L. lactis BACTERIOPHAGES AND METHODS OF THEIR ELIMINATION FROM DAIRY PRODUCTS

OLGA SHYDLOVSKA, YULIIA KHMELNYTSKA
Kyiv National University of Technology and Design, Kyiv, Ukraine

Dairy products are important in human diet and nutrition. That is why dairy production is critical not only economically, but also socially and medically. In recent decades, dairy production has had problems with disturbances in fermentation processes caused by bacteriophage contamination. It is important to note that every year there are new reports about newly discovered bacteriophages that disrupt fermentation processes in the production of kefir, yogurt, and various types of cheese. *Lactococcus lactis* strains are of particular importance in dairy technology, as they are used for the production of various yogurts and cheeses. The study of the spectrum of bacteriophages infecting this strain can help to monitor the evolutionary changes of viruses and the horizontal transfer of genes. In this paper, an analysis of phages infecting L. lactis was carried out. Most bacteriophages belong to the *Siphoviridae* and *Podoviridae* families. Moreover, the authors analyzed approaches that can be used to reduce bacteriophage contamination in the production of dairy products. It has been shown that the use of disinfectants, such as ethanol on sodium hypochlorite, can reduce the titer of bacteriophages and protect products from the development of viral infection. It is also possible to use membrane filtration with UV irradiation. Moreover, all these approaches can be combined to achieve the most effective result.

Keywords: bacteriophages, *Lactococcus lactis*, biotechnology

INTRODUCTION

Bacteriophages are the most common viruses on planet Earth. This is because their hosts – bacteria – inhabit the most diverse ecological niches, namely soil, water, thermal springs, the bottom of the ocean, the surface of human skin, the gastrointestinal tract of animals, etc. It is clear that where bacteria live, we can also find bacteriophages (Batinovic *et al*., 2019).

This work is devoted to the review of bacteriophages infecting *Lactococcus lactis*, their characteristics and properties. The authors of the paper paid special attention to the analysis of possible methods of combating bacteriophage contamination of dairy production. This work shows that the development of effective methods of combating bacteriophage contamination is an important issue and requires in-depth study, since a universal and effective method has not yet been invented.

The main source of phages entering dairies is through raw milk, which can contain up to 104 IU/ml. Milk often contains phages for Lactococcus lactis and Streptococcus spp. The temperature and time combinations commonly used for pasteurization, i.e., low temperature/long time (63°C, 30 min) or high temperature/short time (72°C, 15 s), are largely insufficient to eliminate most LAB phages. Work surfaces such as floors, walls, stairs, doorknobs, office desks, equipment, detergents, and pipes can also be a source of contamination. Phages cannot be eliminated in the dairy environment because they are naturally present in raw milk, withstand most common heat treatments, spread among production facilities through liquid splashes and airborne particles, persist on equipment surfaces and biofilms, and can be found in high titers in cheese whey or other industrial effluents (Pujato *et al*., 2019).

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REVIEW OF BACTERIOPHAGE STRAINS INFECTING *Lactococcus lactis*

L. lactis strains are widely used in the production of numerous fermented dairy products and are among the most economically important lactic acid bacteria (LAB) (Yerlikaya, 2019). All lactococcal phages belong to the order *Caudovirales*, families *Siphoviridae* (most lactococcal phages) and *Podoviridae* (few lactococcal phages). Bacteriophages infecting *L. lactis* have been divided into 10 species, and those belonging to c2, 936 or P335 species are more common in dairies. At present, 28 genomes of lactococcal bacteriophages isolated during the last 3 decades as a result of failed fermentation in dairies around the world have been identified. The GC content of the analyzed bacteriophages ranges from 34 to 36.4%. The genome length of isolates ranges from 20 to 23.2 kb for c2 phages, from 25.3 to 32.6 kb for species 936 phages, and from 25.3 to 32.6 kb for Bk5-T members. The number of putative ORFs ranges from 34 to 42 among c2 species, from 46 to 62 among 936 phages, and from 51 to 60 for Bk5-T isolates (Marcelli *et al*., 2020).

RESISTANCE OF *L. lactis* TO BACTERIOPHAGES

The type III-A system provides persistent phage immunity in part due to the absence of PAM sequence requirements (adjacent protospacer motif), tolerance of nucleotide mismatches in the target sequence (protospacer), and the efficiency of inhibition from a single spacer, especially when targeting important genes. The genetic similarity between the *L. lactis* type III-A CRISPR and other type III-A systems may predict a similar function. The *in vivo* functionality of the type III-A lactococcal system is consistent with the mechanism of action of similar systems found in *S. epidermidis*, *S. thermophilus*, and *Thermus thermophilus*. The flexibility in spacer targeting and the strength of immunity derived from a single spacer, especially when targeting essential genes, offer significant advantages for the generation of industrial strains with enhanced and programmed phage resistance (Millen, 2019).

In the study of the authors Marcelli *et al*. (2019) showed that the resistance mechanism of four bacteriophage-insensitive mutants of *L. lactis* involves changes in the phage receptor. For example, phage CHPC971 shows a low or very low adsorption rate for all four bacteriophage-insensitive mutant *L. lactis* strains compared to its sensitive strains CH LC01 and CH LC02. The studied phage is able to adsorb on L. lactis CH LC01 and CH LC02 strains, while on the other four *L. lactis* strains, adsorption was not observed or was very weak. It is important to note that rhamnose plays a direct role in the work of this receptor and may be a key molecule that provides resistance to bacteriophages.

METHODS OF REDUCING BACTERIOPHAGE CONTAMINATION IN FERMENTED *Lactococcus lactis* PRODUCTS

Complete inactivation of lactococcal phages can be achieved by treatment at 90°C for 5 minutes. Among alcohols (ethanol and isopropanol), ethanol in a concentration of 75% or more has an effective antiviral effect. Sodium hypochlorite in concentrations above 100 ppm (the maximum commercial concentration used is 200 ppm) is also capable of completely suppressing viral activity. The most effective agent widely used in the dairy industry is peracetic acid. At a concentration (0.15%, 40 °C) rapidly

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inactivates phage suspensions (the number of viruses below the detection limit, <10 PFU/ml) after 5 minutes of treatment (Marcó *et al*., 2019)

Authors Michel *et al*. (2021) investigated an orthogonal process strategy (cross membrane filtration combined with UV-C irradiation or heat treatment) to dramatically reduce the number of bacteriophages in filtered whey. An effective mode of bacteriophage elimination was membrane filtration followed by UV irradiation at 2.25 J cm^2. At the same time, no option with heat treatment of whey was effective – mild pasteurization conditions (72–75 °C, 15–30 s, whey protein denaturation by approximately 1%) and higher combinations of temperature and time (for example, 216 s at 75 °C for P008 phage or 10.5 min at 95 °C for P680 phage; whey protein denaturation >5% to >90%, respectively). Although this technology is pilot, it can be considered for scaling up the process of purifying serum from bacteriophages on an industrial scale. It is important to note that even in an industrial pasteurizer it is possible to achieve a certain decrease in the titer of bacteriophages, the conditions of industrial pasteurization of milk (72°C for 15 s) are not sufficient for effective inactivation of even the heat-sensitive model phage P008 (Wagner, 2018).

An interesting approach to combating bacteriophage contamination is the development of transgenic strains of *L. lactis* bacteria. The authors performed plasmid transduction and proved the effectiveness of this procedure under certain circumstances. The selected phage must be able to infect both donor and recipient strains and encode a terminase that can bind plasmid DNA and package it into nascent phage heads (Marcelli, 2020).

CONCLUSIONS

Most of the bacteriophages that infect *Lactococcus lactis* belong to the *Sifoviridae* and *Podoviridae* families. Even though *L. lactis* strains resistant to phages have been isolated, the threat of reducing the efficiency of fermentation processes still exists. This is due to the ultra-fast evolution of viruses. However, today there are quite effective approaches to fight against bacteriophage contamination. Among the most common and easy to use is the use of ethanol and sodium hypochlorite solutions. Furthermore, a more serious approach of combining methods of membrane filtration and treatment of raw materials with UV irradiation can significantly reduce the titer of bacteriophages. Among the latest approaches, the development of recombinant strains can be singled out, but this technology can be too expensive for dairy industries. Today, we can conclude that the search for new approaches and the improvement of old approaches to fight against bacteriophage infections on dairy farms needs more attention from scientists and researchers. It can be said that a combination of different approaches and techniques can provide greater efficiency in reducing the phage titer in raw materials. Moreover, it can help in the complete elimination of phage from dairy raw materials. The most promising methods currently are the combination of ultrafiltration with UV irradiation.

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