Thermal and chemical inactivation of *Lactobacillus* virulent bacteriophage

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ABSTRACT

The effect of thermal treatments and several biocides on the viability of *Lactobacillus* virulent phage P1 was evaluated. Times to achieve 99% inactivation (T_{99}) of phage at different treatment conditions were calculated. The thermal treatments applied were 63, 72, and 90°C in 3 suspension media (de Man, Rogosa, Sharpe broth, reconstituted skim milk, and Tris magnesium gelatin buffer). Phage P1 was completely inactivated in 5 and 10 min at 90 and 72°C, respectively; however, reconstituted skim milk provided better thermal protection at 63°C. When phage P1 was treated with various biocides, 800 mg/L of sodium hypochlorite was required for total inactivation (~ 7.3 log reduction) within 60 min, whereas treatment with 100% ethanol resulted in only a ~ 4.7 log reduction, and 100% isopropanol resulted in a 5.2-log reduction. Peracetic acid (peroxyacetic acid) at the highest concentration used (0.45%) resulted in only a ~ 4 -log reduction of phage within 60 min. The results of this study provide additional information on effective treatments for the eradication of potential phage infections in dairy plants.

Key words: *Lactobacillus* virulent bacteriophage, thermal treatment, chemical treatment, inactivation

INTRODUCTION

Lactobacillus plantarum is a versatile mesophilic lactic acid bacterium (LAB) that constitutes parts of the natural microflora of dairy, meat, and vegetables, as well as fermented foods, and it is closely associated with human health (de Vries et al., 2006). Some strains express high resistance to extreme intestinal conditions, including gastric acidity and bile toxicity, and therefore can be utilized as probiotic cultures (Karasu et al., 2010; Briggiler Marcó et al., 2012). In the dairy industry, *L. plantarum* is used as a starter culture

to produce cheese, yogurt, and *Lactobacillus*-based probiotic beverages. When *L. plantarum* is used as a starter culture in Cheddar cheese manufacture, it has been shown to influence the formation of important aroma compounds. In addition, the culture has been demonstrated to accelerate the speed of cheese ripening (Lynch et al., 1999).

Bacteriophage infection is considered an important economic problem worldwide in food fermentation. Phage particles in a food plant can disseminate quickly, lysing starter cultures and resulting in poor quality or unsafe food because slow or complete starter failure results in poor acid production (Zhang et al., 2015). In fermented foods, the phage gains entry via raw milk, contaminated bacterial cultures, air, and processing equipment (Moineau, 1999; Madera et al., 2004). In most cases, raw milk is pasteurized before starter culture addition, and equipment is sanitized to reduce the presence of spoilage bacteria. Decreasing the incidence of phage infection via proper thermal treatment of milk and the use of effective biocides on equipment not only results in greater economic savings but also assures greater safety of the food products. Overall, information on the chemical and thermal resistance of *Lactobacillus* phages used in the dairy industry is limited because it often tends to be phage-specific.

Virulent phage P1 was initially isolated from a slow fermentation containing *L. plantarum* IMAU10120. The phage was subsequently shown to belong to the *Siphoviridae* family. The latent period of this phage was 45 min with a burst time of 90 min; the burst size was 132.88 ± 2.37 phage counts expressed per mL per infective center. This phage exhibited good tolerance ($>95\%$ survival) when treated at 0, 10, 20, 30, 37, 42, and 50°C; however, incubation at 50°C decreased adsorption; maximum adsorption was observed between 30 and 42°C (Chen et al., 2016).

The aim of the present research was to investigate the effect of thermal treatments and biocides routinely used in dairy plants and laboratories to limit phage infection and thereby contribute to the increased awareness of phage resistance.

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MATERIALS AND METHODS

Bacteria Strain, Phage, and Culture Conditions

Lactobacillus plantarum IMAU10120 was used as the host strain for the *Lactobacillus* virulent phage P1 and was obtained from the Lactic Acid Bacteria Collection Center of the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, P.R. China.

The organism was grown at 37°C in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Becton Dickinson and Co., Franklin Lakes, NJ) for 24 h. For phage amplification, MRS was supplemented with 10 mM CaCl₂. Phage stocks were prepared as previously described (Neviani et al., 1992) and stored as lysates at 4°C. Phage counts, expressed as plaque-forming units (pfu) per milliliter, were obtained using a plaque assay method with modification (Quiberoni et al., 2011). In brief, 100 µL of phage suspension was mixed with host bacterial suspended in a top layer of melted MRS agar (20 mL, 0.7% wt/vol agar) containing 10 mM CaCl₂ and maintained at 46°C. The top layer was immediately poured into petri dishes (90 mm) containing a bottom layer of MRS agar (1.5% wt/vol agar). All plates were incubated at 37°C for 16 to 18 h before they were examined for plaques.

Thermal Treatments

To evaluate the heat resistance of phage, 3 temperatures (63, 72, and 90°C) were used together with 3 suspension media: (1) MRS broth; (2) reconstituted skim milk (RSM; 10%, wt/vol); and (3) Tris magnesium gelatin buffer (TMG; 10 mM Tris-HCl, 10 mM MgSO₄, and 0.1% (wt/vol) gelatin, pH 7.4; Briggiler Marcó et al., 2009). The temperatures selected were based on routine pasteurization treatments used in the dairy industry. The suspension media used in the present investigation are commonly used in laboratories or dairy plants for starter culture preparation.

Phage P1 (approximately 10⁸ pfu/mL) was mixed with each suspension medium, and 1.0-mL mixtures were distributed into a series of capped tubes and incubated at 1 of the 3 temperatures described above. At predetermined time intervals, the tubes were removed and cooled quickly in ice water. The surviving phages were counted using a plaque assay, and results were expressed as the concentration of active viral particles and plotted against time. Time (min) to achieve 99% inactivation (T_{99}) was calculated graphically from survival curves, as described by Capra et al. (2004). Similar phage suspensions without heat treatment were used as controls.

Biocide Treatments

The biocides used included ethanol (10, 20, 30, 50, 75, and 100%, vol/vol; Tianjin Fengchuan Chemical Reagent Technologies Co. Ltd., Tianjin, China); isopropanol (10, 30, 50, and 100%, vol/vol; Tianjin Fengchuan Chemical Reagent Technologies Co. Ltd.); commercial sodium hypochlorite (100, 200, 400, 800 mg/L; Shandong Lircon Medical Technology Inc., Shandong, China); and peracetic acid (0.15, 0.25, and 0.45%, vol/vol; Tianjin Fengchuan Chemical Reagent Technologies Co. Ltd.). Sodium hypochlorite was diluted in phosphate buffer (pH 7). The alcohols and peracetic acid were diluted in distilled water. The resulting pH of the peracetic acid solution was 2.7. All assays were carried out at 25°C.

For assessment of biocide efficacy, phage P1 (~10⁷ pfu/mL) was mixed with a biocide solution and incubated at 25°C. Similar phage suspensions without biocide addition but with pH adjustment were used as controls. At predetermined time intervals, the tubes were removed and the surviving phages counted as previously described. Results are expressed as the concentration of active viral particles and plotted against time. The T_{99} was calculated from the survival curves.

Statistical Analysis

All data were analyzed using the Originpro software (version 8.6; Originlab, Northampton, MA). Experiments were replicated 3 times. Means were compared using a one-way ANOVA followed by SPSS Statistics 20 (IBM Corp., Armonk, NY); significance was declared at $P < 0.05$.

RESULTS

Thermal Treatment

Survival of phage heated at 63 and 72°C in MRS broth, RSM, and TMG is shown in Figure 1. The greatest resistance was observed at 63°C, regardless of suspension medium used. The T_{99} values for phage at 72 and 90°C, regardless of suspension medium, were <5 min. At 63°C, T_{99} values ranged from 7.55 to 9.89 min, depending on the suspension broth (Table 1). Overall, thermal resistance of phage appeared greatest in RSM.

Survival of phage P1 is shown in Figure 1; RSM and MRS provided the maximum and minimum protection to phage P1, respectively. Regardless of medium used, phage suspensions heated at 72 and 90°C were completely inactivated within 10 and 5 min, respectively.

Figure 1. Thermal inactivation kinetics of phage P1 ($\log_{10}$ plaque-forming units/mL) at 25°C (control), 63°C, and 72°C in de Man, Rogosa, Sharpe medium (MRS); reconstituted dry skim milk (RSM); and Tris magnesium gelatin buffer (TMG). Values are the mean of 3 determinations. Error bars are not shown and are ± 0.11 .

Table 1. Thermal resistance of *Lactobacillus* virulent phage P1 in different suspension media

Medium ¹	T_{99} ² (min)		
	63°C	72°C	90°C
MRS broth	7.55 ± 0.07^c	<5	<5
RSM	9.89 ± 0.01^a	<5	<5
TMG buffer	9.42 ± 0.06^b	<5	<5

^{a-c}Within a column, means with different letters are significantly different ($P < 0.05$).

¹MRS = de Man, Rogosa, Sharpe medium; RSM = reconstituted dry skim milk; TMG = Tris magnesium gelatin.

²Time required for 99% inactivation of phage particles. Values are the mean $\pm$ SD of 3 determinations.

Chemical Treatments

The T_{99} values of phage when treated with different biocides are shown in Table 2. Variations in the time to achieve T_{99} depended on the nature of the biocide and its concentration. Overall, increasing the biocide concentration resulted in a progressive increase in time required to achieve a 2-log phage reduction.

Although 100% ethanol resulted in the shortest time required to achieve T_{99} inactivation of phage (Table 2), this concentration was unable to achieve a complete kill within 60 min and resulted in only a ~ 4.7 -log reduction (Figure 2). Phage reductions with 10, 20, and 30% ethanol were <1.70 log with a $T_{99} > 60$ min. As expected 75% ethanol ($T_{99} = 8.15$ min) was more effective than 50% ethanol ($T_{99} = 16.86$ min).

Isopropanol (100%) resulted in the highest phage inactivation (Figure 3); however, similar to 100% ethanol, it did not result in complete elimination of phage within 60 min (~ 5.2 -log reduction). With 10% isopropanol, phage populations were observed to remain relatively unchanged ($T_{99} > 60$ min). As expected, 50% isopropanol ($T_{99} = 24.21$ min) was more effective than 30% isopropanol ($T_{99} = 30.83$ min).

Sodium hypochlorite at 100 mg/L exhibited little effect on phage P1 ($T_{99} > 60$ min; Figure 4). At 200 mg/L, the T_{99} remained > 60 min and resulted in only a 1.37-log reduction after 60 min. Increasing the concentration to 400 mg/L resulted in a T_{99} value of 46.44 min and a 2.15-log reduction. In contrast, phage treated with sodium hypochlorite at 800 mg/L exhibited a $T_{99} = 7.70$ min, with total inactivation within 60 min.

The use of peracetic acid at 0.15% had little effect on the phage population ($T_{99} > 60$ min, Figure 5). With 0.25% peracetic acid, a ~ 2.5 -log reduction was observed with a T_{99} value of 51.83 min. Increasing the concentration to 0.45% further increased lethality, resulting in a ~ 4.0 -log reduction after 60 min and a T_{99} of 21.74 min.

DISCUSSION

Phage infection, which can occur at any time during processing and after pasteurization, is the most prevalent reason for starter culture failure in the fermentation production of dairy and meat products. These failures and poor activity result in economic losses for the manufacturer and are a potential cause for foodborne illness. Research on methods to control the incidence of phage infection has therefore become important espe-

cially in the development of new fermentation products including functional foods. The vast majority of intervention methods rely on good manufacturing practices (GMP) and, in particular, the application of sanitizers or biocides and heat to eliminate phage from equipment and air. In this respect, various criteria are used to evaluate efficacy. One of the most commonly used is T_{99} , which is the time required to reduce 99% of the most sensitive phage in a population under specified conditions. This criterion has been reported to yield a very good approximation of phage resistance to inactivation treatments (Guglielmotti et al., 2012).

In dairy plants, raw milk is most often subjected to thermal treatment to eliminate many microorganisms, including spoilage pathogens, thus assuring safety and a longer shelf life (Guglielmotti et al., 2012). In this respect, the thermal resistance characteristics of *Lactobacillus* virulent phage P1, isolated from a starter culture failure vat, would be of importance when used as an intervention strategy.

In general, mesophilic LAB phages have been reported to exhibit a wide range of heat resistance. This may indicate that, at least for some, phage thermal treatment is phage-specific. For example, Ebrecht et al. (2010) investigated the efficiency of thermal treatments normally used in dairy plants, on 3 virulent (BYM, YAB, and Ib3) and 2 temperate (Cb1/204 and Cb1/342) *Lactobacillus delbrueckii* bacteriophages. The authors reported that the 2 temperate phages showed relatively low heat resistance, with a $T_{99} < 2$ min at 63°C in MRS broth, and a $T_{99} < 2.4$ min for all phages at 72°C. In contrast, Briggiler Marcó et al. (2009) reported that 4 phages (ATCC8014-B1, ATCC8014-B2, FAGK1, FAGK2) specific to *L. plantarum* expressed relatively high heat resistance at 63°C ($T_{99} > 45$ min) and much higher resistance at 72°C when reconstituted skim milk was used as heating medium. Pujato et al. (2014) reported that 9 *Leuconostoc mesenteroides* phages isolated from blue cheese manufacture generally showed high resistance at 63°C and moderate resistance at 72°C. However, using 80°C for 30 min or 90°C for 2 min resulted in complete inactivation of phages (Pujato et al., 2014). In comparison, in the present study, phage P1 exhibited a relatively low resistance to heat treatment because it was completely inactivated in 5 and 10 min at 90 and 72°C, respectively. As previously reported, most phages cannot survive heating at 90°C. Mercanti et al. (2012) reported that 2 temperate phages, Φ ILp84 and Φ ILp1308, isolated from mitomycin-C induction of *Lactobacillus paracasei* strains 84 and CNRZ1308, were susceptible to 90°C when heated for 2 min. Increasing the temperature from 63 to 90°C therefore improved phage inactivation. Heat treatment at 90°C for 5 min was sufficient for inactivation of lactobacilli phages

Table 2. Resistance of *Lactobacillus* virulent phage P1 to biocides

Biocide	Concentration	T_{99}^1 (min)
Ethanol (% vol/vol)	10	> 60
	20	> 60
	30	> 60
	50	16.86 ± 0.16^a
	75	8.15 ± 0.07^b
Isopropanol (% vol/vol)	100	6.48 ± 0.05^c
	10	> 60
	30	30.83 ± 0.32^a
	50	24.21 ± 0.24^b
Sodium hypochlorite (mg/L)	100	8.10 ± 0.12^c
	200	> 60
	400	46.44 ± 0.88
	800	7.70 ± 0.18
Peracetic acid (% vol/vol)	0.15	> 60
	0.25	51.83 ± 1.43
	0.45	21.74 ± 0.26

^{a-c}Means within a column and within the same biocide with different letters are significantly different ($P < 0.05$).

¹Time required for 99% inactivation of phage particles. Values are the mean $\pm$ SD of 3 determinations.

Figure 2. Inactivation kinetics of phage P1 ($\log_{10}$ plaque-forming units/mL) with ethanol [0 (control) to 100%]. Values are the mean of 3 determinations; some error bars are too small to be visible and are ± 0.07 .

(Briggiler Marcó et al., 2009; Guglielmotti et al., 2012) but not effective for some *Lactococcus* phages. Atamer et al. (2009) reported that *Lactococcus lactis* phages P1532 and P680 could not be completely inactivated when heated for 5 min at 97 and 95°C, respectively.

For *Lactobacillus* phage P1, we observed that the nature of the suspension medium provided varying protective effects. In the present study, RSM provided the maximum protective effect. It has been suggested that some milk properties, including buffering capacity and protein or salt content, may play a protective role during heating and therefore be responsible for the

higher thermal resistance observed for some phages (Quiberoni et al., 2003). Briggiler Marcó et al. (2009) also reported high thermal resistance for *L. plantarum* phages (ATCC8014-B2, FAGK1, FAGK2) in RSM and EM-glucose media, whereas Ebrecht et al. (2010) reported that RSM could provide protective effects to *L. delbrueckii* bacteriophages (Cb1/204, Cb1/342, BYM, YAB, Ib3). In contrast, Capra et al. (2004) evaluated the thermal resistance of 2 *Lactobacillus casei* and *Lactobacillus paracasei* bacteriophages PL-1 and J-1, using MRS broth, RSM, and TMG buffer but reported that no clear difference in resistance was demonstrated by

Figure 3. Inactivation kinetics of phage P1 ($\log_{10}$ plaque-forming units/mL) with isopropanol [0 (control) to 100%]. Values are the mean of 3 determinations. Some error bars are too small to be visible and are ± 0.12 .

any of the suspension media. Binetti and Reinheimer (2000) evaluated the thermal resistance of 5 autochthonal bacteriophages of *Streptococcus thermophilus* isolated from Cuartirolo cheese whey and yogurt, using enriched tryptic soy broth, reconstituted commercial nonfat skim milk, and Tris magnesium gelatin buffer. Those authors reported that none of the media resulted in significant differences in phage resistance. It is pos-

sible, therefore, that protective effects exhibited by different media are phage-specific.

In this study, the effects of several common biocides used in the dairy industry and in laboratory research were evaluated using phage P1. In our previous study (results not published), we reported that phage activity at 25 and 40°C did not appear to differ; therefore, a treatment temperature of 25°C was arbitrarily chosen.

Figure 4. Inactivation kinetics of phage P1 ($\log_{10}$ plaque-forming units/mL) with sodium hypochlorite [0 (control) to 800 mg/L]. Values are the mean of 3 determinations. Some error bars are too small to be visible and are ± 0.14 .

In addition, it is well recognized that higher temperature use with some biocides (e.g., peracetic acid and chlorine) increases their volatility, contributing to eye and skin irritation of plant personnel. Among the biocides tested, sodium hypochlorite was the most effective; 800 mg/L completely inactivated phage P1 in 60 min, with $T_{99} = 7.70$ min. However, this chlorine concentration is 2 to 3 times higher than that allowed in the food industry (Briggiler Marcó et al., 2009) and therefore could not be used for commercial purposes. However, it could be of use in future pilot- or

laboratory-scale studies. Briggiler Marcó et al. (2009) also reported that a 30-min exposure to 800 mg/L of residual free chlorine was necessary to totally inactivate *L. plantarum* phages B1, FAGK1, FAGK2, whereas 15 min was necessary for phage B2 (Briggiler Marcó et al., 2009). For total activation of *L. casei* and *L. paracasei* phages, 5 min at 800 mg/L was necessary (Capra et al., 2004). In contrast, some *L. delbrueckii*, *Lactobacillus helveticus*, *Lactococcus lactis*, and *S. thermophilus* phages could be completely eliminated using 100 mg/L of free residual chlorine (Quiberoni et al., 1999; Binetti

Figure 5. Inactivation kinetics of phage P1 ($\log_{10}$ plaque-forming units/mL) with peracetic acid [0 (control) to 0.45%]. Values are the mean of 3 determinations. Some error bars are too small to be visible and are ± 0.07 .

and Reinheimer, 2000; Suárez and Reinheimer, 2002; Ebrecht et al., 2010). Of note, *L. bulgaricus* phage Ib3 represents one of the most resistant phage to sodium hypochlorite, requiring at least 45 min of exposure and a minimum concentration of 1,200 mg/L of residual free chlorine for complete inactivation (Quiberoni et al., 2003). Maillard et al. (1998) suggested that sodium hypochlorite may cause phage inactivation via aggregation of tail proteins or that it could result in structural alterations to the capsid, possibly releasing nucleic acid into the surrounding medium.

Compared with other LAB phages, phage P1 appeared less sensitive to both ethanol and isopropanol. During a 60-min treatment, ethanol (100%) resulted in a ~ 4.7 -log reduction, whereas 100% isopropanol re-

sulted in a ~ 5.2 -log reduction. Complete inactivation was therefore not achieved. Treatment of phage using 10, 20, or 30% ethanol, and 10% isopropanol resulted in minimal inactivation. Similar results have been reported for *L. casei* and *L. paracasei* phages (Capra et al., 2004), *L. plantarum* phages (Briggiler Marcó et al., 2009), and *L. delbrueckii* (Ebrecht et al., 2010). However, for some LAB phages, ethanol or isopropanol was more effective. For example, the T_{99} value for *S. thermophilus* phages CYM, 0BJ, and 031-D in 75% ethanol was less than 5 min, and the phages were undetectable after a 15-min incubation with 75 or 100% ethanol (Binetti and Reinheimer, 2000). *Lactobacillus delbrueckii* phage LL-H could also be completely inactivated after a 30-min treatment with 75% ethanol (Quiberoni et

al., 2003). It has been suggested that the antibacterial property of alcohol is because it alters the lipid fraction of the plasma membrane and that naked phages are likely to be naturally more resistant than bacteria to an ethanol challenge (Maillard et al., 1996; Guglielmotti et al., 2012). Ethanol has been used in the sanitation of airflow units or on laboratory surfaces; however, to increase effectiveness, it is suggested that it be used together with other biocides (Briggiler Marcó et al., 2009).

Peracetic acid is viricidal and has been widely documented as an economical and practical agent for LAB phage inactivation, including those targeting *L. plantarum* (Briggiler Marcó et al., 2009), *L. delbrueckii* (Ebrecht et al., 2010), *L. casei*, *L. paracasei* (Mercanti et al., 2012), *S. thermophilus* (Binetti and Reinheimer, 2000), and *L. lactis* (Suárez and Reinheimer, 2002). Major advantages include its ability to quickly and efficiently inactivate most vegetative microorganisms, its resistance to catalase and peroxidase, and degradation into environmentally friendly products. Peracetic acid maintains its activity over wide temperature (0–40°C) and pH (3.0–7.5) ranges and in hard water, and it is not affected by protein residues. These properties allow for its safe use in the food industry including cleaning-in-place (CIP) practices (Guglielmotti et al., 2012). The low pH (~1.5–2) has been shown to inactivate phage (Mercanti et al., 2012). Peracetic acid is a powerful oxidizing agent that causes irreparable damage to various microbial macromolecules including proteins. It has the ability to penetrate cellular membranes, causing internal pH changes that eventually lead to death. In phage, complete rupture of nucleic acid is accomplished, resulting in inactivation. Whether this occurs inside the capsid or after the DNA is released due to capsid structural damage remains unclear (Guglielmotti et al., 2012). In the present investigation, peracetic acid at 0.15% for 60 min exhibited little inactivation toward phage P1, and increasing the concentration to 0.45% for 60 min resulted in only a ~4.0-log reduction. Thus, it appears that phage P1 was somewhat resistant to peracetic acid, which further highlights the importance of recognizing phage-specific biocides. Higher concentrations of peracetic acid could be used, but similar to chlorine, it is a strong irritant, affecting eyes, skin, and the respiratory system. Workers would therefore be adversely affected.

CONCLUSIONS

We evaluated the resistance of *Lactobacillus* virulent phage P1, isolated from a slow fermentation containing of *L. plantarum* IMAU10120, with respect to various thermal and biocide treatments routinely used in dairy

plants and laboratories. Phage P1 showed a relatively low resistance to heat and could be completely inactivated in 5 and 10 min at 90 and 72°C, respectively. Among the media examined, reconstituted skim milk provided the best heat protection. Resistance to biocides appeared high; sodium hypochlorite at 800 mg/L was required for total inactivation within 60 min. During the same period, treatment with 100% ethanol and isopropanol did not result in complete lethality. Phage P1 also expressed high resistance toward peracetic acid, surviving treatment at 0.45%. The results of this study should prove helpful in future studies on phage resistance, especially in the design of intervention strategies for the reduction of phage infections in laboratories and dairy plants.

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