Cheese bioconservation technologies using listeriosis phage during the stages of cheese head ripening

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Abstract. The analysis of bacteriophages usage in cheese production to reduce food listeriosis risk was performed. Products based on listeriosis bacteriophages are recommended in the United States and Western Europe for cheese processing. An increase in the activity of domestic cheese producers causes the relevance of phage-containing compositions use on the basis of listeriosis bacteriophages, as well as the development and testing of the technology of cheese bioconservation at the stage of cheese head ripening using a phage-containing composition. Technologies of cheese bioconservation at the stages of cheese head ripening using a phage-containing composition have been designed and tested.

1. Introduction
Nowadays, the creation of a safe food space is a top target of the research and technical sphere of the world community.

One of the most important economic and industrial challenges facing humanity in the modern world is the problem of preserving the food product quality, which can be exposed to a variety of microorganisms - bacteria, yeast and mold fungi. Every year, millions of people throughout the world are infected because of low-quality food consumption. Especially dangerous in this case are pathogenic microorganisms, the ingestion of which in the human body can cause serious diseases. According to WHO, 2.2 million people die from foodborne infections a year. Global economic losses due to this factor are more than 30% of the total losses. This review presents data concerning the peculiarities of microbiological spoilage of various food products and promising ways to combat this phenomenon [19].

On the world market, cheese is a high-value natural food product. In most developed countries, the average per capita consumption of cheese is 10-15 kg, and in Italy, France and Israel – more than 20 kg per year. In Russia, this number is slightly more than 6 kg per year, with the recommended rate of consumption of cheese for the human body of 6.5 kg per year. The largest part of the cheese market is taken by hard cheeses (65 %), the second place is taken by whizzed cheeses (24 %) and only 11% is accounted by soft and lactic cheeses [20].

Milk for cheese making is regarded as cheese-making if it has an optimal content of not only proteins, fat, nonfat milk solids, calcium, but also has specific microbiological indicators allowing it to be used without pasteurization.
Bacterial load of milk for cheese production is characterized by both the total amount and the qualitative composition of the microflora, namely, the presence of gas-forming bacteria. The latter content in milk leads to various defects in the cheese ripening.

Today, one of the most pressing tasks of ensuring microbiological safety of food products is to decrease food listeriosis risk – a disease caused by Listeria monocytogenes bacteria. Particularly susceptible to this disease are pregnant women, elderly people, as well as ones with immunodeficiency. Listeriosis is characterized by high mortality in children and people with immunodeficiency [16].

In the case of fermented dairy products, cheeses are the most susceptible to L. monocytogenes contamination. According to the FDA, the probability of the presence of L. monocytogenes in cheese made from unpasteurized milk is 50-160 times higher compared to cheese made from pasteurized milk [14]. Nevertheless, during cheese head ripening, contamination of the product by L. monocytogenes is also likely [2].

In Western Europe and the United States of America, agents for the biocontrol of L. Monocytogenes have already been designed and produced [3,4]. Intralytix company (USA) manufactures Listshield, and Micreos company (Netherlands) produces Listex. These tools are designed taking into account the L. monocytogenes isolates circulating in the territories of these countries. However, currently, the import of cheeses from Western Europe and the United States to the territory of the Russian Federation is excluded. Thus, it is possible to predict an increase in the activity of domestic cheese producers, which will result in the need to improve the control of listeriosis.

The improvement and subsequent introduction into practice of the technology of cheese head bioconservation at the stage of its ripening will not only fully preserve the nutritional value and taste qualities of cheese, but also lower the risk of sporadic cases and breakouts of listeriosis, and, therefore, fatal risk cases.

This study aims to develop and test cheese bioconservation technology at the stage of cheese head ripening using a phage-containing composition.

The goal was fulfilled by solving the following issues:
- dosage regimen development of a phage-containing composition for cheese bioconservation at the stage of cheese ripening;
- testing the efficiency of the developed technology of cheese bioconservation in the process of cheese head ripening.

2. Materials and Methods
The phage-containing agent was a composition consisting of listeriosis bacteriophage in a titer of 109 BFU / ml in a sodium phosphate buffer with a neutral pH.

A non-pathogenic strain of L. innocua was used as a host strain for the bacteriophage’s cultivation. For infection of the cheese head, L. monocytogenes was used as a test strain. Daily cultures of L. innocua and L. monocytogenes strains from a dense nutrient medium were inoculated into test tubes with meat-peptone broth and cultured in a thermostat at 37°C for 18 hours. Further, the broth cultures were dissolved with sterile saline solution according to the turbidity standard of 2 McFarland units, which corresponds to 5 units of the turbidity standard-2.5-5.0 x 108 CFU / ml (turbidity OSO, Scientific Centre for Expert Evaluation of Medicinal Products).

Bacteriophage titration was done using the Appelmann and Grazia methods.

The Appelman method is based on the inclusion of different amounts of titrated bacteriophage in broth seeded with the same dose of homologous microbe culture, for obtaining the bacteriophage phenomenon [1, 13, 18].

Twelve test tubes containing 4.5 ml of meat infusion broth were set in a rack in a single row. 0.5 ml of the test phage was injected into the first test tube with a sterile pipette. Test tube contents were stirred and 0.5 ml of liquid was transferred to the third and then to the tenth inclusive. From the tenth test tube, the extra 0.5 ml was poured out. The eleventh and twelfth test tubes are test ones. Liquid transfer from one test tube to another was performed each time using a separate sterile pipette with a
capacity of 1 ml. Therefore, in 10 test tubes, dilutions of the bacteriophage were obtained from 1:10 до 1:10^10.

All 10 test tubes of the prepared series of dilutions were filled with 0.2 ml of an 18-24-hour broth culture of bacteria of the same name as the titrated phage. The additional culture did not contain phage-resistant cells. Microbial suspension density added to the test tubes with titrated bacteriophage was not relevant, since the change in microbial concentration in the range from 100 thousand to 4.5 million in 1 ml did not have a significant effect on titration findings of the bacteriophage.

The 11th test tube was a test culture. It contained 5 ml of broth and 0.2 ml of broth culture of bacteriophage.

The 12th test tube was a sterility control and contained 5 ml of broth without the addition of culture and phage.

A rack with test tubes was placed in a thermostat at 27°C for 18-20 hours. These results were recorded immediately after the tubes were taken out of the thermostat. It was assumed that the maximum number at which the complete lysis of the culture sensitive to it was observed was the titer. This matched the last test tube in the row, in which the broth was still completely clear. In a number of studied bacteriophage dilutions, for example, the broth in the first 7 test tubes remained completely transparent. Starting from the 8th test tube, turbidity of the medium appeared, which increased in further test tubes. Consequently, the bacteriophage titer in this case corresponded to the contents of the 7th test tube and could be expressed 10^7.

The Grazia agar layer method is based on the inclusion of various dilutions of the titrated bacteriophage in the corresponding bacterial culture in the agar layer on a dense nutrient medium, for obtaining negative colonies (plaques) bacteriophage [1, 13, 18].

Before the day of the experiment, nutrient media were prepared. 1.5% meat infusion (heart-brain) agar was poured into Petri dishes in an amount of 25-30 ml. The agar was covered with sterile filter paper and dried for 30 minutes in a thermostat or 2-3 hours at room temperature, after which it was covered with a lid and left at night in the room. Agar prepared for use should be completely dry, since even a slight moistening can change the quantitative readings of the content of phage corpuscles in the test liquid.

0.7% meat infusion (heart-brain) agar, poured into 2.5 ml test tubes and sterilized in them, can be prepared a few days before the experimentation.

The number of Petri dishes, as well as tubes with 0.7% meat infusion agar, should match the number of tubes with phage dilution.

In the experimentation day from the liquid containing the titrated phage, a series of consecutive ten-fold dilutions was prepared in test tubes. Sterile meat infusion broth or saline solution was used to dilute the phage.

Then, 1 ml of dilutions of the titrated phage were poured into test tubes with 0.7% meat infusion (heart-brain) agar, melted in a water bath and cooled to 44-46°C. Together with the phage, 0.1 ml of the same phage-sensitive culture was added to each tube.

Test tube contents were stirred by rapidly rotating the test tubes between the palms, avoiding the formation of air bubbles, and poured a second layer into cups with 1.5% agar. After the medium cooled, the seeding cups were placed in a thermostat at 27°C.

These results were recorded after 24 hours of incubation of the crops in a thermostat, counting the number of plaques on 2-3 cups sown with the highest dilution phage. The arithmetic mean number of colonies was obtained from these data and the resulting value was multiplied by the degree of maximum dilution.

Cultivation of the bacteriophage strain of the phage-containing agent was performed using a technology based on the method described by the authors in Patent No. 2525141 of the Russian Federation [12].

To do this, the bacterial culture of the host strain in a titer of 108-109 CFU / ml was seeded into a culture vessel on slant meat infusion (heart-brain) agar and cultured for 3-3. 5 hours at 27°C. After that, a uterine bacteriophage was sown on the resulting lawn of the host strain culture in a titer of 105-
106 BFU/ml, a culture vessel was hermetically sealed and cultured for 13-15 hours at 27°C. Afterwards, the phagolysate was received by suspending the bacteriophage from the surface of a dense nutrient medium with a buffer solution with a pH of 7.0-7.2, the phagolysate was evacuated to a sterile container, and centrifuged for 30-45 minutes at 5000-6000 rpm. The resulting phagolysate supernatant was sterilized by filtration through a filter with a pore diameter of 0.22 microns.

Microbiological indicators of the phage-containing agent were evaluated according to TR CU 021/2011 (Annex 2, section 1.8, 1.9), and individual requirements which are dictated by the special product features (Table 1) [17].

| Name of the indicator | Indicator’s value | Control method |
|-----------------------|-------------------|----------------|
| QMAFAnM, CFU/g, no more | 10 | GOST 10444.15-94 |
| Coliforms in 10.0 g | Is not allowed | GOST R 52816-2007 |
| E. coli in 50.0 g | Is not allowed | GOST R 52830-2007 |
| S. aureus in 25.0 g | Is not allowed | GOST R 52815-2007 |
| Yeast and mold CFU / g, no more | Is not allowed | GOST 10444.12-88 |
| Staphylococcal enterotoxins, in 5 samples of 25.0 g | Is not allowed | MUK 4.2.2429-08 |
| Verocytotoxins, in 5 samples of 25.0 g | Is not allowed | MUK 4.2.2963-11 |
| Living cells of producers (S. aureus, E.coli), in 50.0 g | Is not allowed | Direct inoculation method (MUK 4.1/4.2-588-96) |

Sanitary and microbiological studies were also conducted in accordance with GOST 10444.12-88, GOST 30347-97, GOST R 52814-2007, GOST R 52816-2007 and MUK 2.3.2.721-98 [5,6,7,8,15].

In the sanitary and microbiological study of all samples, indicator, conditionally pathogenic and pathogenic microorganisms were not seeded.

Russian Camembert cheese according to GOST R 53379-2009 was applied as a research object [14].

The study of cheese samples was performed in accordance with GOST 32031-2012, TR CU 021/2011, GOST R 52054-2003 and GOST R 52090-2003 [5,9,10,11,17].

In order to determine the dosage regimen of the phage-containing composition, contamination of the L. monocytogenes cheese head was carried out at the rate of 104 CFU/cm2. The contaminated cheese head was dipped in a phage-containing composition with a different bacteriophage titer per unit surface area. This control sample was dipped in a sodium phosphate buffer with a neutral pH. Treated cheese head was incubated at 27°C. The results were recorded after 24 hours. For this purpose, flushes were made from the surface of the cheese heads. After that ten-fold dilutions of flushes were prepared, accompanied by sowing from the received dilutions on a selective nutrient medium for the isolation of listeria.

3. Results and Discussion

Findings of determining the optimal dosage regimen are given in the table 2.

| The titer of the bacteriophage, the BFU/cm2 | Control | 1.0×10^3 | 1.0×10^6 | 1.0×10^7 |
|---------------------------------------------|---------|----------|---------|---------|
| Titer of L. monocytogenes after 24 hours, CFU / cm2 | 3.80×10^6± 0.72×10^6 | 4.16×10^4± 0.48×10^4 | 2.20×10^2± 0.80×10^2 |
Data from Table 2 indicate that the bacteriophage titer of $1.0 \times 10^7$ BFU/cm$^2$ was the most efficient for cheese bioconservation.

Then the developed technology of cheese bioconservation was tested during the cheese head ripening process.

The test technology of bioconservation of cheese heads, L. monocytogenes was infected at the rate of 10^4 CFU/cm$^2$. Cheese heads were divided into an experimental and control group. Contaminated cheese heads from the experimental group were dived into a phage-containing composition at the rate of 10^7 BFU/cm2. Control cheese heads were dipped in a sodium phosphate buffer with a neutral pH. These cheese heads were kept for 21 days at 4°C. Every 3 days, cheese heads were tested for contamination with L. monocytogenes. Test findings are given in Table 3.

Table 3. Testing of cheese bioconservation technology.

| Sampling control points | Content of L. monocytogenes, lg CFU / cm$^2$ |
|-------------------------|---------------------------------------------|
|                         | Experimental group | Control group |
| 0 days                  | 4                 | 4             |
| 3 days                  | 0                 | 4             |
| 6 days                  | 0                 | 5             |
| 9 days                  | 0                 | 5             |
| 12 days                 | 0                 | 6             |
| 15 days                 | 0                 | 6             |
| 18 days                 | 0                 | 7             |
| 21 days                 | 0                 | 7             |

Note: number of samples at each control point – n=5

Table 3 shows that cheese biodecontamination using a phage-containing composition was successful during the ripening period of cheese head.

Development and further implementation of phage bioconservation technology of the cheese head at the stage of its ripening will not only fully preserve the nutritional value and taste qualities of the cheese, but also lower the risk of sporadic cases and breaks of listeriosis. Consequently, it will reduce the risk of fatal cases. Furthermore, listeriosis bacteriophages can considerably expand the range of source raw milk materials.

It is particularly important to mention the genetic control of the strains of listeriosis bacteriophages used to exclude phages carrying integrase genes, that encode antibiotic resistance and other objectionable genes.

Therefore, it is essential to adapt phage-containing compositions based on listeriosis bacteriophages, as well as to develop and test the technology of cheese bioconservation at the stage of cheese head ripening using a phage-containing composition.

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