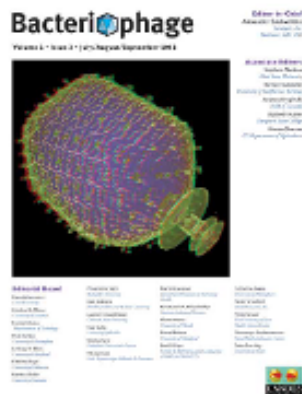


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Bacteriophage

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Bacteriophages and dairy fermentations

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Bacteriophages and dairy fermentations

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Keywords: bacteriophage, dairy industry, milk fermentation, lactic acid bacteria, phage control strategy

This review highlights the main strategies available to control phage infection during large-scale milk fermentation by lactic acid bacteria. The topics that are emphasized include the factors influencing bacterial activities, the sources of phage contamination, the methods available to detect and quantify phages, as well as practical solutions to limit phage dispersion through an adapted factory design, the control of air flow, the use of adequate sanitizers, the restricted use of recycled products, and the selection and growth of bacterial cultures.

Performance of Starter Cultures in Dairy Fermentations

The growth of dairy starter cultures can be influenced by a number of factors including the raw milk quality, presence of antibiotics or sanitizers, bacterial interactions, and phages.^{6,7}

Raw milk composition. LAB have generally complex nutritional requirements. As a consequence, most LAB species can grow only in media where constituents like amino acids and vitamins are freely available. Even if milk provides this ideal growing environment, other components may act as inhibitors of LAB.⁸ The lactoperoxidase-thiocyanate-hydrogen peroxide system, as well as immunoglobulins naturally present in milk are among the known inhibitors affecting LAB activity. If hydrogen peroxide is present in milk as a metabolite of some microorganism, it combines with the lactoperoxidase to oxidize thiocyanate into products (sulfate, carbon dioxide, ammonia, and water) that will inhibit some LAB. Some bacteria, including LAB species, may also agglutinate in raw milk. Antibodies found in the globulin fraction of milk cause this effect. As a consequence bacteria can form clumps and sediment on the bottom of vats, causing slow or heterogeneous acid production. However, the inhibitory role of these compounds is mainly relevant when raw milk is used in cheese manufacture, since agglutinins and immunoglobulins are inactivated by heat treatments or homogenization process.

Leucocytes and lysozyme, also present in milk, have antimicrobial properties, the last being particularly resistant to thermal treatments. Normally, their levels are very low in milk, but increases due to mastitis and high somatic cell counts. An antibacterial activity is also frequently associated with lactoferrin, an iron-binding glycoprotein present in milk. Finally, antibiotics, which may enter milk due to the treatment of cows for bacterial infection of the udder, can also affect LAB growth and activity. Good quality raw milk should not contain antibiotic residues, but some reports point to these molecules as responsible for slow acidification during milk fermentation processes. The sensitivity of dairy starters to antibiotics will vary although in general, *Lactococcus* spp are much more resistant to penicillin, *Lactobacillus* spp to tetracycline and *Streptococcus thermophilus* to streptomycin.⁶

Bacterial interactions and phages. The acid production rate of some LAB strains might be increased in the presence of other microorganisms, such as *Micrococcus* spp, which either remove H₂O₂ or produce stimulating metabolites.⁹ In contrast low concentrations of free fatty acids may also be inhibitory to several

Introduction

In a fermentative dairy process, lactic acid bacteria (LAB) growth and metabolic activities are needed to assure a high-quality final product. These microorganisms produce lactic acid via lactose fermentation, which leads to a rapid decrease in pH. Cheese and fermented milk manufacture depends, largely, on this factor, which is also crucial for ensuring control of pathogenic and spoilage microorganisms.¹

Bacteriophages or “phages” are viruses that infect bacteria. They are now believed to represent the most abundant biological entities with an estimated range of 10³⁰ to 10³² total phage particles on earth, assuming that they outnumber bacteria about 10-fold.² These bacterial viruses are present in ecosystems where bacteria have been found, including man-made ecological niches such as food fermentation vats. The industry has been dealing with this biological phenomenon for many years now and has relied on a variety of practical approaches to control phages, which include adapted factory design, improved sanitation, adequate ventilation, process changes, improved starter medium, and culture rotation.^{3–5} Despite extensive efforts, however, phage infection of starter LAB cultures remains the most common cause of slow or incomplete fermentation in the dairy industry, and both researchers and industrial technologists are aware of regular, although unpublished, cases where phage infections actually cause product downgrading. Thus, the goal of this review is to make the reader aware of the relevance and implication of phage attacks in dairy fermentations, with special emphasis on the daily and practical aspects related to this problem in the dairy fermentative industry.

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LAB strains. Low levels of these organic acids are present in fresh raw milk, with increasing concentration as consequence of the activity of psychrotrophic bacteria, such as *Pseudomonas* spp.¹⁰ Even if the ability of some dairy starters to produce bacteriocins is generally considered as a positive attribute for food safety reasons, this feature may be problematic when the antimicrobial spectrum includes LAB species.

Despite of the above, phage infection represents the most significant biological factor affecting industries that rely on bacterial growth and metabolic activities. Depending on the process stage in which the infection proceeds, consequences may vary from slow acid production to completely lost batches.¹¹ High pH values, high residual lactose concentration and insufficient lactic acid content are the result of phage attacks occurring during the early stages of the fermentation. In particular, the residual lactose might be the substrate for the growth and metabolic activity of spoilage bacteria that negatively affect the quality of the product. Besides the inadequate overall product quality, all these factors may constitute an optimum ecosystem for the growth of pathogens, with the serious consequences on the consumer health.

The recognized ubiquity of phages in dairies is the basis for studies aimed to control rather than to eradicate them.² For several reasons, cheese manufacture is the most affected process. Worldwide, large volumes of raw milk are daily fermented by LAB starters, with *Lactococcus lactis* being the most extensively used. Consequently, *Lactococcus lactis* phages are the best studied and documented over the world, followed by *S. thermophilus* phages.^{2,12} The number of reported *Lactobacillus* (*Lb.*) phages is notably lower, possibly due to the characteristics of processes involving this genus. However, several phages affecting fermentation processes driven by *Lb. helveticus*, *Lb. delbrueckii* subsp *bulgaricus* or *Lb. delbrueckii* subsp *lactis* were isolated and documented.^{13,14} Lastly, emerging data suggests an increasing occurrence of phages for specific probiotic LAB strains, especially *Lb. plantarum*, *Lb. acidophilus*, *Lb. casei* and *Lb. paracasei*, which are increasingly used in several fermented products.¹⁵

Phage Entry into Dairy Environments

Raw milk. It is now acknowledged that the most permanent source of new phages within dairy environments is through raw milk, with their concentration ranging between 10^1 and 10^4 phages per ml.^{4,16–23} Madera et al.⁴ reported that almost 10% of raw milk samples collected from different dairies in Spain contained infectious lactococcal phages. Several research groups have also reported that many dairy phages are able to survive milk pasteurization.^{18,22,24–26} Moreover, the concentration of phages is even higher if only thermized or raw milk are used to manufacture fermented milk products. Consequently, phages might enter the manufacturing process and accumulate rapidly during fermentation if phage-sensitive strains are used, reaching concentrations up to 10^9 phages per ml of cheese whey or per g of product,^{27–30} up to 10^8 plaque-forming units (PFU) per ml in brine,²² and up to 10^8 PFU per m³ in air.^{31–33} Taken altogether, a great diversity of phages is naturally present in the raw milk ecosystem, thus the absence of phages in dairies is unreachable.

Factory environment. Although raw milk is the most logical source of phages in the industrial environment, several dispersion pathways may be occur in dairies. Aerosolization is currently recognized as an important route of dispersion.³⁴ Personnel movements or transport of equipment and/or raw materials might cause the dispersion of phage particles as an aerosol. The consequences of this aerosolization are even worse if dispersion is unrestricted between contaminated and uncontaminated zones. In addition, phages present in recycled by-products may also spread to the entire factory environment, since bioaerosols can remain in the air for long periods.^{34,35} Additional underestimated sources of phage contamination are the working surfaces in the dairy facilities. In a recent study,³³ a qPCR assay found evidence for the presence of genetic material from c2-like and 936-like lactococcal phages on a variety of surfaces, such as floors, walls, stairs, door handles, office tables, equipment, cleaning materials and pipes. Although it is unclear whether these phages were active or inactive at sampling, these data emphasize the relevance of correct sanitation measures as well as personal training to diminish the risks of phage infection.³³

Recycling of milk by-products. The dairy industry, particularly cheese manufacturing, recycles whey protein concentrates (WPC) to increase product yield and/or enhance attributes of the final product.^{36–40} However, such a process is risky due to the possible presence of phages in these ingredients.²⁹ Indeed, phage remained present in liquids (whey, WPC, etc) subjected to pasteurization and even stronger heat treatments, such as 95°C for several minutes.^{22,30,41} Moreover, salts, fat, saccharides, and whey proteins may protect phages from thermal damage, thus increasing the risk of this recycling practice. To compound the risk associated with WPC, whey is frequently concentrated (ultrafiltration or microparticulation), thereby increasing the phage levels due to the possible retention of virions by the membranes. A general recommendation to minimize problems associated with WPC should consider its addition only to a fermentation involving the use of significantly different starter cultures, such as mesophilic and thermophilic bacteria. It must also be noted that whey products derived from manufactures using natural undefined (unknown composition) starters should not be added to processes driven by defined (known composition) strain cultures. Natural starters often contain phages and those viruses represent a serious threat to the limited number of strains composing the defined starter cultures.¹

Prophages. Genome sequencing projects has confirmed that many LAB strains contains prophages.⁴² In fact, lysogeny is widely distributed among dairy lactococci and lactobacilli.^{43–49} A significant lower incidence of lysogeny was demonstrated in *S. thermophilus* species, as only a few strains (1–2%) were induced by mitomycin C, although others reported much higher frequencies (25%).⁵⁰ A recent study showed that 25 out of a collection of 30 probiotic strains of *Lactobacillus* contained inducible prophages.⁵¹ Putting these lysogenic LAB under certain environmental conditions such as heat, salt, antimicrobials, or starvation, may activate the induction prophages that will replicate, leading to the release of new virions. The latter can potentially infect sensitive strains if present in starter cultures.⁴⁴ Capra et al.⁵² isolated

two lytic phages for the strain *Lb. paracasei* A from pure cultures, indicating that both phages could most probably have evolved from a lysogenic state. Whenever possible, the presence of prophages as well as the risk of their spontaneous induction should be carefully investigated when selecting strains and designing cultures for specific industrial fermentation processes.²

It should be noted that detecting the presence of inducible prophages in lysogenic strains might involve several assays. Ideally, culture treatment with an inductor leading to cell lysis and the subsequent plaque formation is the first evidence of lysogeny. However, suitable indicator strains may be hard to find and thus, a negative result is not proof for the absence of inducible prophages. Observation under an electron microscope to visualize induced phages in a lysate may be an option.^{52,53} Even prophage remnants that have lost most of the phage genome, are not inert entities within bacterial chromosomes. Indeed, defective (and functional) prophages are source of genes to recombine with infecting virulent phages.^{53–58}

Interestingly, lysogenic strains may not always result in detrimental consequences. Studies of controlled lysis of lysogenic bacteria have shown positive effects, such as a decrease in bitterness for some ripened cheeses, where the hydrolysis of casein-derived hydrophobic peptides is performed by intracellular bacterial peptidases released by phage lysis.⁵⁹ Prophages might also be responsible for the resistance of a lysogenic strain against infection by virulent phages. The protection is conferred by prophage genes, particularly superinfection exclusion genes, which might encode repressor molecules.⁶⁰

Classification of Dairy Bacteriophages: An Overview

According to the International Committee on Taxonomy of Viruses (ICTV), all known phages infecting LAB are tailed phages and members of the *Caudovirales* order. Tailed phages are, in turn, organized into three families: *Podoviridae*, *Myoviridae*, and *Siphoviridae*. *Podoviridae* members have short and noncontractile tails; myoviruses have tails with a contractile sheath and a central tube while siphophages have noncontractile tails.⁶¹

As previously stated, *Lactococcus* is the most extensively LAB used by the dairy industry and phages infecting this genus are the most studied. Lactococcal phages belong mainly to the *Siphoviridae* family, with a few being *Podoviridae*. Lactococcal phages are currently classified into 10 groups based on morphology and genomic sequence analyses. At least one genome from each lactococcal phage group is available.^{62,63} However, most lactococcal phages isolated from dairy fermentations belong to one of the three main groups: 936, c2, and P335.

A recent review of *Lactobacillus* phages reported 231 phages, 186 of them morphologically characterized.¹⁴ A total of 109 were siphophages, 76 were myoviruses, and only one belonged to the family *Podoviridae*. Before the availability of genomic sequences, the classification of *Lactobacillus* phages was based mainly on morphological observations and DNA homology, *Lb. delbrueckii* phages being the first to be classified in the 1980s.⁶⁴ Later, several completely sequenced *Lactobacillus* genome phages were assigned

to a classification scheme based on the organization of the structural gene module of the siphophages.⁶⁵ Further proposals for classification of *Lactobacillus* phages were based on the deduced proteomic trees, disregarding phage morphology, but the underrepresentation of *Lactobacillus* phages in these schemes might distort the impact of those phylogenetic trees.⁶⁶

All *S. thermophilus* phages reported to date are members of the *Siphoviridae* family, and can be assembled into two distinct groups according to their DNA packaging mechanism (*cos* or *pac*) and the number of major structural proteins.⁶⁷ Although a third group of *S. thermophilus* phages may have been uncovered recently.⁶⁸ A strict correlation exists between the presence of a particular set of major structural phage proteins and the mechanism of DNA packaging, demonstrating that *cos*-containing phages possess two major structural proteins in contrast to the *pac*-containing phages, which possess three major structural proteins. Moreover, a great diversity of streptococcal phages is often observed in cheese making, in contrast with a more homogeneous phage population in yogurt production facilities.¹² The diversity of phage populations in cheese making may be due to the rotation of multiple strains of *S. thermophilus* in starter cultures, as compared with yogurt starters.^{69,70} Genomic sequences are available for a few isolates of each group.¹²

Leuconostoc strains are present in some dairy mesophilic starters, most often mixed with lactococci.⁷ This combination is essential for most applications since *Leuconostoc* grows slowly in milk as compared with lactococci but its addition still provides specific dairy flavors. Very scarce information is available on the biology of *Leuconostoc* phages, possibly because few phage problems have been reported in the literature.^{30,71–74} On the other hand, *Leuconostoc* phages have been isolated during in coffee fermentation⁷⁵ and in sauerkraut fermentation brines.⁷⁶ Globally, most of these phages were assigned to the *Siphoviridae* and to the *Myoviridae* families. More recently, the first complete phage genome sequence from a *Leuconostoc* phage was reported.⁷⁷ Bioinformatic analysis revealed low similarity with other phage genomes, pointing out that this phage is a rather unique.

Detection and Quantification of Dairy Bacteriophages: A Brief Survey

Early phage detection in raw milk, ingredients or the dairy environment is designed to diminish and control phage attacks during the fermentation processes.⁷⁸ Two general types of phage detection methods are available: direct and indirect. Direct detection methods focus on detecting the presence of lytic phage particles or their components (DNA, proteins) in a sample. Standard microbiological methods, i.e., plaque assays, spot tests and activity tests, are usually applied to milk or fermented products (cheese whey and fermented milks). One of the advantages of this type of technique is discrimination between phage and non-phage inhibitors. Disadvantages include the requirement for a sensitive indicator strain and the relatively long time needed to obtain results. Therefore, molecular detection is a preferred method, especially because the assay time is much shorter. Several assays based on the

polymerase chain reaction (PCR) have been designed and successfully applied to detect, or even classify, *Lactobacillus*, *Lactococcus* and *Streptococcus* phages in different dairy matrixes, including cheese whey, cheese whey starters, and milk samples.⁷⁹ The detection limit of a classical one-step PCR method usually ranges from 10^4 – 10^7 PFU ml⁻¹, depending on the phage type and sample, but an additional phage concentration step will allow detection of as little as 10^3 PFU ml⁻¹. qPCR-based methods provide highly sensitive, rapid and real time monitoring of specific phages during the fermentation process. Rapid detection assays (no more than 30 min) of *Lb. delbrueckii* and *S. thermophilus* phages were recently reported, with 10^4 PFU ml⁻¹ and 10^5 PFU ml⁻¹ of milk as quantification limits, respectively.^{80,81} In a recent study, this methodology allowed the detection and enumeration of three groups (c2, 936 and P335) of lactococcal phages in goat's raw milk and whey with a low detection limit (10^2 UFP/ml) in about 2 h.⁸² However, it must be noted that these molecular methods do not discriminate between active and non-active phage particles since they detect phage DNA. Molecular detection techniques can also be too expensive and too specific for routine monitoring. Moreover, another major inconvenience common to all DNA-based detection methods is that they can only detect phages whose genome sequences are available. To overcome these limitations, PCR-based methods and classical microbiological assays might be used together to obtain more data about the phages contained in the sample (titers, host range, phage type).⁸³

Of the traditional indirect methods, the activity test is one of the most commonly implemented for routine analysis in dairy plants. The presence of phages in a sample is assessed as a decrease in acid production (compared with a phage-free control sample) by a starter or strain culture in sterile, steamed or pasteurized milk.⁸⁴ Important limitations must be considered for this assay, particularly for mixed strain starters, since phage-insensitive strains will continue to grow and acidify. Other detection methods, called indicator tests, are based on the reduction of an indicator compound (generally, methylene blue or bromochresol purple), due to culture acidification in presence and absence of sample filtrate.⁸⁵ If phages are present in the sample, a time delay or a failure in the color change is observed. As in the activity test, mixed cultures may mask the presence of phages, producing false-negative results.

Another indirect method proposed for monitoring the fermentation process involves flow cytometric analysis. Flow cytometry is based on the detection of cells with low mass that are found late in the lytic cycle. Detection of lactococcal phage infection by flow cytometry was recently reported with limits comparable to classical PCR methods (10^5 PFU/ml).⁸⁶

In this case, the authors observed that during phage infection the typical lactococcal chains are broken up while cells with low-density appeared and could be detected. Results of the study demonstrated that phage infection of *L. lactis* is fast and efficiently detected, in a real time and even at the first signs of phage attack. The detection was evidenced as early as 1 to 2% of the lactococcal cells were infected. On the other hand, the authors argued that large particles such as eukaryotic cells and fat globules should be removed in order to avoid the blocking of the flow cytometer.

Another indirect method is based on the detection of changes in the electrical impedance or conductance of the milk, due to a decrease in lactic acid production when a phage infection occurs.⁸⁷ In a recent study, García-Aljaro et al.⁸⁸ developed a rapid phage detection method based on the evaluation of impedance changes during infection of a host-biofilm established onto metal (platinum and gold) microelectrodes. The infection and subsequent host cell lysis was monitored by non-faradaic impedance spectroscopy in milk samples. In this case, an *Escherichia coli* phage and its host were chosen as models, but the methodology would be applicable to any dairy phage, as long as a suitable bacterial host can grow on the microelectrode surface. The simplicity of the assay and the possibility of miniaturization of the system are among the advantages.

Recently, a method combining epifluorescence microscopy and Atomic Force Microscopy (AFM) was reported to monitor the presence of phages.⁸⁹ Specifically, epifluorescence microscopy allows *Lb. helveticus* phage particles to be enumerated from phage-infected cultures, while AFM allow monitoring changes in phage and bacteria population during the infection process. Phage particles to be enumerated with epifluorescence microscopy require SYBR Green I staining, then the emitting green light results in a bright particle larger than the actual size of the virion, enabling it to be counted with a fluorescence microscope. As disadvantages, authors have highlighted that both virulent and non-virulent phage particles are counted by the epifluorescence microscopy. Considering these facts, the authors suggested a combined phage count approach, including plaque assay and epifluorescence, in order to determine the total viral abundance and host specificity in dairy samples.

As acknowledged the early detection of bacteriophage in milk, raw ingredients or by-products at any point during fermentation, is extremely helpful minimizing the detrimental consequences of phage attacks in dairy factories. Nevertheless, several characteristics must be taken into account when selecting a particular phage detection method, including the volume of milk transformed each day, the type of fermentation process, the starter culture used, the diversity of the phage population, and the risk or frequency of phage infections. Additional considerations include the requirement for rapid results, the quantification limit, and, finally, the cost of the assay.

Control Strategies in Dairy Plants

Since the presence of phages is unavoidable in dairy plant environments, phage control strategies are designed to control rather than eradicate them.²¹ Culture rotation programs, direct vat-inoculation of starters, careful handling and disposal of whey,⁹⁰ use of phage-inhibitory media, optimized sanitation, and use of starter cultures with increased phage resistance⁹¹ are some of the approaches applied to minimize phage spreading in dairy plants (Table 1).

Plant design, airflow, and equipments. The layout of a dairy factory is one of the critical factors for preventing phage infection. Contact between raw materials and waste (whey or water) should be avoided. Some examples include the physical separation of the milk reception from other plant areas, as phage-containing

Table 1. Phage control strategies in dairies discussed in this review

| Phage source | Control strategies | Application methodologies | Remarks |
|---|---|--|---|
| Factory environment | Factory and equipment design | Physical separation of plant areas | |
| | | Use of specific manufacturing areas for distinct technologies | |
| | | Use of filtered air under positive pressure | |
| | | Control of bioaerosols | |
| | Process design | Optimization of the processing steps | |
| | Sanitation | Use of effective sanitizers and disinfectants | Efficiency depending on phage susceptibility, phage initial load and suspension media |
| | | Physical treatments (UV light irradiation, photocatalysis) | |
| Raw milk | Refrigerated storage of raw ingredients | | |
| | Sanitation | Thermal treatments of raw materials and ingredients | Efficiency depending on phage susceptibility, phage initial load and suspension media |
| | | High pressure technologies | Under laboratory tests only |
| | Direct vat-inoculation starters | | Available for all types of processes |
| | Use of starter cultures with increased phage resistance | Bacteriophage-insensitive mutants | Simple methodology, without regulatory restrictions, valid to many LAB species |
| | | Bacteriophage-resistant derivatives | Strains containing natural phage resistance mechanisms |
| | | Genetically Modified Organisms | Available only in a few countries |
| Culture rotation programs | | Suitability for many types of processes excluding probiotic products. Increased phage diversity | |
| Water as ingredient | Use of microbiologically safe water | | |
| Processed or recycled ingredients (whey, i.e.) | Adequate whey handling | Avoiding bioaerosol generation (closed drains, i.e.) | |
| | | Sanitation before recycling (thermal treatments) | Efficiency depending on phage susceptibility, phage initial load and suspension media |
| | | Adequate disposal | |
| | Limiting the recycling of the final waste within the plant | | |
| Lysogenic strains | Assessment of their absence when designing/selecting defined starter cultures | | |

aerosols can be generated during tanker emptying and raw milk spillage. The starter preparation room should be sealed off from the manufacturing area and maintained under a positive pressure of filtered air. Another concern involves the whey tanks and separators, which should be placed in a separate area situated as far as possible from the starter room and the cheese manufacturing vats.

Avoiding the generation of bioaerosols as well as limiting the air microbial count by using spray systems with appropriate disinfectants should help in controlling infections.²¹ Additionally, the air used for positive pressure applications must be filtered to

remove dust particles, which may bind phages, and the air inlet for the filters should be located as far as possible from the milk silos and whey tanks. Finally, the efficiency of the filters should be checked regularly.^{21,92}

Stainless steel with a high grade of polish is the ideal material for all the equipment used in the fermentation processes, as it should be subjected to an efficient cleaning and sanitizing before and after use. The fermentation tanks must be closed, sterilized by heat or sanitizers, with positive pressure filtered air in the headspace; the tanks should be monitored periodically for cracks.⁹²

Factory sanitation. A strict system for cleaning and disinfection of equipment and utensils used during processing are mandatory for maintaining phage levels as low as possible, and minimizing the risk of phage infection and dissemination within the dairy. No compromise should be made here. Several factors should be considered when selecting a sanitizer, including a fast antimicrobial activity, ease of application, low cost, lack of negative impact on the final product, and degradation into harmless final compounds. The effectiveness on phage inactivation is a criterion taken into consideration only recently, which is reflected by an increasing number of studies on their viral effectiveness.

Peracetic acid-containing products are often the most effective, assuring fast and efficient inactivation of phage particles. Sodium hypochlorite, ethanol and isopropanol, typically used for cleaning laboratory surfaces and utensils, are notably less effective in the inactivation of viruses.⁹³ Recently, the effectiveness of several classic biocides used by the dairy industry was evaluated on phages infecting *Lb. delbrueckii*,⁹⁴ *Lb. casei* and *Lb. paracasei*.⁹⁵ Biocides at extreme pH, such as alkaline chloride foam or ethoxylated nonylphenol with phosphoric acid (pH values > 12 and < 2, respectively), were exceptionally efficient, although pH level is not the only factor to take into consideration when choosing a biocide.⁸³ While quaternary ammonium chloride was efficient,⁹⁴ p-toluenesulfonchloroamide showed no reduction in phage numbers.⁹⁵

As phage particles can remain in the air for long period of time, bioaerosols are one of the most important dispersion routes of virions. Very few studies have addressed this issue. For example, little data are available on the viral efficiency of fumigation/fogging systems, ozone treatment, and UV light irradiation in industrial facilities. The photocatalytic properties of TiO₂ have been investigated, but mainly for the photochemical pollutant oxidation. Several advantages of photocatalysis, such as low cost, high abundance and safety of TiO₂, the absence of residues, treatment of pollutant mixtures, broad range and ease of operation, suggest this methodology as an alternative to the traditional chemical disinfection. Semiconductor TiO₂ generates highly oxidizing species (O₂⁻ and ·OH) when photoexcited by UV radiation, thus catalyzing various chemical reactions, including the decomposition of organic compounds. Photocatalysis application has been mostly intended to destroy fungi, bacteria and spores in the air,^{96–105} but its efficiency for inactivating viruses in bioaerosols has been explored only recently. Kakita et al.⁹⁶ and Kashige et al.⁹⁷ have reported the inactivation of LAB phage PL-1 (*Lb. casei*) liquid suspensions using a ceramic preparation coated with a mixture of oxides (TiO₂ and AgO) and black-light (BL) (300–400 nm). Reduction of 6 logs were reported for *Lb. delbrueckii* and *Lb. plantarum* phages after photocatalysis exposure for less than 1 h while 2 h were needed for *Lb. casei* and *L. lactis* phages.^{23,35}

Ingredients treatment and recycling of products. From the time of collection, immediate refrigerated storage of milk is required to diminish the risk of microbial propagation including bacterial viruses. Depending on the type of product to be manufactured, the milk undergoes different heat treatments to reduce microbial load (pathogens and spoilage). These heat treatments also indirectly reduce viral titers.⁹² However, a remarkably

high thermal resistance has been reported for some phages infecting *L. lactis*, *S. thermophilus*, *Lb. casei* and *Lb. paracasei*, even up to 5 min at 95°C.^{15,22} High levels of thermo-resistant phages (10⁹ PFU / ml) have also been found in whey, brine and cream. Thus, the recycling of these by-products should be avoided since the return of even small quantities of phages can lead to constant propagation. Consequently, adequate heat treatment of by-products, prior to recycling, is recommended in order to reduce the problems associated with recontamination.^{30,106} It is recognized, however, that the physical properties and function of whey proteins can be severely affected by treatments to minimize phage load in by-products.⁸³

Inactivation of dairy phages using technologies involving high pressure has been explored.^{107–110} The most studied and applied pressure-based processes are high hydrostatic pressure (HHP) and high pressure homogenization (HPH). Moroni et al.¹⁰⁷ demonstrated a significant difference in sensitivity to HPH between the two morphological types of lactococcal phages: prolate-headed (c2-like) were less stable than isometric-headed (936-P335-like). Others observed reduction of 2 to 6 logs for phages of *Lb. paracasei*, *Lb. casei*, *Lb. delbrueckii*, *Lb. plantarum*, *Lb. belveticus*, *S. thermophilus* and *L. lactis*, after 5 passes at 100 MPa in reconstituted skim milk.^{95,109} The phage inactivation rate was proportional to both applied pressure and number of passes. The influence of suspension media (milk, whey permeate, buffer) was variable, with the results dependent on the phage tested and authors.^{107,109}

Similarly, HHP has been proposed as an alternative to the thermal treatments applied in food preservation.¹¹¹ Little data are available on HHP, although inactivation of some dairy phages has been reported and seems variable from one phage to another. Specifically, *L. lactis* phages P001 (c2-like) and P008 (936-like), suspended in enriched M17-broth, were treated at up to 600 MPa.¹¹² The isometric phage P008 was considerably more resistant, with a 5-log reduction in concentration after treatment for 2 h at 600 MPa, whereas the same titer reduction was obtained for prolate phage P001 during the pressure-build-up time. Only an exhaustive analysis of costs, involving the estimated yield and the desired product characteristics, would help dairies to select alternative treatments to be applied to raw materials and the dairy environment in order to diminish the risk of phage infections.

Phage inhibitory media. Culture media might be designed to contain components that inhibit or delay phage propagation. For example, one strategy use culture media containing chelating agents, such as phosphates or citrates, capable of binding divalent cations, which are often needed to successfully complete the phage lytic cycle.⁷⁸ The use of sodium tripolyphosphate-high solubility (TAS) at low concentrations (0.3–0.5%) in milk was effective at inhibiting the lytic cycle of LAB phages.^{5,113} However, some bacterial strains showed a delayed growth and acidification profile, possibly due to the buffer ability of the added phosphates. Another technology used purified phage peptides as an additive to protect a lactococcal culture, though phages were not inactivated.¹¹⁴ The peptides were able to extend the growth of *Lactococcus* culture in phage-containing L-M17 medium and milk. The culture was even protected from phage infection

through renneting and ripening stages when the starter culture bulk was prepared in a medium containing the phage peptides.

Starter cultures. The use of natural starters composed of an undefined mixture of different strains and/or species is still the key for the production of many traditional cheeses in various countries.¹¹⁵ These artisanal starters are considered to be highly tolerant to phage infection because they are grown in the presence of phages, which lead to the dominance of resistant or tolerant strains. However, the limited reproducibility of their technological performance has led to the replacement of these traditional starters by direct multi strain cultures (DSC) in the production of many industrial large-scale cheese varieties. The strain and/or species in DSC are perfectly defined and their technological performance is highly reproducible. However, as a consequence of the limited number of strains used, a phage infection may cause the disruption of lactic acid fermentations. The use of concentrated DSC, added directly to the vat (Direct Vat Inoculation cultures—DVI) constitutes an alternative without need for on-site starter propagation, therefore, diminishing the risk of infection by phages from the cheese factory environment. Moreover, rotation of these cultures is probably the main basis for an efficient phage control program: avoiding recontamination by the same phage and build-up of high phage levels in a cheese plant. Although this strategy is not suitable for all dairy manufacturing processes, it provides a relatively simple way to minimize fermentation failures due to phages.⁸³ As a consequence, recent efforts have been made to search for potential new starter bacteria from the pool of wild strains recoverable from raw milk, undefined cultures, or traditional dairy fermented products. Hence, LAB strains with dairy-grade (e.g., antibiotic susceptibility) or pro-technological (e.g., broad phage resistance, high acidification activity, lack off-flavor development) traits are highly valued.¹

The extended co-survival of LAB and phages in the same environment has prompted the strains to acquire a variety of native phage defense systems.¹¹⁶ These mechanisms include inhibition of phage adsorption, blocking of DNA injection, restriction/modification systems, CRISPR-Cas systems and abortive infection (for a list of reviews on this subject see Garneau and Moineau⁸³). In lactococci, these mechanisms may be encoded by chromosomal or plasmid genes. Interestingly, natural gene transfer by conjugation of plasmid DNA is a common feature of lactococci. So, the conjugation of native phage resistant plasmids has been a profitable strategy for genetically improving dairy LAB for over 20 y, yielding multiple dairy starter cultures that have been in commercial use for many years, many of them under worldwide patent.^{3,117} However, although the conjugal transfer of phage resistance plasmids represents one of the most convenient, simple, and “natural” strategies to improve starter strains, the isolation of bacteriophage-insensitive mutants (BIMs) is an alternative for bacteria without conjugative plasmids. Several studies have described the isolation of spontaneous phage resistant variants from sensitive strains of lactococci,^{118,119} *S. thermophilus*,^{120–123} *Lb. helveticus*,^{124–127} *Lb. delbrueckii*,¹²⁸ *Lb. casei* and *Lb. paracasei*¹²⁹ strains. Though there are some disadvantages to this methodology (e.g., a high frequency of phenotype reversion and physiological bacterial modifications), the isolation of BIMs has gained

recent popularity because it is simple and involves no genetic manipulation, thus there are no regulatory restrictions to applications in industrial environments. The mechanism involved in BIM generation has been attributed to mutations in the phage receptors,^{120,130,131} even though recent studies have demonstrated that CRISPR-Cas systems^{121,123} or abortive infection systems¹³² play a role in the development of BIMs.

Over the past 25 y, the construction of genetically engineered strains has been intensively studied as an alternative to the development or use of transconjugants or phage-resistant mutants. Several genetic tools, based on the characterization and exploitation of the LAB native phage defense mechanisms as well as some phage genetic elements,^{3,133} have been designed. These anti-phage approaches include origin-derived phage-encoded resistance, antisense RNA technology, phage triggered suicide systems, overproduction of phage proteins, DARPins, and neutralizing antibody fragments.^{11,83} Nevertheless, despite intensive research and economic support, dairy and starter culture industries have not benefited as expected mainly due to modest progress in the development of legislation regarding Genetically Modified Organisms.

Controlling phage infections of probiotic bacteria is starting to be documented and may become a new challenge. The manufacture of certain types of probiotic products involves propagation of the strains as a starter^{134–137} making them particularly vulnerable to phages.¹³⁷ Also, *Lactobacillus* strains have long been known to harbor prophages, yielding the possibility of spontaneous prophage induction during use, or of prophage DNA involvement in the generation of new virulent phages.^{51–53} For probiotic phages, control strategies are limited as strain rotation is likely not possible and specific health claims may not be directly applicable if a phage-resistant derivative is generated.

Concluding Remarks

The risk of phage infection in processes relying on bacterial growth is here to stay. Despite significant progress made over the past decades to reduce the overall problem associated with phage contaminations, improvements are still needed. Ideally, fast and online tools that would detect amplifying phages would be a welcomed addition for most industries relying on bacterial growth. New technologies to remove phages from raw materials and by-products as well as air and equipments are still needed. Finally, a better understanding of phage-host interactions is an ongoing venture to appropriately select or develop bacterial strains for long-term industrial use in phage-contaminated environments.

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