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Review

Lactococcus lactis phages from the perspective of their diversity, thermal and biocidal resistance

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ABSTRACT

Lactic acid bacteria (LAB), particularly *Lactococcus lactis*, are of great significance in dairy fermentations. Many LAB strains are susceptible to attack by phages that affect their technological, biochemical and physiological functions. Phages of *L. lactis* are a serious concern because of the economic importance of this bacterium in the dairy industry. Members of *L. lactis* phages belonging to the P335, 936 and c2 groups are more problematic for the dairy industry. Many phages of the 936 group are resistant to various thermal and biocidal treatments commonly used in the dairy industry. This article reviews the diversity of *L. lactis* bacteriophages of the P335, 936 and c2 groups and discusses their interaction with their bacterial hosts. In addition, this review provides an overview of the resistance of *L. lactis* phages to thermal treatments and chemical biocides, and highlights some novel strategies to destroy these phages.

Contents

1. Introduction

Bacteriophages or, more simply, phages, are bacteria-infecting viruses that are the most ubiquitous entity in the biosphere. These phages affect bacterial cells through successful viral infection that leads to the incorporation of the viral genome into the host-cell DNA, where the viral genome becomes incorporated into the bacterial genome as a prophage. As a result of this process, the physiology, metabolism, functionality (Holt et al., 2017) and global cellular gene expression (Poranen et al., 2006) of phage-infected cells are seriously affected, leading to altered cellular activities.

Among all food industries in which phage issues are documented, the dairy industry is probably the most affected industry. Fermentation of milk using lactic acid bacteria (LAB), commonly belonging to the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Weissella*, and *Leuconostoc*, is one of the oldest milk

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preservation methods. Robust starter cultures are a prerequisite for the successful and consistent production of dairy products. Unwanted or premature lysis of LAB cells may cause several fermentation issues of varied severity, leading to loss or the production of low quality products (Marcó, Moineau, & Quiberoni, 2012). Since the first report (Whitehead & Hunter, 1945) on the effect of phages on a dairy starter culture, lactic streptococci, there has been a lot of progress in understanding the genetics, ecology, and functionality of phages in relation to LAB, owing to the commercial importance of these bacteria and the products produced using them. Still, phages are an obstinately persistent problem in today's mega-scale productions where dairy fermentations are performed routinely and extensively. Technologically robust starter cultures are a prerequisite for consistent production. *Lactococcus lactis* is a dominant mesophilic bacterial species that is widely used for the production of cheese and butter milk and it is considered as economically the most important LAB species (Song, In, Lim, & Rahim, 2017). A blend of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* strains is widely used in defined industrial and artisanal starter cultures for dairy fermentations throughout the world (Ruggirello, Cocolin, & Dolci, 2016). Defined starter cultures are more susceptible to bacteriophage attack as compared with complex and undefined starter cultures coming from traditional cheese production farms because of varied phage sensitivities among different species and strains (Frantzen, Kleppen, & Holo, 2018).

Bacteriophages attacking *L. lactis* subsp. *lactis* and *L. lactis* sbsp. *cremoris* strains are present almost everywhere in the dairy processing environment and may negatively affect production processes (Kleppen, Bang, Nes, & Holo, 2011). Genomes of many *L. lactis* strains are known to contain prophages and prophage-like elements, which can be released in the form of intact phage particles and cause premature cell lysis that may lead to complete fermentation failure (Garneau & Moineau, 2011). A recent study reported that up to 10^9 prophages per mL of the lysogenic strain culture are usually permanent residents of these starter strains where they also play important roles in the evolutionary success of these lysogens by providing competitive advantage to hosts, and interestingly the number of these prophages may increase if the culture is subjected to stress conditions like elevated temperature and salinity (Alexeeva, Martinez, Spus, & Smid, 2018). Also, prophages significantly affect the physiology and adaptability of the host strains to environmental and cultivation conditions (Aucouturier, Chain, Langella, & Bidnenko, 2018).

Phages of lactococcal species, particularly belonging to three groups (936, P335 and c2), are among the most resistant phages towards heat and different biocidal treatments (Atamer et al., 2009; Hayes et al., 2017) and thus pose a serious threat to milk fermentations. To improve fermentation efficiency and stability, and to avoid phage predation in the dairy processing environment, it is imperative to understand their diversity and sensitivity to biocidal and thermal treatments. The objective of this manuscript is to review the diversity and prevalence of *L. lactis* phages in dairy products and milk processing environments, and discuss their resistance towards heat and commonly used sanitisers and disinfectants. In addition, we have highlighted some novel strategies to destroy bacteriophages in food industries.

2. Diversity of lactococcal phages in dairy fermentations

There are several LAB of substantial economic importance that are constantly threatened by phage infections, causing phage-induced fermentation problems in the milk transformation industry. Out of these bacteria, *L. lactis* is an intensively and globally exploited lactic acid bacterial species in large-scale dairy food fermentations. It is extensively used for the production of a variety of cheeses, sour-cream, kefir, and buttermilk (Moineau et al., 1996). The dairy environment is ideal for the proliferation of virulent lactococcal phages, as these phages, being present in raw milk, can survive pasteurisation and become abundant causing production inconsistencies with concomitant economic losses (Madera, Monjardin, & Suarez, 2004).

There are 10 genetically unrelated known *L. lactis* phage groups based on morphology and genome sequence relatedness: c2, P335, 936, P034, P087, 949, 1706, KSY1, Q54 and 1354. Out of these, members belonging to the c2, P335, and 936 groups are the most prevalent and frequently reported phages accounting for 5%, 10% and 80%, respectively, of all phages in the dairy fermentation industry (Spinelli, Veesler, Bebeacua, & Cambillau, 2014). The rest of the seven groups are less frequently reported and their presence is only reported in raw milk rather than in failed fermented products (Deveau, Labrie, Chopin, & Moineau, 2006). Table 1 summarises previous reports of *L. lactis* phages belonging to these ten groups in dairy products or manufacturing facilities throughout the world. Eight of these ten phage groups belong to the *Siphoviridae* family, members of which contain long non-contractile tails, while the remaining 2 groups belong to the *Podoviridae* family, the members of which contain short tails (Ackermann, 2007; Deveau et al., 2006).

Table 1
Diversity of *Lactococcus lactis* phages reported in dairy products or the dairy environment throughout the world.[a]

Host	Number of phages (if known)	Abundance of three main phage groups (%)			Other phage group (if ;any; %)	Product/sampling point	Region	Reference
		936	C2	P335				
L. lactis subsp. *lactis*	–	24	72	4	N/A	Raw milk	Spain	Madera et al. (2004)
L. lactis	20	100	none	none	N/A	Cheese-production facilities	Ireland	Murphy et al. (2013)
L. lactis subsp. *lactis*/ *cremoris*	34	50	50	none	N/A	Whey samples	Poland	Szczepańska, Hejnowicz, Kołakowski, and Bardowski (2007)
L. lactis	23	17.5	65	none	P034 (17.5)	Dairy products	Republic of Belarus	Raiski and Belyalsova (2009)
L. lactis	137	61.3	15.3	23.3	N/A	Whey samples	16 different countries	Oliveira et al. (2018)
L. lactis	18	11	none	none	949 (55.5); P087 (33.3)	Artisanal cheeses	Sicily (Italy)	Mahony et al. (2017a)
L. lactis	30	37	57	6%	N/A	Cheese plant	Canada	Moineau et al. (1992)
L. lactis	27	81.4	11.1	7.4	N/A	Buttermilk plants	USA	Moineau et al. (1996)

[a] Abbreviation: N/A, not applicable.

All known *L. lactis* phages contain a double stranded DNA genome and are characterised by a non-contractile tail. According to the International Committee on Taxonomy of Viruses, *L. lactis* phages belong to the *Caudovirales* order, which is a very large, biologically, genetically and morphologically diverse and complex group that includes more than 95% of all known phages (Maniloff & Ackermann, 1998).

Among the three main groups (c2, 936, and P335), members of the phages belonging to the polythetic P335 phage group are genetically more diverse and contain a total of five genetically distinct lineages based on their overall nucleotide similarity and virion morphology (Kelleher et al., 2018). The whole genome sequences of 27 phages belonging to the P335 group are publicly available. Until now, 936 and c2 groups only comprise lytic bacteriophages, which contain a small isometric capsid (60 nm in diameter) and a prolate capsid (60 nm by 40 nm), respectively. Members of the 936 group have a long non-contractile tail, the length of which ranges from 140 nm to 200 nm (morphotype B1), whereas, members of the c2 group have a 100 nm long non-contractile tail (morphotype B2). Similar to the 936 group phages, P335 phages have a small isometric capsid; however, they can either be lytic or temperate (Moineau, Fortier, Ackermann, & Pandian, 1992; Murphy et al., 2013).

The 936-type phages are the most commonly encountered and problematic lactococcal phages in the dairy fermentation environment throughout the world (Chmielewska-Jeznach, Bardowski, & Szczepankowska, 2018; Mahony, Murphy, & van Sinderen, 2012; Moineau et al., 1996). In a recent study, 61% of 137 *L. lactis* phage isolates recovered from 100,000 samples of whey belonged to the 936 group (Oliveira, Mahony, Hanemaaijer, Kouwen, & van Sinderen, 2018). Heat resistance is one of the factors that determines the abundance of only selected phages belonging to the three groups. For instance, phages belonging to the groups 949 and P087 are sensitive to heat as compared with phages belonging to the 936 group (Mahony et al., 2017a; Wagner et al., 2018). Thus, the difference in heat resistance of 949 and P087 group phages may explain their rare abundance in today's modern dairy fermentations.

3. Phage-host interactions in *L. lactis* phages

Phage infection is initiated by the adsorption of a phage virion on the cell surface of a susceptible bacterial host, followed by penetration and injection of the phage genetic material into the cytoplasm of the host cell. The phage DNA inside the host bacterial cell is replicated as a part of the host cell genetic material, leading to the production of new phages inside the cell that are released from the infected cell through cell lysis, resulting in the death of the bacterial cell. The complex initial interactions between phages and their bacterial hosts are tightly regulated by bacteriophage adsorption kinetics and depends on physico-chemical characteristics of the medium, surface hydrophobicity, type of the phage receptor binding protein (RBP), the physiology of the host cells, phage orientation on the host cell surface, biochemical interactions between viral surface proteins and host cell receptors, diffusion-induced conformational changes in receptor proteins (Hosseinidoust, Olsson, & Tufenkji, 2014; Storms & Sauvageau, 2015) and various environmental factors like temperature, pH and ionic strength of the immediate environment. The interaction between phages and hosts is also affected by the presence of any chemical detergents or disinfectants in the immediate vicinity (Denes & Wiedmann, 2014; Ly-Chatain, 2014).

The structural elements of *L. lactis* phages not only determine their morphotypes, but also dictate their initial attachment on bacterial cell surface. The adhesion apparatus of a phage comprises several genes encoding a number of phage-encoded proteins like RBP (a protein of the phage tail base-plate that helps phages in recognition and binding to the bacterial cell wall of a susceptible host cell), predicted tail tape measure protein (TMP), distal tail (Dit) protein and tail-associated lysin (Tal) (Bebeacua et al., 2013; Mahony et al., 2016). The tail tape measure protein (TMP) of bacteriophages (with a tail) is an inner membrane protein that determines the tail length and facilitates the phage in injecting its genome into the cytoplasm of the host cell. The TMP of *Staphylococcus aureus* phages has been reported to exhibit peptidoglycan hydrolytic activity as well (Rodriguez-Rubio et al., 2012), which may have a role in the host's cell wall degradation. Such activity has not yet been reported in the TMP of *L. lactis* phages. However, the Tal of the two members of the P335 group, Tuc2009 and TP901-1, have been reported to contain a peptidoglycan hydrolase (M23 peptidase) that helps in efficiently infecting stationary phase cells through the endopeptidase activity on the surface (Stockdale et al., 2013).

The attachment of a lactococcal phage on the bacterial cell surface is mediated by a phage-carrying RBP, which allows the specific recognition of the host-encoded target receptor material. A RBP of a phage recognises a specific receptor on the surface of the host and allows attachment with the correct orientation on the surface. The primary receptor material for the members of *L. lactis* (subsp. *cremoris* and *lactis*) phage groups (P335, 949, 936 and P087) is saccharidic in nature (Mahony, Randazzo, Neve, Settanni, & van Sinderen, 2015; Tremblay et al., 2006), and various biosynthetic operons, localised to variable genetic loci, encompassing these cell wall polysaccharides (CWPS) have been identified and characterised in various bacterial strains (Mahony et al., 2013). Recently, a conserved carbohydrate binding module has been reported among baseplate Dit components of members of the 936 group phages. The binding capacity of this module to the host surface gave the possibility of its role in specific phage-host adhesion process. However, the exact role of its involvement in phage attachment as a prelude to DNA release remains unknown (Hayes et al., 2018).

Bioinformatics and functional genomics of many lactococcal strains have revealed variations in gene cluster presumed to be involved in CWPS biosynthesis, evidencing the existence of a correlation between CWPS-based genotype and host-range (Mahony et al., 2013). A total of three CWPS-based genotypes, CWPS A, CWPS B and CWPS C, of lactococcal strains have been identified based on sequence analysis of the *cwps* gene cluster (Mahony et al., 2013). Studies on structural complexity and diversity of CWPS among CWPS type-C strains have further classified them into five sub-types (C_1 to C_5) having specific host ranges. It was also revealed that despite similar genetic loci associated with the biosynthesis of CWPS in two different lactococcal strains, a substantial difference in chemical properties and CWPS structures may be responsible for markedly different phage host ranges of lactococcal strains (Ainsworth et al., 2014). Recently, the CWPS structure of *L. lactis* UC509.9, a *cwps* type-A strain, was found to contain complex rhamnose-rich polysaccharide with branching oligosaccharides (Vinogradov et al., 2018b). Similar CWPS component of *L. lactis* subsp. *lactis* (IL1403), a strain possessing a B-type CWPS genotype, was recently found to contain high rhamnose content having glycerophosphate as a major component (Vinogradov et al., 2018a).

Currently, little is known about the CWPS structural chemistry and diversity among type-A and B strains. In parallel with these studies, other studies involving the 3D analysis of structures of lactococcal phage tail components have given evidence of their binding ability to these saccharidic receptor moieties (Bebeacua et al., 2010). However, phages may require conformational changes in their RBP prior to adhesion. Veesler et al. (2012) showed that RBP in P335 phages (TP901-1-like phages) is pointed towards

the host and does not need any conformational changes before adhesion to the host surface, whereas p2-like phages have their RBP towards the capsid rather towards the host, which requires a Ca^{2+}-dependent activation of RBP. The RBP orientation in p2-like phages requires a 200° rotation downwards for host adhesion and infection. Thus phage infection in case of p2-like phages is a multistep process involving a reversible attachment, Ca^{2+}-dependent activation of RBP, specific attachment of the RBPs to the carbohydrate moiety and DNA ejection (Bebeacua et al., 2013).

Mahony et al. (2017b) classified 17 *L. lactis* phages of the P335 group into four different morphotypes (sub-groups I–IV) based on morphology and sequence relatedness of the genomic region encoding the adhesion apparatus and RBP. It was revealed that some members of the sub-group IV also need a Ca^{2+}-dependent activation of RBP.

The fifth morphotype of P335 phages was recently revealed when whole genome nucleotide alignment of all intact prophages from the 30 completely sequenced genomes of lactococcal strains were analysed and compared with the nucleotide sequences of all previously known lactococcal prophages and representative phages of all four morphotypes of the P335 group. The analysis was based on the lysis module including the lysis cassette, comprising a lysin and a holin gene, in combination with the adhesion module of each prophage and phage. A low amino acid similarity and highly conserved predicted functional synteny among the structural regions was observed. Members of the sub-group V (i.e., C10E, UC063C, 98,201) display TMPs of varying lengths and possess a well conserved Dit and large RBPs. The members of morphotype V, like members of morphotype I, do not possess a distinct Tal and RBP, but encode a protein with a fused Tal and RBP. Among the five morphotypes, the genomes of only I and V morphotypes contain fused Tal-RBP. Interestingly, a member (89,201) of the morphotype V also displays a long tail fibre protruding from the tail tip, which is reminiscent of the morphotype I members. Despite sufficiently distinct sequences of the RBPs of the phages belonging to the morphotypes I and V, there are still common structural characteristics and functional roles among them which seem conserved between these phage types (Kelleher et al., 2018).

The attachment mechanism of phages belonging to the c2 group is completely different from that of phages belonging to groups 936 and P335. In contrast to the carbohydrate preference of phages belonging to the P335 and 936 groups, c2-like phages recognise a membrane-anchored protein of the host cell termed “phage infection protein or Pip” (Mooney, Jann, & Geller, 2006). The binding of phages of the c2 group to saccharidic receptors is reversible and is rendered irreversible only as a result of their interaction with Pip, which also leads to phage DNA ejaculation into the host (Monteville, Ardestani, & Geller, 1994). It was reported that as a result of inactivation of the lactococcal protein Pip, *L. lactis* C2 became resistant to phages of the c2 group (Kraus & Geller, 1998). Later on, another host trans-membrane protein YjaE, a protein of 799 amino acids which is encoded by a 2400 nucleotide gene, was recognised as a complementary receptor protein for the infection of a phage (bIL67) belonging to the genus *C2virus*, and a mutation in *pip* did not affect its ability to infect the lactococcal strain. Now, members of the *C2virus* genus requiring Pip for infection are classified as c2-type and that requiring YjaE for infection as bIL67-type (Millen & Romero, 2016).

4. Thermal and chemical resistance of *L. lactis* phages

L. lactis strains are economically and industrially the most important microorganisms that are frequently used in various milk fermentation processes. These strains, despite having various sophisticated defence systems (Klaenhammer, 1989; Kong & Josephsen, 2002), are highly susceptible to attack by a variety of phages, thus posing a constant threat to the dairy industry in terms of the quality, safety and value. Raw milk is a potential reservoir of phages that subsequently affect dairy processing. In a survey analysing the presence of phages in 900 raw milk samples, 9.2% of the samples proved to contain phages (Madera et al., 2004). Raw milk may contain high number of phages, up to 10^4 plaque forming units (pfu) mL^{-1} (McIntyre, Heap, Davey, & Limsowtin, 1991), which may increase during fermentation and reach up to 10^9 pfu mL^{-1} in the final product like cheese (Atamer et al., 2009). Certain dairy phages can persist in a dairy processing plant for several months. For instance, Rousseau and Moineau (2009) reported the persistence of a phage (CB13) in a cheese factory for more than a year.

Dairy ingredients, processing equipment, fermentation products and by products may all contain phages and contribute towards the overall phage count of the final product. Therefore, elimination of phages present in raw milk, dairy ingredients, recycled by-products and dairy products is crucial for optimised fermentation processes. Resistance to thermal treatments and biocidal agents is an important survival strategy of these phages. In the following section, resistance of lactococcal phages to thermal treatments and biocidal agents will be discussed.

4.1. *Thermal resistance of* L. lactis *phages*

Phages are ubiquitous in dairy environments and it is hard to completely eliminate them. Heat treatment is one of the most commonly used methods to control lactococcal phages in the dairy industry. Heating causes structural damage to phages and may decompose phages into head and tail or release DNA from phage capsids (Atamer & Hinrichs, 2010). However, many phages are resistant to commonly applied heat treatments in the dairy industry. It is well established that the majority of phages affecting dairy strains are resistant to pasteurisation temperatures (Madera et al., 2004).

Several studies have reported the thermal resistance of lactococcal phages in raw milk focussing on temperatures (63 °C, 72 °C and 90 °C) commonly used in the dairy industry to kill most pathogens and microorganisms to elongate the shelf life of milk. Buzrul, Öztürk, Alpas, and Akcelik (2007) studied the heat resistance of 10 lactococcal phages and reported that the commonly used heat treatments in the dairy industry, 72 °C for 15 min and 90 °C for 5 min, are not sufficient to inactivate phages. Ultra-high-temperature (UHT) treatment of milk is carried out at 135–138 °C for 2–3 s to extend the shelf life of milk. This treatment is somewhat effective against spore forming bacteria and phages, but imparts negatively on the organoleptic characteristics of milk as a result of the Maillard reaction.

According to the International Dairy Federation (IDF Bulletin 263, 1991), a heat treatment of 90 °C for 15 min is sufficient to inactivate all phages when a medium containing phages is heated in standard glass test tubes. Emergence of highly heat-resistant lactococcal phages overcoming this thermal hurdle has invalidated this phage monitoring assay. It was first revealed when a phage (P1532) of *L. lactis* subsp. *lactis*, belonging to the 936 group, was heated in a thin walled borosilicate glass tube at 90 °C for 15 min and only showed a minor 2-log reduction following this treatment. Similarly, another *L. lactis* phage (P680) of the 936 group also survived this thermal treatment with a residual titre of more than 10^2 pfu mL^{-1}. A revised thermal treatment of 90 °C for 45 min has since been proposed (Capra et al., 2013). P1532 and P680 are now considered as model heat resistant phages of *L. lactis*. There are several reports where these two phages of the 936 group have been identified as the most heat resistant among all phages of dairy

bacteria. Atamer et al. (2009) studied the thermal resistance (80 °C for 5 min) of 56 lactococcal phages comprising members of all three commonly reported lactococcal phage groups (11 from the c2 group, 42 from the 936 group and 3 from the P335 group). Highly heat resistant phages belonged only to the 936 group. P1532, isolated from a sour cream sample, survived 90 °C for 20 min and 97 °C for 5 min; whereas P680, isolated from Quarg, survived 95 °C for 5 min. Two other phages (P656 and P4565) also survived 90 °C for 5 min in skim milk.

The two pasteurisation conditions, i.e., low temperature long time pasteurisation (LTLT, 62–65 °C, 30 min) and high temperature short time pasteurisation (HTST, 72–75 °C, 20–40 s), cannot warrant 9-log inactivation of these two heat resistant phages and calculations showed that 9-log inactivation of P1532 would require milk to be heated at 95 °C for 7 min, which would result in denaturation of milk proteins (Atamer et al., 2009). The heat resistance of these two extremely heat resistant phages was also tested in a pilot-plant pasteuriser by suspending phage titres (10^7 pfu mL^{-1}) in raw milk (Wagner et al., 2018), where the thermal inactivation values for P1532 proved to be much lower than that predicted by Atamer et al. (2009) (Table 2). Survival of *L. lactis* phages of the 936 group at 95 °C for 5 min has been reported for other phages (P956, P963, P983 and P959) isolated from whey powder as well. Interestingly, P956 survived the heat treatment of even 95 °C for 20 min and 97 °C for 10 min, which makes this phage the most thermo-resistant among all phages of *L. lactis* (Wagner et al., 2017).

Dairy phages of *L. lactis* exhibit the highest heat resistance among all dairy bacteriophages. Many LAB phages belonging to other species are relatively less heat resistant (Wagner et al., 2017). The most heat resistant phage (973), out of 77 *Leuconostoc* phages isolated from a variety of dairy products (cheese, whey, brine, butter cream and butter milk sample) required 1.2 min at 90 °C for 9-log inactivation (Atamer, Ali, Neve, Heller, & Hinrichs, 2011) in skim milk.

Table 2 shows the heat resistance of all previously reported *L. lactis* phages in different heating media at various temperatures. However, phages of the c2 group are sensitive to heat, which may be a reason why they are not prevalent in dairy products. However, these phages are usually prevalent in raw milk samples. Most of these phages are susceptible to the heat treatment set by the IDF (90 °C for 15 min). Nine lactococcal phages of the c2 group were subjected to heat treatments at different combinations of temperature and time. $D_{70\ °C}$ values of 16.6 min for the most heat resistant phage and 12 s for the most heat sensitive phage were calculated (Marvig et al., 2011), which were significantly less than the $D_{70\ °C}$ values calculated for the phages belonging to the 936 group. Similarly, Madera et al. (2004) reported that c2 strains are 35 times less heat resistant than 936-like phages at 72 °C for 15 s in a plate heat exchanger.

There are several structural features of phages and heating medium characteristics that confer advantage to phages and enhance their survival ability in the case of heat treatment. For instance, milk and its components exert a protective effect on phages during heating. The positive effect of milk on the heat resistance of phages has been reported in many studies (Chen et al., 2017; Muller-Merbach, Neve, & Hinrichs, 2005; Quiberoni, Guglielmotti, & Reinheimer, 2003; Wagner et al., 2017). For instance, the whey component of milk proteins exerted a strong protective effect on the survival of P1532 phage at pH 5 (Geagea, Gomaa, Remondetto, Moineau, & Subirade, 2017). In another

Table 2
Thermal activation of *Lactococcus lactis* phages after different thermal treatments in different media.[a]

Phage	Heating protocol	Initial phage concentration (pfu mL^{-1})	Phage survival	Decimal reduction time	Reference
001	72 (5), RSM		99% inactivation	20 min (72 °C), milk	Suarez and Reinheimer (2002)
046	72 (1.9), RSM		99% inactivation	–	Suarez and Reinheimer (2002)
QF12	72 (1.5), RSM		99% inactivation	–	Suarez and Reinheimer (2002)
QP4	72 (3.2), RSM		99% inactivation	–	Suarez and Reinheimer (2002)
P1532 (sour cream)	90 (5), SM	10^7	1.5-log reduction	112 min (72 °C)	Atamer et al. (2009)
P680 (quarg)	90 (5), SM	10^7	0.5-log reduction	–	Atamer et al. (2009)
P680	140 (0.03), whey, WPC, WC	10^8 to 10^9	9-log reduction	–	Atamer and Hinrichs (2010)
P680	90 (15), whey, WPC, WC	10^8 to 10^9	6-log reduction	–	Atamer and Hinrichs (2010)
P1532	90 (15), SM	10^7	2-log reduction	–	Capra et al. (2013)
P956 (whey powder)	95 (5), SM	Nearly 10^7	2.3-log reduction	105.4 min (75 °C), milk 45.6 min (75 °C), water	Wagner et al. (2017)
P956 (whey powder)	95 (5), water	Nearly 10^7	Complete inactivation	–	Wagner et al. (2017)
P680 (whey powder)	–	–	–	76.8 min (75 °C), milk	Wagner et al. (2017)
P963, P983 and P959 (whey powder)	95 (5), SM		3-log reduction		Wagner et al. (2017)
P1532	97 (0.6)	10^7	3.5-log reduction	11.3 s (95 °C), milk 11.9 s (97.5 °C), milk	Wagner et al. (2018)
P959	80 (1), SM	10^6	1-log reduction		Wagner et al. (2017)
P1006	80 (1), SM	10^6 to 10^7	1-log reduction		Wagner et al. (2017)
P951	80 (1), SM	10^7	7-log reduction		Wagner et al. (2017)
P994 and P1003	80 (1), SM	10^7	7-log reduction		Wagner et al. (2017)
P1007	80 (1), SM	10^8	4-log reduction		Wagner et al. (2017)
P558	80 (1), SM	10^8	3-log reduction		Wagner et al. (2017)
P975	80 (1), SM	10^5	0.5-log reduction		Wagner et al. (2017)
P977	80 (1), SM	10^7	1-log reduction		Wagner et al. (2017)
P962	80 (1), SM	10^7	1-log reduction		Wagner et al. (2017)
P993	80 (1), SM	10^7	1.5-log reduction		Wagner et al. (2017)
P985	80 (1), SM	10^5	1-log reduction		Wagner et al. (2017)
P986	80 (1), SM	10^5	1.5-log reduction		Wagner et al. (2017)
P990	80 (1), SM	10^6	1-log reduction		Wagner et al. (2017)
P889	80 (1), SM	10^6	2-log reduction		Wagner et al. (2017)
P008	–	–	–	8.4 min (72 °C), milk	Muller-Merbach et al. (2005)

[a] Heating protocol is given as temperature (°C) with time (min) in parenthesis and heating medium. Abbreviations are: RSM, reconstituted skim milk; SM, skim milk; WPC, whey protein concentrate; WC, whey cream. Decimal reduction time is given only for phages for which the relevant data were available; temperature of treatment given in parenthesis.

study, a significantly lower inactivation (9-log reduction) of phage P680 was observed in whey, whey protein concentrate and whey cream as compared with milk, showing that whey is responsible for protecting phages against heat in milk (Atamer & Hinrichs, 2010). The thermal conductivity of the heating material also affects the heat resistance of phages. Glass materials have shown to have low inactivation efficiency for lactococcal phages P680 and P1532 as compared with stainless steel tubes (Capra et al., 2013).

There has been a great progress in understanding the factors affecting the heat resistance of lactococcal phages. However, it is only recently that a genetic determinant of heat resistance in phage CB14 belonging to the lactococcal phage group 936 (now *Sk1virus*) has been determined. The heat resistance of a virulent *Sk1virus* phage was attributed to the phage structural protein known as TMP. Two CB14 derivatives with a slightly high heat resistance as compared with other phages of the same group were selected. Their genome sequences revealed a 120-bp deletion in the gene encoding the TMP. This 120-bp deletion in the same gene (*tmp*) was also found in extremely heat resistant phages P680 and P1532 (Geagea, Labrie, Subirade, & Moineau, 2018). However, reasons behind variation in heat resistance between CB14 and the two 936 species P680 and P1532 phages are still not known.

4.2. *Resistance of* L. lactis *phages against sanitisers and biocides*

The use of sanitisers, disinfectants, and biocides is common practise to control bacteriophages, particularly LAB phages, in food industries. There is a plethora of work over decades on the application of traditional and commercial food contact sanitisers against bacteriophages (Bennett & Nelson, 1954; Chen et al., 2017; Guglielmotti, Mercanti, Reinheimer, & Quiberoni, 2011). In the dairy industry, cleaning in place (CIP) procedures are employed to eliminate organic materials and microbial contaminations from food contact surfaces including pipelines and floors. Food contact sanitisers are usually applied after CIP to properly sanitise a surface. Food sanitisers include, but are not limited to, chlorine compounds including bleach, quaternary ammonium compounds, sodium hypochlorite, anionic acids, hydrogen peroxide and iodine compounds (Guglielmotti et al., 2011; McDonnell & Russell, 2001). These sanitisers can only be approved by health authorities if they meet a minimum set of criteria that include the innocuousness of their residues, low human toxicity, lack of negative impact on food, low cost, and high antimicrobial efficiency against bacteria or viruses (antimicrobials should be able to reduce at least 3-log population of targeted bacteria or virus in 5 min; whereas, disinfectant should show a 5-log reduction in 30 s) (Guglielmotti et al., 2011). In Europe, LAB phage reduction claims are used and a disinfectant must show a 4-log reduction of LAB phages in a set time limit (European Committee for Standardization: CEN, 2002).

There is a lot of work on the efficiency of these sanitisers in inactivating bacteria and viruses associated with human diseases. When biocides are to be used in the food industry, it is important to take into account the efficiency of these biocides in inactivating bacteriophages of concern for the industry as well. There is a plethora of reports on the characterisation of the biocidal inactivation of LAB phages other than *L. lactis* phages (Binetti & Reinheimer, 2000; Ebrecht, Guglielmotti, Tremmel, Reinheimer, & Suarez, 2010; Quiberoni et al., 2003; Quiberoni, Suarez, & Reinheimer, 1999), but there is limited work assessing the efficiency of commonly used biocides against *L. lactis* phages.

As discussed earlier, some phages are resistant to heat and despite many thermal treatments used in the dairy industry viral particles survive in milk. These phages may persist on various surfaces in the dairy industry such as processing lines, doors, floors and can disseminate in the production facility through air as well (Verreault et al., 2011). Therefore, elimination of these phages using effective biocides is essential for a smooth fermentation process. Suarez and Reinheimer (2002) reported the effectiveness of different biocides against *L. lactis* phages (QF12, P001, QP4 and 046) with an initial concentration of 10^6 pfu mL^{-1}. Ethanol turned out to be effective at a concentration of 75% and inactivated 99% of QF12 and QP4 population after 4.9 and 1.3 min, respectively. Generally, it is believed that higher the concentration of a biocide, greater will be the inactivation of phages. However, this was not true for ethanol as 75% ethanol proved to be more lethal that 100% ethanol, in agreement with a previous study (Quiberoni et al., 1999). Ethanol proved to be a better biocide against *L. lactis* phages than iso-propanol, which remained ineffective against all of the four phages. The suitability of 75% ethanol against ten *L. lactis* phages has also been reported in another study (Buzrul et al., 2007).

Peracetic acid proved to be the most effective biocide against all four phages and a very low concentration of peracetic acid (0.15%, v/v) reduced their population below the detection limit (<10 pfu mL^{-1}) after 5 min of exposure. Sodium hypochlorite, at a specific concentration of 100 ppm, turned out to be effective in reducing the population of *L. lactis* phages QF12 and P001 to below the detection limit (<10 pfu mL^{-1}) after 5 min of exposure. The same concentration of sodium hypochlorite resulted in 99% inactivation (T_{99}) of the two phages QP4 and 046 after 8.5 and 32.7 min, respectively. A higher concentration of sodium hypochlorite proved to be ineffective in eliminating phages. For instance, phages QP4 and 046 required 45 min and 30 min for complete inactivation after the exposure of sodium hypochlorite at the concentration of 300 and 200 ppm, respectively (Suarez & Reinheimer, 2002).

Other studies involving the use of the same biocides against phages of other LAB have also reported phage- or formulation-dependent efficiency of these chemical biocides (Guglielmotti et al., 2011; Mercanti, Guglielmotti, Patrignani, Reinheimer, & Quiberoni, 2012). As an example, only 100 ppm of sodium hypochlorite was found to be effective against many *L. lactis* phages (Suarez & Reinheimer, 2002), but remained ineffective at a higher concentration (300 ppm) against many *L. lactis* phages of unknown groups in another study (Avsaroglu, Buzrul, Alpas, & Akcelik, 2007). Phages of the 936 group (phi43, phi93, jj50, *Viridis*JM2 and *Caseus*JM1) retained their 99% activity even after the exposure of 800 ppm of sodium hypochlorite for 30 min (Murphy, Mahony, Bonestroo, Nauta, & van Sinderen, 2014). In a prior study, several *L. lactis* phages remained unaffected by 300 ppm of sodium hypochlorite and required up to 5000 ppm of sodium hypochlorite for complete inactivation (Avsaroglu et al., 2007). Several phages belonging to LAB other *L. lactis* have also shown high tolerance towards sodium hypochlorite (Briggiler Marco, De Anton, Reinheimer, & Quiberoni, 2009; Quiberoni et al., 2003). It can be implied that the effectiveness of sodium hypochlorite highly depends on phages rather than its concentration.

Murphy et al. (2014) evaluated the robustness of 11 *L. lactis* phages of the 936 group in response to biocidal treatments and surface disinfectants. Sanitation chemicals showed a varied and phage-specific potential to reduce phages. All 11 phages were quickly inactivated by 0.015% peracetic acid and 0.2% sodium hydroxide. The application of the surface disinfectant Virkon (1%) completely inactivated all phages within 10 min. Similar results were obtained by using a blend (termed Spor-Klenz) of peracetic acid, acetic acid and hydrogen peroxide.

Campagna, Villion, Labrie, Duchaine, and Moineau (2014) evaluated the efficiency of 23 chemical biocides, including 21 food-grade sanitisers, against various LAB phage, including those of *L. lactis*, and determined the effect of milk and whey on the efficacy of biocides as milk and whey are often left after the CIP step.

Iodine-based compounds, isopropanol, amphoteric hypochlorite, and chlorinated compounds just showed 2–3 log reduction of all *L. lactis* phages including phage P008 and P1532 in both milk and whey and thus proved ineffective. Oxidising agents (peroxide and peroxyacid mixtures) and quaternary ammonium compounds (QAC) proved to be the most effective (5-log reduction) chemical biocides against phages CB13, Q54 and 1358 after 2 min of exposure. Phage 1532 showed resistance against all sanitisers except QAC and oxidising agents after 15 min of exposure (Campagna et al., 2014). Similar results were obtained in a recent and the most comprehensive study on the effects of 18 commonly used biocides on 36 *L. lactis* phages of the 936 group at the structural and proteomic levels (Hayes et al., 2017). In congruence with previous studies, QAC proved to be the most efficient disinfectant in pure form as well as a part of a mixture of commercially available chemicals. Nevertheless, detection of some resistant phages for QAC-based compounds show that all QAC are not equally suitable for all dairy phages. In addition, there is an increasing concern about the residual activity of QAC during dairy processing (Danaher & Jordan, 2013). The efficacy of sodium hydroxide in two sodium hydroxide-based sanitisers was also observed, which is in agreement with another recent study (Murphy et al., 2014).

The effect of different biocides on the structure of phages was elucidated for the first time and several electron microscopy images showed that biocides had great effect on the structure of phages. Fig. 1 shows effects of four biocides on the structure of a *L. lactis* phage 93 of the 936 group.

Despite the effectiveness of many biocides against *L. lactis* phages, these phages continue to persist in the dairy environment, probably because of variation in their resistance and the selection of highly resistant phages from the evolutionary perspective. A combination of recommended concentration and time may not inactivate all phages and thus some phages resistant to some biocides, which are otherwise effective against others, may survive and persist in a processing operation. Routine and steady use of biocides may select biocide-resistant phages such as P1532 and CB13. The fact that a single phage exhibits resistance to multiple chemically distinct biocides indicates that phages do not have a specific resistance mechanism against a specific biocide, but they exhibit resistance to biocides because of their intrinsic robustness. Genetic comparison based on nucleotides sequences between resistant and non-resistant phages also showed that resistance to biocides in phages was an intrinsic property that was a result of the cumulative effect of many genetic factors (Hayes et al., 2017). Different chemical biocides, in different concentrations, have varied and phage strain-specific ability to eliminate phages. It is hard to select and optimise the concentration of a single biocide rendering it effective against all phages of the dairy industry. 936 phages are of the major concern for the dairy industry and Virkon has yet been reported as the best surface disinfectant effective against them. Similarly, peracetic acid and sodium hydrochloride are considered as effective biocidal agents against these phage species.

5. Novel approaches to control LAB phages in the dairy industry

Despite stringent control measures including sanitation practises, microbial inactivation of raw ingredients, use of aerosol disinfectants, starter/strain rotation or culture rotation regimes, use of phage inhibiting components (Garneau & Moineau, 2011), phages still persist in the dairy industry. Thermal and chemical treatments are among traditional methods to inactivate bacteriophages. These methods are effective against almost all bacteriophages, but only if applied regardless of their impact on the quality or safety of foods. For instance, increased heat treatment may have a negative impact on the quality of foods as it may cause milk protein denaturation, non-enzymatic browning and loss of nutrients like vitamins and aromatic volatiles. Similarly, increased concentration of disinfectants on food contact surfaces may raise safety concern due to leftover residues of disinfectants and cleaning agents on food contact surfaces. Phages resistant to commonly applied thermal and chemical treatments have evolved as a result of natural selection which is a serious issue for the food industry. Thus bearing in mind, undesirable side-effects of heating and the excessive biocide applications, considerable effort during the last few years has been devoted to finding other ways to destroy bacteriophages.

Novel strategies to control bacteriophages include the application of ultraviolet radiation (Bae & Shin, 2016; Gunter-Ward et al., 2018; Hazem, 2002), high hydrostatic pressure (Zhang et al., 2015), non-thermal plasma (Mizuno, 2017), photo-catalysis (Doss, Carré, Keller, André, & Keller, 2018), pulsed light (Belliot et al., 2013) and nano- or microfiltration membranes (Samtlebe et al., 2015). Many of these techniques are not yet applied in the dairy industry for the inactivation of LAB phages in milk, whey, or whey products.

Treatment of skim milk with ultraviolet light (UV-C) has been proved as an effective technique for the eradication of some bacteriophages (MS2 and T1UV), yet its efficiency against *L. lactis* phages is not yet known (Gunter-Ward et al., 2018).

Whey is widely used in many dairy applications, particularly in fermented products to increase product yield and improve nutritional characteristics and texture. Drained cheese whey can be contaminated with phages during the cheese manufacturing process and it may subsequently contaminate products in which it is added (Madera et al., 2004). Membrane separation techniques have been successfully used in the dairy industry to remove phages from whey. Samtlebe et al. (2015) reported that polyethersulfone membrane with a cut-off of 300 kDa is capable of retaining 3.7 log pfu of the *L. lactis* phage P008 from whey proteins, with whey permeation of 40 and 60% for β-lactoglobulin and α-lactalbumin, respectively. The use of flat sheet membrane (500 kDa cut-off) to remove common phages belonging to the 936, c2 and P335 groups in whey was recently shown (Samtlebe et al., 2017).

High hydrostatic pressure has been successfully used for the inactivation of *L. lactis* phages (Avsaroglu, Bozoglu, Akcelik, & Bayindirli, 2009; Avsaroglu, Buzrul, Alpas, Akcelik, & Bozoglu, 2006). However, this treatment has not yet been applied on raw milk to inactivate bacteriophages. The effect of high hydrostatic pressure on the inactivation of phages has been well reviewed (Guglielmotti et al., 2011).

The ability of bacteriophages to remain suspended in air and disseminate through bio-aerosols is another concern for the dairy industry. All currently available technologies for the inactivation of phages are ineffective against bacteriophages in the air contaminating the dairy environment. Photolytic inactivation of microorganisms is a well-known method to kill bacteria and fungi. The photolytic properties of titanium dioxide (TiO_2) have long been exploited to kill bacteria, fungi (Gogniat, Thyssen, Denis, Pulgarin, & Dukan, 2006; Kuhn et al., 2003; Venieri et al., 2017) and bacteriophages on surfaces or liquids (Briggiler Marco et al., 2009; Cho, Cates, & Kim, 2011; Kashige, Kakita, Nakashima, Miake, & Watanabe, 2001; Sjogren & Sierka, 1994). Its efficiency in destroying phages of LAB in bioaerosols is scarcely reported. TiO_2 generates active oxygen species like superoxide anions (O_2^-) and hydroxyl radicals (.OH) upon photoexcitation with UV radiation (Konaka et al., 1999). Recently, TiO_2/β-SiC solid alveolar foams with light emitting diodes as a source of irradiation have successfully used to kill air-suspended T2 bacteriophage viruses (Doss et al., 2018). Versoza et al. (2018) have recently employed a non-contact ultrasound transducer to produce shock waves in air to kill bacteriophages that either caused genetic mutation or

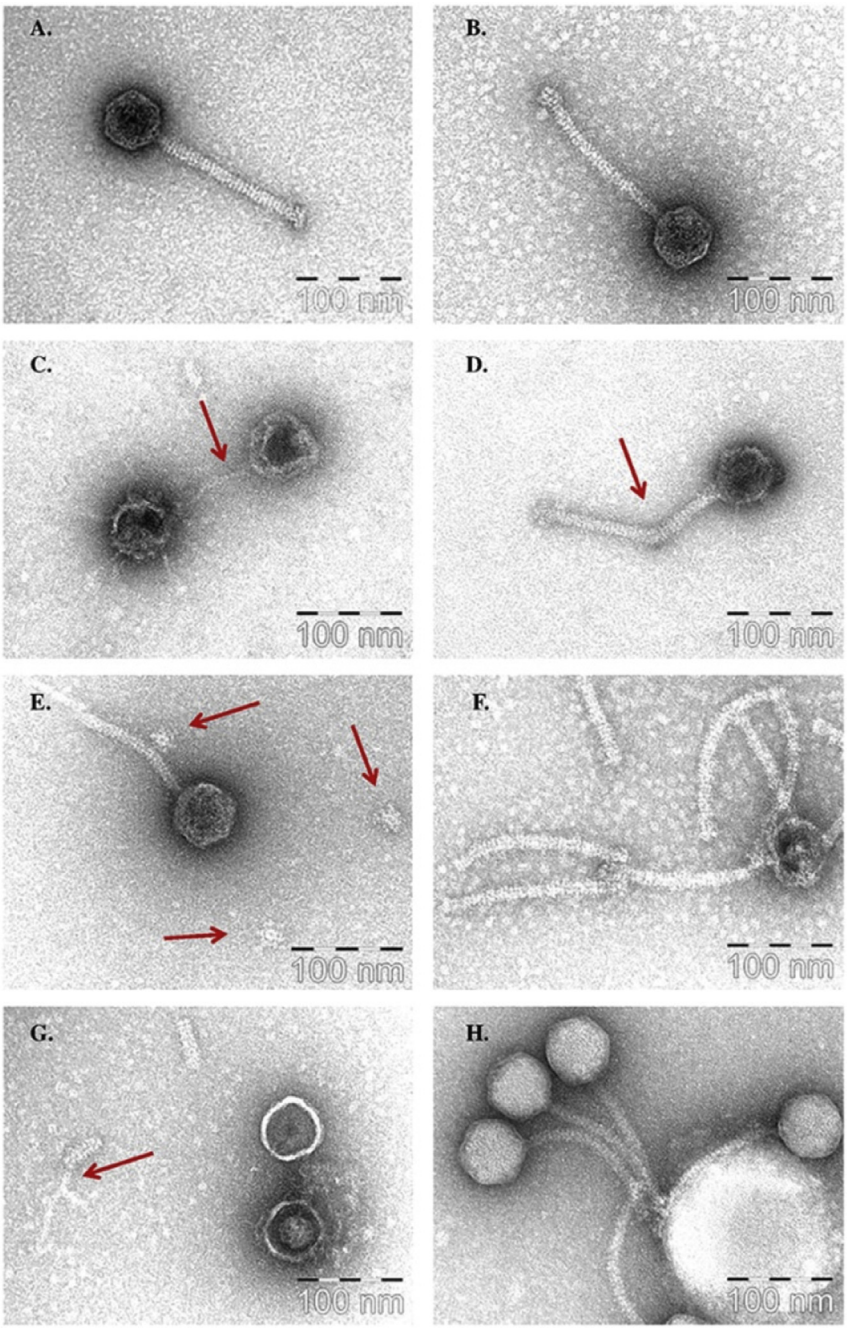

Fig. 1. Electron microscopy images of four biocides on the *Lactococcus lactis* phage 93 (936 group); visible damage caused by biocides to phages are indicated by arrows. Panel A, control; panel B, neutralisation buffer control; panel C, 3% polyvinylpyrrolidone-iodine (PVP) – caused DNA damage; panel D, 1% PVP – caused damaged to the tail; panel E, 1% PVP – caused detachment of the baseplate; panel F, 0.5% sanitiser A (C8-C18 alkyldimethyl chloride ammonium compound); panel G, 0.5% sanitiser B (mixture containing 30–60% sodium hydroxide; 0.06% at concentration employed) – caused the degradation of the major tail protein (MTP) and possibly the tape measure protein (TMP) remains intact; panel H, 3% sanitiser D (ethanol 10%, chlorhexidine digluconate 10%, tetradecyl-trimethyl-ammonium-bromide < 1%). Reproduced from Hayes et al. (2017).

produced dangerous by-products (Versoza et al., 2018). Pulsed ultraviolet light has also been shown to have potential to destroy virus-laden air (Lin et al., 2017).

6. Concluding remarks

LAB are economically very important bacteria for the dairy industry because of their enormous applications in dairy fermentation. Among LAB, *L. lactis* is of great significance in the manufacture of buttermilk and a variety of cheeses. This bacterium is considered as a workhorse for biotechnological applications in dairy fermentation. Success of dairy fermentations highly depends on the robustness of starter cultures. *L. lactis* is susceptible to attack by lytic bacteriophages, ubiquitous in the fermentation and dairy environment, which renders it unsuitable for fermentation causing substantial economic losses due to fermentation failure and product losses. Phages of *L. lactis* belong to 10 phage groups, out of which members of the three phage groups 936, P335 and c2 are

most prevalent and have previously been reported in raw milk, cheese and whey samples and many other dairy products throughout the world.

Bacteriophage infection is initiated by the recognition of bacterial surface by phages using their adhesion apparatus and the overall process is controlled by several complicated factors related to the immediate environment, phage orientation and surface properties of bacteria. Phages of *L. lactis* have RBP that specifically targets a substrate on the host surface which is usually saccharidic in nature for most of *L. lactis* phages. Classification of *L. lactis* phages is mainly based on morphology and nucleotide sequence of phage adhesion apparatus. Some phages of *L. lactis* like P1532 and P680 are resistant to commonly applied heat treatments and biocide applications in the dairy industry which further exacerbate the situation. Among many biocides that have been tested to inactivate *L. lactis* phages, variable effectiveness in phage inactivation depending on phage and chemical composition of biocides has been noted. Overall, alcohols, in virtually all cases, have remained ineffective against *L. lactis* phages. In turn, QAC, oxidising agents, peracetic acid and sodium hydroxide have proven to be very fast and efficient disinfectants even in low concentration and less exposure time. Peracetic acid has several advantages in terms of its efficiency in low concentration and the production of non-toxic compounds.

It should remain clear that all disinfectants are not equally effective in inactivating all phage groups. Also, the application of biocides in a specific dairy industry should be based on a prior survey leading to the identification of specific bacteriophages present as the effectiveness of these biocides in many cases is phage strain dependent. It is advisable to use a combination of phage inactivation or removal techniques (thermal treatment, biocides, high hydrostatic pressure, membrane filtration, ultraviolet radiation, etc.) to completely eliminate phages. For instance, cheese vats and processing lines should be first rinsed with water at high temperature (95 °C) for 5 min to effectively remove most of the phages before applying any biocides on the surface.

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