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ASSESSMENT OF ANTIMICROBIAL POTENTIAL OF SUBSTANCES ISOLATED FROM SOME WASTES OF MEAT PROCESSING INDUSTRY

Elena Kotenkova, Ekaterina Polishchuk

ABSTRACT

The slaughter of farm animals generates a large number of by-products. Meat waste management includes various methods, but cost-effective technologies are still in priority. This manuscript reports the results of the study of antimicrobial activity of substances isolated from such wastes of meat processing industry as bovine and pork mucous membranes and epithelial tissues. Proteomic study included two-dimensional electrophoresis with following mass spectrometric identification. Antimicrobial activity against *L. monocytogenes*, *P. aeruginosa* and *S. aureus* of neutralized native extracts and after enzymatic treatment as well as its ultrafiltrates was determined by flow cytometry with EvaGreen and PI dyes. It was shown that a large number of histones were found in bovine mucous membranes as well as several tissue-specific proteins, which would be a precursor of bioactive peptides. Bovine mucous membranes of the tongue and nasal cavity possessed the greatest activity in relation to *P. aeruginosa*, the rate of surviving cells decreased to 22.0%. Bovine mucous membranes of the rectum and the oral cavity, submandibular lymph nodes, pig mucous membranes of the larynx, tongue, lips, and rectum increased dead cells count up to 40% of all cells. Bovine nasal mucosa and pork mucous of labial cavity possessed the greatest activity against *S. aureus*, the rate of surviving cells did not exceed 10.0%. Determination of antimicrobial action against *L. monocytogenes* of native samples and treated with trypsin showed that bovine mucous membranes of the rectum and oral cavity, pork mucosa of the lips and submandibular glands were the most active. Treatment with trypsin or ultrafiltration demonstrated different effects on activity of samples. It was shown the perspectivity of recycling of such type of by-products into effective and demanded substances which can be used, for example, in the food industry as an alternative to chemical preservatives.

Keywords: AMP; antimicrobial activity; slaughter wastes; flow cytometry; mucous membranes

INTRODUCTION

The slaughter of farm animals generates a large number of by-products. The amount of such kind of wastes averages approximately about 10% to 15% of the value of the live animal in developed countries, in other countries this rate can reach for about two-third of the animal after slaughter **(Alao et al., 2017)**. The yield of animal by-products is high and ranges between 50-60% of the live weight **(Irshad and Sharma, 2015)**, including carcasses, hides, hoofs, and heads, offal, viscera, bones, fat and meat trimmings, blood **(Helkar, Sahoo and Patil, 2016)**. Non-carcass parts of animal (by-products) are divided into edible or inedible parts **(Barbut, 2015; Alao et al., 2017)**. Some internal organs, e. g. liver, kidney, hearts, tongue etc. could be used for humans food but it depends on traditions and religion **(Jayathilakan et al., 2012)**, as well as legacy regulations **(Jędrejek et al., 2016)**. Therefore, effective by-product utilization is a quite sharp problem.

Meat waste management included such methods as composting, aerobic and anaerobic digestion **(Banks and Wang, 2004; Arvanitoyannis and Ladas, 2008)**. Usage of by-products for animal feed and pet food and biofuel or solid fuel production is also widely developed **(Virmond et al., 2011; Jędrejek et al., 2016; Hamawand et al., 2017; Adhikari et al., 2018)**. On the other hand, for a safety reasons most produced feed materials are in severe restrictions in their use for feed farm animals, because slaughterhouse wastes are potentially contaminated by several pathogens **(Arvanitoyannis and Ladas, 2008; Jędrejek et al., 2016; Adhikari, Chae and Bressler, 2018)**.

Nevertheless, animal by-products are a good source of nutrients and bioactive substances, therefore its widely used in food industry, e.g. as functional ingredients, for technological applications and biopeptides production as well as for medical and pharmaceutical applications **(Toldrá, Mora and Reig, 2016; Alao et al., 2017; Chernukha et al., 2018)**. Some kind of by-products also recycled for fertilizer and chemical applications **(Irshad and Sharma, 2015; Toldrá Mora and Reig, 2016; Helkar Sahoo and Patil, 2016)**.

Concerning meat waste management, cost-effective technologies are in priority, therefore the developing progressive technologies, which can be based on by-

products as a source of certain bioactive substances is in demand.

This manuscript reports the results of the study of antimicrobial activity of substances isolated from some wastes of meat processing industry.

Scientific hypothesis

Most of the studied mammalian antimicrobial peptides (AMP) and proteins were isolated from neutrophils, but some such compounds were found in the small intestine, tongue, myeloid and epithelial cells **(Wang, Li and Wang, 2016)**. Therefore, not only the granular apparatus can be considered as a source of AMP, but also mucous membranes and epithelial tissues. These tissues due to its border position are constantly in contact with a wide range of pathogenic and opportunistic microorganisms and viruses, fungi, and therefore can potentially contain a set of substances with antimicrobial action.

MATERIAL AND METHODOLOGY

Pork mucous membranes of the larynx, tongue, labial and nasal cavities, rectum, and submandibular glands, bovine mucous membranes of the tongue, larynx, nasal and oral cavities, rectum and submandibular and lymphatic glands were selected as objects of study.

Proteomic study

Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE). The subsequent detection of the proteins was carried out by staining with Coomassie R-250 (Applichem, USA) and silver nitrate (PanReac, Spain) as described previously **(Kovalyov et al., 2006)**. The resulting digital images were edited in a graphic editor and the quantitative protein content was calculated using ImageMaster 2D Platinum version 7 (GE Healthcare, Switzerland).

Protein fractions were excised from the gel, grinded and undergone trypsinolysis (Sigma, Germany) **(Zvereva et al., 2015)**. Obtained peptides were investigated by MALDI-TOF MS and MS/MS mass spectrometry on Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser(336 nm) in the positive ion mode in molecular weight range of 500 – 8000 Da with calibration according to known peaks of trypsin autolysis.

Bioinformatics analysis

Analysis of obtained tryptic peptides mass spectra was performed using Peptide Fingerprint option in Mascot software (Matrix Science, USA) with MH+ mass determination accuracy of 0.01%; search was performed in databases of the National Center for Biotechnology Information, USA (NCBI). Comparative analysis of obtained proteomic profiles was carried out with use of information module "Proteins of skeletal muscle of cows (Bos Taurus)" of the Database "Proteomics of muscle organs" (http://mp.inbi.ras.ru).

Preparing testing samples

Grinded mucous membranes were extracted with 10% acetic acid solution at ratio 1:5, stirring speed of 400 rpm, for 5 hours at 4 – 5 °C at Laboratory dispersing equipment (Labotex, Russia). Then extracts were centrifuged (Sigma 3K30, Germany) at 15,000 rpm and 4.0 °C for 5 min. The supernatant was neutralized to pH = 6 with a 4N sodium hydroxide solution. Neutralized extracts were subjected to trypsinolysis (PanReac, activity 328 USP $U.mg^{-1}$). Ultrafiltrates were obtained by centrifugation on centrifuge ultrafilters Amicon Ultra-4 (50kDA, Millipore). Native extracts and extracts after enzymatic treatment were subjected to a sterilizing filtration on syringe filters with a pore size of 0.22 µm (Nylon L. E., Teknokroma).

Antimicrobial activity study

The activity of antimicrobial substances contained in native extracts after neutralization and after enzymatic treatment, and ultrafiltrates were studied by flow cytometry. *L. monocytogenes* ATCC 13932, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 strains were obtained from the State Research Center for Applied Biotechnology and Microbiology (Obolensk, Moscow region, Russia). Suspensions with an approximate concentration of 1 × 106 $cells.mL^{-1}$ were used as positive controls. To obtain negative control of *L. monocytogenes* ATCC 13932, the resulting suspensions were heated at 100 °C for 10 min.

Neutralized native samples and ultrafiltrates analysis protocol

A total of 10 µL of tested sample was mixed with 90 µL *P. aeruginosa* ATCC 27853 or *S. aureus* ATCC 25923 (an approximate concentration of 1×10^6 $cells.mL^{-1}$) and incubated overnight in thermostat at 37ºC. A total of 20 µL overnight incubated mixture of tested sample and bacterial cells was mixed with 5 µL of EvaGreen (Synthol, Russia), 365 µL of deionized water, and 10 µL of DMSO (Biolot, Russia); then, samples were incubated in the dark for 15 min and green fluorescence signals corresponding to live cells were measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) up to 5000 events. A total of 20 µL overnight incubated mixture of tested sample and bacterial cells was mixed with 3 µL of PI (Logos Biosystems, Republic of Korea), 377 µL of 0.9% sodium chloride solution; then, samples were incubated in the dark for 15 min and red fluorescence signals corresponding to dead cells were measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) up to 5000 events. Survived cells were calculated in relation to control and expressed in percentage, dead cells were calculated in relation to the survived cells and expressed in percentage.

Native extracts and after enzymatic treatment analysis protocol

A total of 50 µL of tested sample was mixed with 50 µL *L. monocytogenes* ATCC 13932 (an approximate concentration of 1×10^6 $cells.mL^{-1}$) and incubated overnight in thermostat at 37 ºC. A total of 20 µL overnight incubated mixture of tested sample and bacterial cells was mixed with 5 µL of EvaGreen (Synthol, Russia), 365 µL of deionized water, and 10 µL of DMSO (Biolot, Russia); then, samples were incubated in the dark for 15 min and green and red fluorescence signals were measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) up to 5000 events **(Kotenkova et al., 2019)**.

All cells corresponded to survive were taken as 100 percent, live and dead cells were calculated in percentage of survived cells.

RESULTS AND DISCUSSION

In previous study it was shown that a large number of histones were found in bovine mucous membranes as well as several tissue-specific proteins, which would be a precursor of bioactive peptides **(Kotenkova et al., 2019)**.

The proteomic study of porcine tissues was also carried out. A large number of histones were found too, such as H2B type 1-like, HIST1H2BB, H2B ½ and H2B type 1-N as well as proteins S100-A12 and AGR2, which were previously identified in bovine mucous membranes. Lysozyme C was identified in the mucous membranes of the tongue and rectum.

Bovine mucous membranes of the tongue and nasal cavity were the most active against *P. aeruginosa*, the proportion of survived cells decreased to 22.0%. In some cases, there was noticed an increase in the number of cells by almost 2 times when extracts of bovine lymphatic glands, mucous membranes of oral cavite and rectum, porcine mucous membranes of the larynx, tongue, labial cavity and rectum were added to the cell culture. However, a large amount of dead cells was noticed in these samples, the ratio of dead cells reached 40.0% of all cells. Presumably, this observation could be explained by the fact that AMP is "packed" into a protein molecule, which can be used by microorganisms initially as a substrate, and only after release demonstrate an antimicrobial effect **(Abaturov, 2011; Pasupuleti, Schmidtchen and Malmsten, 2012; Wang, 2014)**. The noted observation was confirmed by the fact that no such effect was observed while ultrafiltrates addition. In addition, addition of some ultrafiltrates with removed high molecular weight substances led to increase of antimicrobial activity. Native extracts of bovine mucous membrane of the larynx and submandibular glands salivary glands, pork mucous membrane of the nasal cavity did not have a significant antimicrobial effect against *P. aeruginosa*. Ultrafiltration of extracts of bovine mucous membranes of the larynx, oral cavity and rectum, pork mucous membranes of the larynx, tongue, nasal cavity, rectum and submandibular glands led to increasing of antimicrobial activity, in the case of bovine lymphatic glands and pork mucous membrane of labial cavity – did not affect on the activity, and in relation to bovine mucous membrane of the tongue and submandibular glands – on the contrary, reduced. Figure 1A shows the type of cytogram using Eva Green and PI dyes when antimicrobial activity was determined against *P. aeruginosa.*

Almost all native extracts were active against *S. aureus*. The greatest antimicrobial effect was observed when extracts of bovine mucous membrane of the nasal cavity and pork mucous membrane of the labial cavity were added to cell culture, the proportion of surviving cells did not exceed 10.0%. An increase of survived cells by more than 1.5 times was observed when pork rectum mucosa extract was added to cell culture. However, this sample showed a similar observation as in case *P. aeruginosa*: the proportion of dead cells reached 46.5% of all cells. It should be noted that the proportion of dead cells in all samples was significantly higher than in the experiment with *P. aeruginosa*. This observation indicated a higher activity of samples against gram-positive bacteria. Ultrafiltration of extracts in most cases did not lead to an increase in activity, except pork mucous membranes of the larynx, tongue and rectum – on the contrary, there was an increase in activity. Figure 1B shows the type of cytogram using Eva Green and PI dyes when antimicrobial activity was determined against *S. aureus.*

It was observed that EvaGreen dye, which is commonly used in PCR analysis, stained live cells of *L. monocytogenes* ATCC 13932 and fluoresced in green and red spectra; the dye also stained dead cells and only demonstrated red fluorescence **(Kotenkova et al., 2019)**. Bovine mucous membranes of the rectum and oral cavity, porcine mucous membranes of labial cavity and submandibular glands demonstrated the highest activity against *L. monocytogenes*, the proportion of living cells decreased to 2.7%. Enzymatic treatment with trypsin of extracts of bovine mucous membranes of the tongue, and submandibular and lymphatic glands, pork mucous membranes of the larynx, labial and nasal cavities, rectum resulted in increased activity, in the case of bovine mucous membranes of the larynx, nasal cavity and rectum, pork mucous membranes of the larynx, tongue and submandibular – did not effect on activity, and in relation to bovine mucous membrane of the oral cavity - on the contrary, reduced. Figure 2 shows the type of cytogram using Eva Green dye when antimicrobial activity was determined against *L. monocytogenes*.

In mammals, AMPs are most frequently found in blood, less often - in the saliva, mucous membrane of gingivae, tongue, cheeks and lips, submandibular gland and small labial glands, neutrophils, Paneth cells, tissues of small intestine, epithelial cells of nose and bronchi, and tracheae **(Kokryakov, 1995; Shamova, 1995; Jarczak et al., 2013; Shamova, 2013; Bosch-Marcé et al., 2014; Wang, 2014; Zhao and Lu, 2014; Wang et al., 2015; Zharkova, 2016)**. According to an analysis of the International UniProt Protein Database and Antimicrobial Peptide Database, porcine and bovine tissues have the high content of both AMPs and other substances with antimicrobial and antiviral action. Protegrins are determined in pigs; bovine tissues are characterized by cathelicidins and defensins. Different isoforms of lysozyme present in both animal species.

Nevertheless, in our pilot study we confirmed that mucous membranes and epithelial tissues of farm animals could be also a good source of such substances.

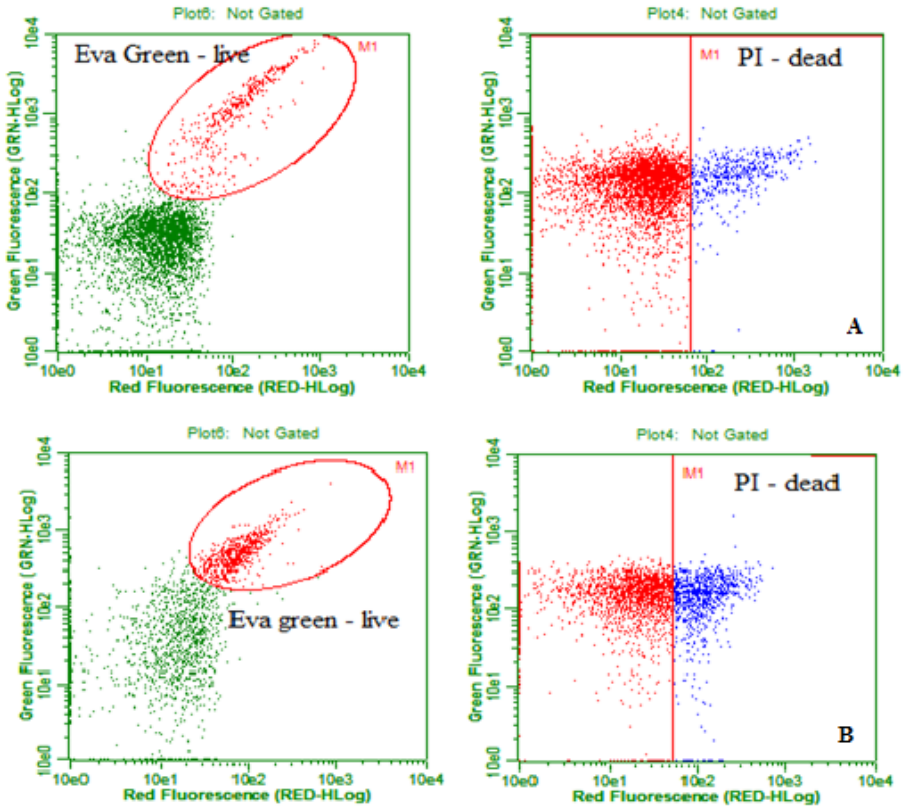

Figure 1 The type of cytogram using Eva Green and PI dyes when antimicrobial activity against *P. aeruginosa* (A) and *S. aureus* (B) of pork rectum was determined.

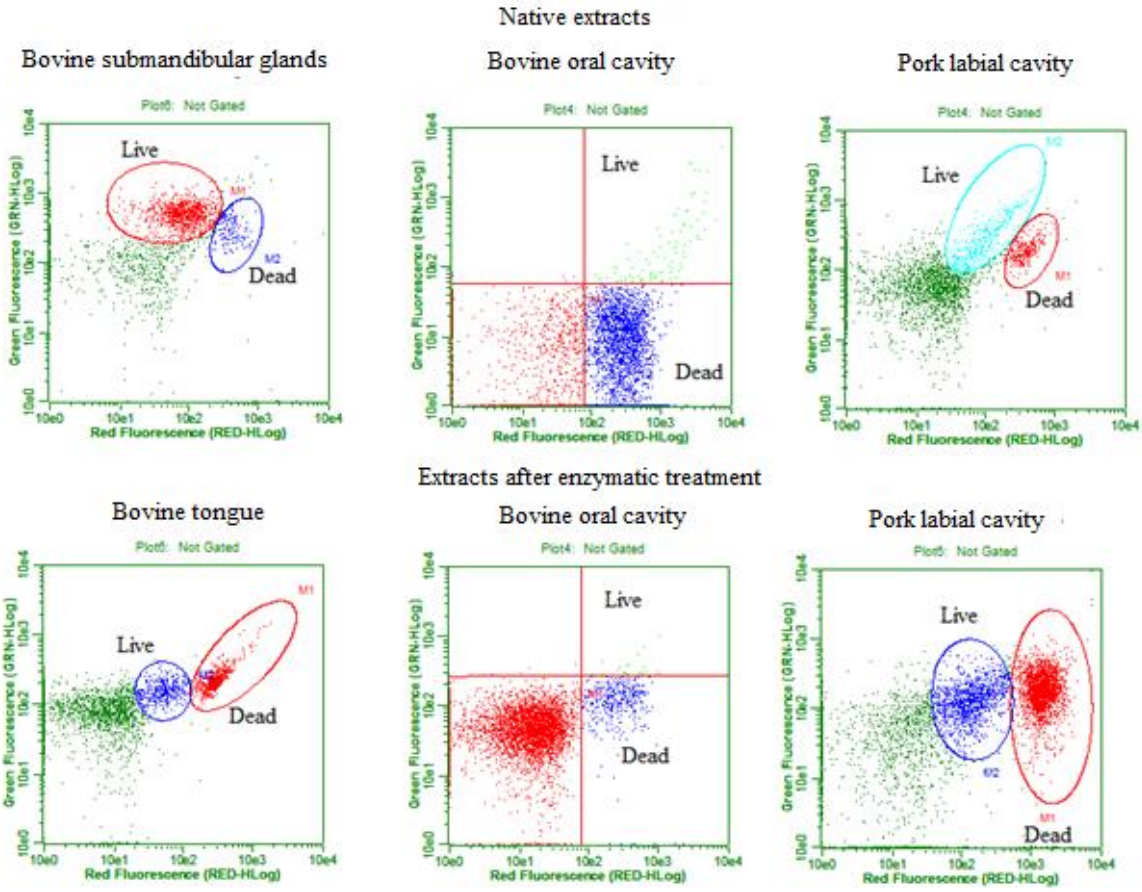

Figure 2 The type of cytogram using Eva Green dye when antimicrobial activity against *L. monocytogenes* of native extracts and after enzymatic treatment were determined.

CONCLUSION

Results of pilot study confirmed bovine mucous membranes of the tongue and nasal cavity, pork mucous membrane of labial cavity as a most promising source of antimicrobial compounds. The samples were the most active against gram-positive bacteria. It was also interesting observations found in respect of bovine submandibular lymph nodes and pork mucosa of the rectum. In connection with the revealed observation of "unpacking" of AMP from a high molecular weight protein molecule by a culture or trypsin, it is planned to consider in more detail the expediency of removal of high molecular weight substances from extracts, as well as the effects of enzyme treatment.

Moreover, it was shown the perspectivity of recycling of such type of by-products into effective and demanded substances which can be used, for example, in the food industry as an alternative to chemical preservatives.

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Contact address:

*Elena Kotenkova, V. M. Gorbatov Federal Research Center for Food Systems of RAS, Experimental-clinical research laboratory of bioactive substances of animal origin, 109316, Talalikhina st., 26, Moscow, Russia, Tel.: +79031684478,
E-mail: lazovlena92@yandex.ru
ORCID: https://orcid.org/0000-0003-1864-8115

Ekaterina Polishchuk, V. M. Gorbatov Federal Research Center for Food Systems of RAS, Experimental-clinical research laboratory of bioactive substances of animal origin, 109316, Talalikhina st., 26, Moscow, Russia, Tel.: +79260389927,
E-mail: kat.1997@mail.ru

Corresponding author: *