Original scientific paper
UDC 637.5:614.31
664.91:614.31

APPLICATION OF BIOPROTECTORS IN MEAT INDUSTRY

Slavica Vesković Moračanin[1*], Srdjan Stefanović[1], Lazar Turubatović[1]

[1]Institute of Meat Hygiene and Technology, Kaćanskog 13, 11000 Belgrade, Republic of Serbia

***e-mail: slavica@inmesbgd.com**

Abstract

Modern concept of food production and processing is based on application of various protective technologies with the aim to ensure and preserve product safety, as well as acceptable and unchanged product quality from the moment of production to the moment of consumption. From the other side, consumers show the need for the food that did not undergo extensive preservation processes and free from chemical preservatives. Such trend from one side (so-called «green technology») and continous development of modern protective technologies in XX and XXI century induce the development and application of achievements in the field of bio-protection of food.

Growing needs for natural safe food led to increased interest for utilisation of bacteriocin-producing species of lactic acid bacteria (LAB), that are used in production of fermented products as protective cultures. The principle on which bio-protection is based is decreasing risk for the consumers by acting on undesirable spoilage bacteria or foodborne pathogens, preserving quality parameters at the same time.

This paper presents the part of long-term research carrie out by the authors with the aim to define conditions for application of protective cultures and/or bacteriocins in meat industry during the manufacture of fermented sausages.

Leuconostoc mesenteroides ssp. mesenteroides IMAU:10231 has been isolated from traditionally fermented „sremska" sausage, its antimicrobial properties were determined *in* vitro, and after the adequate preparation, bacteriocin was applie as additive in manufacturing the same sausage from which it was isolated. The results showed that traditional fermented sausage manufactured in this way is a safe product with certain quality parameters improved over the classical manufacturing process. At the same time, such production procedures contribute significantly in the area of novel methods for bioprotectors development, that should be further improved.

Key words: *Food safety, bacteriocins, lactic acid bacteria, meat industry.*

1. Introduction

Despite the huge progress in the field of modern technologies, production, processing and storage of safe food still occupies the attention of all subjects involved in this chain, both in developing countries and in developed and highly industrialized countries. One of the biggest issues in modern food industry of today are significant economic losses due to revoking of food from the market, ever-present trend of decreasing the production expences, decreasing and elimination of risk of food-borne pathogens spreading, and finally, the increasing efforts aimed in satisfying growing needs of the consumers for ready to eat food characterized by fresh flavor, high nutritional value, and at the same time, minimally processed and artificially preserved. Empirical usage of sertain microorganisms and/or their natural metabolites (bioprotectors) in food production, has found its application throughout history of mankind throughout past several thousand years (Ross *et al*. [1]). Lactic acid bacteria (LAB) are considered to be especially important due to its essential role in production of fermented meat or milk products, preservation of vegetables et sim. By the means of its metabolic activity, these bacteria modify ripening processes resulting in formation of desirable sensory properties, simultaneously inhibiting the growth of undesirable microflora. For the reason of their dominant activity during fermentation and long tradition in their utilisation, LAB are designated as „safe" microflora. Bioprotection of LAB, regardless whether these bacteria are already present in food of intentionally added, is achieved through production of non-specific metabolites (lactic, acetic and other organic acids, diacetyl, etc), and specific metabolites, i.e. bacteriocins (Lindgren and Dobrogosz [2] and De Vuyst and Vandamme [3]).

Bacteriocins are natural peptides or proteins with antibacterial properties with the wide range of potential applications in food industry, with the aim of consumers' healh improvement; their effect on increasing the shelf-life of foodstuffs, and sometimes, improvement of quality parameters is considered highly significant as well (Caplice and Fitzgerald [4], Cleveland *et al*. [5] and Turcotte *et al*. [6]).

This paper presents a part of long-term research of the authors with the aim to determine conditions for application of protective cultures and/or bacteriocins in meat industry, namely in manufacturing of fermented sausages. Bacteriocin isolated from *Leuconostoc mesenteroides ssp. mesenteroides* IMAU:10231 has been added during the production of traditional "sremska" sausage in industrial conditions. This LAB strain has been isolated from traditional fermented Serbian sausage, and after adequate laboratory treatment, re-entered manufacturing cycle of the same sausages, in the role of additive.

The obtained results show that traditional fermented sausages manufactured in this way, are safe products with some quality parameters improved. Furthermore, such manufacturing procedures significantly contribute development of novel methods of bioprotection which needs to be further elaborated and improved.

2. Materials and Methods

2.1. Bacterial strains

Ln. mesenteroides ssp. mesenteroides IMAU:10231, isolated from Serbian dry fermented sausage ("Sremska" sausage) was used throughout this study (Vesković *et al*. [7]).

2.2. Isolation of semi purified bacteriocin from *Ln. mesenteroides ssp. mesenteroides* IMAU:10231

Isolation of semi purified bacteriocin from *Ln.mesenteroides* ssp. *mesenteroides* IMAU:10231 was carried out by the method of saturated precipitation with ammonium-sulphate (Schillinger and Lücke [8]) modified according to the available laboratory conditions (Veskovic [9]). Several days plating of the broth culture, with the aim of achieving adequate concentration of *Ln. mesenteroides* (10^{10} - 10^{11} $cfug^{-1}$), was done by centrifuging at 10000 g for 30 minutes at 4 ^{0}C (MSE, "High Speed 18", England). After the separation and neutralization up to pH 6,5 – 7,0 of the supernatant with 10 M NaOH, the precipitation of bacteriocin was achieved using ammonium-sulphate. Separated bacteriocin in the form of whitish pellets was suspended in 0.05 M sodium- phosphate buffer-pH 7. This suspension was applied in aerosol form in sausage filling before the stuffing in natural casings.

2.3. Sausage production and sampling procedure

"Sremska" sausage was produced in industrial conditions ("YUHOR" meat industry, Jagodina, Serbia), using the basic principles of traditional production. Raw material consisted of pork (I and II category) and frozen firm fat. Additives were nitrite salt and dextrose, while added spices were chopped garlic and grinded sweet and hot paprika. The produced filling was split into two equal parts – control batch and experimental batch. To the latter, semi-purified bacteriocin isolated from *Ln. mesenteroides* ssp. *mesenteroides* IMAU 10231, was added (bacteriocin strenght was 2560AU/kg of filling). Both batches were stuffed into pork small intestine.

2.4. Laboratory analysis

2.4.1 Determination of potency range of bacteriocin isolated from Ln. mesenteroides ssp. mesenteroides IMAU 10231.

Antimicrobial activity of Isolated and semi-purified bacteriocin from *Ln. mesenteroides* ssp. *mesenteroides* IMAU 10231, was determined against three pathogenes: *Listeria monocytogenes* NCTC 10527, *Staphylococcus aureus* NCBF 1499 and *Escherichia coli* 0157:H7 NCTC 12079. Liquid culture (18- hours old) was added to BHI agar containing 0.5% of agar. Final concentration was 10^7 – 10^8 cells/mL of medium. Isolated bacteriocin (50 mL) was added to previously made agar wells. After one hour incubation at 4 ^{0}C, the plates were incubated at 30 ^{0}C for 24h, in order to stimulate diffusion of bacteriocin.

2.4.2. Microbiological analysis

Each sample (25 g) was transferred to a sterile stomacher bag and 225 mL of saline-peptone water was added and mixed for 30 seconds in stomacher. Further decimal dilutions with the same diluents were made and the following analyses were carried out on duplicate agar plates: (a) total viable count on Peptone Agar incubated under aerobic conditions for 48-72 h at 30 ^{0}C; (b) LAB on MRS agar (Oxoid, UK), incubated with a double layer for 48 h at 30 ^{0}C; (c) total colliforms and *Escherichia coli* on VRB agar (Merck, GmbH, Darmstadt, Germany) incubated with a double layer for 24 - 48 h at 37 ^{0}C; (d) fecal streptococci on Kanamycin Aesculin agar (Oxoid, UK) incubated for 24 - 48 h at 37 ^{0}C; (e) *Staphylococcus aureus* on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37°C for 24 - 48 h; (f) yeast and moulds on Sabouraud-4% Maltose agar (Merck, GmbH, Darmstadt, Germany) incubated for 24 - 48 h at 37 ^{0}C. Determination of *Listeria* spp. was performed following the procedure of the ISO 11290-1:2004 [10] and *Salmonella* spp. according to ISO 6579:2002 [11].

2.4.3. Physico – chemical analysis

Determination of pH was carried out using pH – meter MA-5730 (PAT N° 35398, Iskra, Slovenia), with glass electrode in accordance with ISO 2917/2004 [12]. *Water activity* (a_w value), was determined using a_w-meter (Wert-Messer, Durotherm) at the constant temperature of 25°C. Water content was determined according to SRPS ISO 1442 method (1998) [13]. Sodium chloride content was carried out using Volhard's method - SRPS ISO 1841-1, 1999 [14]. Sodium nitrite concentration was determined spectrophotometrically according to SRPS ISO 2918 (1999) [15]. Protein content was determined using Kjeldahl's method on Kjeltec Auto 1030 Analyzer. Determination of fat content was carried out according to SRPS ISO 1443/92 [16].

2.4.4. Sensory analysis

Sensory properties of sausages were determined using quantitative-descriptive test (SRPS ISO 6658 [17]), at the end of fermentation (evaluated parameters were color, consistency, odor, fat quality, acidity, juiciness, overall taste, aftertaste and overall acceptability). Panel consisting of five evaluators was formed for this purpose. The results were expressed as mean values for each property ± standard deviation, SD.

3. Results and Discussion

3.1. Antimicrobial activity of isolated bacteriocin

Characterisation of antimicrobial metabolites isolated from *Leuconostoc* spp. show that isolated bacteriocins belong to subclass IIa (pedocin-like bacteriocins) with pronounced antilisterial effect (Stiles [18] and Ennahar*et al*. [19]). Species from genus *Leuconostoc* are significant bacteriocin producers, e.g. *Ln. mesenteroides* ssp. *mesenteroides* produces mesenterocine Y 105, *Ln. mesenteroides* UL5 produces mesenterocine 5, *Ln. gelidium* produces leucocine A-UAL 187 (Hechard *et al*. [20] and Daba *et al*. [21]). Investigations of Leuconostoc bacteriocin potency in respect to test microorganisms (Table 1) showed pronounced antimicrobial activity for *L. monocytogenes*, while the activity on *S. aureus* and *E. coli* was not recorded. This is confirmed by other authors (Schillinger [22], Abbe [23], Veskovic [9]) who point out that inhibitory activity of bacteriocins isolated from LAB is mainly directed to Gram positive bacteria.

Table 1. Potency range of bacteriocin isolated from *Ln. mesenteroides ssp. mesenteroides* IMAU 10231

Test microorganism	Potency of bacteriocin
L. monocytogenes NCTC 10527	+++
S. aureus NCBF 1499	-
E. coli 0157:H7 NCTC 12079	-

+++ = pronounced activity; - = without activity

Table 2 shows the results of aerobic mesophilic bacteria and LAB enumeration and presence of investigated pathogens and spoilage microorganisms. Results are expressed as log cfu g^{-1}± SD.

Table 2. Results of microbiological investigations of "sremska" and "sremska plus" sausage

Experimental groups	Total viable count (log cfu* g^{-1}± SD)	LAB (log cfu g^{-1}± SD)	Other microorganisms
"Sremska plus" sausage	5.328±0.020	6.159±0.123	Not detected
"Sremska" sausage	5.893±0.090	6.514±0.163	Not detected

*cfu – colony forming unit

It is obvious that bacteriocin posesses certain inhibitory properties to LAB too, however, these results have no statistical significance. At the end of ripening and fermentation process, presence of *L. monocytogenes*, *Salmonella* spp, *S. aureus, E. coli*, coliformes, fecal Streptococci, yeasts and molds could not be detected. From the aspect of microbiological risk, final products were safe for consumption in every respect.

Table 3 shows the results of physico-chemical investigations of "sremska" sausages produced without and with added bacteriocin isolated from *Ln. mesenteroides* ssp. *mesenteroides* IMAU:1023 („sremska plus" sausage).

Table 3. Results of physico-chemical investigations of „sremska" sausages and „sremska" sausage with added bacteriocin („sremska plus" sausage)

Parameters	**„Sremska" sausage**	**„Sremska plus" sausage**
Water content (%)	18.78±0.145	19.82±0.025
Fat content (%)	45.82± 0,17	43.82± 0,11
Protein content (%)	22,46 ± 0.10	24,17 ± 0.11
NaCl (%)	3.91±0.017	3.77±0.000
Sodium nitrite (mg/kg)	6.31± 0,14	6.50± 0,01
a_w value	0.855	0.857
pH value	6.31± 0,14	6.50± 0,01

Certain differences in quality parameters can be observed by analyzing the obtained results of sausage samples with and without added bacteriocin. "Sremska plus" sausage has somewhat higher water content and decreased salt content, while nitrite content is higher in "sremska" sausage. From the aspect of microbiological stability, this kind of sausage is safer for the consumer.

Observed trend of pH value variation results from the influence of several factors, major ones being quantity and kind of added sugars, type of muscle groups, filling composition, type of epiphytic microflora and finally, added Leuconostoc bacteriocin, that shows inhibitory

activity towards LAB as well (resulting in higher pH). It can be said that the added bacteriocin acted as «small factory» in sausage filling. Its antimicrobial activity, and its potential influence on quality parameters should be interpreted as one segment of complex interactions taking place in sausage filling. It is evident that further determination of optimal media conditions and environmental conditions is necessary in order to optimize bacteriocins activity.

"Sremska plus" sausage had better grades in 6 sensory parameters, two parameters (acidity and cut surface) were graded the same, while coherence and fat quality were higher graded in control batch (Table 4). Overall impression was better in "sremska plus" sausage (8.45 ± 0.69) than in original "sremska" sausage (8.05 ± 0.37).

Table 4. Sensory properties of investigated sausages

Sensory properties	"Sremska" sausage (control)	"Sremska plus" sausage
	X± SD	*X± SD*
Color	8.00±0.37	8.15±0.69
Cut surface	7.40±0.32	7.40±0.41
Coherence	8.25±0.64	8.20±0.71
Rancidity	9.55±0.76	9.70±0.48
Fat quality	7.45±0.44	7.40±0.32
Acidity	8.35±0.47	8.35±0.49
Juiciness	8.00±0.34	8.15±0.43
Tenderness	8.25±0.79	8.30±0.63
Overall flavour	8.20±0.48	8.65±0.53
After taste	8.15±0.47	8.40±0.42
Overall impression	8.05±0.37	8.45±0.69

Acknowledgement

The results presented in this paper are the part of scientific project in the area of integral and interdisciplinary research, under the title" Improvement and development of hygienic and technological procedures in production of animal originating foodstuffs with the aim of obtaining safe products competitive on the global market", financed by the Ministry of Education and Science of Serbia.

4. Conclusions

– The results obtained in this research, as well the results from previous investigations show that application of bacteriocin in food production has numerous advantages over traditional processes: a) Increased shelf-life of foodstuffs; b) Additional protection of products in undesirable storage conditions; c) Decreased risk of pathogens transfer; d) Decrease of economic losses due to spoilage; e) Decreased utilization of chemical preservatives; f) Usage of lower temperatures during food processing procedures, thus preserving nutritive, protective and sensory properties of food; g) Availability of novel products with lower acidity, lower salt content and higher water content. All elements are in the best interest of both food industry and consumers.

– Regardless of the fact that beneficial properties of bacteriocins are utilised for thousand years, only the usage of nizine is officially permitted for the purposes of bioprotection. The question arises, why other bacteriocins are not used in the bioprotection processes. The answer lies in certain difficulties related to more widespread use in industry and implementation on the food market, not on the lack of scientific evidence that justify their application in food production. Naturally, bacteriocins are not the answer for all the problems that arise in food safety. Their application represents primarilly a good alternative, especially combined with other natural food protectors.

– Further investigations should improve understanding of their nature, activity, possibilities of application, as well as discovery of new bacteriocins, which, if used properly can become natural food bioprotectors.

5. References

[1] Ross R.P., Morgan S., Hill C. (2002). *Preservation and fermentation: past, present and future*. International Journal of Food Microbiology, 79 pp. 16.

[2] Lindgren S.E., Dobrogosz W.J. (1990). *Antagonistic activities of lactic acid bacteria in food and feed fermentations*. FEMS Microbiology Reviews, 7 pp. 149-163.

[3] De Vuyst L., Vandamme E.J. (1994). *Antimicrobial potential of lactic acid bacteria*. In: De Vuyst, L., Vandamme, E.J., (Eds.), Bacteriocins of Lactic Acid Bacteria, Blacke Academic and Professional, London, pp. 91-149.

[4] Caplice E., Fitzgerald G.F. (1999). *Food fermentations: role of microorganisms in food production and preservation.* International Journal of Food Microbiology, 50, pp. 131-149.

[5] Cleveland J., Montville T.J., Nes I.F., Chikindas M.L. (2001). *Bacteriocins: Safe, natural antimicrobials for food preservation*. International Journal of Food Microbiology, 71, pp. 1-20.

[6] Turcotte C, Lacroix C., Kheadr E., Grignon L., Fliss I. (2004). *A rapid turbidometric microplate bioassay for accurate quantification of lactic acid bacteria bacteriocins*. International Journal of Food Microbiology, 90, pp. 283-293.

[7] Vesković Slavica, Borovic B., Velbert B., Karan D., Turubatović L., Obradović D., Okanović Đ. (2009). *Phenotypisation of LAB strains isolated from "Užička" sausage*, Proceedings, 888-892, 55th International Congress of Meat Science and Technology, (ICoMST), 16-21 August, 2009. Copenhagen, Denmark,

[8] Schillinger U., Lücke F.K. (1989). *Antibacterial activity of Lactobacillus sakei isolated from meat*. Applied and Environmental Microbiology, 55, pp. 1901-1906.

[9] Veskovic S. (2005). *The influence of bactericides isolated from Leuconostoc mesenteroides E 131 and Lactobacillus sake I 154 on Listeria monocytogenes during production of "Sremska" sausage.* Msci. Thesis, Agriculture Faculty, University of Belgrade.

[10] ISO 11290-1 (2004). *Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of Listeria monocytogenes -- Part 1: Detection Method*.

[11] ISO 6579 (2002). *Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of Salmonella spp.*

[12] SRPS ISO 2917 (2004). *Meso i proizvodi od mesa. Merenje pH* (Referentna metoda).

[13] SRPS ISO 1442 (1998). *Meso i proizvodi od mesa. Određivanje sadržaja vlage*.

[14] SRPS ISO 1841-1 (1999). *Meso i proizvodi od mesa. Određivanje sadržaja hlorida. Deo 1: Metoda po Volhardu*.

[15] SRPS ISO 2918 (1999). *Meso i proizvodi od mesa. Metoda za određivanje sadržaja nitrita.*

[16] SRPS ISO 1443 (1992). *Meso i proizvodi od mesa. Određivanje sadržaja ukupne masti.*

[17] SRPS ISO 6658 (2002). Senzorske *analize, metodologija – Opisni kvantitativni testovi i testovi senzorskog profila.*

[18] Stiles M.E. (1994). *Bacteriocins Produced by Leuconostoc species*. Journal of Dairy Science, 77 pp. 2718-2724.

[19] Ennahar S., Sashihara T., Sonomoto K., Ishizaki A. (2000). *Class IIa bacteriocins: Biosynthesis, structure and activity*. FEMS Microbiology Reviews, 24 pp. 85-106.

[20] Hechard Y., Derijard B., Letellier F., Cenatiempo Y. (1992). *Characterization and purification of mesentericin Y105, ann anti-Listeria bacteriocin from Leuconostoc mesenteroides.* Journal of General Microbiology, 138 pp. 2725-2731.

[21] Daba H., Pandian S., Gosselin J.F., Simard R.E., Huang J., Lacroix C. (1991). *Detection and activity of a bacteriocin produced by Leuconostoc mesenteroides.* Applied and Environmental Microbiology, 57 (12) pp. 3450-3455.

[22] Schillinger U. (1990). *Bacteriocins of lactic acid bacteria*, In D. D. Bills and S. Kung (ed.), Biotechnology and food safety, Butterworth-Heinemann, Boston, pp. 55-74.

[23] Abbe T. (1995). *Pore-forming bacteriocins of gram-positive and self-protection mechanisms of producer organisms*. FEMS Microbiology Letter, 129 pp. 1-10.