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Development and evaluation of technology for preserving hard cheese with staphylococcal bacteriophage

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ABSTRACT

Despite the control of dairy products by microbiological indicators, this category of products is among those that most often cause alimentary infections and poisonings among consumers. At the same time, the most dangerous food pathogens are *Campylobacter*, *Salmonella*, *Listeria monocytogenes* and toxin-producing pathogens – *Staphylococcus aureus*. Therefore, the issue of increasing the microbiological safety of dairy products, including hard cheese, is constantly relevant. This search aimed to determine S. aureus contamination of raw milk and hard cheeses and assess the influence of isolated bacteriophages on the development of staphylococci and lactic acid microorganisms during cheese ripening. *S. aureus* was not detected in 25.5% of raw milk samples received for processing. In comparison, the bulk of the milk – 52.9% of the samples – was contaminated with coagulase-positive staphylococci up to 500 CFU/ml. 21.6% of raw milk samples at the processing plant contained *S. aureus* more than 500 CFU/ml. Hard cheeses sold in the trade network did not contain *S.aureus* in 70.4% of samples; in 22.2%, its amount did not exceed 5 ×102 CFU/g, and in 7.4% of cheese samples, the content of *S.aureus* was higher than the standard norm of 5 $\times 10^2$ CFU/g. From 30.4 to 60.8% of raw milk samples contained virulent phages that lysed *S. aureus*, which was isolated from hard cheeses and dairy raw materials. Two phages (No. 4 and No. 8) were isolated from raw milk, which showed 80.0 – 90.0% virulent activity in four crosses against both *S. aureus* isolated from milk and rennet cheeses. These phages were introduced into the technology of hard cheese production. The addition of virulent staphylococcal bacteriophages to the milk mixture (2 ml per 1 l at a concentration of 10^8 CFU/ml) during the production technology of hard cheese allows its preservation against the development of *S. aureus*. Thus, a technology for the biocontrol of *S. aureus* in hard cheese has been developed, almost wholly neutralizing it during production.

Keywords: staphylococcal bacteriophages, phage control, rennet cheeses, cheese technology

INTRODUCTION

Hard rennet cheeses are characterized by high nutritional value and biological composition and are a source of well-digested protein **[1]**, and **[2]**. Typically, hard rennet cheeses are produced from cow's milk, and the technological process is lengthy **[3]**, and **[4]**. The main microflora of this dairy product is the lactic acid microbiota of the added starter, a small part is the residual microflora of pasteurized milk, technological equipment and objects that have contact during the entire technological process **[5]**, and **[6]**. Therefore, high-quality hard cheese is considered valuable not only due to the nutritional components of milk **[7]**, but also thanks to the vital activity of its starter microflora, which is selected and survives during the complex technological process of maturation **[8]**, and **[9]**. According to **[10]**, the following microbiological safety indicators are monitored in hard cheeses: coliform bacteria, *S. aureus*, *Salmonella*, and *Listeria monocytogenes*. In particular, coliform bacteria are not

allowed in 0.01 g, S. aureus in 1 g, no more than 5 ×10^2 CFU, and salmonella and listeria in 25 g **[10]**. Despite the control of hard cheese, as well as other dairy products, by microbiological indicators, this category of products is classified as one of those that most often cause alimentary infections and poisonings among consumers **[11]**, **[12]**, and **[13]**. According to the European Food Safety Administration and the European Centre for Disease Prevention and Control, 4,005 food outbreaks were reported across the EU and the UK in 2021, affecting 32,543 people **[14]**. Similar statistics in the US show that approximately 56,000 people are hospitalized yearly due to alimentary illnesses **[15]**. WHO estimates that unsafe food causes 600 million cases of foodborne illness worldwide each year, resulting in 420,000 deaths **[16]**. The most dangerous food pathogens are *Campylobacter*, *Salmonella* and toxin-producing pathogens - *Staphylococcus aureus* **[17]**, **[18]**, and **[19]**.

Given this trend, the issue of increasing the microbiological safety of dairy products, including hard cheese, is constantly relevant, and the development of new safe methods of inhibiting the growth of pathogenic microflora in it or reducing them during storage and sale is always promising.

Several technological approaches are used to increase the safety of perishable food products during their production and sale. In particular, these are temperature processing (pasteurization, sterilization, freezing) **[20]**, **[21]**, **[22]**, and **[23]**, processing under high pressure **[24]**, with ultrasound **[25]**, with ultraviolet radiation **[26]**, etc. At the same time, the use of antimicrobial substances of synthetic or natural origin to influence the microflora of a food product (chemical preservatives, natural essential oils, etc.) is quite widespread **[27]**, **[28]**, and **[29]**. Each of these methods has its advantages and disadvantages regarding the influence on the product microbiota and the main ingredients of the food matrix.

One of the generally recognized and common disadvantages of all these methods is that they affect all microorganisms, i.e. inhibit both pathogenic and potentially beneficial autochthonous lactic acid bacteria of the “normal” microflora of fermented products **[30]**, and **[31]**. Furthermore, even using these available methods of microflora neutralization, foodborne outbreaks still occur relatively frequently **[32]**. The above factors collectively illustrate the need for a targeted antimicrobial approach that can be used in food technology alone or with other methods to set up additional barriers to prevent foodborne bacterial pathogens from reaching consumers.

An ecologically safe way to suppress and destroy microbiota in a food product can be the technology of "biological preservation" with the help of lytic phages specific to a particular pathogen of the product without harmful influence on the normal sourdough microflora. This approach is called “bacteriophage biocontrol” or “phage biocontrol” **[33]**, **[34]**, and **[35]**.

Bacteriophages are viruses common everywhere in nature and infect only bacterial cells. These organisms are characterized by high specificity, an important feature that allows them to be used in the food industry **[36]**. Phages are used in three sectors of the food industry: primary production, bio sanitation, and biopreservation. In bio sanitation, phages or the enzymes (endolysins) they produce are mainly used to prevent the formation of biofilms on the surfaces of equipment used in production facilities. In biopreservation, phages extend the shelf life of products by controlling pathogenic bacteria that spoil food **[37]**. In addition, in recent years, the trend of consuming healthy food products without chemical preservatives has been increasingly spreading, and bacteriophages can be a good alternative to this process **[38]**.

Therefore, the use of specific bacteriophages against specific pathogens of food-borne infections and toxicoses in the production technology of dairy products to increase their safety without harmful influence on the products themselves and beneficial microflora is relevant and requires thorough research.

The work aimed to determine S. aureus contamination of raw milk and hard cheeses and assess the influence of isolated bacteriophages on the development of staphylococci and lactic acid microorganisms during cheese ripening.

Scientific Hypothesis

The added lytic staphylococcal bacteriophages to the hard cheese production technology are expected to neutralize staphylococci, increasing the safety of the finished product and its storage stability during sale.

Objectives

The main objective of this study was to develop and test a biopreservation technology for hard cheese using virulent staphylococcal bacteriophages to suppress Staphylococcus aureus without affecting the beneficial lactic acid microflora. Additionally, the study aimed to evaluate the occurrence of S. aureus in raw milk and hard cheeses and determine the effectiveness of selected bacteriophages during cheese ripening.

MATERIAL AND METHODOLOGY

Samples

Samples description:

Raw milk samples were collected at milk processing plants in Ternopil region (Ukraine), and hard cheese samples were collected at supermarkets in Ternopil and Kamianets-Podilsk (Ukraine). Approbation of technology for producing hard cheese with staphylococcal bacteriophage was tested at the Chortkiv Cheese Plant (Chortkiv, Ukraine).

Raw milk samples were collected in sterile 200 ml flasks during delivery to the processing plant from tanker trucks, and cheese samples were collected in 200 g plastic trays in supermarkets.

Samples collection:

The collected milk and cheese samples were placed in a refrigerated bag and transported to the laboratory for analysis. The time between sample collection and delivery to the laboratory for analysis did not exceed two hours.

Samples preparation:

In the laboratory, the samples were unpacked, weighed at 10 ml/g, the milk was stirred, the cheeses were homogenized, heated in a water bath to +30 °C, and prepared for microbiological examination.

Number of samples analysed:

51 samples of raw milk were investigated; 27 samples of hard cheeses; 10 isolates of *S. aureus* from raw milk and 10 from hard cheeses; 10 staphylococcal phages isolated from raw milk; 5 samples of Dutch cheese with staphylococcal bacteriophage and 5 samples of cheese without bacteriophage (control).

Chemicals

Chloroform (Klebrig, Austria, pharmaceutical); sodium chloride (Klebrig, Austria, pharmaceutical); sodium citrate (Klebrig, Austria, pure for analysis).

MRS-agar (HiMedia, India); Baird-Parker agar (Merck KGaA, Germany); meat peptone agar, meat peptone broth (Pharmaktiv, Ukraine).

Animals, Plants and Biological Materials:

In this study, 10 isolates of *S.aureus* were isolated from raw milk and 10 from hard cheeses; 10 staphylococcal phages from raw milk were isolated.

Instruments

Bacterial filters with pores of 0.45 and 0.22 microns (Johnson Test Papers, Great Britain); laboratory water bath Zenithlab HH-S4 1000W (Zentithlab, China); laboratory thermostat (Mizma, Ukraine).

Laboratory Methods

S. aureus contamination of raw milk and cheeses was determined using standard methods by inoculating selected samples and performing tenfold dilutions on Baird-Parker agar selective medium with subsequent incubation at a temperature of +37 for 48 hours according to the national standard of Ukraine DSTU 7357:2013. A similar method determined Lactic acid microorganisms in cheese during its ripening, but using MRS-agar DSTU 7357:2013.

According to general methods, bacteriophages from dairy raw materials were isolated using S. aureus bacterial cells as specific hosts **[39]**. Bacteriophages were separated from microbial cells by filtration through bacterial filters with 0.45 μm pores **[40]**.

The lytic activity of the isolated bacteriophages was determined against staphylococcal cultures using a generally defined method **[40]**. For this purpose, 3 – 4 drops of an 18 – 24 hour broth culture of the studied microorganisms were pipetted onto the surface of meat peptone agar. The optical density of the inoculum was 0.5 McFar-Land units (control using a densitometer), corresponding to 1.5×10^8 microbial cells/ml. Then, the cultures were evenly distributed over the surface of the medium with a sterile spatula. The plates with the inoculated media were dried in a thermostat for 15 – 20 minutes. After that, a drop of the phage under investigation was applied to the surface of the medium with a pipette, the cup was tilted so that the drops flowed down, and then incubated at a temperature of 37 °C; the results were evaluated after 18 – 24 hours. As a control, sterile nutrient broth was applied to the surface of the dense nutrient medium with the culture. The degree evaluation of lysis was carried out by four crosses, where "++++" is a drainable (full) lysis; "+++" is a semi-complete lysis, growth of the culture in the lysis zone; "++" is the presence of more than 50 phage colonies (lysis spots) at the site of application of the phage drop; "+" is the presence of from 20 to 50 phage colonies at the site of application of the phage drop; "+ /-" is the presence of less than 20 phage colonies at the site of application of the phage drop; – complete absence of lysis. The results from "+++" to "++++" were considered as positive reactions.

Sample preparation:

Classical technology was used during the development of the technology for preserving Dutch cheese with staphylococcal bacteriophage, but in the experimental samples, during a technological operation such as adding a leavening agent, calcium salts, and rennet to the milk mixture, a composition of staphylococcal bacteriophage was additionally added in an amount of 2 ml per 1 liter of milk mixture. Subsequently, all technological operations did not differ from each other.

Quality Assurance

Number of repeated analyses: All measurements of instrument readings were performed three times.

Number of experiment replication: The number of repetitions of each experiment to determine one value was three times.

Description of the Experiment

At the first stage, contamination with *Staphylococcus aureus* of raw milk supplied to the dairy plant to produce hard cheeses and cheeses of various brands purchased in supermarkets was determined. At this stage, cultures of Staphylococcus aureus were isolated, which served as hosts for the isolation of lytic bacteriophages. At the second stage, lytic phages were isolated from samples of raw milk and hard cheese and their anti-staphylococcal effect was determined. The most promising active phage strains were selected for use in hard cheese technology and their accumulation to the appropriate concentration was carried out. Dutch cheese was produced at the third stage, during the technology of which a bacteriophage composition was used and the effect of the added phages on the dynamics of changes in staphylococci and lactic acid bacteria during cheese ripening was investigated. At the last stage, statistical processing of the obtained results and the reliability of our hypothesis were carried out.

Laboratory accreditation

The experiments were conducted in an accredited laboratory. Certificate of compliance of the measurement system with the requirements of DSTU ISO 10012:2005 No. 02-0044/2023 dated June 7, 2023. Permit to work with pathogens of pathogenicity groups III - IV No. 01/67 dated January 13, 2022.

Statistical Analysis

Statistical processing of the results was carried out using methods of variation statistics using Statistica 9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the mean (SE) standard error were determined. The difference between the comparable values was considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

Along with the problem of food toxicosis caused by enterotoxigenic staphylococci **[12]**, and **[41]**, these bacteria pose an even more significant threat to public health as they are resistant to antimicrobial preparation **[42]**, and **[43]**. After all, the resistance of microorganisms to antimicrobial preparations has become one of the main threats to public health in the twenty-first century **[44]**, and **[45]**. According to the latest estimates, 1.27 million deaths were caused by antibiotic-resistant bacteria in 2019 **[46]**. Among them, *S. aureus* ranks second in the list of microorganisms responsible for mortality due to antibiotic resistance in high-income countries **[19]**, **[47]**. Since *S. aureus* is responsible for various septic processes, ranging from nosocomial infections associated with high morbidity and mortality in humans to infections in productive animals used by humans **[48]**, and **[49]**. Therefore, taking into account the consequences that dairy products that are excessively contaminated with this ubiquitous microorganism can cause while developing the technology for preserving Dutch cheese with staphylococcal bacteriophage, we determined the level of *S. aureus* contamination of raw milk and hard cheeses in Ukraine. At the same time, this milk is accepted according to the state standard **[50]** with a number of mesophilic bacteria not exceeding 1×10^5 CFU/g and is used to produce hard cheeses.

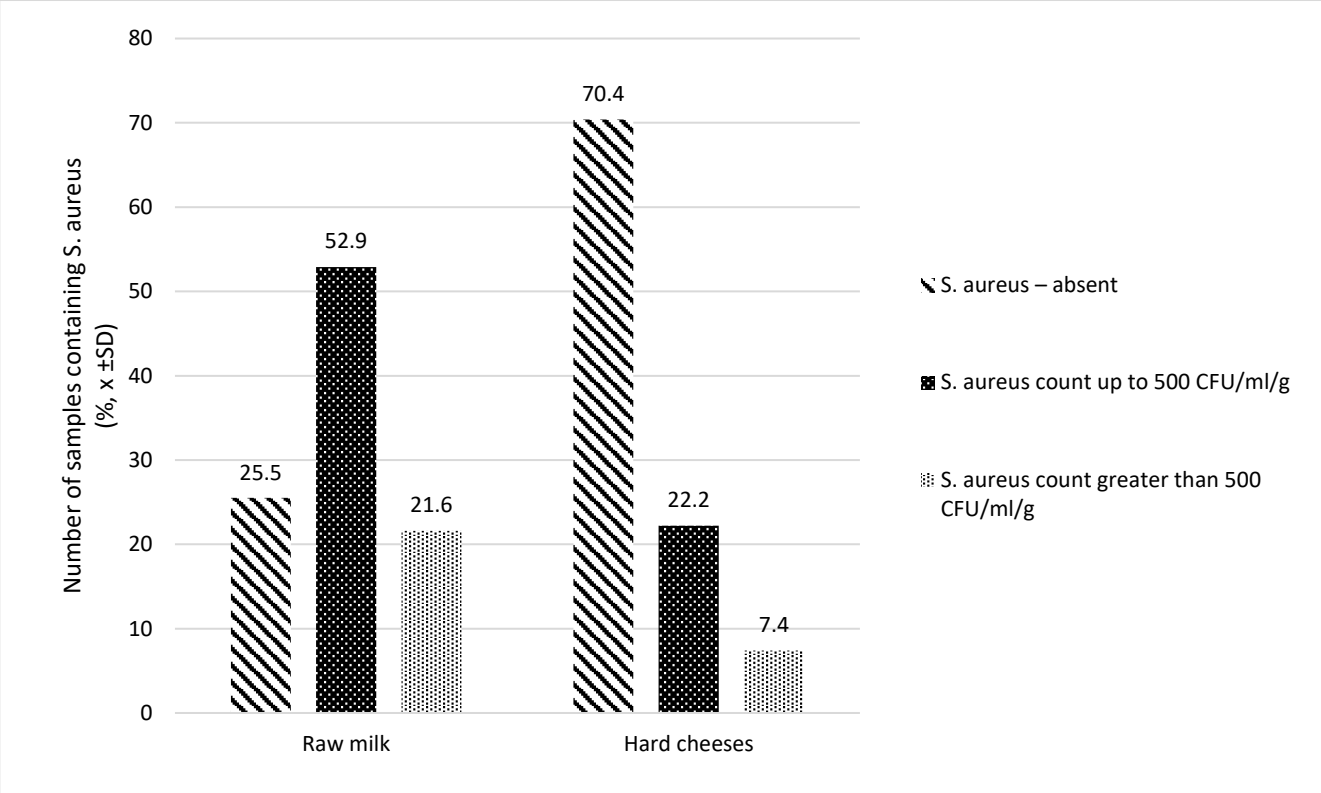

Figure 1 Contamination of raw milk and hard cheeses with *Staphylococcus aureus*.

It was found in Figure 1 that in 25.5% of raw milk samples received for processing, *S. aureus* was not detected. In comparison, the bulk of the milk – 52.9% of the samples – was contaminated with coagulase-positive staphylococci up to 500 CFU/ml. In addition, 21.6% of raw milk samples at the processing plant were recorded with a *S. aureus* count of more than 500 CFU/ml. That is, even high-quality milk by the number of mesophilic microorganisms accepted for processing and used for the production of hard cheese was contaminated with *S. aureus* in a total of 74.5% of samples. The results of these investigations can be explained by the fact that the genus *Staphylococcus* belongs to the autochthonous microflora of the skin of cow teats, from where they enter raw milk **[51]**, **[52]**. At the same time, coagulase-positive staphylococci (*S. aureus*) are much less frequently isolated from the teat skin in 5 – 26% of cases **[18]**. Therefore, it is practically impossible to get raw milk without contamination with staphylococci **[53]**. In addition, *S. aureus* will be isolated from a certain number of samples. Therefore, during the production of dairy products, measures such as pasteurization are aimed at destroying bacteria of the genus Staphylococcus. However, in finished products such as hard cheeses, secondary contamination with S. aureus occurs due to the complexity of the production process **[54]**. In this regard, in hard rennet cheeses according to DSTU 6003:2008 **[10]** *S. aureus* can be present at 5 $\times 10^2$ CFU/g (500 CFU). Therefore, raw milk is considered the main source of S. aureus contamination of Dutch cheese. Therefore, the development of bio-preservation technology using phages to control this pathogen in the finished product is promising.

The evaluation of *S. aureus* contamination of hard cheeses revealed (Figure 1) its absence in 70.4 ±2.4% of samples, and 22.2 ±0.4% of cheese samples were contaminated with *S. aureus* in an amount from 1 to 5 $\times 10^2$ CFU/g. That is, on average 92.8% of rennet cheese samples met the requirements of the standard **[10]** regarding S. aureus contamination. However, 7.4 ±0.2% of cheese samples were found to have *S. aureus* counts higher than the standard norm of 5 $\times 10^2$ CFU/g. This may result from staphylococcal multiplication during production technology, transportation, and implementation in the trade network. At the same time, researchers believe **[13]**, **[55]** that for staphylococcal toxicosis to occur due to the development of enterotoxigenic *S. aureus*, their number in the food product must reach $10^5 – 10^6$ CFU/g/ml. However, we believe that what matters is not the final content of S. aureus in the food product, but the initial number from which they multiply. In particular, in the first case, the number of *S. aureus* 10^6 CFU/g/ml may be the result of contamination at the final stage of the technological process or product sales, in which case, staphylococci will not have time to accumulate a sufficient amount of enterotoxin, which causes toxicosis. In the second case, when favorable conditions are created for developing S. aureus (appropriate temperature, time), even with a small initial number of 10^{1-2} CFU/g, they gradually multiply and accumulate enterotoxins.

Therefore, rennet cheeses available in the trade network may cause staphylococcal toxicosis in 7.4% of samples because they are contaminated with *S. aureus* in quantities exceeding the standard norm.

The biggest technical problem in biocontrol of food products using phages is the effectiveness of the latter against pathogens, i.e. highly lytic (virulent), rather than temperate (lysogenic) bacteriophages, should be used to process raw materials or food products **[33]**, **[34]**, and **[35]**. That is, virulent phages effectively reduce the content of host bacteria in food products. In contrast, lysogenic phages do not influence these bacteria, since they do not destroy bacterial cells **[36]**, and **[37]**. In addition, for the active action of phages, they must come into contact with specific bacterial cells, which requires the introduction of high concentrations of phages into the medium, at least 10^7 CFU/ml. Under such conditions, passive immunization of bacterial cells will occur. Suppose there is a large number of target bacteria in the phage environment. In that case, their intensive infection will occur, and many viruses will be released, infecting new bacteria – active immunization **[56]**. At the same time, phages best demonstrate bacterial lysis if they are isolated from the same biotope as their target cells **[46]**.

Considering this lytic process, we isolated 10 isolates of S. aureus from raw milk and 10 from hard cheeses. These staphylococcal isolates were chosen as hosts for the isolation of staphylococcal bacteriophages from raw milk, since it is generally accepted that dairies are the primary natural reservoir from which lactic acid phages spread as technically harmful bacteriophages **[57]**, and pathogenic bacteria **[33]**, and **[40]**. In this regard, raw milk samples were tested for specific bacteriophages to *S. aureus* isolates isolated from hard cheeses and raw milk. This is because only highly lytic bacteriophages are suitable for bio-preservation of food systems.

Table 1 Number of raw milk samples with the presence of phages against *S.aureus*, %.

Isolates of *S. aureus* from raw milk No.	**Milk samples with phages n=23**	***S. aureus* isolates from hard cheese No.**	**Milk samples with phages n=23**
1	11 (47.8%)	1	7 (30.4%)
2	10 (43.4%)	2	7 (30.4%)
3	8 (34.8%)	3	11 (47.8%)
4	10 (43.4%)	4	12 (52.2%)
5	7 (30.4%)	5	14 (60.8%)
6	8 (34.8%)	6	10 (43.4%)
7	12 (52.2%)	7	8 (34.8%)
8	10 (43.4%)	8	8 (34.8%)
9	6 (26.1%)	9	7 (30.4%)
10	8 (34.8%)	10	9 (39.1%)

It was set up (Table 1) that raw milk contains bacteriophages that lysed *S. aureus* cells isolated from milk and rennet cheeses. In particular, from 30.4 to 52.2% of raw milk samples were contaminated with bacteriophages active against staphylococcal isolates from raw milk, and from 30.4 to 60.8% of samples against *S. aureus* from cheese. This gives reason to believe that raw milk was the primary natural reservoir for S. aureus isolated from rennet cheeses. The investigations have reported on the dependence of phages' lytic activity on their hosts' biotype **[58]**, which found the absence of an infectious process in staphylococcal bacteriophages isolated from humans against staphylococci from cows and dogs.

Thus, *bacteriophages circulating in raw milk mostly neutralized S. aureus isolated from rennet cheeses*. Therefore, raw milk can be used as a biotope for isolating bacteriophages in developing biocontrol technology for staphylococci in rennet cheeses.

According to the research results (Table 1), 10 strains of bacteriophages were isolated, which showed lytic activity against S. aureus isolates from hard cheeses and raw milk. Among them, 48.8% produced SEC, SED, SEC/D. Table 2 gives the characteristics of these phages' lytic activity.

Table 2 Characterization of the lytic activity of isolated phages against *S.aureus* isolates from hard cheeses and raw milk.

Phage strains No.	Lytic activity of phages in the "dripping drop" method							
	S. aureus from raw milk n=10				*S. aureus* from hard cheese n=10			
	+	++	+++	++++	+	++	+++	++++
Phage 1	10	20	30	40	–	20	20	60
Phage 2	20	20	20	40	10	20	40	30
Phage 3	20	30	20	30	10	20	30	40
Phage 4	–	–	20	80	–	–	10	90
Phage 5	–	10	20	70	–		30	70
Phage 6	10	20	20	50	–	20	20	60
Phage 7	–	20	20	60	10	20	20	50
Phage 8	–	–	20	80	–	–	10	90
Phage 9	–	–	30	70	–	–	20	80
Phage 10	–	30	10	60	–	10	20	70

The 10 bacteriophages isolated were virulent to all isolates of *S. aureus*, while different intensities characterized their lytic action. In particular, phages No. 4, No. 8, and No. 9 in 100% of cases lysed *S. aureus* isolates, which were isolated from raw milk in four and three crosses. *S. aureus* cells from rennet cheeses were also actively lysed by the isolated phages, since the lysis rate of four and three crosses was in the above-listed phages and in phage No. 5. This makes it possible to use these phages in the technology of biological preservation of dairy products contaminated with *S. aureus*. In this case, it is most effective to use a phage composition consisting of highly lytic bacteriophages No. 4 and No. 8.

Thus, phage No. 4 and phage No. 8 exhibited 80.0 – 90.0% virulent activity in four crosses against *S. aureus* isolated from milk and rennet cheeses, so these phages were introduced into the technology of hard cheese production.

While producing rennet cheese with staphylococcal bacteriophage, we used the Dutch cheese's classical technology as a basis. It provided for the following standard technological operations and modes. Acceptance of raw milk: evaluation of quantity and fat content, protein, acidity, etc., followed by determination of microbiological indicators and cheese suitability. Purification, cooling, and temporary preservation of milk with subsequent maturation for up to 12 hours at temperatures from +8 to +10 °C. Normalization with homogenization and pasteurization at t ≈ + 77 °C for 20 s, then the pasteurized mixture is cooled to the curdling temperature (37 ±2 °C). At the same time, the difference in the technology was that during such a technological operation as adding a leavening agent, calcium salts, and rennet to the milk mixture, we additionally added a composition of staphylococcal bacteriophage in the amount of 2 ml per 1 liter of milk mixture. At the same time, the concentration of phages in the bacteriophage composition was not less than 10^8 CFU/ml. This concentration of bacteriophages for biocontrol and food preservation is proposed by many scientists **[35]**, **[37]**, and **[56]**, who used anti-salmonella and anti-listeriosis bacteriophages to ensure the microbiological stability of food products. Subsequently, the technology for producing hard Dutch cheese had standard technological operations. In particular, it checks the readiness of the curd, its processing, and setting the grain to heat (second heating temperature 38 – 42°C). Later, the heads were formed, and the cheese was pressed. Salting was performed at 9 – 11°C with a salt concentration of 20%. After the salting process, the cheese was dried at a temperature of +11 °C for two days, and the ripening process was 60 days. The technological operations in the control cheese samples were similar, but without adding bacteriophage.

The main microbiota in Dutch cheese during its maturation is lactic acid fermentation. Due to its development and biochemical activity, enzymatic processes occur to transform the milk mixture and form specific organoleptic properties inherent in this cheese. Considering this process, the investigation was conducted to determine the influence of the developed bacteriophage on the change in lactic acid microbiota during 60 days of cheese maturation (Figure 2).

The experimental and control cheeses had the highest number of lactic acid bacteria on the first day of ripening (10.11 – 10.14 log CFU/g). In contrast, no significant difference between them was observed.

During 15 days of ripening, lactic acid microbiota gradually decreased in both cheese samples, associated with a decrease in carbohydrates **[59]**. At the same time, the process of reducing lactic acid bacteria occurred in the same way in cheese preserved with staphylococcal bacteriophage (test sample) and in the control sample.

During 30 days of ripening of two cheese samples, the total content of lactic acid microorganisms decreased, on average, five times, while no significant difference in the number of these bacteria was noted – 9.27 versus 9.29 log CFU/g. A similar trend in the development of lactic acid bacteria in two cheeses was observed on the 45th and 60th day of their ripening. This is evidence that the staphylococcal bacteriophage added to the test cheese sample does not disrupt the microbiological process with the participation of lactic acid microflora of starter cultures. Since bacteriophages have species specificity for bacterial cells, as indicated by a number of investigations **[46]**, and **[60]**.

Thus, staphylococcal bacteriophages isolated from raw milk and added to the Dutch cheese technology do not lyse starter microorganisms.

Adding staphylococcal bacteriophages to the technological process of Dutch cheese production aims to increase its safety and prevent the multiplication of S. aureus to the amount considered critical for staphylococcal toxicosis. In addition, the number of coagulase-positive staphylococci in the cheese does not exceed the standard requirements of 5 $\times 10^2$ CFU/g **[10]**. Therefore, it was essential to conduct a research to determine the change in *S. aureus* during the 60-day ripening period of the cheese (Figure 3.).

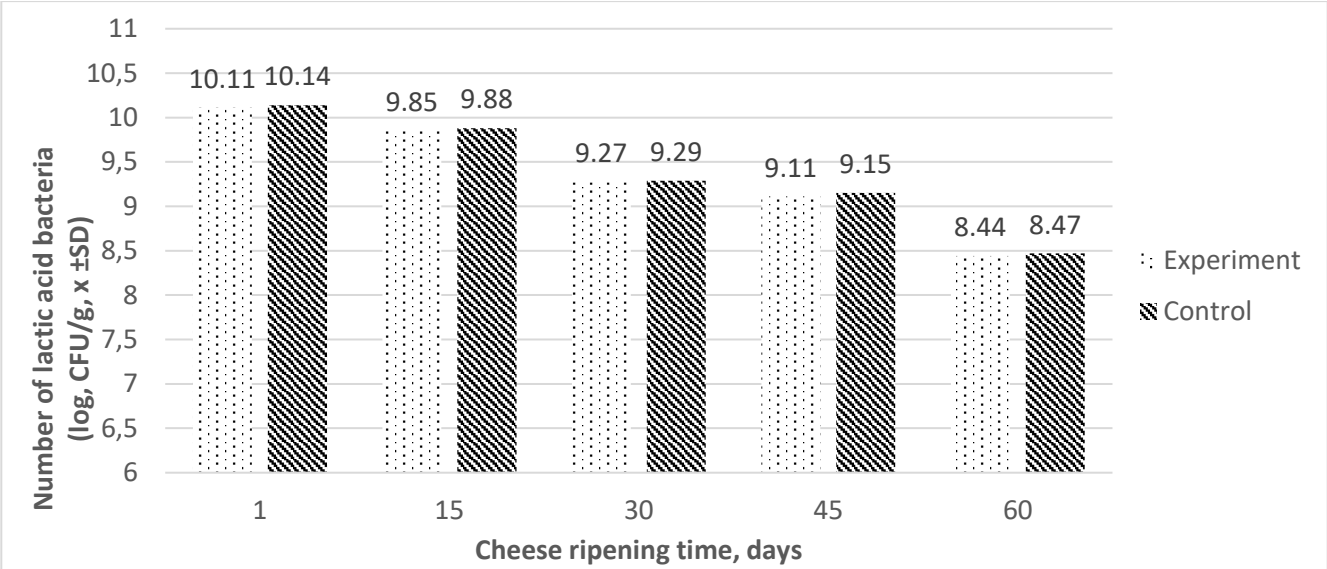

Figure 2 Development of lactic acid microorganisms during ripening of hard Dutch cheese with staphylococcal bacteriophage.

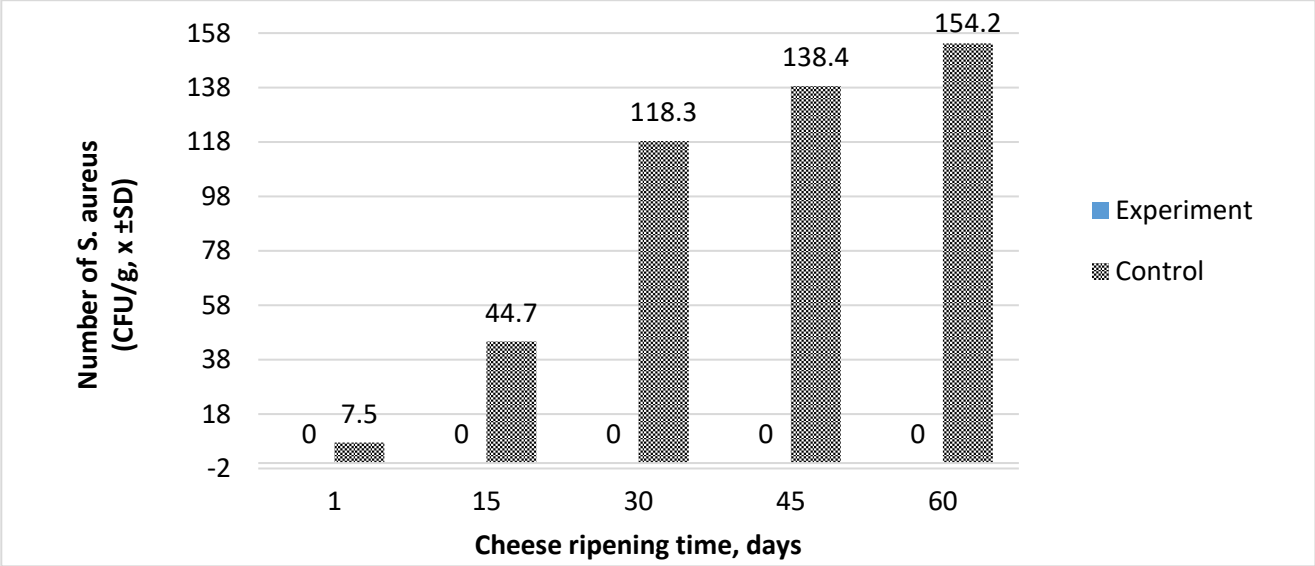

Figure 3 Development of *S. aureus* during ripening of hard Dutch cheese with staphylococcal bacteriophage.

Figure 4. Samples of Dutch cheese with staphylococcal bacteriophage during production.

In the experimental cheese sample with staphylococcal bacteriophages during the 60-day ripening period, no *S. aureus* cells were detected by standard microbiological methods. At the same time, in the control sample of cheese during ripening (1 day), *S. aureus* was isolated in an amount of up to 10 CFU/g. During the first 30 days of ripening, we note the process of *S. aureus* reproduction, since its number increased by 15.7 times to 118.3 ±9.1 CFU/g. In the next 30 days of cheese ripening, the dynamics of staphylococci development in cheese slowed down significantly, probably due to a change in the pH of the environment, and its number on the 60th day was 154.2 ± 11.6 CFU/g. Even though the content of *S. aureus* in the control cheese increased over the entire ripening period, this amount was 3.2 times less than the permissible norm according to the standard - 5×10^2 CFU/g **[10]**. Thus, adding lytic staphylococcal bacteriophages to the milk mixture during the production technology of Dutch cheese allows for its preservation against the development of *Staphylococcus aureus* in the cheese (Figure 4).

Good results in inhibiting the growth of staphylococci during biocontrol of dairy products have been reported by several researchers, in particular, the anti-staphylococcal bacteriophage lysin LysH5 achieved an 8-

logarithmic decrease in 6 hours, and CHAP-SH3b could eliminate *S. aureus* in 15 minutes in different grades of cow's milk **[61]**. The bacteriophage endolysins CHAPK_CWT-LST and Ami2638A acted similarly, and also showed rapid antistaphylococcal activity in milk, and the Lys109 enzyme achieved the same effect, but required 45 minutes of incubation **[62]**. Endolysin LysRODI and its derivative LysRODIΔAmi showed good efficacy against *S. aureus* in milk and during fresh cheese production in laboratory conditions **[63]**. Therefore, we agree with many researchers **[35]**, **[37]**, and **[56]**, which indicate broad prospects for the introduction of bacteriophages against various pathogens in the production technology of various types of food products.

Therefore, lytic bacteriophages for biocontrol of *Staphylococcus aureus* allows for its almost complete neutralization in Dutch cheese during production.

CONCLUSION

Staphylococcus aureus was not detected in 25.5% of raw milk samples received for processing. In comparison, the bulk of the milk – 52.9% – was contaminated with coagulase-positive staphylococci up to 500 CFU/ml. 21.6% of raw milk samples at the processing plant contained more than 500 CFU/ml of *Staphylococcus aureus*. Hard cheeses sold in the trade network did not contain *S. aureus* in 70.4% of samples, in 22.2% of samples its amount did not exceed 5×10^2 CFU/g, and in 7.4% of cheese samples the content of *S. aureus* was higher than the standard norm of 5×10^2 CFU/g.

From 30.4 to 60.8% of raw milk samples contained virulent phages that lysed *S. aureus* isolated from hard cheeses and dairy products. Two phages (No. 4 and No. 8) were isolated from raw milk, which showed 80.0–90.0% virulent activity in four crosses against both *S. aureus* isolated from milk and rennet cheeses. These phages were introduced into the technology of hard cheese production.

The addition of virulent staphylococcal bacteriophages to the milk mixture (2 ml per 1 l at a concentration of 108 CFU/ml) during the production technology of hard cheese allows its preservation against the development of Staphylococcus aureus.

Thus, a technology for the biocontrol of *S. aureus* in hard cheese has been developed, almost wholly neutralizing it during production.

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