of these strains/bacteriocins to enhance the safety and quality of raw milk cheese.

**Conflict of interest.** The authors declare that they have no conflicts of interest.

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Numbers in-text and in tables/figures. Use decimal points, not decimal commas. Avoid starting and ending sentences with numbers (re-write the sentence).

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The author should apply the International System of Units (SI system) and current regulation on measuring units and measuring instruments. Symbols for units derived by division are given as negative exponents, e.g. 10 g $L^{-1}$; 250 V $cm^{-2}$.

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- CFU colony forming units, capitalised, common and so is never spelled out
- kg kilogram, common and so is never spelled out
- L litre, common and so is never spelled out
- Longissimus dorsi (LD) is redundant and so is not used. For the whole muscle, use Longissimus thoracis et lumborum (LTL). The correct terms for the two parts of this muscle are Longissimus thoracis (LT) or Longissimus lumborum (LL).
- mL millilitre, common and so is never spelled out
- µm micrometre, common and so is never spelled out
- mol mole, common and so is never spelled out
- M molar, common and so is never spelled out
- PCR polymerase chain reaction, common and so is never spelled out
- SD standard deviation, capitalised, common and so is never spelled out
- SE standard error, capitalised, common and so is never spelled out
- sp. species (singular), common and so is never spelled out (not capitalised, full-stop)
- spp. species (plural), common and so is never spelled out (not capitalised, full-stop)
- UV ultraviolet, capitalised, common and so is never spelled out
- aw water activity
- h hour(s)
- min minute(s)
- 25°C (no gap after the numeral)
- 20±1°C (no gaps between numbers, sign and unit in-text and in tables/figures)
- $p<0.05$, $p<0.01$ (not italicised, not capitalised, no gaps)
- $n=120$ (no gaps between the letter, sign and numerals in-text and in tables/figures)
- found in 20.05% of cats…(no gap in-text)

**Conclusion** This section provides a review of the most important facts obtained during the research.

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*Laws, regulations, decrees:*

**Serbia. (2010)**. Regulation on general and special conditions of hygiene of food at any stage of production, processing and transport. *Official Gazette of the Republic of Serbia*, 72.

**European Union. (2013)**. **Commission regulation (EU) No 1019/2013 of 23 October 2013 amending Annex I to Regulation (EC) No 2073/2005 as regards histamine in fishery products. *L 282*, 46–47.**

*Symposiums, Congresses:*

**Harvey, J. (1992).** Changing waste protein from a waste disposal problem to a valuable feed protein source: a role for enzymes in processing offal, feathers and dead birds. Alltech's 8th Annual Symposium, Nichdasville, Kentucky, Proceedings, 109−119.

*Citations with organisations as authors:*

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*Software:*

**STATISTICA (Data Analysis Software System) (2006).** v.7.1., StatSoft, Inc., USA (www.statsoft.com).

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**Technical report on the Food Standards Agency project G010008 (2002).** Evaluating the risks associated with using GMOs in human foods, University of Newcastle, UK (http://www.foodsafetynetwork.ca/gmo/gmnewcastlereport.pdf).

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