PRODUCTION OF BACTERIOCINS AND BIOGENIC AMINES ISOLATED FROM TRADITIONALLY FERMENTED „SREMSKA“ SAUSAGE

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Abstract - **One of the priorities of every country is, among the other, investigation of natural resources and available potentials. Until now, Serbia has not conducted extensive research in the field of diversity and properties of autochtonous microflora isolated from traditionally fermented sausages. The consequence is the lack of scientific data necessary to apply the potential of these microorganisms in food industry. Increased number of meat products manufacturers use active starter cultures in production of fermented products. However, since Serbia does not produce its own cultures, the producers are forced to import this product choosing the most acceptable ones for consumers.**

The aim of this paper is to determine some properties of lactic acid bacteria (LAB) derived from the LAB collection isolated from traditionally fermented «sremska» sausage and to determine whether some of these properties might be considered as advantageous for use in manufacturing of meat products. The investigations that were carried out were the ability of production of secondary metabolites – bacteriocins as well as the ability of cultures to produce biogenic amines *in vitro*. Agar-difusion method in BHI medium with inoculated *L .monocytogenes* was used to determine bacetriocin activity. Investigation of biogenic amines production (histamine, putrescine, cadaverine, spermine, spermidine, tyramine, tryptamine) was carried out using high performance liquid chromatography with UV detection.

The established LAB collection with determined technologically positive properties would be the base for further research with the aim of potential production of national starter cultures.

Index-Terms - **fermented sausages, LAB, bacteriocins, biogenic amines**

I. INTRODUCTION

The number of meat products manufacturers that apply imported starter cultures in Serbia following modern trends steadily increases. As a rule, impotred stated cultures are adapted to the needs of other markets. This results in products that lack traditional sensory properties which are most acceptable for the domestic consumers.
Therefore, we started the series of investigations with the aim of selection of LAB species isolated from autochtonous fermented sausages. This will be the basis for the second stage – production of national starter cultures. Application of these cultures in manufacture of sausages would result in specific national products with characteristic and unique sensory properties which Serbian population is accustomed to, and at the same time, it would be possible to improve quality parameters of such products.

However, in order to apply certain microorganisms as starter cultures, it is necessary to undertake series of investigations in respect to their genetic, biochemical and functional properties (Vesković&Martinovic, 2005). Besides exact taxonomical identification, pure culture of starter microorganism has to exhibit stable and desirable physiological properties which will be also preserved in meat which is the substrate for LAB activity. At the same time, these microorganisms should direct and accelerate biochemical processes by its metabolic activity resulting in desirable structural and chemical changes in meat. Above all, starter culture has to be safe (must not produce toxins, biogenic amines or other metabolites harmful for humans, and must not be pathogenic). At the same time, it is expected from the ideal culture used in meat industry as starter and/or protective culture, to produce bacteriocins during fermentation that are stabile in meat matrix and are not inactivated by the components of the filling (*Hames & Hertel,* 1996). Activity of bacteriocins also has to be preserved in contact with other possible bacteriocin-producing starter microorganisms.

Biogenic amines (BA) are organic bases with alifatic, aromatic or heterocyclic structure that primarilly emerge by microbial decarboxylation of amino acids and can be found in number of foodstuffs (Silla S., 1996). Many microbial species typical for fermented products inevitably leads to accumulation of biogenic amines, especially tyramine, 2-phenylmethylamine, tryptamine, cadaverine, putrescine and histamine. Intake of considerable amounts of biogenic amines poses a health risk due to the effect of these compounds on gastrointestinal and nervous system and the effect on blood pressure.

Bacteriocins are extracellular released peptides or protein molecules, produced by some LAB that shows certain anti-bacterial properties towards some microorganisms, usually congenial to the producing bacteria. Bacteriocins production by LAB enables selective and competitive effect on microflora present in the product that may contain spoilage or pathogenic microorganisms.Today, bacteriocins, as naturally produced antimicrobial peptides or proteins, have rather interesting potential of application in food industry and act as a factor in humans' health preservation with the additional effect on increase of shelf-life of food.

II. MATERIALS AND METHODS

2.1. Cultures

For the investigation of potential bacteriocin-producing properties, we used 50 isolated and taxonomically identified LAB strains. Test microorganisms in Agar Well Diffusion Assay (AWDA) method, we used *L. monocytogenes* NCTC 10527, *Staphylococcus aureus* NCBF 1499 and *Escherichia coli* 0157:H7 NCTC 12079.

2.2. Determination of the ability of bacteriocine production by the Ln.mesenteroides ssp. mesenteroides IMAU 10231 and Ln.mesenteroides ssp. mesenteroides STRAIN J9

Test microorganisms were added to BHI agar (with 0,5% of agar) in the amount that ensured the concentration of $10^7 – 10^8$ cfu/mL in the medium. Incubated activity of created H_2O_2 was eliminated by the addition of catalase enzyme (5 mg/mL), and experiment confirmation was done by proteinase test (50 μL of proteinase with strength of 10-25 mg/mL, was added to 50 μL of the examined, neutralized broth culture). After the one hour incubation at 37°C the antimicrobial activity was determined. The existence of the test microorganism growth inhibition zone was considered sa positive result.

2.3. Biogenic amines production by LAB isolated from „sremska" sausage

We investigated the potential metabolic activity and existence of enzymatic systems for decarboxylation of amino acids which results in biogenic amines production, on 50 strains of LAB isolated from traditional "sremska" sausage. Determination was carried out on 18h old liquid LAB cultures (MRS broth, Oxoid). Concentration of the following amines were determined: histamine (HIS), putrescine (PUT), cadaverine (CAD), tryptamine (TRY), spermine (SPE), spermidine (SPD) and tyramine (TYR). Amines were determined by high performance liquid chromatography with UV detection. The analytical method is based on pre-column derivatization of amines by benzoyl chloride. One ml of the LAB culture was deproteinised by one ml of 6% trichloracetic acid in the 15ml centrifuge tube. One ml of 2M of sodium hydroxyde was added followed by one mL of 2% benzoyl chloride in acetonitrile. Derivatization mixture was left for 20 minutes at room temperature and the process was stopped by adding 2ml of saturated sodium chloride. Extraction of amines derivatives was carried out with 2ml of diethyl ether. Extraction step was repeated twice. The organic layer was transfered into clean centrifuge tube and evaporated to dryness in the stream of nitrogen at 40C. Dry residue was reconstituted in 1ml of acetonitrile, and filtered into HPLC vial through 0.45um nylon syringe filter. 10ul was injected into HPLC system.

The system consisted of Waters Alliance 2695 separation module, Waters dual lambda 2487 UV detector recording chromatograms at 254nm. Phenomenex Luna C18, 150x4.6mm, 5um column was used heated at 35C. The mobile phase consisted of acetonitrole (A) and water (B). Gradient elution was applied in order to separate 7 biogenic amines.

Blank sample (MRS broth) and fortified blank at three levels (5, 10 and 20mg/kg) were injected with each batch of samples. Recovery was in the range of 78-115%. Stadndard solution of biogenic amines derivatives was injected at the beginning of the analysis and after every 10 samples in order to monitor the stability of the derivatives during analysis.

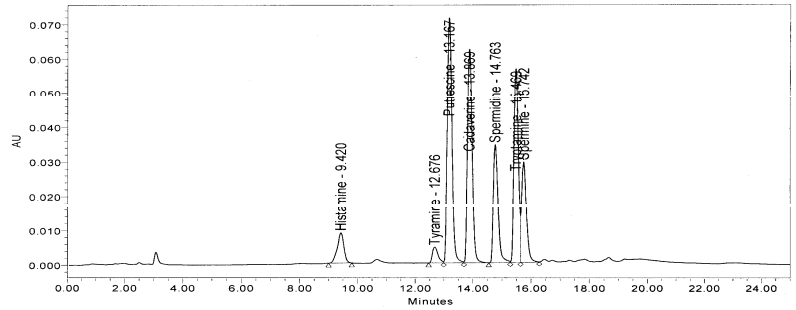
III. RESULTS AND DISCUSSION

Compared to the confirmative tests of bacteriocine activity, investigated strains of *Ln. mesenteroides ssp. mesenteroides IMAU 10231 and Ln. mesenteroides ssp. mesenteroides STRAIN J9*, in medium with *L. monocytogenes* NCTC 10527, showed the typical profile of bacteriocin producing strain (broth culture, neutralized broth and catalase reaction – positive antibacterial activity; proteinase test – negative antibacterial activity).

Investigation of bacteriocin activity of *Ln. mesenteroides ssp. mesenteroides IMAU* 10231 and *Ln. mesenteroides ssp. mesenteroides STRAIN J9* in respect to test microorganisms show pronounced bacteriocin activity of both strains to *L. monocytogenes*, while in the case of *S. aureus* and *E. coli* bacteriocin activity was not observed, i.e. bacteriocins of these LAB have no effect on these two pahogens. Other authors (*Abee,* 1995, Veskovic-Moracanin 2007, 2009) results are in accordance with our findings and suggest that the inhibitory action of bacteriocins of LAB is mostly expressed towards Gram positive bacteria. This is the main reason for using mainly *L. Monocytogenes* as test microorganism in the research of antimicrobial action of LAB (*Deaschel et al.,* 1988; *Schillinger, Lucke,* 1989). Technological utilisation of *Leuconostoc spp* is limited to the direct application of their synthesized and purified bacteriocins due to certain physiological specificities that are unacceptable from tha aspect of quality in meat industry (production of slime, acetoine, diacetyl, ethanol) (*Vesković,* 2005).

The results of investigation of biogenic amines production by LAB showed low concentrations that can be considered as non-significant from both technological and safety aspect. Various strains of *Ln.mesenteroides* produce low quantities of HIS (up to 8.0 μg/ml) and SPE (up to 7.8 μg/ml). The exception are strains obtained in the final days of fermentation where the production level of SPE is up to 15.8 μg/ml and TYR from 16.6 μg/ml to 51.9 μg/ml. In only one strain of *Ln. mesenteroides*, CAD and PUT are observed in extremely low quantities, near the analytical method detection limit. *Pediococcus pentosaceus* produced HIS and TYR up to 16.8 μg/ml, while *Lb.sakei* showed the highest level of TYR production (up to 43.7 μg/ml). This microorganism produced other amines in concenytations up to 12.6 μg/ml. Other LAB strains (*Lb. plantarum, Lb. carnosus, Lb. alimentarius)* showed very low levels of biogenic amines production *in vitro.* Investigations of other authors confirm these findings.

Fig.1 Chromatogram of biogenic amines standard



Although decarboxylation enzymes are not widely present in bacterial enzymatic systems, species of several genera such as *Bacillus, Citrobacter, Clostridium, Klebsiella, Escherichia, Proteus, Salmonella, Shigella, Photobacterium* and lactic acid bacteria *Lactobacillus, Pediococcus* and *Streptococcus* are able to produce biogenic amines by one or more amino acids decarboxylation (Santos, 1996). Food-fermenting LAB are generally non-toxic and non-pathogenic. However, some LAB strains exhibit biogenic amines production.

This property was the motive for extensive studies regarding decarboxylation activity of LAB. Straub et al., 1995 found that Lactococci, Pediococci, Streptococci (*Streptococcus thermophilus*) and *Leuconostoc* spp are not displaying this activity. Some strains of *Lactococcus* and *Leuconostoc* are described as tyramine producers (Choudhury et al., 1990). *Lactobacillus* strains *L. buchneri*, *L. alimentarius, L. plantarum*, *L. curvatus*, *L. farciminis*, *L. bavaricus*, *L. homohiochii*, *L. reuteri* and *L. sakei* were amine-positive with tyramine as the most significant produced biogenic amine (Bover- Cid et al., 2001).

IV. CONCLUSION

Fermented sausages are products of high demand all over the world, but are also the potential source of biogenic amines. Regardless of the lack of legislation defining acceptable levels of biogenic amines in fermented

sausages and other fermented products, numerous research are carried out with the aim of detemining biogenic amines quantities, having in mind their potential health effects.
The investigation results presented in this paper showed that LAB isolated from „sremska“ sausage are not significant producers of biogenic amines *in vitro.* Along with the other, technologically favourable properties, primarilly bacteriocins production, these microorganisms can be potential starter microorganisms in manufacture of meat products.

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