J. Dairy Sci. 100:1–10
https://doi.org/10.3168/jds.2016-12451

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.

Received December 14, 2016.
Accepted May 12, 2017.
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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 m*M* $CaCl_2$. 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 m*M* $CaCl_2$ 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 m*M* Tris-HCl, 10 m*M* $MgSO_4$, 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 propagation.

Phage P1 (approximately 10^8 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 ($\boldsymbol{T_{99}}$) 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 ($\sim 10^7$ 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.