SCREENING OF STARTER CULTURES WITH ASSESSMENT OF SAFETY ON VERTEBRATE CELLS IN VITRO

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Abstract – The paper examines the method for determining the safety of starter cultures for production of uncooked smoked and air-dried products. The method consists of assessing the viability of cells of higher animals *in vitro* upon introducing into a culture medium probiotic microorganisms inactivated by heating or a supernatant obtained by centrifugation of bacteria. Two cell lines were examined as a test-system: NCTC clone 929, murine subcutaneous connective tissue MH-22a, hepatoma of C3HA mice. Ten strains of different microorganisms were chosen: *Lactobacillus curvatus* 111-1 (B-8949), *Lactobacillus sakei* 306-1 (B-8952), *Lactobacillu scasei* 10 (B-8890), *Lactobacillus plantarum* 2П (B-1616), *Pediococcus acidilactici* 3 (B-8891) and *Pediococcus acidilactici* 38 (B-8902), *Pediococcus pentosaceus* 39 (B-8898) and *Pediococcus pentosaceus* 55 (B-8955), *Staphylococcus carnosus* 111-2 (B-8951), *Staphylococcus xylosus* P-1 (B-8944). It was established that all strains were safe and can be used in food product manufacture. The highest effect regarding the oncocells of the MH-22a line had microorganisms *Lactobacillus curvatus* 111-1 (B-8949), *Lactobacillus plantarum* 2П (B-1616) and *Pediococcus acidilactici* 3 (B-8891).

Key Words – biotesting, cell test systems, safety of probiotics, starter cultures

I. INTRODUCTION

On October 26, 2015, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) gave an evaluation of the carcinogenicity of red meat and processed meat. The IARC Working Group of 22 experts from 10 countries after a thorough review of 800 scientific reports performed over 20 years classified red meat as probably carcinogenic to humans (Group 2A) based on limited evidence. This was mainly observed for colorectal cancer, but the correlations were also noted for pancreatic and prostate cancer. Processed meat was classified as carcinogenic to humans (Group 1) on the basis of sufficient evidence that consumption of processed meat causes colorectal cancer in humans [1,2].

The difficulty in establishing a role of a diet resides in the multifactorial nature of food systems as well as in the complex character of the oncological diseases. Comprehensive studies of the role of the biologically active components on tumor cell growth and development opens the possibilities to use food products for prevention and minimization of the cancer risk, as well as for better nutrition [3].

Nowadays, an idea of the target use of specially selected microorganisms with specific properties for production of dried and raw smoked meat products has been actively developing. Moreover, many studies describe the possibility of forming in meat the biologically active compounds with the hypotensive, opioid, anticarcinogenic properties under the action of the inherent or added enzymes [8]. As a modern trend, it is proposed to use starter cultures that have probiotic properties along with their technological characteristics.

Starter cultures are defined as preparations, which contain high numbers of live microorganisms as pure or mixed cultures and are used for achieving set goals by improving metabolism. Microorganisms contained in starter cultures should have a large variety of properties being, at the same time, safe for human health [4].

Since cells *in vitro* react on the impact of chemical and biological factors adequately to an original organism, the research work proposes the method for assessing safety of probiotic microorganisms used as starter cultures using cellular testing systems.

II. MATERIALS AND METHODS

The following cell lines (BioloT Ltd., Saint-Petersburg) were chosen as the cellular testing

systems: NCTC clone 929, murine subcutaneous connective tissue, which presents the cells of an adult animal and allows assessing the response of the normal cells, and MH-22a, hepatoma of C3HA mice, which is the murine hepatocellular carcinoma cell line and gives an opportunity to characterize the impact of objects on tumor cells.

The animal cells were cultivated by the monolayer methods in plastic polysterol flasks with the surface area of 25 cm^2. For culture cultivation, the standard Minimum Essential Medium (MEM) and bovine serum were used in a proportion of 9:1.

As the essential substances depleted and pH changed, the medium in the culture flasks was replaced every 2-3 days. After formation of a monolayer, it was taken out and inoculated. For this, the culture medium was removed, the monolayer was washed twice with the phosphate-buffered saline (PBS) solution, the solution was poured out, cells were disaggregated into a suspension: 1 ml of 0.02% Versen solution and 0.25% trypsin were added in the ratio of 3:1. For better cell detachment the flasks were placed on the horizontal temperature controlled shaker at 37 °C and rotation speed 40–50 rpm for 10 min. After achieving detachment of 90–100 % of cells, they were inoculated in the quantity of 6000–7000 cells/cm^2 using 10 ml of the culture medium with serum. The flasks were placed into the CO_2 incubator (SANYO, MCO-15 AC) at 37 °C and the gas concentration of 5.0 %. Using staining with the 0.4% trypan blue solution and Gorjaev's chamber, the viability of lines and the proliferation index were determined. The proliferation index was calculated as the ratio of the end cell concentration (N_e) to the initial cell concentration (N_i).

The proposed method for assessing the safety of microorganisms included several stages.

1. Preparation of the object under investigation.

For analysis of the toxicity, the microbial cells were grown on the solid culture medium MRS at 37 °C for 24 hours. The microbial cells, which were washed off from agar, were diluted with the sterile 0.9% physiological saline solution to the necessary amount using the turbidity standard and inactivated by heating at 100 °C for 1 hour. To study the toxigenicity, the microbial cells were grown in the liquid culture medium MRS at 37 °C for 10 days. After that, they were centrifugated at 4000 rpm for 30 min.

2. Preparation of the test-system. The monolayer of cells was grown in a flask with an area of 25 cm^2 on the culture medium EMEM, which included 10% of bovine serum. The culture medium was removed from the formed monolayer. After that, the cells were washed with the phosphate-buffered saline (PBS) solution, which was then poured out.

3. Detection of the object safety for a test-system. For analysis of the toxicity, the monolayer of cells was treated with the inactivated bacteria in amounts of 10^7, 10^8, 10^9 CFU / 0.5 ml. To study the toxigenicity, the supernatant of the bacterial cells was added to the flasks with the cell monolayer at 0.25, 0.5 ml and 1.0 ml. In both cases, the EMEM medium with 10% serum was added, held in the CO_2-incubator at 37°C and a gas concentration of 5.0% for 1 hour. Then, the medium was replaced by a fresh one, the flasks were placed into the CO_2-incubator at 37°C and 5.0% CO_2. After that, the culture medium was removed, the cells were disaggregated, the monolayer was removed, staining was carried out and the viability was assessed.

The obtained data were compared to the control, and the result (viability - V) was expressed in percentages according to the equation (1) as a ratio of the number of live cells grown on the medium with addition of an object under investigation to the total number of cells:

$$V = (N_l / N_o) \times 100 \quad (1)$$

Where, N_l is the mean number of live cells at the end of the experiment, units;

N_o is the mean total number of cells at the beginning of the experiment, units

100 is a factor for converting into percentage

If the viability was more than 50% in all experiments, a strain was considered non toxic and non toxigenic and safe upon using in the food industry. If 50% or more of the cell population died, the experiment was repeated. In the case of confirming a result, we determined the cytopathic activity (CA_{50}), which characterizes the number of microorganisms in a culture medium that leads to a reduction in a cell population by 50%, and made a conclusion that a strain was not safe and not recommended for using as a starter culture [5,6].

Ten strains belonging to 8 different species of microorganisms from the MGUPP collection were studied in this work: *Lactobacillus curvatus* 111-1 (B-8949), *Lactobacillus sakei* 306-1 (B-8952), *Lactobacillus casei* 10 (B-8890), *Lactobacillus plantarum* 2П (B-1616), *Pediococcus acidilactici* 3 (B-8891) and *Pediococcus acidilactici* 38 (B-8902),

Pediococcus pentosaceus 39 (B-8898) and *Pediococcus pentosaceus* 55 (B-8955), *Staphylococcus carnosus*111-2 (B-8951), *Staphylococcus xylosus* P-1 (B-8944) [7].

The lactic acid bacteria were cultivated on the rich MRS medium with the following composition (g/L): Peptone 10, Meat Extract 10, Yeast Extract 5, Glucose 20, Tween®80 1, Di-amonium Citrate 2, Sodium Acetate 5, K_2HPO_4 2, $MgSO_4 \times 7H_2O$ 0.2, $MnSO_4 \times 4H_2O$ 0.05. Agar in an amount of 12 g/l was used as a thickening agent. pH of the medium was set at 6.2–6.4 and the medium was sterilized by autoclaving at 121 °C for 15 min. The microorganisms were cultivated by alternating inoculation into the liquid and agar media at 37 °C for 20–24 hours.

III. RESULTS AND DISCUSSION

From a variety of lactic acid microorganisms in the MGUPP collection, 10 strains were chosen, their morphological, cultural and tinctorial properties were studied.

The chosen microorganisms are mesophilic facultative anaerobic gram positive rods or cocci, which form circular colonies on the MRS agar having paste-like consistency, white or yellow-white color and entire margins. The description of the appearance of the bacteria is given in Table 1.

In the *in vitro* systems, different cell lines have specific morphology and growth characteristics.

In the line of the mouse hepatoma MH-22a, formation of the monolayer in a flask occurred during 13 days; that is, the monolayer on the proliferation area of 1 cm^2 was formed for 13 hours. Proliferation index ($PI_{iMH-22a}$) was 10.9. The density of the monolayer was high, the cells were fairly small, round or square. The microscopic picture of the MH-22a cell line is shown in Fig.1.

In the line of subcutaneous connective tissue of a NCTC mice, formation of the monolayer in a culture flask occurred during 19 days; that is, the monolayer on the proliferation area of 1 cm^2 was formed for 18 hours. Proliferation index (PI_{iNCTC}) was 3.8. The cells of this line were fairly large, round or rectangular, and had close intercellular contacts. The microscopic picture of the NCTC cell line is shown in Fig.2.

Effect of the lactic acid bacteria strains on the viability of cell test systems was assessed.

Table 1 Microscopic picture of the selected strains

Species of microorganisms	Microscopic picture
Lactobacillus curvatus	bean-shaped rods, size: 0.7–0.9 x 1.0–2.0 μm
Lactobacillus sakei	rods with rounded ends, size: 0.6-0.8 x 2.0-3.0 μm
Lactobacillus casei	rods with square ends, occur in chains, size: 0.7–1.1×2.0–4.0μm
Lactobacillus plantarum	straight rods with square ends size: 0.9–1.2 x 3.0–8.0 μm)
Pediococcus acidilactici	cocci, size 0.6–1.0 μm in diameter, divide along two planes of symmetry forming tetrads
Pediococcus pentosaceus	
Staphylococcus carnosus	cocci, size 0.8–1.0 μm in diameter, occur in grapelike clusters
Staphylococcus xylosus	

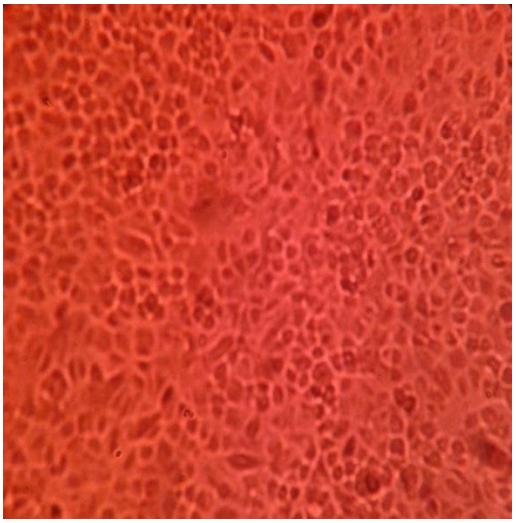

Fig. 1 Monolayer of the MH-22a line; Magnification: 10X

As in all experiments the viability of animal cells was more than 50%, all strains of the microorganisms were safe and could be used in the meat industry. Thus, the strains with the highest effect on the tumor cells of the MH-22a line, which confirmed their functional anticarcinogenic potential, were chosen.

When studying the toxicity, a dose dependent decrease in the cell line viability was established. Regarding the cells of mouse hepatoma, the highest effects had the strains B-1616 and B-8949. The microorganisms inactivated by heating did not have a significant effect on the cells of the connective tissue of a NCTC mouse; with that, the strains B-1616 and B-8949 showed the most pronounced effect.

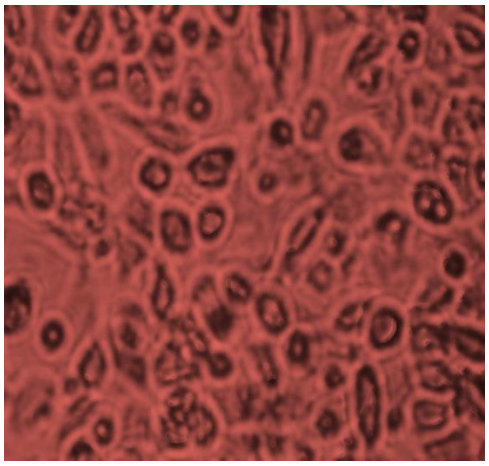

Fig. 2 Monolayer of the NCTC line;
Magnification: 10X

When studying the toxigenicity, a significant influence of the bacterial supernatants on the cellular test systems was established. A decrease in the animal cell viability occurred even at addition of 0.25 ml of the supernatant of several bacterial strains. The bacterial strain B-8891 affected significantly NCTC cell lines, while strains B-1616 and B-8949 showed higher cytotoxic activity against the tumor cell line MH-22a.

Therefore, the highest effect on the oncocells MH-22a had the bacterial strains *Lactobacillus curvatus* 111-1 (B-8949), *Lactobacillus plantarum* 2П (B-1616) and *Pediococcus acidilactici* 3 (B-8891).

From them, two strains of different species (*Lactobacillus plantarum* 2П (B-1616) and *Pediococcus acidilactici* 3 (B-8891)) were chosen in order to develop a bacterial composition.

IV. CONCLUSION

The basis for establishing a relationship between the components of a diet and the key events associated with cancer prevention is based on the knowledge about the corresponding tissue and cell. This research aimed at assessing the cell response to probiotic microorganisms. Ten strains of different lactic acid microorganisms were chosen from the MGUPP collection, pure lines of these microorganisms were obtained and their morphological properties and the microscopic picture were studied.

NCTC clone 929 (subcutaneous connective tissue, mouse) and MH-22a Cell Line (Mouse C3HA, hepatoma) were chosen as the cell test systems.

Toxicity and toxigenicity of bacterial cells for the test cultures were determined. Based on the indicators of animal cell viability the conclusion was drawn that the tested strains of probiotics were safe.

The cytostatic effect manifested itself more significantly for the hepatoma cells, which suggested a possible anticarcinogenic action of the microorganisms. The highest effect on the oncocells of the line MN-22a had the bacterial strains *Lactobacillus curvatus* 111-1 (B-8949), *Lactobacillus plantarum* 2П (B-1616) and *Pediococcus acidilactici* 3 (B-8891).

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