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ИССЛЕДОВАНИЕ БАКТЕРИОФАГА В МОЛОЧНОЙ ПРОМЫШЛЕННОСТИ РОССИИ

STUDY OF BACTERIOPHAGES IN RUSSIAN DAIRY INDUSTRY

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Аннотация. *Около 100 лет в России для производства используются чистые культуры молочнокислых микроорганизмов. Около трёх десятков лет их использования в условиях отечественных молочной промышленности использовали с целью изучения феномена - фаголизиса заквасочных культур. Публикация посвящена исследованиям использования бактериофага в молокоперерабытывающей промышленности Российской Федерации.*

Материалы и методы. *В эксперименте были использованы тест-культуры Lactobacillus, термофильные молочнокислые стрептококки, бактериофаги, лизирующие тест-культуры 375 вирионов бактериофагов лактококков, изолированных на сыродельных заводах России, Белоруссии, Казахстана, Молдовы; 1056 коллекционных культур лактококков вида Lactococcuslactis подвидов: lactis, cremoris, diacetylactis. В качестве питательных сред для культивирования и хранения лактококков и их бактериофагов в присутствии индикаторных культур использованы стерильное обезжиренное молоко (СОМ), бульон из гидролизованного панкреатином молока (БГМ), полужидкий (0,65 %) и плотный (2,0 %) агар-агар из гидролизованного молока.*

Использовался двуслойный метод индикации бактериофага на плотной питательной среде с помошью тест-культур. Титр бактериофагов определяли путем посева на газон тест-культур и подсчета негативных колоний (НКОЕ в 1 мл); фагорезистентность лактококков устанавливали путем нанесения на газоны исследуемых штаммов капель фаговых суспензий с содержанием не менее $1 \cdot 10^6$ НКОЕ/мл; способность бактериофага лизировать культуру определяли через 24 ч культивирования по наличию зоны лизиса в местах нанесения капель; морфологию бактериофагов изучали с помощью электронной микроскопии при ускоряющем напряжении 80 кВ и рабочем увеличении на экране в 60000 раз.

Результаты. *Исследования показали, что в настоящее время остаётся актуальным определение бактериофага в сыворотке (творожной, подсырной) как условие определения риска (идентификации опасного фактора) и разработки предупреждающих воздействий, снижающих риск генетических мутаций между бактериофагами, лизирующими штаммы отечественных и зарубежных бактериальных концентратов.*

Заключение. *Фаговый мониторинг в силу особенностей существующего метода индикации, требующего наличие специфичных тест-штаммов, предлагается закрепить за внешними лабораториями – производителями заквасок (бактериальных концентратов) для молочной промышленности.*

Ключевые слова: молочная промышленность, бактериофаг, фаговый мониторинг, тест-штамм.

Abstract. *For about 100 years in Russia, pure cultures of lactic acid microorganisms have been used for production. About three decades of their use in the conditions of the domestic dairy industry were used to study the phenomenon of phagolysis of starter cultures. The publication is devoted to research on the use of bacteriophage in the milk processing industry of the Russian Federation.*

Materials and methods. *The experiment used Lactobacillus test cultures, thermophilic lactic acid streptococci, bacteriophages, lysing test cultures of 375 lactococcal bacteriophage virions isolated at cheese factories in Russia, Belarus, Kazakhstan, Moldova; 1056 collection cultures of the lactococcus species Lactococcuslactis subspecies: lactis, cremoris, diacetylactis. Sterile skimmed milk (COM), broth from pancreatin hydrolyzed semi-liquid milk (0.65%) and dense (2.0%) agar are used as culture media for the cultivation and storage of lactococci and their bacteriophages in the presence of indicator cultures. hydrolyzed milk agar.*

The two-layer method of bacteriophage indication on a dense nutrient medium with the help of test cultures was used. The titer of bacteriophages was determined by sowing test cultures on a lawn and counting negative colonies (NCOE in 1 ml); phage resistance of lactococci was established by applying droplets of phage suspensions to the lawns of the strains under study with a content of at least 1 × 106 NECL / ml; the ability of the bacteriophage to lyse the culture was determined after 24 hours of cultivation by the presence of a lysis zone at the sites where the drops were applied; The morphology of bacteriophages was studied using electron microscopy with an accelerating voltage of 80 kV and a working magnification of 60,000 times on the screen.

Results. *The studies have shown that at present, the determination of bacteriophage in serum (cottage cheese, cheese) as a condition for determining risk (identifying a hazard) and developing preventive effects that reduce the risk of genetic mutations between bacteriophages that lyse strains of domestic and foreign bacterial concentrates remains relevant.*

Conclusion. *Phage monitoring due to the characteristics of the existing method of indication, which requires the presence of specific test strains, is proposed to be assigned to external laboratories – producers of starters (bacterial concentrates) for the dairy industry.*

Key words: dairy industry, bacteriophage, phage monitoring, test strain.

The history of using pure cultures in the Russian dairy business dates back to the beginning of the 19th century. According to the opinion of S.A. Severin, Head of the Bacteriological and Agronomical Station of the Animal and Plant Acclimatization Society, stated in the Dairy Farm magazine in 1913, thanks to wide using the method of pure cultures in producing sour cream, cottage cheese, and even koumiss from cow's milk now (i.e. in 1913) "... it is time for introducing the method of pure cultures in our cheese making" [1].

By that time, there had already been separated attempts of using pure cultures in making some types of cheese in Russia. In 1901-1902 specialists of the Yur'ev Dairy Laboratory studied the bacterial flora composition of the local Knappkase (Liflyandskiy) cheese curd and used pure cultures in its making, which resulted in a success. The laboratory began producing these cultures.

In 1908 S.V. Parashchuk, Head of the Yaroslavl Laboratory improved the method of developing dry cultures by pre-centrifuging the liquid starter before drying. This technique allowed to increase the cell concentration and made it possible to distribute dry cultures in small portions, which significantly affected their cost.

The experiments of using pure cultures that were not described in special literature had been carried out not only in scientific research, but also in practical cheese making. For example, S.A. Severin writes about the spread of practical using the pure culture of the usual lactic acid bacterium *Bacterium lactis acidi* (nowadays known as *Lactococcus lactis subsp. Lactis*) [1].

S. A. Korolev (1874-1932), Professor, Head of the Microbiology Department of the Vologda Dairy Farming Institute puts forward prerequisites for the rational selection of cultures for cheese making. In his opinion, the species and race (strain) composition of the starter microflora is to be of great importance, since energy, and partly the process direction, are determined not only by the number of active cells, but also by their specific features [2, 3].

D.I. Ivanovsky (1864-1920) is well-known in Russia, since he discovered a filtering virus and became the founder of virology. The scientist paid his attention to the problems of soil microbiology.

Thus, on an industrial scale, pure starter cultures have been actively used since the 1920s and 1930s. Only in the late 50's, i.e. almost 30 years after the beginning of using pure cultures in milk processing enterprises, cases of fermented microflora phagolysis have been recorded. N. N. Belousova gives the first detailed description of the virus that affects lactic acid cultures, i.e. bacteriophage [4, 5].

Lactic acid and fermented products of high quality can be produced only if the starter microflora is developed in them. One of the reasons for its weakening is bacteriophage [151, 162, etc.]. 5–15% of the total amount of products lose quality due to the lysis of microorganisms by phage fermenting.

At the end of the last century, the phenomenon study was actively carried out in Ukraine [6, 7, 8] and, mainly, in the following four laboratories in Russia:

1. All-Russian Scientific Research Institute of Butter and Cheese Making, (VNIIMS) in Uglich – nowadays it is a branch of Federal State Budgetary Scientific Institution Federal Scientific Centre of Food Systems named after V. M. Gorbatov of the Russian Academy of Sciences [9, 10];

2. Federal State Unitary Enterprise Experimental Biofactory of the Russian Academy of Agricultural Sciences [10];

3. State Scientific Institution Siberian Research Institute of Cheese Making of the Russian Academy of Agricultural Sciences, in Barnaul [11];

4. Federal State Budgetary Scientific Institution All-Russian Research Institute of Dairy Industry (VNIMI), in Moscow [12].

Nowadays the most considerable systematic bacteriophage collection resulted from the long-term collaboration of VNIIMS and the Experimental Biofactory experts, with our participation as well, includes 375 virions of lactococci bacteriophages isolated at milk processing plants in Russia, Belarus, Kazakhstan and Moldova [13]. Among the bacteriophages, there are no repeated isolates of the same phage, as it has been demonstrated by DNA / DNA hybridization meth-

ods as well as the restriction analysis using EcoR I and EcoR Y endonucleases. The detected phages of thermophilic streptococcus are *Streptococcus salivarius subsp. thermosphilus* and some lactic acid rods, such as *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus helveticus.*

There is no information about phages lysing bifidobacteria.

Classifications of lactococci bacteriophages in milk processing plants are most often described in earlier works, including our publications [13–17]. It should be noted that B2 phages were mostly widespread in the 90s, B1 phages [13, 15] were spread abroad, but in the 21st century the collection has been increased with a comparatively large number of B1 morphotype phages [14]. There are phages, characterized by a morphological diversity, relating to the collar, basal plate and tail.

This publication aims to inform the world scientific community and producers of pure cultures for the Russian dairy industry about the starter microflora phagolysis cases that we have registered while using imported starter cultures (DVS).

On the one hand, all these cases could be attributed to an insufficient level of antiphage activities and the need for toughening mandatory phage monitoring [18–20].

On the other hand, it is a fact that the synthesis of restrictases and specific bacteria receptors belonging to the phage resistance mechanisms can be encoded not only by the bacterium chromosome, but also by bacterium plasmils, which can be relatively easily lost and acquired by cells of lactic acid microorganisms. The phage DNA can also be modified and become inaccessible to restrictases [14].

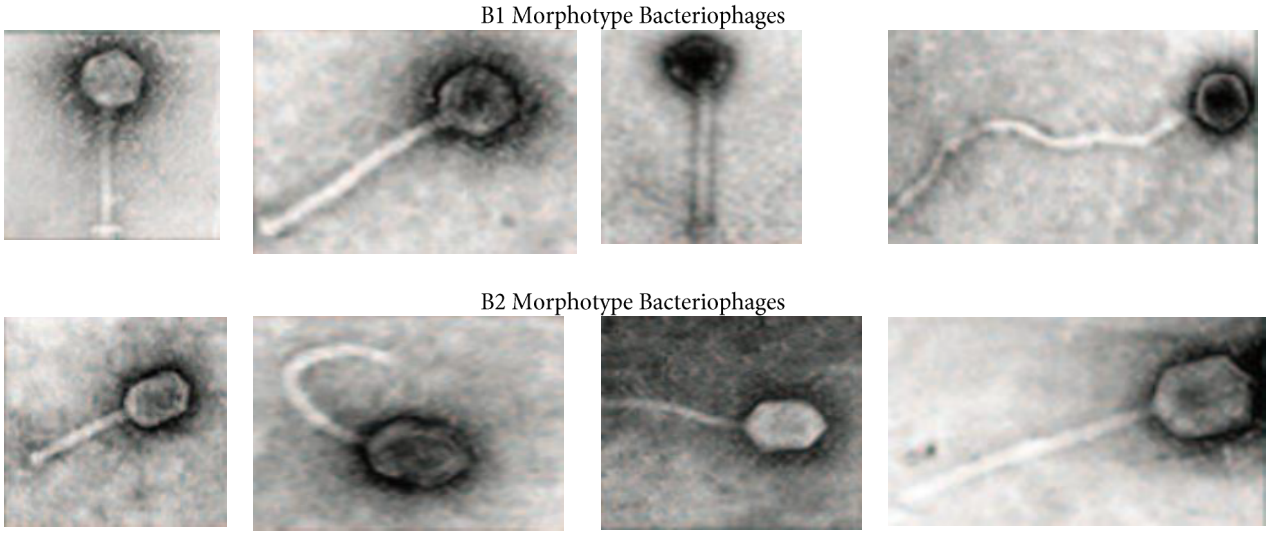

Fig. 1. Lactococci bacteriophages, isolated at the milk processing plants in Russia, Belarus, Kazakhstan and Moldova.

At the turn of the century, DVS starters were considered a panacea for solving the problem of starter microflora phagolysis at dairy plants [14], but the problem remained unsolved.

When fermented milk mixtures with a clear sign of phagolysis of DVS imported starter cultures in domestic test cultures were brought to dairy testing laboratory, the bacteriophage was not detected. Nevertheless, while using isolated clones from the starter applied in these cases as test cultures, the bacteriophage lysed 80-100% of the clones.

Domestic and foreign starter strains of lactic acid cultures are used for a long time sequentially or in parallel (in manufacture of different products) in one enterprise, which does not exclude the possibility of genetic material exchange and generating phages, having a new and broader spectrum of lytic activity. At present, it is no longer sufficient to carry out systematic monitoring of bacteriophages in production, where domestic test-cultures are used.

It is using test cultures of each pure culture supplier that will allow a qualitative assessment of the phagolysis hazard. Another way is to use pure cultures, which a multistrain starter consists of, instead of test strains. However, this measure significantly complicates phage monitoring and the timely replacement of starter cultures by phage-resistant ones [20].

The common methods of developing such phage-resistant cultures are the following [21, 22]:

1) Natural selecting of practically valuable forms. A strain of microorganisms that possesses useful properties for humans is isolated from a natural or production source. Then the most productive strains are selected among them.

2) Using of artificial mutagenesis, which allows increasing various mutations. As mutagens ionizing radiation, some chemicals, as well as ultraviolet radiation are used, although the latter has a low penetrating ability, but it is sufficient for generating mutations in microorganisms.

3) Genetic methods of recombination between shared species (intraspecific conjugation, transformation, transduction, use of protoplasts, etc.) [23, 24].

4) Genetic engineering that uses a plasmid vector of a wide range of hosts, or a bacteriophage vector, etc. for developing starter cultures with desired properties [25–27].

5) Using of lysogenic cultures.

A brief description of the bacteriophage's lytic cycle is as follows: phages adsorbed on the surface of the host cells make a hole in the bacterial cell with the help of their enzymes. The phages inject phage DNA stored in the phage head through the hole. After injection of the nucleic acid in the host cell, the synthesis of bacterial substances ceases, the cell's biosynthetic apparatus begins synthesizing phage components: nucleic acid, envelope proteins and lysozymes or endolysins [14]. There are no visible changes in the acid-forming activity of lactic acid bacteria cells, or this activity rises. This period is known as latent. Only when new phage particles are formed in the cell, the host cell walls become lysed and it dies. The lactic acid process stops before phage-resistant mutants are formed.

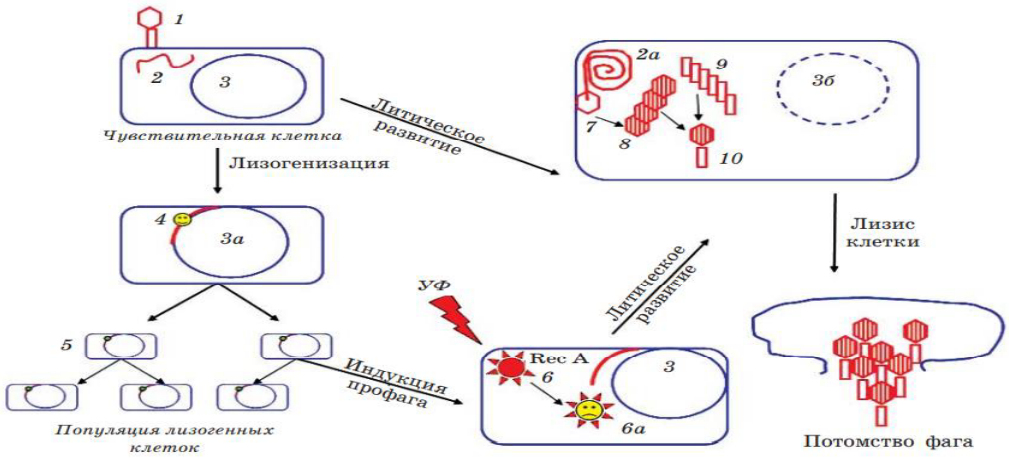

Fig. 2. Scheme of possible bacteriophage development ways

Figure 2 shows the following: bacteriophage (1) becomes attached to the receptor of the bacterial sensory cell (3) and penetrates the nonphage DNA (2). This results in bacterial cell lysogenization (3a). The phage exists as a prophage (4). The lysogenic cell continues breaking down and forms lysogenic cell population (5). Under the action of the inductive factor (6) the lytic development of the sensory cell takes place (3). As a result, the phage DNA (2a), using the cell apparatus (3a) synthesizes heads (7, 8) and cerci (9) of new phage particles (10).

However, bacteriophage lysogeny is a well-known phenomenon, which has been studied in the lambda-phage model quite thoroughly. In this case, DNA penetration into the bacterial cell is not accompanied by the formation of new phage particles inside it and there is no cell lysis. Such phages are known as moderate and cells carrying moderate phages (prophages) are lysogenic (Fig. 2). Lysogenic cells are protected from infecting by a homologous bacteriophage.

If the lysogenic culture undergoes any stress (lack of nutrients, chemical or physical factors), the frequency of prophage release from the lysogenic cell increases hundreds of times. It should be noted, that the average size of lactococci bacteriophages is approximately 100 nm. If we transfer the nano degree to the macro level, it can be figuratively said that, in the event of war, a population group capable of giving offspring migrates, in search for safer living conditions.

Using the scientific and practical approach, it is necessary to study the phenomenon of lysogeny and bacteriocinogenicity of lactic cultures simultaneously. It is known that bacteriocins, which suppress the development of putrefactive and pathogenic microflora in the product and our body, are "defective" phages, formed from prophages. It is assumed

that there is a natural mechanism that has existed, and maybe still exists, by which phages, which are active against different species and families, could exchange successful "evolutionary findings" [29, 30]. Thus, the study of bacteriophages is also one of the steps in increasing the immunological value of fermented functional food products [31, 32].

In recent years, the situation analysis shows great importance of bacteriophages, primarily lactophages as factors that continue disrupting the technological, microbiological and biochemical processes of producing fermented dairy products, including cheeses.

An important requisite in developing methods of preventing damage to fermented microflora by bacteriophages is to study their biological characteristics. This problem seems to be a transnational one due to complexity of the tasks being solved [33, 34, 35].

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