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Article type : Review Article

Bacteriophages on Dairy Foods

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Running headline: Phages in Dairy Foods

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SUMMARY

This review focuses on the impact of bacteriophages on the manufacture of dairy foods. Firstly, the impact of phages of lactic acid bacteria in the dairy industry, where they are considered enemies, is discussed. The sources of phage contamination in dairy plants are detailed, with special emphasis on the rise of phage infections related to the growing use of cheese whey as ingredient. Other topics include traditional and new methods of phage detection, quantification and monitoring, and strategies of phage control in dairy plants, either of physical, chemical or biological nature. Finally, the use of phages or purified phage enzymes as allies to control pathogenic bacteria in the food industry is reviewed.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jam.14062

Keywords: Bacteriophages, Lactic acid bacteria, Dairy foods, Fermentation failure, Phage detection and control, Pathogen biocontrol.

Introduction

Bacteriophages, usually refereed as "phages", are viruses that infect bacteria, and they are present in every ecosystem where bacteria exist. Phages are the most prevalent and ubiquitous biological entities on Earth, outnumbering bacteria by an estimate of 10-fold (Emond and Moineau 2007). Phages can have different morphologies, but all known phages infecting lactic acid bacteria (LAB) belong to the *Caudovirales* order, characterized by a genome of double stranded DNA packed in a head (capsid) connected to a tail (Ackermann 1998). Phages replicate through infection of the host cell and subsequent arrest of host metabolism, replication and assembly of phage particles, and release of the virions after cell lysis, in a process known as lytic cycle. Phages can be classified as virulent and temperate. While the former can use only the lytic cycle, temperate phages can integrate their genomes into the bacterial chromosome and multiply in that dormant state, known as prophage, through multiple cell divisions, until external conditions trigger the lytic cycle (Capra and Mercanti 2012). A strain containing one or more prophages is a lysogenic strain or lysogen. Phages play a key role in the balance of bacterial populations, and they can move between different environments, promoting horizontal gene transfer and therefore forcing bacteria to fast evolve (Breitbart and Rohwer 2005).

Phages have been recognized for decades as "enemies" in diverse industrial environments. Phage-related problems were not only reported in the food industry, but also in pharmaceutical, chemical and pesticide factories. However, the largest problems originated by phage presence have been described in dairy industries (Garneau and Moineau 2011), where the first report was made more than 80 years ago for phages infecting lactic streptococci (Whitehead and Cox 1935). LAB comprise different genera with some common features, i.e. Gram-positive, low-GC, aerotolerant, non-motile, non-sporulating and strictly fermentative bacteria that produce lactic acid as the major

metabolic end product of carbohydrate fermentation (Björkroth and Koort 2011). The most important strains used in the dairy industry are circumscribed to certain LAB genera and species, mainly *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc* sp., and/or *Lactobacillus* sp. (Stiles and Holzapfel 1997). The correct manufacture of cheese, yogurt and other fermented milks rely on the acidification carried out by LAB cultures added to milk after pasteurization. The main sign of phage infection in a milk fermentation process is the decrease of the starter culture activity, leading in worse cases to a total arrest of acidification. However, phage attacks to adjunct cultures, such as probiotic bacteria added to dairy products, may not be easily evidenced (Mercanti *et al.* 2011). On the other hand, phages can also be considered "friends", when they infect unwanted bacteria. Consequently, the use of phages as biocontrol agents of pathogenic bacteria in the dairy industry will be discussed as well.

Sources of contamination in dairy environments

Phages are ubiquitous and can cause severe problems into fermentative processes in the dairy industry. For this reason, it is crucial to identify the sources or reservoirs of phages within a factory in order to introduce corrective actions to limit their propagation.

Raw milk and ingredients

Although several sources of contamination exist, the main entrance of phages in dairy factories is likely to be through raw milk, which may contain phages up to 10^4 plaque-forming units (pfu) ml^{-1}. It is considered that phage titers about 10^6-10^7 pfu ml^{-1} lead to significant fermentation failures, but concentrations as low as 10^2 pfu ml^{-1} can already cause problems, even if they do not stop the fermentation (Tayyarcan *et al.* 2018). One study on raw milk samples obtained from different dairies in Spain determined that approx. 10% of the samples contained *L. lactis* phages (Madera *et al.* 2004), while lactococcal and streptococcal phages were detected in 37% of raw milk samples used for yogurt (del Rio *et al.* 2007). Phages native from raw milk were still infective after diverse heat treatments (Atamer *et al.* 2013; Capra *et al.* 2013; Tayyarcan *et al.* 2018). Combinations of

temperature and time normally used for pasteurization, i.e. low temperature/long time (LTLT; 63°C, 30 min) or high temperature/short time (HTST; 72°C, 15 s), are well known to be largely insufficient for the elimination of most LAB phages (Binetti and Reinheimer 2000; Suárez and Reinheimer 2002; Capra *et al.* 2006; Mercanti *et al.* 2012; Capra *et al.* 2013; Tayyarcan *et al.* 2018). A particularly high thermal resistance was observed for phages infecting *L. lactis, Lactobacillus helveticus*, *Strep. thermophilus, Lactobacillus casei, Lactobacillus paracasei, Leuconostoc pseudomesenteroides, Leuconostoc mesenteroides* and *Lactobacillus delbrueckii*. The killing of 99% of the phages by heat treatment of milk at 72°C required long times, between 2 and 300 min (Quiberoni *et al.* 1999; Suárez and Reinheimer 2002; Quiberoni *et al.* 2003; Müller-Merbach *et al.* 2005; Guglielmotti *et al.* 2012; Atamer *et al.* 2013; Capra *et al.* 2013; Pujato *et al.* 2014; Wagner *et al.* 2018). Most studies, however, tested heat resistance of phages in glass or stainless-steel tubes in a water bath, whereas a pilot-plant pasteurizer showed much improved efficiency using the same combination of time and temperature, due to better heat transfer achieved by the continuous, turbulent flow through plate heat exchangers (Wagner *et al.* 2018).

Factory environment

With regard to the levels of contamination in air, Atamer *et al.* (2013) reported phage counts close to 10^8 pfu m^{-3}. Liquid splashes and air displacement around vats and other contaminated surfaces generate aerosols, which remain in air for long periods and are recognized as a major route of phage dissemination in dairies (Verreault *et al.* 2008; Verreault *et al.* 2011; Briggiler Marcó *et al.* 2017). In this way, phages present in recycled by-products or any other material subjected to aerosolization may also spread to the entire factory environment. Working surfaces constitute an underestimated source of phage contamination in dairy facilities. The presence of genetic material from lactococcal phages was evidenced in many surfaces, such as floors, walls, stairs, door handles, office tables, equipment, cleaning materials and pipes (Verreault *et al.* 2011). Although it is unclear whether these phages were active or inactive at sampling, these data emphasize the relevance of applying correct

sanitation measures as well as an adequate training of the personnel to diminish the risk of phage infections (Briggiler Marcó *et al.* 2017).

Whey and whey products

Whey from cheese making or casein processes can have different fates, from simple discharge into natural waterways to the isolation of important products. The former is a low cost method that was commonly applied several decades ago, but due to the high impact of whey as an environmental pollutant, it has been replaced by alternative treatments (McIntyre *et al.* 1991; Carvalho *et al.* 2013; Yadav *et al.* 2015). Thus, whey has been progressively used as is for spray irrigation of pastures and animal feeding, concentrated by evaporation or, more recently, fractionated into specific whey components using centrifugation, chromatography, ion exchange or membrane filtration (Masotti *et al.* 2017). Alternatively, biomass and defined metabolites, or specific molecules of interest can be obtained by fermentation or chemical reaction, respectively.

Processed whey come in diverse forms: native whey extracted directly from skim milk, whey protein concentrate (WPC), isolate (WPI) or hydrolysate (WPH), microparticulated whey protein, whey cream and whey powder (Yadav *et al.* 2015). Due to the fact that whey proteins are among those with the highest nutritional value, there has been a growing interest in their use as additive in nutritional supplements, cereals, infant formulae, beverages and dairy products, where they are also expected to improve yield, texture and/or quality (Yadav *et al.* 2015; Ipsen 2017; Masotti *et al.* 2017). However, when processed whey or whey derivatives are to be incorporated during the manufacture of cheese or other fermented dairy products before the fermentation step, it should be taken into account that these additives may contain phages at high concentrations.

Phages can survive and multiply in cheese whey, reaching titres as high as 10^9 pfu ml^{-1} after cheese manufacture (Atamer *et al.* 2009; Atamer *et al.* 2011). Phage numbers might even increase up to about 1 log order if whey is concentrated by means of evaporation or membrane filtration (Samtlebe *et al.* 2017a). On the other hand, the presence of phages in dried milk products (e.g., skim milk powder, whey powder, WPC, WPI) and their characterization have not received much attention until

recently (Wagner *et al.* 2017a; Wagner *et al.* 2017b). Nevertheless, this could be a significant problem in economic terms, given the ever high frequency of phage infections in dairy plants and the growing volumes of whey that are being recycled and incorporated in different forms into milk products.

It has been already mentioned that many LAB phages survive pasteurization. Furthermore, the reports of LAB phages with very high or extreme thermo stability noticeably increased over the last few years, very likely due to the selective pressure exerted by the growing use of thermally treated whey in dairies (Samtlebe *et al.* 2015; Samtlebe *et al.* 2017a; Samtlebe *et al.* 2017b; Wagner *et al.* 2017a; Wagner *et al.* 2017b). Recently, Wagner *et al.* (2017a) found that dairy phages were able to survive at high titers after four years of storage in either whey powder or whey powder formulations, beyond the high initial temperatures reached by whey pasteurization and spray-drying, and the tough conditions during storage of the powder, such as low water activity and high osmotic stress. In that study, a total of 35 powder samples were screened for the presence of phages infective for *Strep. thermophilus*, *L. lactis*, *Leuc. mesenteroides* and *Leuc. pseudomesenteroides*, which could be isolated from 100%, 50% and 40% of the samples, respectively. The heat resistance of these phages was assessed (Wagner *et al.* 2017b), and most *L. lactis* phages (88%) and *Leuc. pseudomesenteroides* phages (83%) survived a heat treatment at 80°C for 5 min. What is more, 16% of all *L. lactis* phages from whey dried samples could not be completely inactivated after heat treatment at 95°C for 1 min. These data confirmed that phages isolated from spray-dried whey powders and whey powder formulations are significantly more thermo resistant than those isolated from other dairy sources.

Lysogenic bacteria

Prophages are found in most commercial strains of lactococci and lactobacilli currently used in the dairy industry (Zaburlin *et al.* 2017), and constitute a source of genes susceptible of recombination with either host DNA or another co-infective phage. In this way, phages may increase both phage and host diversity through horizontal gene transfer (Baugher *et al.* 2014; Mercanti *et al.* 2016).

Recent studies analyzed *Lact. casei* group strains, and found that 92% of their genomes contained prophages or prophage remnants, while the prophages could be induced with mitomycin C in 71% of the strains (Mercanti *et al.* 2011; Zaburlin *et al.* 2017). When exposed to certain environmental stresses such as heat, salt, antimicrobials, or starvation, lysogenic strains may lead to the release of prophages, which can potentially lose their lysogenic gene cluster and become virulent if they find sensitive strains in starter cultures (Capra *et al.* 2011). Even if this event is not usual, it should still be considered a hazard and an extra source of phage contamination (Samson and Moineau 2013).

Methods of phage detection: advantages and disadvantages

There have been substantial recent improvements in the strategies aimed to detect phages, forced in part by the high economic impact of phage infections in the food industry. In spite of that, the occurrence of new virulent phages is still significant. Table 1 summarizes the main advantages and disadvantages of different phage detection methods. Classical methods detect infective phage particles with a high sensitivity; they are economic and relatively simple to carry out, and comprise plaque assay, spot test, turbidity test and activity test. On the other hand, these methods are often time-consuming and yield non-reproducible results among laboratories (Anderson *et al.* 2011). Pujato *et al.* (2017) observed that turbidity and spot tests were only positive for 90% and 61%, respectively, of a group of samples for which lysis plaques were actually observed on plaque assays. The spot test uses smaller volumes of sample, which makes it more practical but at the same time it reduces its limit of detection. Additionally, lysis plaques (plaque assay, spot test) are hardly observed with some phages otherwise well evidenced in liquid media (turbidity test, activity test). Regardless of these disadvantages, plaque assay and spot test does not need special equipment and remain the tools recommended by the International Dairy Federation (IDF 1991).

The decreasing costs of molecular biology reagents and techniques, together with the steady increase of available phage DNA sequences, allowed the design and improvement of numerous molecular methods for phage detection (Muhammed *et al.* 2017). Numerous PCR-based assays

(including Simplex PCR, Multiplex PCR, one-step PCR, qPCR and MLST) have been studied to detect *Lactobacillus*, *Lactococcus, Streptococcus* and *Leuconostoc* phages. These techniques allow a rapid detection of bacteriophages in different dairy matrices (Binetti *et al.* 2008) with a detection limit in the range of 10^3– 10^7 pfu ml^{-1} (Muhammed *et al.* 2017). Traditional PCR (Simplex PCR or Multiplex PCR) is a specific and sensitive technology used to detect and identify phages DNA from different samples. As this method generates DNA fragments of specific size, several protocols have been developed not only to detect but also to identify, by fragment size discrimination, bacteriophages that infect the main dairy LAB species used in the dairy industry such as *Lact. casei, Lact. paracasei*, *Lact. delbrueckii, Lact. helveticus, Strep. thermophilus, L. lactis, Leuc. pseudomesenteroides* and *Leuc. mesenteroides* (Labrie and Moineau 2000; Binetti *et al.* 2005; Quiberoni *et al.* 2006; Zago *et al.* 2008; Ali *et al.* 2013). One step ahead is the use of quantitative real-time PCR (qPCR), which enables the faster and more sensitive quantification of phage particles. Besides, the use of distinctive fluorochromes allows discrimination among different phage species in the same reaction (Verreault *et al.* 2011). In a recent study, qPCR was successfully applied for the simultaneous quantitative detection (with a detection limit of 10^3 pfu ml^{-1}) of phages of *L. lactis* and *Leuconostoc* in samples obtained from a dairy plant that employed traditional mother-bulk-cheese vat system (Muhammed *et al.* 2017). Multilocus sequence typing (MLST) is a PCR and sequencing based method also tested for phage detection. This method is highly discriminatory and allowed differentiate the 936 group of lactococcal phages by the sequencing of specific phage genes (Moisan and Moineau 2012). Nevertheless, it should be kept in mind that as molecular methods are based only on the presence of phage DNA, they cannot distinguish between infective and inactive phage particles. Despite this, these techniques might be useful to quickly (a few hours) classify the batches of raw milk received in a fermentation plant according to their content of phage DNA, and consequently optimize their distribution to different processes (del Rio *et al.* 2012). For example, if DNA of mesophilic LAB phages is detected in the raw milk, this milk would be better redirected to elaborate yoghurt instead of cheese, thus allowing for an efficient distribution of the raw material.

On the other hand, Raman spectroscopy is a fast and non-destructive method that provides information about specific properties of molecules, and can be applied to living organisms (Das and Agrawal 2011; Butler *et al.* 2016). An innovative tool combining Raman spectroscopy with chemometric analysis was developed for selective detection of *Strep. thermophilus* and *Lact. delbrueckii* phages in raw milk (Tayyarcan *et al.* 2018). In that study *Strep. thermophilus* phages were detected in a short time at concentrations as low as 10^2 pfu ml^{-1}. Thus, Raman spectroscopy is a promising alternative to help designing quick phage detection tests to be applied to raw milk in dairy factories (Tayyarcan *et al.* 2018). Additional methods for phage detection involve monitoring of infected cells through flow cytometry, based on the relatively lower mass of cells at advanced stages of the lytic cycle. Adding a fluorescent phage DNA staining (e.g., with SYBR green) to this method enabled a direct enumeration of phage particles. Using this approach, Michelsen *et al.* (2007) detected phage infection of *L. lactis* in real time and with high efficiency. Another methodology based on epifluorescence and atomic force microscopy has been tested to detect phages, but the need for expensive and sophisticated equipment as well as specialized staff has hampered their actual application in the dairy industry (Zago *et al.* 2012).

Strategies of phage control in dairy plants

Phages are impossible to eliminate in dairy environments given that, as detailed in the previous section, they are naturally present in raw milk, survive most common heat treatments, disseminate among production facilities via liquid splashes and aerosolization into airborne particles, persist in equipment surfaces and biofilms, and can be found at high titres in cheese whey or other industrial effluents. Thus, control strategies are aimed to reduce phage levels and to halt phage dissemination in dairy factories. Strategies applied in this sense can be of physical (heating, filtration, high pressure, UV, electro-impulse treatments), chemical (biocides) or biological (strain rotation, use of strains with improved phage resistance) nature, and will be subsequently discussed.

Plant design and hygiene

Dairy factory design, adequate hygiene, strict and regular cleaning of equipment, and appropriate airflow, are key factors to reduce phage proliferation. First, plant personnel should be trained in the correct application of phage control procedures. Concerning plant design, the risk of cross-contamination between bacterial media, fermentation by-products and factory equipment should be minimized, for example using independent manufacturing areas for each process (Briggiler Marcó *et al.* 2012). Peracetic acid, quaternary ammonium-products and other biocides with extreme pH such as alkaline chloride foam (pH 12.7) and ethoxylated nonylphenol with phosphoric acid (pH 1.7) showed good phage removal; no phages were detected after 5 min of incubation, using the biocides at the concentrations suggested by the manufacturers (Ebrecht *et al.* 2010; Mercanti *et al.* 2012; Pujato *et al.* 2014). It is important to take into account that phages are usually quite sensitive to acid (pH < 4) and alkaline (pH > 11) conditions (Moineau and Lévesque 2005). Comparatively, sodium hypochlorite, ethanol and isopropanol failed to achieve good phage reductions in several studies (Guglielmotti *et al.* 2012; Mercanti *et al.* 2012; Pujato *et al.* 2014). Sodium hypochlorite showed good efficacy only at concentrations higher than those permitted in food industry (Guglielmotti *et al.* 2012). Concerning the presence of phages in air, Atamer *et al.* (2012) reported counts close to 10^8 pfu m^{-3}, highlighting the necessity of limiting the generation of bioaerosols as much as possible. With this purpose, air can be filtered, and bacterial starter cultures must be prepared in a positively pressurized room. On the other hand, photocatalysis (UV-A irradiation using TiO_2 as catalyst) possess a demonstrated efficiency in the inactivation of bacteria, spores, fungi and viruses, both in liquid medium and in gaseous phase. Therefore, this technology could be used to reduce phage loads (Kashige *et al.* 2001; Ditta *et al.* 2008; Sanchez *et al.* 2012; Zacarías *et al.* 2015). In this regard, *Lact. casei* phage PL-1 was efficiently inactivated by radiation UV-A in presence of TiO_2 in liquid medium (Kakita *et al.* 2000). Similarly, photocatalytic treatments were efficient for the inactivation of different phages of LAB when simulating bioaerosol generation in a laboratory-scale reactor (Briggiler Marcó *et al.* 2009; Briggiler Marcó *et al.* 2011). Recently, a semi-pilot-scale photocatalytic

reactor (UV-A/TiO_2) was designed to study the inactivation of phages contained in bioaerosols (Briggiler Marcó *et al.* 2017). Using this reactor, a decrease of 2.7 log orders was achieved after only 1 h of photocatalytic treatment.

Adequate handling of raw milk, ingredients and by-products

In general, raw milk is cooled after collection to avoid bacterial growth, and maintained refrigerated until pasteurization or other heat treatments are applied immediately after milk discharge in dairy factories. These measures are intended to eliminate pathogens and significantly reduce spoilage bacteria, but indirectly decrease phage loads as well (Guglielmotti *et al.* 2012). Nonetheless, as discussed earlier, many dairy phages survive pasteurization or harsher heat treatments, and although phage titres in milk are usually low, they can propagate up to 10^8-10^9 pfu ml^{-1} if they encounter a sensitive bacterial host during the fermentation step.

Water added to dairy processes should be microbiologically safe. Brine and cream have also been reported to contain high concentrations of thermo resistant phages (Briggiler Marcó *et al.* 2012). Therefore, the recycle of these by-products into dairy processes before fermentation, as well as that of whey or whey formulations must be avoided unless effective actions are taken for phage removal. In this sense, heat treatments are still the most commonly applied, but they cannot assure complete phage elimination and, at the same time, strong heating have negative effects on the functional properties of native whey proteins, the most valuable and thermo sensitive components of whey. Conventional spray-drying of whey (and other dairy products and by-products) has many technological and economic advantages, as reviewed by Písecký (2005), and is helpful to discreetly reduce phage titres in whey. However, this process cannot warrant efficient phage removal. This can be explained by the fact that, although inlet temperatures of air reach about 180-200°C, water is quickly vaporized during the process, with the consequent decrease of temperature within whey droplets to 60°C or below (Wagner *et al.* 2017b). Consequently, considerable attention has been paid to alternative methodologies of phage control during the last years, such as application of high pressure, membrane filtration or UV irradiation.

For lactococcal phages, sensitivity to dynamic high-pressure (DHP) treatments was found to be significantly higher for a prolate-headed phage (c2-like) than for isometric virions of 936 and P335 phage species (Moroni *et al.* 2002). Another approach, high pressure homogenization (HPH), has been also assayed for the elimination of LAB phages (Capra *et al.* 2009; Mercanti *et al.* 2012). Results were variable among phages of *Lact. casei* group, *Lactobacillus plantarum*, *Lact. delbrueckii*, *Lact. helveticus*, *L. lactis* and *Strep. thermophilus*, but reductions attained after 5 passes at a pressure of 100 MPa were only moderate (from 2 to 6 log orders), demonstrating the inappropriateness of HPH, at least if it is not used in combination with heat treatments or other phage control measures (Mercanti *et al.* 2012). For both DHP and HPH treatments, phage reduction was proportional to the number of passes and applied pressure, and significantly varied in different suspension media (buffer, milk, culture broth). Data available on the use of high hydrostatic pressure (HHP) for phage inactivation is scarce. Again, phage morphology was related to the efficiency of high pressure treatment; *L. lactis* phage P001 (with a prolate head) was more sensitive than *L. lactis* phage P008 (isometric phage) to the same HHP treatment (Müller-Merbach *et al.* 2005). Bearing in mind their cost of application, the high pressure approaches tested do not seem convincing and reliable to actually reduce phage infections in raw or processed milk products, and a comprehensive analysis of cost should be conducted to justify their use with this aim.

Considering ultrafiltration, a key issue is that not only whey proteins but also phages are concentrated when using a standard membrane with a cut-off of 20 kDa (Mistry and Kosikowski 1986). This is due to the fact that phage particles are somewhat larger than whey proteins molecules. Recently, filtration of cheese whey through different types of membranes has been investigated as an alternative to diminish phage levels without compromising nutritional value of cheese whey (Samtlebe *et al.* 2015; Samtlebe *et al.* 2017a; Samtlebe *et al.* 2017b). Samtlebe *et al.* (2015) optimized a cross-flow membrane-based filtration process in order to find a balance between good permeation of α-lactalbumin and β-lactoglobulin, the main whey proteins, and high phage retention. In that study, a polyethersulfone membrane with a cut-off of 300 kDa showed the best

compromise between retention of lactococcal phage P008 and protein permeation, whereas low protein permeation or low phage retention were achieved when using membranes with a cut-off of 100 or 500 kDa, respectively. Samtlebe *et al.* (2017b) determined the influence of phage shape and size, and the effect of adding differently treated bacterial host cells to native whey, on the efficiency of *L. lactis* phage retention through polyethersulfone membranes. While no clear effect of phage morphology was perceived, the presence of mechanically disrupted lactococcal host cells significantly enhanced phage retention. Samtlebe *et al.* (2017a) filtered cheese whey through 0.1 mm microfiltration ceramic membranes with a tubular design, either at laboratory or pilot plant scale. The latter was best in terms of phage reduction and protein transmission, which was attributed to a longitudinal permeability gradient only present in the pilot plant system membranes. Treatment of surfaces, drinking water and waste water by non-ionizing UV-C irradiation was proposed as a good alternative to heat treatments for the control of pathogenic and indicator bacteria, as well as for the elimination of viruses, including bacteriophages (Chang *et al.* 1985; Sommer *et al.* 2001; Hazem 2002; De Roda Husman *et al.* 2004; Wang *et al.* 2004; Schmidt and Kauling 2007). Sommer *et al.* (2001) compared the effect of non-ionizing (UV-253 .7 nm; dose of 750 Jm^{-2}) and ionizing (gamma) radiation, on the inactivation of phages with different nucleic acids (ssRNA, ssDNA and dsDNA). Due to their different mechanisms of inactivation, ionizing and non-ionizing radiation cannot be compared directly and absolutely, and sensitivity to UV-253 .7 nm was variable among phages. However, UV-253 .7 nm produced a 4-log reduction for the most heat resistant phage in tap water. One limitation of UV rays is their poor penetrability through most materials, including turbid solutions, which restricts the use of UV irradiation. Another drawback is that proteins are also damaged by UV rays. To overcome these limitations, a novel helical UV reactor, UVivatec®, was specifically designed for viral inactivation (though it can also inactivate bacteria) in cloudy solutions (Schmidt and Kauling 2007). The UVivatec® system possesses a fixed central Hg lamp surrounded by one channel where the liquid sample runs helically instead of linearly, with a hydrodynamic design optimized in such a way that residence time and UV irradiation

of each particle in the solution are uniform, with no over/under exposure. Wavelength is fixed at 254 nm to maximize viral damage (DNA and RNA absorption) and minimize protein damage. Although UVivatec® was not specifically designed for the food industry, the possibility of scaling up the coiled tube technology would permit its use on a high-volume scale, for example to treat cheese whey to be recycled as an ingredient in dairy processing (Schmidt and Kauling 2007). Nevertheless, photoreactivation has been verified in UV-Inactivated phages (Rodriguez *et al.* 2014). This light-mediated mechanism involves the photolyase enzyme, and can efficiently restore in hours the phage ability to infect bacterial host and multiply (Bohrerova and Linden 2007). Consequently, this phenomenon should be carefully considered when evaluating the application of UV methodologies for phage control.

In summary, membrane filtration or UV irradiation might be useful to help eliminate or at least reduce thermo-resistant phages in whey. However, it is suggested that these technologies be combined, at moderate intensities, rather than used alone at extreme conditions that will produce loss and/or damage of whey proteins. Beyond the mentioned techniques to reduce contamination with phages, there are some basic rules that should be taken into consideration to minimize their impact; for example, the recycle of whey from a process involving the use of a given starter (i.e., thermophilic bacteria) into a fermentation process driven by a non-related starter (i.e. mesophilic bacteria). Moreover, vats in which cheeses are made using DVS cultures should never be added of cheese whey produced using natural starters, as the diversity of phages naturally present in the latter makes very likely the infection of the few strains present in selected cultures.

Correct selection and use of dairy starters

Cultures of LAB used for cheese making can be composed of undefined (natural starters) or defined (selected starters) strains (Spus *et al.* 2015). The former possess a main microflora dominated by different LAB strains, but also contain a secondary, complex microflora composed of low numbers of *Lactobacillus* sp. and other bacteria (e.g. *L. lactis*, *Enterococcus faecium*) (Powell *et al.* 2011). The bacterial biodiversity of natural starters produces cheeses with taste and flavor similar to those

made with raw milk, and makes the starters highly tolerant to phage infection, given that bacterial and phage populations co-exist in a shared and self-regulated environment (Spus *et al.* 2015). In Canada, many aged Cheddar cheeses are made with mixed starter cultures because defined starters have failed to provide the expected flavor and quality (Bissonnette *et al.* 2000). Nevertheless, the variable composition and activity of natural starters does not allow controlling key parameters of milk fermentation, and for that reason they have been extensively replaced by selected, direct-vat-set (DVS) cultures. DVS starters usually contain a few selected strains that produce lactic acid from lactose at a predictable and controlled rate, rendering products that retain homogeneous characteristics and quality over time, while decreasing the risk of occurrence of uncontrolled contaminations. The correct selection of strains for DVS cultures is a long and complicated process based on biochemical, microbiological and technological criteria, which will rely on the specific product to be manufactured. As only a few strains are present in DVS cultures, if specific phages are present in the milk (raw or pasteurized), equipment surfaces or in the environment of a dairy plant, phage infections can promptly become evident (Verreault *et al.* 2011). Consequently, phage resistance becomes one of the main criteria for strain selection.

Phage-inhibitory media containing chelating agents, sodium polyphosphates or purified phage peptides have been used to inhibit or delay phage adsorption and propagation (Hutmacher *et al.* 2001; Mahony *et al.* 2015). Culture rotation is another simple alternative to hinder phage propagation through successive cheese making batches (Mahony *et al.* 2013). Strains rotated in such schemes should possess similar acidifying activity but different phage resistance profiles, and must be free of inducible prophages. However, this approach is generally useless in the case of probiotics, given that such strains possess unique traits and cannot be easily replaced (Capra and Mercanti 2012). Some LAB strains are naturally insensitive to phages via diverse mechanisms, such as inhibition of phage adsorption, blocking of DNA injection, restriction/modification (R/M) systems, abortive infection (Abi), and clustered interspaced short palindromic repeats (CRISPR) systems (Moineau and Lévesque 2005; Doron *et al.* 2018). In this sense, several studies describe the isolation

of bacteriophage-insensitive mutants (BIMs) from sensitive strains of *Strep. thermophilus*, *Lactococcus*, *Lact. delbrueckii*, *Lact. plantarum*, *Lact. helveticus, Leuc. mesenteroides* and *Leuc. pseudomesenteroides* (Quiberoni *et al.* 1998; Guglielmotti *et al.* 2006; Guglielmotti *et al.* 2007; Briggiler Marcó *et al.* 2014; Pujato *et al.* 2018). In some cases, the mechanism of BIM generation involves mutations in the phage receptors (Viscardi *et al.* 2003) or the presence of Abi systems (Haaber *et al.* 2008). In the past two decades, however, it has been unraveled the essential role played by CRISPRs systems in the generation of BIMs of *Strep. thermophilus* (Barrangou *et al.* 2007; Haaber *et al.* 2008; Mills *et al.* 2010; Hynes *et al.* 2016), which would provide the basis for the production of food-grade BIMs of starters for the dairy industry. This system allows obtaining natural BIMs after challenging sensitive strains with virulent phages (de Melo *et al.* 2018). However, it is also possible obtaining BIMs resistant to multiple phages in a fast and single assay today, by programming CRISPR-Cas systems (McDonnell *et al.* 2018). This is carried out by transforming strains that have an active CRISPR-Cas system with a high copy plasmid containing both protospacers contained in diverse phages and specific PAM (protospacer adjacent motifs) sequences (Hynes *et al.* 2016). The creation of a bank of BIMs differing only in the CRISPR region may be useful in the application of culture rotation schemes (Mills *et al.* 2010). In this sense, the DuPont group (DuPont Nutrition Biosciences ApS) supported many studies to generate BIMs using a CRISPR approach, finally obtaining a European Patent (EP 2 325 332 B1, European Patent Specification, 31.10.2012 Bulletin 2012/44) (Barrangou and Horvath 2012). Currently, another CRISPR-based application offers starter cultures composed of three to six *Strep. thermophilus* strains for pizza cheese manufacture (CHOOZITTM SWIFT, DuPontTM) (Barrangou and Horvath 2012). Besides, the rotation of strains obtained through this technology could be extended to probiotic cultures, provided that genomes remain intact outside the CRISPR region. Alternatively, newly discovered phage resistance systems with still unknown mechanisms have been described in bacteria and archaea. These systems include BREX (Goldfarb *et al.* 2015), prokaryotic Argonautes (pAgos) (Swarts *et al.* 2014), and DISARM (Ofir *et al.* 2018). Additionally, nine new families of confirmed anti-phage defense systems were recently

discovered in the microbial pangenome, three of which contain membrane-associated proteins (Doron *et al.* 2018). Many protein domains present in these newly discovered systems are similar to domains found in CRISPR-Cas and RNA interference (RNAi) systems, including helicases, nucleases, and nucleic acid binding domains. Decrypting the mechanism through which these systems operate will help in the development of new molecular tools for the generation of BIMs.

Nevertheless, and despite the great advances in the engineering of LAB strains (antisense RNA technology, cloning of replication origin, neutralizing antibody fragments, phage triggered suicide systems, overproduction of phage proteins) (Samson and Moineau 2013), there are still many legislation concerns that should be addressed prior to freely and widely use genetically modified organisms in foods.

Phages as friends: biocontrol of unwanted bacteria

The interest on phages as remarkable therapeutic agents exist since phage discovery by William Twort and the elucidation of their potential to kill bacteria by Félix d'Hérelle, about a century ago, and was still relevant during the pre-antibiotic era (Kutter *et al.* 2010; Abedon *et al.* 2011; Clokie *et al.* 2011). One major cause of severe bacterial infections in humans is the ingestion of contaminated food. Thus, phages are being considered as biocontrol agents of either pathogenic or spoilage bacteria in foods (García *et al.* 2008; Gill 2010). Fig. 1 summarizes the use of phages for biocontrol in dairy foods.

Phages possess several advantages over antibiotics and commercial biocides. Firstly, on the long term they will probably be the only reliable alternative against emergent multidrug resistant (MDR) bacteria. Besides, phages are specific and attack single species or even single strains, using a lytic mechanism totally different from antibiotics action. As a consequence, phage treatments are completely safe and do not disrupt non-targeted microbiota (Bruttin and Brussow 2005; Sarker *et al.* 2012). In this regard, all the existing evidence points out that oral consumption of even high doses of phages is completely nontoxic. In fact, phages are ubiquitous in foods, and therefore human-phage

contacts are regular and persistent (Hagens and Loessner 2010). Finally, phages do not ruin surfaces or equipment, do not modify the organoleptic quality of food and usually remain stable over long periods of storage (Fernandez *et al.* 2017).

On the other hand, there is a series of hurdles to overcome for phage biocontrol to be successful. Firstly, the high phage specificity, though advantageous with respect to antibiotics, may be a significant problem to guarantee the killing of pathogenic strains with different sensitivity to phages. With this aim, only polyvalent phages with broad host spectra should be considered. It is also advised to combine diverse phages into cocktails, which increase the efficacy and prevent the incidence of bacterial resistance. Ideally, the genomic sequence of any phage intended to be used for pathogen biocontrol should be determined to warrant absence of undesirable traits, such as lysogeny module, antibiotic resistance genes, virulence factors and genes encoding allergenic proteins. The use of strictly lytic phages is mandatory. Temperate phages might be less effective and, more significantly, they can also act as vectors of horizontal gene transfer (HGT) among bacteria, for example of virulence factors or antibiotic resistant genes (ARGs) (Haaber *et al.* 2016; Subirats *et al.* 2016; Cui *et al.* 2017; Lekunberri *et al.* 2017). Indeed, a recent study concluded that phages could largely contribute to ARGs dissemination among non-human sources (Lekunberri *et al.* 2017). For obvious reasons, anti-phage mechanisms developed by bacteria are also detrimental of phage biocontrol. In this context, however, the existence of evading mechanisms such as anti-CRISPR proteins, reported in the genomes of prophages (Rauch *et al.* 2017) and virulent phages (Hynes *et al.* 2017) would be useful, and should be taken into consideration at the time of phage isolation for pathogen biocontrol. Phages replicate only in the presence of bacterial hosts. On one hand, this could be advantageous, so phage dose would be self-regulated ("auto dosing") (Loc-Carrillo and Abedon 2011; Yen *et al.* 2017). However, foods are usually complex matrices with a micro-structure that markedly influences phage treatment efficacy, either by limiting phage diffusion movements (solid or gelatinous/viscous media) or by interaction of phages with other food components that

limit or inhibit encounters with target bacteria. These issues, as well as the analysis of the costs of production vs. phage efficacy, need to be address before real-life application (Hagens and Loessner 2010).

Once properly selected, phages can be processed distinctly in order to enhance survival and infectivity, considering the route of application. Although phages have been incorporated in different foods, the number of scientific reports of applications on dairy products is significantly lower in comparison with those on meat and other foods. As comprehensively reviewed by Cooper (2016), phages can be introduced at the starting point of the food chain (live animals, either through oral, intragastric, skin, muscle or vascular delivery), post-laughter (meat) or directly in the product (milk), or later, during or after processing in a food processing plant. In the latter case, phages can be either added directly to foods or embedded in the food packaging. It has been suggested that the best moment for phage usage would be at the same time that the pathogenic or spoilage bacterial target is expected to enter the food matrix (Hagens and Loessner 2010). Numerous strategies and carrier systems have been studied to supplement food systems with phages (Anany *et al.* 2011; Gouvêa *et al.* 2015; Gouvêa *et al.* 2016; Lone *et al.* 2016; Samtlebe *et al.* 2016). Phages destined to farm animals showed better performance when they were microencapsulated within different matrices (Samtlebe *et al.* 2016; Colom *et al.* 2017). Alternatively, diverse ways of phages incorporation into bioactive packaging materials were studied for delivery into food products (Lone *et al.* 2016).

In meat products, phage biocontrol tools have focused mainly on *Escherichia coli* O157:H7, but also on *Campylobacter jejuni*, *Listeria monocytogenes* and *Salmonella* spp. (Hudson *et al.* 2013; Tomat *et al.* 2013a; Tomat *et al.* 2013b; Liu *et al.* 2015; Yeh *et al.* 2017). Interestingly, phages of *L. monocytogenes* eliminated bacterial biofilms in the surface of industrial equipment (Iacumin *et al.* 2016). These results are promising, as bacteria in biofilms are protected by a surrounding matrix and show higher resistance to biocides treatment, representing a stable source of re-contamination in food environment (Gutierrez *et al.* 2016; Pires *et al.* 2016).

In dairy processing, there are some critical points at which phages can be included (Cooper 2016). The addition of phages after milk collection, for example, could reduce *Staph. aureus* in raw milk coming from mastitic cows and/or contamination of milking equipment. Although *Staph. aureus* does not survive pasteurization, it can produce very thermo stable toxins that can still remain active after cooking of food (Schelin *et al.* 2011). The application of phages against *Staph. aureus* have been reported in UHT and traditionally pasteurized whole milk (Garcia *et al.* 2009), during cheese manufacture and ripening (García *et al.* 2007; Bueno *et al.* 2012; El Haddad *et al*, 2016) and to eliminate biofilms, a major cause of pathogenic bacteria re-growth in dairies (Soni and Nannapaneni 2010; Alves *et al.* 2014). *L. monocytogenes* is another key pathogen found in dairy products (Perera *et al*. 2015). ListShield™ is a commercial phage (Intralytix Inc., Baltimore, USA) proposed as sanitizer for the surface treatment of both industrial equipment and cheeses. Guenther and Loessner (2011) observed a reduction of *L. monocytogenes* in artificially contaminated white mold (Camembert-type) and red-smear surface (Limburger-type) cheeses. Soni *et al*. (2012) obtained a complete 9 log cfu ml^{-1} reduction of *L. monocytogenes* after treatment with P100, a safe and efficient *Listeria* phage thoroughly characterized for use in biocontrol (Carlton *et al.* 2005; EFSA BIOHAZ Panel 2016), combined with lauric arginate in tryptic soy broth. In queso fresco cheese, however, the pathogen was not completely eliminated and showed regrowth at 4°C, supporting the already mentioned effect of the food matrix on phage efficacy. Besides, Fister *et al*. (2016) isolated *L. monocytogenes* strains resistant to phage P100 from Austrian dairy plants, and their appearance was linked with phage treatments; this contraindicates the routine application of a single phage due to the likely occurrence and spread of insensitive strains. In other study, phage treatment of the surfaces of soft cheeses was effective only when using high doses of phages, and highly dependent on the initial concentration of *L. monocytogenes* (Silva *et al.* 2014). Applications of *L. monocytogenes* phages with good results have been also reported in ready-to-eat foods (Guenther *et al.* 2009), as well as for the elimination of biofilms (Soni and Nannapaneni 2010).

Regarding regulations on the actual use of phage-based products in foods, ListShield™ was the first commercial phage cocktail approved (United States, year 2005). Afterwards, a few countries around the world (Israel, Canada, Switzerland, Australia, New Zealand, among others) also issued approvals for phage-based products. Although the use of phages in foods is not officially authorized by the European Union, it is allowed in some of its countries under national regulations. Recently, Moye *et al*. (2018) updated a list of phage products approved for food safety applications.

In the final stage of most phage infections, holin proteins facilitate the passage of endolysins through the bacterial cell membrane, where they attack the peptidoglycan layer, leading to cell burst and release of recently synthesized virions (Wang *et al.* 2000). This natural process is called lysis "from within", but in the case of most Gram-positive phages, their isolated endolysins are capable of lysing bacterial cells "from without", with no phage infection required. These enzymes normally possess cell wall-binding domains (CBD), involved in an extremely specific recognition of distinctive molecules, and enzymatically active domains (EAD), actually responsible of cell wall degradation (Loessner 2005; Schmelcher and Loessner 2016). This modular structure allows domain-shuffling approaches to obtain chimeric molecules with desired specificity and improved activity (Fig. 1). In Gram-negative bacteria, an outer membrane (OM) acts a barrier for exogenously applied endolysins. However, recently developed endolysin-based antimicrobials, called Artilysin®s, are able to pass through the OM (Gerstmans *et al.* 2016). Besides, endolysins are considered safe, not likely to generate bacterial resistance, and promising anti-biofilm agents (Coffey *et al.* 2010; Schmelcher and Loessner 2016). For all these reasons, the application of purified phage endolysins against pathogenic bacteria has attracted considerable interest over the last decade. Schmelcher and Loessner (2016) published an extensive list of both natural and chimeric endolysins with activity against foodborne pathogens or spoilage bacteria, but most of them were not tested in foods. In this sense, some obstacles include reduced activity and possible inactivation within food matrices, and the existence of commercially developed phage cocktails with proved efficiency and lower cost

(Coffey *et al.* 2010). Nevertheless, endolysins remain particularly interesting as an alternative to whole phage preparations for biocontrol in foods.

Acknowledgements

All the authors were supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; project PIP 112-201201-00046; Argentina), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT; Project PICT 2015-0079; Argentina) and the Universidad Nacional del Litoral (UNL; Project CAI+D 2011 Nº PI 501 201101 00039).

Conflict of Interest

None.

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Figure legends

Figure 1. Flow chart summarizing the uses of phages against pathogenic bacteria in the dairy industry

Table 1 Methods of phage detection

Type	Advantages	Disadvantages	Examples	References
Microbiological methods	Require simple and economic materials Enumerate only infective particles Daily use in research and industrial laboratories Quantitative* High sensitivity Suitable for different dairy matrices	A sensitive strain is needed Long incubation time Lack of reproducibility among laboratories Starter growth may be inhibited by non-phage inhibitors (false positives)† Difficulty of observing plaques with some phages Positive results from some samples should be confirmed using a second method	Activity test Plaque assay Turbidity test Spot test	Pujato *et al*. 2017; Anderson *et al*. 2011
PCR-based methods	Rapid phage detection Suitable for different dairy matrices Allow specific identification of phage groups Allow evaluating phage evolution and epidemiology‡ Good reproducibility among laboratories§	Non-quantitative¶ Low and variable sensitivity** Only detect phage DNA; do not discriminate between infective and non-infective phages particles Require expensive equipment and trained staff Phages with unknown DNA sequence might not be detected	Simplex PCR Multiplex PCR qPCR MLST RAPD	del Rio *et al*. 2012; Muhammed *et al*. 2017
Microscopy	Allow immediate phage detection Allow phage morphology characterization Actual observation of phage particles Semi-quantitative	Variable (usually high) detection limit Require very expensive equipment and trained staff Do not discriminate between infective and non-infective phages particles Phage counts may be overestimated	Electron microscopy Fluorescent microscopy Atomic force microscopy	Zago *et al*. 2012
Others	Rapid phage detection Suitable for different dairy matrices Highly specific and sensitive Quantitative	Require expensive equipment and trained staff Other studies are necessary to improve the developed method	Flow cytometry Biosensors Raman spectroscopy Immunodetection	Michelsen *et al*. 2007; Tayyarcan *et al*. 2018

[*]Plaque assay and spot test; [†]Turbidity test and activity test; [‡]MLST; [§]Except for RAPD; [¶]Except for qPCR; [**]PCR has the lowest detection limit