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# Inactivation of dairy bacteriophages by commercial sanitizers and disinfectants

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ABSTRACT

Many commercial sanitizers and disinfectants have been used over the years to control microbial contamination but their efficacy on phages is often unknown. Here, 23 commercial chemical products, including 21 food-grade sanitizers were tested against virulent dairy phages. These food-grade chemicals included oxidizing agents, halogenated agents, alcohols, quaternary ammonium compounds, anionic acids, iodine-based acids, and an amphoteric chemical. Phage P008 was first exposed to each sanitizer for 2 and 15 min at room temperature and at two different concentrations, namely the lowest and highest no-rinse sanitizing concentrations. Organic matter (whey or milk) was also added to the testing solutions. At the end of the exposure period, the test solution was neutralized and the number of infectious phages was determined by plaque assays. The five most efficient sanitizers against phage P008 (<4 log of inactivation) were then tested against virulent lactococcal phages P008, CB13, AF6, P1532 of the 936 group, P001 (c2), Q54, and 1358 as well as *Lactobacillus plantarum* phage B1 and *Streptococcus thermophilus* phage 2972 using the same protocol. The oxidizing agents and the quaternary ammonium compounds were the most efficient against all phages although phages CB13 and P1532 were less sensitive to these chemicals than the other phages. This study may help in the selection of appropriate chemicals for controlling phage contamination in industrial factories and research laboratories.



## 1. Introduction

Virulent phages infecting lactic acid bacteria (LAB) still represent a significant risk for milk fermentation failures during the production of cheeses and a variety of other fermented dairy products. These phages can also reduce product quality (Coffey and Ross, 2002; Émond and Moineau, 2007). Strains of *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus* sp. are the most important LAB used by the dairy industry (Hols et al., 2005).

Many antiphage strategies have been devised to control lactic phage populations (Samson and Moineau, 2013). These include, among others, the use of starter culture rotation as well as phage-resistant strains (Émond and Moineau, 2007; Labrie et al., 2010). Others have also proposed reducing the number of bacterial strains to limit phage biodiversity within any given cheese factory (Quiberoni et al., 2006). These approaches have been successfully used for reducing phage contamination in large-scale industrial fermentations (Émond and Moineau, 2007). However, these selective pressures also led to the emergence of novel phages (Mahony et al., 2012; Rousseau and Moineau, 2009).

In dairy processing plants, novel LAB phages can be introduced and dispersed through various sources (Émond and Moineau, 2007; Briggiler Marcó et al., 2012a,b; Verreault et al., 2011): i) raw milk in which they are found; ii) ingredients added to the milk, iii) re-used dairy by-products such as whey protein concentrates; iv) movement of employees within the plant; v) ineffective cleaning of the equipments; vi) water used for rinsing equipment or for the dilution of cleaners and disinfectants; and vii) ambient air.

Heat is the primary treatment used to inactivate most microorganisms traditionally encountered in raw milk. However, the majority of virulent phages infecting LAB can resist pasteurization (Guglielmotti et al., 2011; Murphy et al., 2013). High-pressure treatments have also been suggested but some LAB phage species can resist pressures up to 100 MPa (Capra et al., 2009; Mercanti et al., 2012). Numerous commercial chemical products are also used in food processing plants for disinfecting and sanitizing contact surfaces. To be approved by health authorities, food contact sanitizers must meet several criteria, such as

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minimum residue levels, low human toxicity and antimicrobial efficacy (minimum of 3 log reduction of specific bacteria or viruses in 5 min, or, for a sanitizer with a disinfectant claim, 5 log reduction in 30 s (Gaulin et al., 2011).

In dairy processing plants, cleaning in place procedures (CIP) are used on equipment and surfaces (including floors) as the first step of a sanitization program to physically and chemically remove organic and microbiological contamination (Cords et al., 2001). This step is important since organic matter (such as milk or whey residues) may inactivate or lead to decreased effectiveness of sanitizers (Gaulin et al., 2011; Gelinas and Goulet, 1983). A food contact sanitizer is then applied to the equipment to properly sanitize or disinfect the surfaces. In Canada, for example, approved food contact sanitizers include chlorine compounds (e.g., bleach), peroxide and peroxyacid mixtures, carboxylic acids, quaternary ammonium compounds, anionic acids, and iodine compounds (Gaulin et al., 2011). For the sanitizing step of the CIP treatment, the US FDA has approved over 40 different compounds for the food industry (US FDA, 2012). Although food contact sanitizers with disinfecting claims are effective in reducing or eliminating food microorganisms (including viruses) linked to human diseases, little is known about their efficiency in inactivating LAB phages. In Europe, such LAB phage reduction claims exist and must provide a 4 log reduction of the number of viable units (or plaque forming units in case of phages) in an established time (European Committee for Standardization (CEN), 2002).

In the past decade, a few studies have attempted to evaluate the efficiency of biocides on a few LAB phages. In the case of phages of *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, and *Lactobacillus paracasei*, the efficiency of chemical biocides (peracetic acid, sodium hypochlorite, ethanol, isopropanol) varied and was phage- or formulation-dependent [(Capra et al., 2004; Ebrecht et al., 2010; Quiberoni et al., 2003; Quiberoni et al., 1999), reviewed in Guglielmotti et al. (2011) and Mercanti et al. (2012)]. In general, as shown with *L. lactis* phages, peracetic acid (0.15% (v/v)) is an efficient sanitizer while sodium hypochlorite requires prolonged contact time and alcohols are not efficient (Suárez and Reinheimer, 2002; Murphy et al., 2013). Taken together, it is rather difficult to compare the effectiveness of these products since the methodologies vary between the studies. Factors influencing the efficacy of disinfectants that are realistically found in the processing plant environment, such as organic matter and hard water, are not always included in these phage inactivation protocols.

The aim of this study was, therefore, to measure the efficiency of traditional and commercial food contact sanitizers on representative LAB phages (infecting *L. lactis*, *Lactobacillus* or *S. thermophilus*) in a worst-case scenario of a dairy plant environment (i.e., in the presence of organic contamination and hardened water), using a standardized protocol. Our underlying goal was to determine the most efficient sanitizers against phages for the food industry and research laboratories.

## 2. Materials and methods

### 2.1. Strains, phages and growth conditions

The virulent lactococcal phages P008, CB13, AF6, P1532, P001, 1358 and Q54 as well as *Lactobacillus plantarum* phage B1 and *S. thermophilus* phage 2972 were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca). The bacterial hosts used to amplify them were *L. lactis* IL1403, *L. lactis* SMQ-404, *L. lactis* SMQ-1001, *L. lactis* HER1142, *L. lactis* SMQ-388, *L. lactis* SMQ-562, *L. plantarum* ATCC8014 and *S. thermophilus* DGCC7710, respectively. Phage P008 (Loof et al., 1983) was selected as a representative of the lactococcal 936 group, which is the most predominant group in cheese factories worldwide (Mahony et al., 2012; Rousseau and Moineau, 2009), and is also suggested in European standards (European Committee for Standardization (CEN), 2002). Lactococcal phages CB13 and AF6, belonging to 936 group, were recently isolated from whey samples from a Canadian cheese plant (Moisan and Moineau, 2012; Rousseau and Moineau, 2009) and phage CB13 was found to be persistent for over one year in the same cheese factory (Rousseau and Moineau, 2009). Phage P1532 (936 group) was selected because it was shown to be highly resistant to heat treatment (Atamer et al., 2009). Phage P001 was selected as a representative of the lactococcal phage group c2, and is also a reference virus in the European standards (Braun et al., 1989; European Committee for Standardization (CEN), 2002). Phages 1358 and Q54 belongs to lactococcal phage groups rarely encountered in milk fermentation facilities (Jarvis, 1984; Fortier et al., 2006; Deveau et al., 2006). Virulent phage 2972 (Lévesque et al., 2005) was used as a reference for streptococcal phages since it represents one of the two main groups of *S. thermophilus* phages (Le Marrec et al., 1997; Quiberoni et al., 2010) encountered in dairy environments and phage B1 was selected as the representative of *Lactobacillus* phages (Briggiler Marcó et al., 2012a,b).

Bacterial strains were cultured in M17 (Oxoid) supplemented with either 0.5% glucose (GM17) or 0.5% lactose (LM17) at 30 °C for the lactococcal strains or with LM17 at 42 °C for the streptococcal strain. *Lactobacillus* strains were cultured in MRS (Difco) at 37 °C. When propagating phages, 10 mM $CaCl_2$ was added to the medium. For the plaque assays, an aliquot of phage solution was mixed with an appropriate volume of an overnight culture of the host strain in soft agar at 45–50 °C using the appropriate medium for the bacterial host strain (GM17, LM17 or MRS) supplemented with 0.75% agar and 10 mM $CaCl_2$. The inoculated soft agar was then poured over a 1% agar medium (of the same composition) in a Petri dish. The plates were incubated overnight at the appropriate temperature for the bacterial host strain.

### 2.2. Sanitizers

Five different chemical companies accredited by the Canadian Food Inspection Agency to sell sanitizing products to the Canadian dairy industry provided samples of commercial sanitizers (between 1 and 20 L). These sanitizers were chosen on the basis of their relevance to the food and dairy industries and were certified by their respective companies to be effective for the inactivation of enteric and environmental microorganisms. Different sanitizers ($n = 21$) were chosen among the following chemical families: chlorinated agents, peroxide and peroxyacid (PPA) mixtures, amphoteric compounds, quaternary ammonium compounds (QAC; benzalkonium chloride-based), anionic acids (phosphoric acid-based), and iodine compounds (iodine-based acids). As traditional disinfectants, ethanol and isopropanol were also included. Each sanitizer is described in Table 1, as per the Material Safety Data Sheets (MSDS) provided by the respective chemical companies. Note that the lists of active ingredients composing the different sanitizers listed in Table 1 may be incomplete, since only toxic ingredients are listed in the MSDS.

The chemical concentrations used for the phage inactivation assays were determined according to the recommended concentration interval for a sanitizing procedure described in the technical sheet of each sanitizer. Concentration 1 was selected as the lowest sanitizing concentration not requiring water rinse, and concentration 2 was either the highest no-rinse sanitizing concentration or the disinfecting concentration, depending on the product, since some companies did not specify a range of sanitizing concentrations for the product. Although not approved as contact sanitizers per se, we also tested two other chemicals, sodium dichloro-S-triazinetrione and bromochlorodimethylhydantoin (BCDMH), which are both solid tabs used in water treatment systems in food industries and commonly used in drains (wastewater). All concentrated sanitizers were diluted in hardened water (1.26 mM $MgCl_2$, 2.52 mM $CaCl_2$, 3.36 mM $NaHCO_3$, pH 7.0; for a desired concentration of 300 mg/kg $CaCO_3$).

**Table 1**
Composition and concentration of the food contact sanitizers used in this study.

| Family | Sanitizer (Abbreviation) | Concentration used 1 | Concentration used 2 | Relative composition of the concentrated products sold in Canada (chemical concentration %) |
|---|---|---|---|---|
| *Chlorinated agents* | | | | |
| | $Na^{2+}$ hypochlorite | 200 ppm | 500 ppm | Sodium hypochlorite 12% |
| | Chlorine dioxide | 20 ppm | 50 ppm | Sodium chlorite 1–5% |
| *Peroxide and peroxyacid mixtures* | | | | *Peracetic acid %; acetic acid %, $H_2O_2$ %; others* |
| | Oxi-A | 0.2% | 0.5% | 5–10%; 10–20%; 10–20% |
| | Oxi-B | 0.2% | 0.35% | 5–10%; 7–13%; 15–40%; |
| | Oxi-C | 0.5% | 1.4% | 15–17%; 33–38%; 9–11% |
| | Oxi-D | 0.13% | 0.25% | 3–7%; 15–40%; 5–10%; L-octanesulfonic acid, sodium salt 3–7%; octanoic acid 1–5% |
| | $Na^{2+}$ percarbonate | 200 ppm | 500 ppm | Sodium percarbonate 30–60%, solid |
| *Quaternary ammonium compounds* | | | | *Benzalkonium chloride %; others* |
| | QAC + EtOH | 200 ppm | 500 ppm | 7–13%; ethanol 1–5% |
| | QAC | 200 ppm | 500 ppm | 10–20% |
| *Anionic acids* | | | | *Phosphoric acid %; others* |
| | Anionic-A | 200 ppm | 500 ppm | 10–30%; oleic acid, sulfonated sodium salt 1–5%; propylene glycol 3–7% |
| | Anionic-B | 200 ppm | 500 ppm | 10–30%; octanoic acid 1–5%; lactic acid 1–5%; propylene glycol 5–10% |
| | Anionic-C | 200 ppm | 500 ppm | 10–30%; dodecyl benzene sulfonic acid 1–5% |
| | Anionic-D | 200 ppm | 500 ppm | 15–40%; oleic acid, sulfonated, sodium salt 7–13% |
| | Anionic-E | 2000 ppm | 5000 ppm | 10–30%; 2-hydroxypropanoic acid 1–5%; sodium alkylnaphtalene sulfonate 10–30%; octanoic acid 3–7%; decanoic acid 0.5–1.5% |
| *Iodine-based acids* | | | | *Iodine; others* |
| | Iodine-A | 25 ppm | 50 ppm | 1–5%; nitric acid 20–30% |
| | Iodine-B | 25 ppm | 50 ppm | 1–5%; phosphoric acid 10–30%; isopropanol 1–5% |
| | Iodine-C | 12.5 ppm | 25 ppm | 1–5%; phosphoric acid 10–30%; sodium iodide 1–5%; methyloxirane, polymer with oxirane 10–30% |
| | Iodine-D | 12.5 ppm | 25 ppm | 1–5%; phosphoric acid 10–30%; methyloxirane, polymer with oxirane, monobutyl ether 7–13%; dipropylene glycol monomethyl ether 1–5% |
| *Alcohols* | | | | |
| | Ethanol | 70% | 80% | Ethanol anhydrous |
| | Isopropanol | 70% | 80% | Isopropanol anhydrous |
| *Amphoteric* | | | | |
| | Amphoteric | 200 ppm | 500 ppm | Alkylaminocarboxymethylaminopropane, sodium salt 5–10% |
| *Treatment of water system or drains* | | | | |
| | $Cl_2$-triazinetrione | 200 ppm | | Dichloro-S-triazinetrione, sodium salt, solid |
| | BCDMH | 100 ppm | | Bromochlorodimethylhydantoin 60–100% |

Abbreviations: $Na^{2+}$, sodium; ppm, parts per million; $H_2O_2$, hydrogen peroxide; EtOH, ethanol; $Cl_2$, dichloro.

### 2.3. Phage inactivation protocol

The phage inactivation protocol was adapted from the European standard EN 13610:2002 (European Committee for Standardization (CEN), 2002). Briefly, the phage lysate, standardized at a final concentration of about $1 \times 10^8$ to $1 \times 10^9$ pfu/mL, was exposed to the sanitizer for 2 or 15 min at the selected concentration in the presence of 1% (v/v) organic contaminant (milk or whey) that was added 5 min to the phage before the beginning the experiment at room temperature (about 21 °C). The whey stock solution was made of milk acidified with 0.3% DL-lactic acid (Sigma) for 30 min at room temperature, then centrifuged at 4000 *g* for 30 min and passed through a 0.45 μm filter before storing at −20 °C. At the end of the exposure period, the test solution was immediately neutralized by diluting 1/50 (v/v) in a neutralization solution, composed of M17 medium supplemented with 3% polysorbate 80 (Tween 80® — Sigma), 0.3% sodium thiosulfate (Sigma), 0.3% L-cysteine (Sigma) and 0.3% L-histidine (Sigma). The remaining number of infectious phages was then determined by plaque assays using serial dilutions of $10^0$, $10^{-1}$ and $10^{-2}$ in phage buffer (50 mM Tris–HCl at pH 7.5, 100 mM NaCl and 6 mM $MgSO_4$). Since the aim of this study was to identify the most efficient sanitizers against LAB phages in the presence of organic contamination, we incorporated an elimination procedure. In the first step, all chemicals (n = 21) were tested against phage P008 in the presence of whey. In the second step, the eight most efficient sanitizers were selected and retested against phage P008 but in the presence of milk. Finally, the sanitizers with the five best inactivation results were tested at their lowest effective concentrations against a set of eight phages (CB13, AF6, P1532, P001, Q54, 1358, B1, and 2972) in the presence of 1% milk or whey (v/v).

### 2.4. Controls

For each experiment, a hard water control (positive control, with phages) was processed in parallel as the reference titer for the inactivation potential. Negative controls without phage were also included to verify the sterility of the different solutions (hard water, milk, whey, phage buffer and neutralization solution). During each experiment, the titers of the phage stock solutions were estimated to verify the validity of the positive control. The neutralization solution was also tested to validate its non-toxicity against the phages and bacterial strains used. The effectiveness of the solution for neutralizing the different sanitizers was also tested. The sanitizing solutions without phages were left to react for 2 or 15 min at the selected concentration in the presence of 1% (v/v) organic contaminant (milk or whey) at room temperature (21 °C). The test solution was immediately neutralized by diluting 1/50 (v/v) in a neutralization solution. The phage lysate was added to this solution, left to react for 30 min, and processed for plaque assays.

### 2.5. Statistical analysis

Each experiment was repeated three times with technical duplicate for four phages (P008, 2972, CB13, and B1) while the experiments with the remaining five phages (AF6, P1532, P001, Q54, and 1358) were repeated twice with technical duplicate. Inactivation ratios were calculated by dividing treatment titer by the hard water control titer. To analyze the relative efficiency of sanitizers against each phage, all the sanitizers were compared to Oxi-D as it is widely used by the dairy industry in Canada. The log-transformed inactivation ratios were analyzed under repeated measures two-way ANOVA followed by a Bonferroni tests to correct the p-values for the multiple comparisons. To compare the overall phage sensitivity to the sanitizers, the data were analyzed under a two-way ANOVA followed by a Turkey test to correct the p-values for the multiple comparisons. All the statistical analyses were done using the software GraphPad Prism 6 (GraphPad software, Inc. San Diego, Calif.).

## 3. Results

### 3.1. Inactivation of lactococcal phage P008 in whey

The inactivation potential (expressed in $\log_{10}$ units reduction) of each sanitizer on phage P008 is detailed in Table 2. Chlorinated compounds, isopropanol, iodine-based compounds and the amphoteric compound were not effective for inactivating phage P008 (less than 2 to 3 log units reduction; $p < 0.01$ compared to Oxi-D control) in the presence of 1% whey. Alcohols were also not very effective, although ethanol had some phage inactivation efficacy (4 log reduction) after 15 min of contact time. All peroxide, peracetic acid and acetic acid mixtures reached at least 4 logs unit reduction after 15 min at the lowest concentration or 2 min at the highest concentration. The two quaternary ammonium compounds and four (A to D) anionic acids were the most effective in inactivating P008 (>5 log inactivation at all time and concentrations) in the presence of whey.

### 3.2. Inactivation of lactococcal phage P008 in milk

To further test the efficacy of the sanitizers, we tested 8 of them against P008 in the presence of 1% milk. The selected sanitizers reduced the titer of P008 by at least 4 log at their respective lowest concentration (concentration 1) and shortest contact time (2 min) in presence of 1% whey (Table 2). All sanitizers achieved close to 5 log reduction of P008 titer at their respective highest concentration in 1% milk and at both contact times (Table 3). At the lowest sanitizing concentration, Oxi-D, the two QACs, and the four anionic acids were all more effective against P008 than Oxi-A ($p < 0.001$). Although not statistically significant, among the commercial anionic acid products, Anionic-B and -D, were slightly more efficient than Anionic-A and -C that were not completely inactivating the phages (Table 3) while the inactivation ratios of Anionic-B and -D were above the detection limit.

### 3.3. Inactivation of a set of eight phages by the most efficient sanitizers

The next step was to confirm the sanitizer efficacy results using eight additional phages, namely three lactococcal phages member of the 936 group (CB13, AF6 and P1532), one lactococcal phage representing the c2 group (P001) used in the European standards (European Committee for Standardization (CEN), 2002), two members of rare lactococcal phage groups (Q54 and 1358), one *Lactobacillus* phage (B1) and one streptococcal phage (2972). Here, we tested five sanitizers at their lowest recommended concentration. We tested two anionic acid-based products Anionic-B and Anionic-D. Since both QACs gave similar results on the inactivation of phage P008, only the formulation without ethanol was tested. Finally, we selected the Oxi-B and Oxi-D products to represent the peroxide and peroxyacid mixtures.

Table 4 shows that all sanitizers were very effective in inactivating the streptococcal phage 2972 (>4 log reduction) without any significant difference ($p = 0.63$). The sanitizers were so efficient against 2972 that

**Table 2**
$\log_{10}$ units reduction of lactococcal phage P008 by commercial food contact sanitizers ($n = 21$) in the presence of 1% whey (v/v).

| Family | Sanitizer (abbreviation) | Concentration 1 | | Concentration 2 | |
|---|---|---|---|---|---|
| | | 2 min | 15 min | 2 min | 15 min |
| *Chlorinated agents* | | | | | |
| | $Na^{2+}$ hypochlorite | <2.0* | <2.0* | 2.3 ± 0.3* | <2.0* |
| | Chlorine dioxide | <2.0* | <2.0* | 2.1 ± 0.1* | <2.0* |
| *Peroxide and peroxyacid mixtures* | | | | | |
| | Oxi-A | 4.8 ± 0.4 | 5.1 ± 0.2 | 4.3 ± 0.8 | >5.4 |
| | Oxi-B | 3.8 ± 0.4 | >5.8 | 4.3 ± 0.8 | >5.8 |
| | Oxi-C | 2.4 ± 0.0* | >5.9 | 5.0 ± 0.4 | >5.9 |
| | **Oxi-D** | **4.9 ± 0.5** | **5.6 ± 0.3** | **5.4 ± 0.3** | **>5.8** |
| | $Na^{2+}$ percarbonate | 2.5 ± 0.3* | 5.6 ± 0.2 | 5.2 ± 0.6 | 5.6 ± 0.2 |
| *Quaternary ammonium compounds* | | | | | |
| | QAC + EtOH | >5.8 | 5.2 ± 0.6 | >5.7 | 5.6 ± 0.2 |
| | QAC | >5.8 | 5.6 ± 0.2 | >5.8 | 5.2 ± 0.6 |
| *Anionic acids* | | | | | |
| | Anionic-A | 5.4 ± 0.4 | 5.5 ± 0.3 | 5.6 ± 0.4 | 5.7 ± 0.1 |
| | Anionic-B | >5.8 | >5.7 | >5.8 | >5.7 |
| | Anionic-C | >5.7 | 5.6 ± 0.2 | >5.7 | 5.6 ± 0.2 |
| | Anionic-D | 5.5 ± 0.2 | 5.5 ± 0.1 | 5.5 ± 0.3 | 5.5 ± 0.1 |
| | Anionic-E | <2.0* | <2.0* | 3.8 ± 1.1 | 5.1 ± 0.7 |
| *Iodine-based* | | | | | |
| | Iodine-A | 2.4 ± 0.4* | 2.4 ± 0.4* | 2.6 ± 0.3* | 4.6 ± 0.9 |
| | Iodine-B | <2.0* | 2.9 ± 0.9* | 3.1 ± 0.6* | 4.3 ± 0.6 |
| | Iodine-C | <2.0* | <2.0* | <2.0* | <2.0* |
| | Iodine-D | 2.4 ± 0.4* | 2.4 ± 0.4* | 2.5 ± 0.3* | 2.6 ± 0.3* |
| *Alcohols* | | | | | |
| | Ethanol | 3.7 ± 0.3 | 4.4 ± 0.3 | 3.2 ± 0.1* | 4.0 ± 0.2* |
| | Isopropanol | 3.3 ± 0.5 | 3.6 ± 0.2* | <1.9* | 2.4 ± 0.2* |
| *Amphoretic* | | | | | |
| | Amphoteric | 2.4 ± 0.4* | 2.4 ± 0.4* | 2.4 ± 0.4* | 2.2 ± 0.5* |

Data are expressed as the average $\log_{10}$ units reduction compared to hard water control ± SEM ($n = 3$).
* Indicates a significant difference ($p < 0.01$) between the sanitizers and the control sanitizer (Oxi-D, in bold characters).

**Table 3**
$\log_{10}$ units reduction of lactococcal phage P008 by 8 commercial food contact sanitizers in the presence of 1% milk (v/v).

| Family | Sanitizer | Concentration 1 | | Concentration 2 | |
|---|---|---|---|---|---|
| | | 2 min | 15 min | 2 min | 15 min |
| *Peroxide and peroxyacid mixtures* | | | | | |
| | Oxi-A | 3.6 ± 0.1* | >5.1 | 4.9 ± 0.4 | >5.2 |
| | **Oxi-D** | **>5.6** | **>5.5** | **>5.3** | **>5.7** |
| *Quaternary ammonium compounds* | | | | | |
| | QAC + EtOH | 5.2 ± 0.1 | >5.1 | 4.9 ± 0.4 | 5.1 ± 0.1 |
| | QAC | 5.2 ± 0.1 | 5.1 ± 0.1 | 5.0 ± 0.3 | >5.2 |
| *Anionic acids* | | | | | |
| | Anionic-A | 5.2 ± 0.2 | >5.1 | 5.4 ± 0.0 | 4.9 ± 0.4 |
| | Anionic-B | >5.3 | >5.1 | >5.4 | >5.2 |
| | Anionic-C | 4.8 ± 0.4 | >5.1 | 5.1 ± 0.2 | >5.2 |
| | Anionic-D | >5.3 | >5.1 | >5.4 | >5.2 |

Data are expressed as the average $\log_{10}$ units reduction compared to hard water control ± SEM ($n = 3$).
* Indicates a significant difference ($p < 0.01$) between the sanitizers and the "control" sanitizer (Oxi-D, in bold characters).

**Table 4**
$Log_{10}$ units reduction of 8 dairy phages by five commercial sanitizers in the presence of 1% milk (v/v).

| Phage | Contact time | *Peroxide and peroxyacid mixtures* | | *Quaternary ammonium compound* | *Anionic acids* | |
|---|---|---|---|---|---|---|
| | | Oxi-B | **Oxi-D** | QAC | Anionic-B | Anionic-D |
| AF6 | 2 min | >3.9 | **2.6 ± 0.4** | 4.3 ± 0.8 | 4.8 ± 0.4* | 5.7 ± 0.3* |
| | 15 min | >5.5 | **4.4 ± 0.7** | >5.5 | >5.5 | >5.5 |
| CB13 | 2 min | 2.9 ± 0.1* | **5.4 ± 0.6** | 5.6 ± 0.4* | <2.0* | <2.0* |
| | 15 min | 5.6 ± 1.0 | >**6.3** | >6.3 | 2.4 ± 0.5* | <2.0* |
| P1532 | 2 min | 2.5 ± 0.1 | **3.4 ± 0.5** | 4.6 ± 1.6 | 1.9 ± 0.2 | 3.5 ± 1.4 |
| | 15 min | 4.6 ± 0.4 | >**5.9** | 5.2 ± 1.0 | >5.1 | 3.7 ± 0.4 |
| P001 | 2 min | 4.2 ± 0.6 | >**4.5** | >3.0 | >4.8 | >4.5 |
| | 15 min | >4.2 | >**4.2** | >4.2 | >4.2 | >4.2 |
| Q54 | 2 min | 3.0 ± 0.9* | >**5.7** | >4.4 | 3.9 ± 0.5 | >5.4 |
| | 15 min | >3.1 | >**5.7** | >5.7 | >5.7 | 5.7 ± 0.4 |
| 1358 | 2 min | >5.7 | >**5.7** | >2.7 | >4.6 | >5.7 |
| | 15 min | >5.8 | >**5.8** | >5.8 | >5.8 | >5.8 |
| 2972 | 2 min | 4.6 ± 0.0 | >**4.6** | 4.2 ± 0.4 | >4.6 | 4.1 ± 0.5 |
| | 15 min | >4.6 | >**4.6** | >4.6 | >4.6 | >4.6 |
| B1 | 2 min | >6.2 | **5.7 ± 0.2** | 4.3 ± 1.3 | <2.0* | >6.2 |
| | 15 min | >5.3 | >**5.3** | >3.1 | 3 ± 0.1* | >5.3 |

The sanitizer concentrations used were: Oxi-B, 0.2%; Oxi-D, 0.13%; QAC, 200 ppm; Anionic-B, 200 ppm; and Anionic-D, 200 ppm. Data are expressed as the average $log_{10}$ units reduction compared to control ± SEM.
* Indicates a significant difference ($p < 0.01$) between the sanitizers and the control sanitizer (Oxi-D, in bold characters).

the contact time did not affect the inactivation potential ($p = 0.19$). However, Oxi-D and Anionic-B were the most efficient as their inactivation ratio exceeded the limit of detection of the test as no plaques were observed after any of the treatments (Table 4). Similarly, all sanitizers were efficient against lactococcal phages Q54, 1358 and P001 ($p > 0.18$), although phage Q54 seems slightly more resistant as contact time can be considered a significant factor in this case ($p = 0.02$). Lactococcal phage AF6 was inactivated efficiently (>4 log reduction after 2 min of contact time) by most of the sanitizer except Oxi-D that reaches only 2.6 log of reduction after 2 min. All sanitizers, except Anionic-B, were highly efficient against the *Lactobacillus* phage B1 (>4 log reduction within 2 min of contact time). Anionic-B efficiency achieved only 3 log reduction after 15 min of contact times ($p = 0.002$ compared to Oxi-D).

On the other hand, lactococcal phages CB13 and P1532 were more resistant to the sanitizers tested (Table 4). Indeed, CB13 was significantly more resistant than phages B1, Q54 and 1358 ($p < 0.038$) whereas statistical analysis revealed that P1532 is more resistant than all phages tested excepted CB13 ($p < 0.014$). The anionic acids were particularly inefficient against phage CB13 (Table 4) while Oxi-D and QAC were able to reduce CB13 titers by more than 5 log with an exposure time of only 2 min. QAC, Oxi-B and Oxi-D were highly efficient against the set of phages tested and their activity was not significantly different from each other, the commercial anionic acid Anionic-B was significantly less efficient than Oxi-D ($p = 0.002$).

### 3.4. Inactivation of phages by water disinfectants

Finally, as phages found in the dairy industry may reside in drains, we tested two products commonly used as water disinfectants against P008, CB13 and 2972 (Table 5). One component, dichloro-S-triazinetrione, could not reach 3-log reduction of P008 at 200 ppm in 1% milk, and was, therefore, not tested against other phages (Table 5). Conversely, BCDMH was efficient against all three phages, including CB13, at a concentration of 100 ppm after only 2 min of exposure in the presence of 1% milk (log reduction between 4.2 ± 0.4 and 5.9 ± 0.2, depending on the phage, Table 5). When placed in water, BCDMH slowly dissolves to 5,5-dimethylhydantoin and to the unstable active products, hypobromous acid and hypochlorous acid. Both are oxidizing agents. BCDMH is highly stable on the shelf and is innocuous in storage (WHO, 2006).

## 4. Discussion

The results obtained in our study shed light on the efficacy of commercially available and regulatory approved sanitizers to inactivate LAB phages, which are a recurrent problem in the dairy industry (Émond and Moineau, 2007). Our results suggest that the dairy industry should perhaps focus on the new generation of peracetic acid and acetic acid mixtures such as Oxi-D as well as quaternary ammonium compounds such as QAC to ensure adequate inactivation of phages during sanitization of factories manufacturing fermented dairy products. However, the use of BCDMH compound is suggested if phage contamination is suspected to come from the wastewater or drains.

In general, several factors must be evaluated in order to choose an adequate sanitizer to use on food contact surfaces in the sanitization step: its regional availability, its effectiveness against microorganisms, its penetrative power in biofilms, its stability over time, its toxicity and odors (affecting workers), the hardness of the local water, and the cost-effectiveness of the product (Gaulin et al., 2011). For the dairy industry, we believe that the effectiveness against phages should also be documented using standardized protocols. Recently, another study analyzed 8 sanitizers for their ability to reduce lactobacilli phages in dairy environments (Mercanti et al., 2012). In that study, the time required to inactivate 99% (2 log reduction) of the phages was calculated (T99). Here, we elected to measure the efficiency of phage inactivation by sanitizers (log units reduction) over time, as this measure is often used in microorganism inactivation studies. In our study, T99 would

**Table 5**
$Log_{10}$ units reduction of lactococcal phages P008 and CB13, and *S. thermophilus* phage 2972 by two commercial sanitizers used for treatment of water systems in food industry, in the presence of 1% milk (v/v).

| Sanitizer | P008 | | CB13 | | 2972 | |
|---|---|---|---|---|---|---|
| | 2 min | 15 min | 2 min | 15 min | 2 min | 15 min |
| $Cl_2$-triazinetrione | 2.4 ± 0.0 | 2.4 ± 0.2 | – | – | – | – |
| BCDMH | >5.3 | >5.1 | 5.9 ± 0.2 | >6.3 | 4.2 ± 0.4 | 3.8 ± 0.1 |

Data are expressed as the average $log_{10}$ units reduction compared to hard water control ± SEM (n = 3). The concentrations used were: 200 ppm $Cl_2$-triazinetrione; 100 ppm BCDMH.

likely not have been sufficient to discriminate between sanitizers since most of them achieved 2 log reduction within 2 min at the lowest concentration.

Here, chlorine dioxide (20–50 ppm), anionic compound E (2000–5000 ppm), all iodine-based acids, the amphoteric compound (200–500 ppm) and alcohols were inefficient against *L. lactis* phage P008, thus they were not further tested against other LAB phages. It was previously observed that lactococcal and lactobacilli phages are resistant to sodium hypochlorite at the concentrations allowed on food contact surfaces (Guglielmotti et al., 2011; Briggiler Marcó et al., 2009; Mercanti et al., 2012; Parada and de Fabrizio, 2001; Quiberoni et al., 2003; Murphy et al., 2013). One study showed that 800 ppm of sodium hypochlorite needed 30 min for complete inactivation of *Lactobacillus* phages (Briggiler Marcó et al., 2009). Iodine-based sanitizers are sometimes used at the dairy farm to disinfect the cow's udder before milking (Gibson et al., 2008) but they are not widely used in dairy factories, mainly due to the color it could leave on the equipment and the low residual activity. Some oxidizing agents, such as sodium percarbonate and Oxi-C mixture were efficient against phages at their lowest sanitizing concentration, but only after a prolonged contact time (15 min), which is likely not suitable for fast-sanitizing steps. Sodium percarbonate has the advantage of dissolving in water, generating hydrogen peroxide and sodium carbonate, with the former acting as an oxidizing agent (OECD SIDS, 2006). Interestingly, Oxi-C had the highest concentrations of peracetic acid (15–17%) and acetic acid (33–38%) but this was still not enough to quickly inactivate phage P008 (Tables 1 and 2).

Although peracetic acid alone has good activity against *Lactobacillus*, *Streptococcus*, *Lactococcus* and *E. coli* phages (Binetti and Reinheimer, 2000; Mattle et al., 2011; Mercanti et al., 2012; Murphy et al., 2013), its combination with acetic acid and hydrogen peroxide enhances the oxidizing power of the sanitizer on proteins or nucleic acids, as well as its storage stability (Kitis, 2004; Maillard et al., 1994; McDonnell, 2007). Indeed, blends of peracetic acid and acetic acid (or PPA) are widely used in the dairy industry and in CIP practices since they inactivate most microorganisms. Although these mixtures can be corrosive to skin, metals and stainless steel (Gaulin et al., 2011), the residual degradation products after dilution are non-toxic for humans after few minutes. Moreover, these chemicals are active at various temperatures and are not influenced by acid or neutral pH, by hard water or organic matter (Cords et al., 2001).

Peroxide and peroxyacid mixtures, which are commonly used in the dairy industry in Canada, effectively inactivated the tested phages but needed a longer contact time. The use of new formulations of PPA mixtures, such as Oxi-D (supplemented with sodium octanesulfonic acid and octanoic acid) were more effective against all phage tested, including CB13 and P1532. A similar formulation also showed greater antifungal activity in the context of fresh cut vegetables (Hilgren and Salverda, 2000).

Finally, although QACs were introduced as disinfectants in 1916 (McDonnell and Pretzer, 2001), they are still highly efficient as ready-to-use, no-rinse sanitizers. As shown by our study, they are also effective against dairy phages. Besides their high antimicrobial and antiphage effects, QACs are non-staining, non-corrosive on surfaces and offer good residual activity, even in the presence of hard water and organic matter (Gaulin et al., 2011). However, one should keep in mind that QAC residues may contaminate milk and inhibit starter culture activity (Cords et al., 2001; Hassan and Frank, 2001). Although bacterial resistance has been observed with another QAC used in contact lens care solutions (Bruinsma et al., 2006), no phage resistance was observed here.

Until recently, peracetic acid was perceived to be the only effective sanitizer against phages. As indicated above, it is even more effective when combined with other oxidizing agents and anionic acids such as Oxi-D. A new oxidizing agent containing potassium ferrate (VI) could also be promising since it was efficient against coliphage MS2 in water and wastewater treatments (Hu et al., 2012). However, to be acceptable to the food industry, its toxicity, efficiency and residual activity must be verified in the presence of organic matter such as milk compounds.

As an alternative to common chemical sanitizers, photocatalysis using $TiO_2$ combined with UV-A radiation was recently proposed to inactivate dairy phages (Briggiler Marcó et al., 2011). However, the time needed to sanitize the environment is rather long (3 h). As stated by the authors, this technology's main advantage is that it is safe enough to use for long periods in the presence of employees. Moreover, it directly reduces phage particles in the air. Other combinations of photosensitizers with UV, such as porphyrin (Jahid and Ha, 2012) or fullerol (Hotze et al., 2009) have also been proposed, although their safety remains to be established. The combination of a photosensitizer, light (UV-A) and molecular oxygen can induce important damage to biological targets (Costa et al., 2012).

Taken altogether, it is important to find an efficient system to reduce the levels of phage particles within a dairy plant to reduce the risk of milk fermentation failures. This is becoming particularly critical with the apparent emergence of LAB phages able to withstand standard thermal treatments used by the dairy industry (Atamer and Hinrichs, 2010). Therefore, the identification and improvement of commercially available sanitizers (Oxi-D, QAC) with potent antiphage activity is a step in that direction.

Finally, it remains to be seen whether the routine and steady use of these chemicals will lead to the selection of phages with increased resistance, such as observed for CB13 and P1532). Clearly, close monitoring of the phage population as well as regular testing of sanitizers against emerging new phages, as proposed here, should be implemented. As another precautionary measure to avoid selecting resistant phage population, a rotation of different sanitizers should also be employed. The development of new performing sanitizers with antiphage activity should also be encouraged.

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