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Inactivation of *Lactobacillus* Bacteriophages by Dual Chemical Treatments

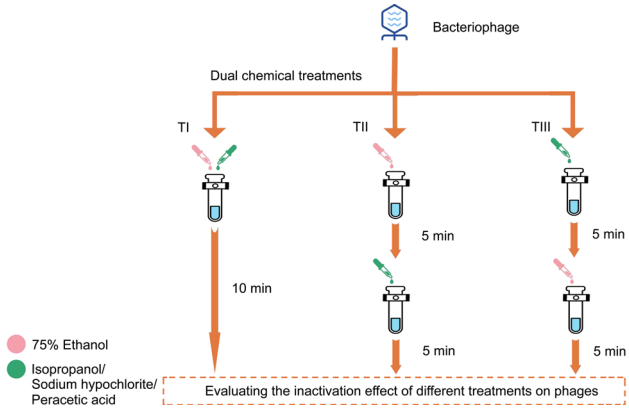
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Abstract

Phage contamination is one of the significant problems in the food fermentation industry, which eventually causes economic losses to the industry. Here, we investigated the viability of *Lactobacillus plantarum* phage P1 and P2 using various biocides treatments (ethanol, isopropanol, sodium hypochlorite and peracetic acid). Results indicated that phage P1 and P2 could be completely inactivated by treatment with 75% ethanol for 5 min, followed by 400 ppm of sodium hypochlorite treatment for 5 min. Phage P2 could be completely inactivated in the reverse sequence, while 800 ppm of sodium hypochlorite was required to achieve a similar effect for phage P1. Moreover, 100% isopropanol could increase the inactivating effect of 75% ethanol. This study may provide basic information on using multiple antimicrobials for phage control in laboratories and food plants.



K e y w o r d s: *Lactobacillus plantarum* phage, combined chemical treatments, successive chemical treatments, alcohol disinfectant, peracetic acid, sodium hypochlorite

Introduction

Lactobacillus plantarum is a recognized probiotic conferring various health effects to the host, including cholesterol lowering, management of gastrointestinal disorders and diarrhea, and prevention of irritable bowel syndrome (Seddik et al. 2017). *L. plantarum* is also widely used as a starter culture in fermented food products, such as cheese, kefir, sauerkraut, grape juice, and other beverages (Wang et al. 2020; Mirmohammadi et al. 2021).

Like other lactic acid bacteria (LAB), *L. plantarum* strains are easily infected by bacteriophages, which can result in fermentation failure. Due to their ubiquitous presence and the constant maintenance in bacterial populations, bacteriophages can pose substantial financial

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losses in the food fermentation industry by altering the quality of finished products or by delaying the manufacturing processes (Połaska and Sokołowska 2019). Diverse strategies to control phages in food plants have been reported, including physical treatments (heating, filtration, high pressure, UV radiation, electro pulsing), chemical treatments (biocides, biological compounds), strain rotation or use of strains with improved phage resistance (Pujato et al. 2018). However, many reports have indicated that the inactivating effect of a single chemical biocide was limited (Guglielmotti et al. 2012b; ; Murphy et al. 2014; Briggiler Marcó et al. 2019).

Methods to improve the effect of biocides on viruses by using complex treatments, such as chemical inactivation with UV radiation, synergistic chemical inactivation, and synergistic inactivation with UV and ozone, have already been reported. Pujato et al. (2014) found that 0.15% peracetic acid and 600–800 ppm of sodium hypochlorite had a good inactivation effect on *Lactobacillus mesenterium* bacteriophage. Kim et al. (2015) reported that the single treatment of 1% trisodium phosphate or 30% ethanol for 30 min was not effective on murine norovirus 1 (MNV-1), but cotreatment showed effective inactivation of MNV-1 on stainless steel after 15 min. Therefore, the combined treatment is an alternative.

L. plantarum phage P1 and phage P2 are phages isolated from the abnormal fermentation liquid of *L. plantarum* IMAU10120. They belong to *Siphoviridae* family. Phage P1 has an isometric capsid (71.7 ± 3.0 nm) and a long noncontractile tail (272 ± 3.0 nm long, 11.3 ± 1.5 nm wide) (Chen et al. 2016). Its whole genome was composed of linear dsDNA, with 73,787 bp in length and 86 predicted CDs. The GC content was 39.17% (Guo et al. 2022). When compared to phage P1, phage P2 was smaller, with an isometric capsid about 66.7 ± 3.0 nm and a long noncontractile tail (216.7 ± 3.0 nm long, 12.3 ± 3.0 nm wide) (Chen et al. 2019). Genome analysis showed that the whole genome of phage P2 was composed of linear dsDNA, with a length of 77,937 bp, 39.28% GC content, and 96 CDs (Zhu et al. 2022). Our previous studies demonstrated that these two phages possessed relatively high resistance to temperature and a single biocide treatment. Even for the most efficient biocide (sodium hypochlorite), a high concentration (800 ppm) and a long time (30 min or 60 min) were required to inactivate the phages (Chen et al. 2017; Chen et al. 2018). In 2007, Scheffler et al. (2007) found that PAA-ethanol (PES) can efficiently inactivate pseudorabies virus (PRV) or porcine parvovirus (PPV). In 2020, Hassaballah et al. (2020) reported that the total disinfection time for the bacteriophages added to the treated wastewater could be reduced from 1 h to 12.5 min through the combined treatment of peracetic acid (PAA) and ultraviolet light. So, the objective of this study was to investigate the inactivating effects of dual chemical treatments for the control of *Lactobacillus* phages P1 and P2 in plants and laboratories.

Experimental

Materials and Methods

Bacterial strain, phages, and culture conditions. *L. plantarum* IMAU10120, phages P1 and P2 were obtained from the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, P.R. China.

L. plantarum IMAU10120 was cultured at 37°C using de Man, Rogosa, and Sharpe (MRS) broth for 24–48 h. The media were supplemented with 10 mmol/l $CaCl_2$ for phage propagation and enumeration using a double-layer plaque titration method (Capra et al. 2018).

Treatment using combined biocides (TI). Ethanol (75%, 450 µl) was added to isopropanol (450 µl) at various concentrations of 10%, 30%, 50%, and 100% in a series of microfuge tubes to obtain a final alcohol concentration as 38.3%, 47.3%, 56.3%, and 78.8%, respectively. The final concentration of the alcohol solution was calculated according to the equation described by Kalua and Boss (2008). Phage (10^8 PFU/ml, 100 µl) was mixed with each suspension (1 ml of final volume) and incubated at 25°C for 10 min. After dilution with saline buffer (0.85%), survivors were counted using a double-layer plaque titration method. Similar protocols were used with sodium hypochlorite (ppm): 100, 200, 400, and 800 or PAA (%, v/v): 0.15, 0.25, 0.45 with a ratio of 1:1.

Successive biocide treatments (TII, TIII). TII: Phages (10^8 PFU/ml, 100 µl) were mixed with ethanol (75%, 450 µl) in microfuge tubes and incubated at 25°C for 5 min. Then, 450 µl isopropanol at different concentrations (v/v, 10%, 30%, 50%, 100%) was subsequently added. After incubation at 25°C for 5 min and dilution with saline buffer (0.85%), surviving phage particles were enumerated using the abovementioned methods. Similar protocols were used with sodium hypochlorite (ppm): 100, 200, 400, 800 or PAA (%, v/v): 0.15, 0.25, 0.45.

TIII: The treatment time was the same as TII, but the inactivation sequence of 75% ethanol and isopropanol, sodium hypochlorite, or peracetic acid solutions at different concentrations, as mentioned above, was reversed, respectively.

Statistical analysis. All experiments were replicated three times. Treatments using a single biocide with the same concentration were used as control. All data were analyzed using the Originpro software (version 8.6; Originlab, USA). Means were compared using a one-

way ANOVA followed by IBM SPSS Statistics 20.0 software (IBM Corp, USA). Significance was determined at $p < 0.05$. Duncan's multiple range test was used to separate the means.

Results and Discussion

Antimicrobial effect of ethanol and isopropanol. Chemical biocides, including alcohols, sodium hypochlorite, quaternary ammonium chloride compound, and peracetic acid, sanitize and control microbial growth on the equipment and food contact surfaces.

As one of the most used biocides, ethanol has been reported to destroy or denature enzymes and microbial proteins in cell walls. The ethanol concentration, similarly to most biocides, determines whether its action is biocidal or static. Low (< 20%) concentrations will limit its effect and are usually biostatic (Kim et al. 2020). Moderate concentrations (60–85%) can quickly solidify microbial proteins resulting in a biocidal effect (Sauerbrei 2020). However, when the concentration is too high (> 90%), it can form films on the microbial surface, thereby influencing its effect (Setlow et al. 2002; Zhang et al. 2012).

Concerning phages, biocides affect the protein structure in the capsid (Sato et al. 2016). It was reported that ethanol and isopropanol could denature proteins, which is destructive to most viruses (Maillard 2002; Mahony and Sinderen 2015; Boyce 2018). However, the previous studies found that neither ethanol nor isopropanol could completely inactivate phage P1 and P2, even after a 60 min treatment. Nevertheless, the inactivation effect of isopropanol was slightly better than that of ethanol (Chen et al. 2017; 2018). To explore whether isopropanol and ethanol could mutually improve the inactivating effect on phages, the survival counts of *L. plantarum* phages P1 and P2 after the combined and successive chemical treatments with ethanol and isopropanol are shown in Fig. 1.

In dairy industries, sanitization between fermentations is a critical step in controlling the phage contamination (Hayes et al. 2017). It employs purpose-made chemical sanitizers for the physical and chemical removal of phages and other microbial contaminations (Pujato et al. 2018). For biocides to be considered eligible to use in the dairy industry, several criteria must be met. For example, in Europe, sanitizers must have a proven ability to reduce phage numbers by at least four logs under the recommended test conditions before they can be deemed suitable for phage inactivation (European Committee for Standardization (CEN), CEN/TE-216) (Bolten et al. 2022).

As illustrated in Fig. 1, combined or subsequent treatments with ethanol and isopropanol could not completely inactivate phages P1 and P2. However, isopropanol could enhance the biocidal effect of 75% ethanol to a certain extent. Ethanol (75%) or isopropanol (100%), when used alone for 10 min, reduced P1 by

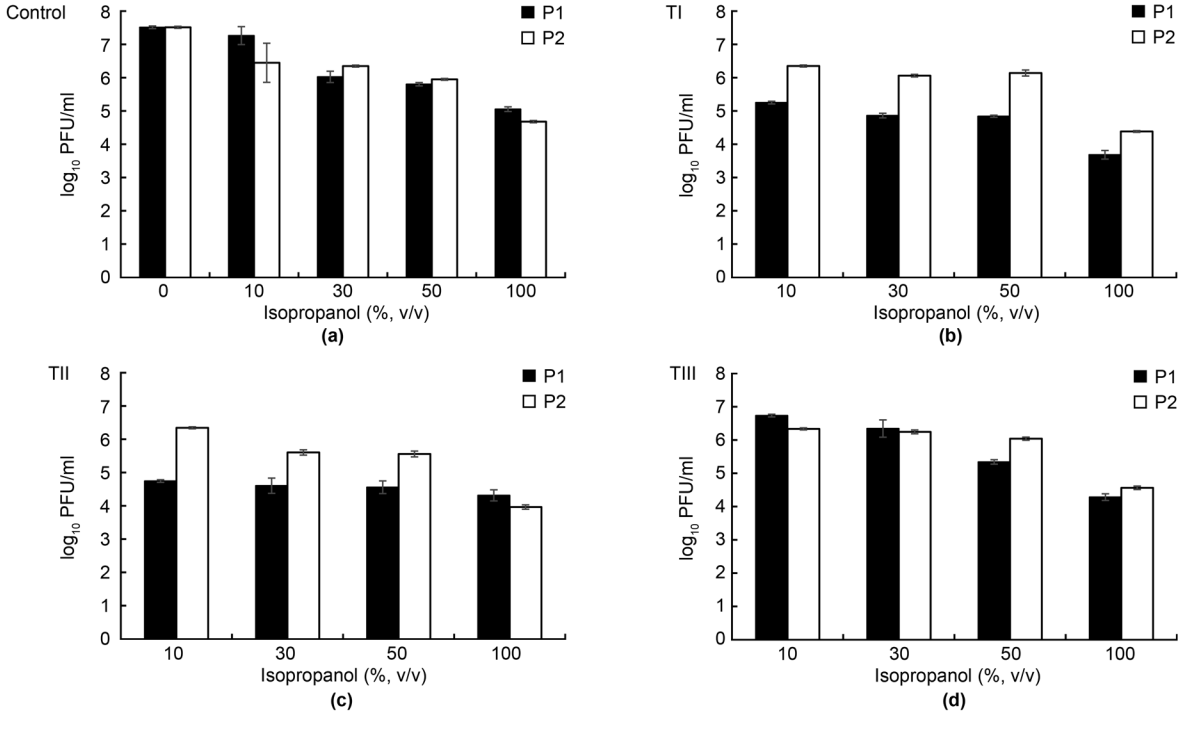


Fig. 1. Viable count of phages treated with 75% ethanol and isopropanol.

a) Treatment with isopropanol for 10 min; b) treatment with 75% ethanol and isopropanol for 10 min; c) treatment with 75% ethanol for 5 min followed by isopropanol for 5 min; d) treatment with isopropanol for 5 min followed by 75% ethanol for 5 min.

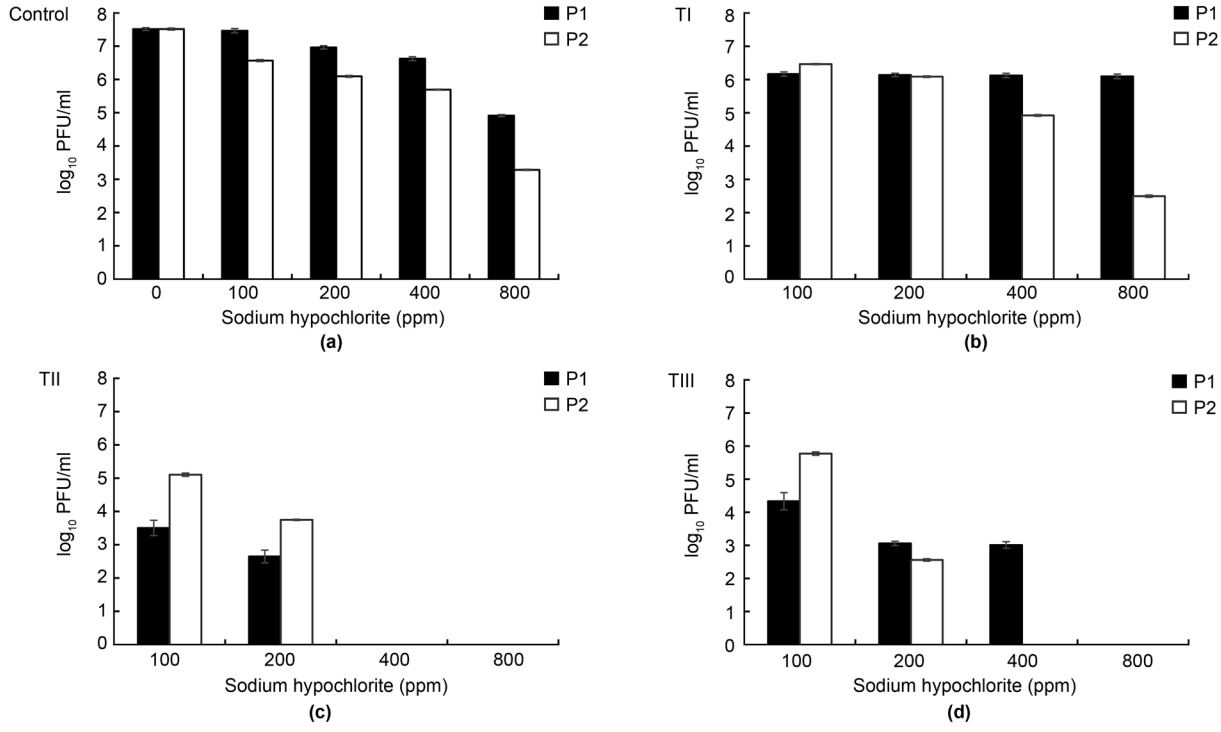


Fig. 2. Viable count of phages treated by 75% ethanol and sodium hypochlorite.
a) Treatment with sodium hypohlorite for 10 min; b) treatment with 75% ethanol and sodium hypohlorite for 10 min; c) treatment with 75% ethanol for 5 min followed by sodium hypohlorite for 5 min; d) treatment with sodium hypohlorite for 5 min followed by 75% ethanol for 5 min.

2.45 and 2.46 log (Fig. 1a), respectively. However, when phages were treated with a mixture of 100% isopropanol and 75% ethanol for 10 min, a 3.83 log reduction was obtained (Fig. 1b). Similar results were observed for phage P2, as the treatment with ethanol (75%) for 5 min, followed by the treatment with isopropanol (100%) for 5 min, was even more effective, resulting in a 3.55 log reduction (Fig. 1c).

Both ethanol and isopropanol have been reported to destroy most lipophilic viruses (Maillard 2002; Mahony and Sinderen 2015). Although isopropanol (100%) can enhance the effect of ethanol (75%) on phages P1 and P2, the augmenting effect is limited. As reported, isopropanol can interact with microorganisms in aqueous solutions to form $(CH_3)_2CHOH(H_2O)_n$, or isopropanol water clusters, where the n value represents the amount of H_2O molecules. The structures of water clusters formed by isopropanol in various electrostatic environments are different, providing differences in the structural stability of water clusters, thus having diverse effects on microorganisms (Han et al. 2017; Zhu et al. 2019). Different phages will produce different electrostatic environments in the solution. The charge density is a significant characteristic of each phage, which mainly depends on the nature of the phage capsid or tail protein and the difference in genetic material (Hernando Pérez et al. 2015; Cooper et al. 2022). In our previous research, the morphology of phages P1 and P2 were similar, but their structures were slightly different. The tail fibrin (CDs18) of phage P1 was different from that encoded in phage P2's genome (Guo et al. 2022). The two electrostatic environments caused by different tail fibrin may enable isopropanol to form isopropanol-water clusters with different stability in aqueous solution, thus affecting the biological efficacy of isopropanol on phages P1 and P2.

Antimicrobial effect of ethanol and sodium hypochlorite. The combined and successive effects of ethanol and sodium hypochlorite on phage P1 and phage P2 are shown in Fig. 2.

The phage treatment with 400 ppm of sodium hypochlorite for 10 min resulted in a 0.89 log reduction for phage P1 (Fig. 2a). As expected, the number of phage P1 survivors decreased significantly ($p < 0.05$) with increased sodium hypochlorite concentrations. Phage P1 was completely inactivated by successive treatment of 75% ethanol and 400 ppm sodium hypochlorite. The successive treatment of 800 ppm of sodium hypochlorite and 75% ethanol could completely inactivate phage P1, regardless of the chemical sequence. Similarly, for phage P2, the number of survivors significantly decreased when 75% ethanol was used with sodium hypochlorite. A 1.82 log reduction was achieved when phage P2 was treated with 400 ppm of sodium hypochlorite alone for 10 min (Fig. 2a). However, it was inactivated by a successive treatment of 75% ethanol and 400 ppm of sodium hypochlorite, regardless of the chemical sequence (Fig. 2c and 2d).

Sodium hypochlorite is a highly effective sanitizer. However, the efficacy of sodium hypochlorite is affected by several factors, such as pH, temperature, and organic matter. Lowering the pH, increasing the temperature, and reducing the organic load increase its antimicrobial effect (Cai et al. 2016). Hypochlorite acts directly on DNA. In this respect, it has been shown that *in vitro*, sodium hypochlorite can interact with DNA, forming nucleotide chloramines, leading to the destruction of the nitrogenous base ring structures (Osinnikova et al. 2019). As mentioned, our previous studies showed that sodium hypochlorite could be considered an efficient biocide that could inactivate phages P1 and P2 under high concentration and long time (phage P1: 800 ppm for 60 min; phage P2: 400 ppm for 50 min or 800 ppm for 30 min) (Chen et al. 2017; 2018). From the results showed in Fig. 2, we could observe that the successive treatments of 75% ethanol and 400 ppm or 800 ppm of sodium hypochlorite provided apparent synergistic inactivating effects on phage P1 and phage P2. It might occur due to the protein structure of the phage capsid, which was affected by ethanol, further exposing the nucleic acid within the virus head to sodium hypochlorite.

Sodium hypochlorite hydrolyzed in water form hypochlorous acid, which is lethal for most microbes. It has been demonstrated to interfere with carbohydrate metabolism, oxidize protein, and target phosphate dehydrogenase (Cho et al. 2010; Sato et al. 2016). Sodium hypochlorite also inactivates *Pseudomonas aeruginosa* phage F116 by causing structural changes in the head, tail, and overall structure. In this respect, nucleic acids were released from the damaged capsid and were detected in the surrounding medium (Maillard et al. 1998). Generally, the food industry application standard for sodium hypochlorite is 200 ppm (Briggiler Marcó et al. 2009).

As reported, 100–200 ppm sodium hypochlorite had a good inactivation effect on some phages, such as *Lactococcus lactis* phages QF12 and P001 and *Lactobacillus delbrueckii* phage Cb1/204 (Suárez and Reinheimer 2002; Ebrecht et al. 2010; Guglielmotti et al. 2012a). However, due to the specificity of phages, different phages express diverse resistance to sodium hypochlorite, and some phages possess higher tolerance to sodium hypochlorite. Quiberoni et al. (2003) reported that *L. delbrueckii* bacteriophage Ib3 isolated from yogurt could be totally inactivated when exposed to 1,200 ppm of sodium hypochlorite for 45 min. In 2010, Capra et al. (2004) showed that *Lactobacillus paracasei* bacteriophage PL-1 and *Lactobacillus casei* bacteriophage J-1 could be completely inactivated in 800 ppm of sodium hypochlorite within 5 min. Reducing the concentration to 700 ppm was insufficient to achieve a complete inactivation effect within 45 min. Murphy et al. (2014) found that nine of 11 936-type phages isolated from cheese had an average survival rate of over 96% after exposure to 800 ppm of sodium hypochlorite for 30 min. Some bacteriophages even can tolerate much higher concentrations. It has been proved that bacteriophage φpll36 isolated from Turkish dairy factory was inactivated after the exposure to 2,000–3,000 ppm of sodium hypochlorite for 20–30 min (Dilek Avsaroglu et al. 2007).

Genomic differences between phages P1 and P2 may account for the variation in resistance. As reported, sodium hypochlorite can inactivate phages by acting on their protein structures or genome. It can cause the inactivation of coliphage MS2 through genome damage. In contrast, the inactivation *Pseudomonas* Phi6 is driven by its reaction with proteins in the nucleocapsid and polymerase complex rather than with genome and lipids (Ye et al. 2018). The sensitivity of viruses to free chlorine depends on the similarity of the amino acid sequences in capsid proteins. Typical amino acid substitutions between genogroup A and genogroup B genomes of CVB4 affect the chlorine reactivity of attachment sites, resulting in different chlorine tolerance of genogroup A and genogroup B viruses of CVB4 (Torii et al. 2022).

Our previous studies have shown 11 gene differences between these two phages, including eight putative proteins, one tail fibrin, and two HNH endonucleases. Moreover, phage P2 encoded more putative proteins with unknown functional gene sequences (Guo et al. 2022; Zhu et al. 2022). The difference in protein structure and the gene sequence coding proteins may be why phages P1 and P2 have different tolerance to sodium hypochlorite. Similar to our study, Briggiler Marcó et al. (2009) reported that sodium hypochlorite (800 ppm) could completely inactivate phage B2 of *L. plantarum* in 15 min but could not inactivate phage B1, FAGK1, and FAGK2 from the same isolation source and host bacteria under the same conditions. Phages B1 and B2 shared the same host strain *L. plantarum* ATCC 8014, belonging to the *Siphoviridae* family. Genome analysis presented that the whole genome of phages B1 and B2 were composed of linear dsDNA, of a length of 38,002 bp and 80,618 bp, and GC content of 47.6% and 37.0%, containing 60 ORFs and 127 ORFs, respectively.

Interestingly, they also had tail fibrin and different HNH endonucleases. So, genomic differences may influence the tolerance of phages to chemical biocides treatments. Phages isolated from the identical source and the same host might exhibit different tolerances to sodium hypochlorite. Inactivation effects could mainly depend on the nature of the phages (Briggiler Marcó et al. 2009).

Antimicrobial effects of ethanol and peracetic acid. Compared with ethanol and chlorine, peracetic acid (PAA) is considered a relatively new sanitizer. PPA is normally used at a concentration of 0.15% (v/v) in

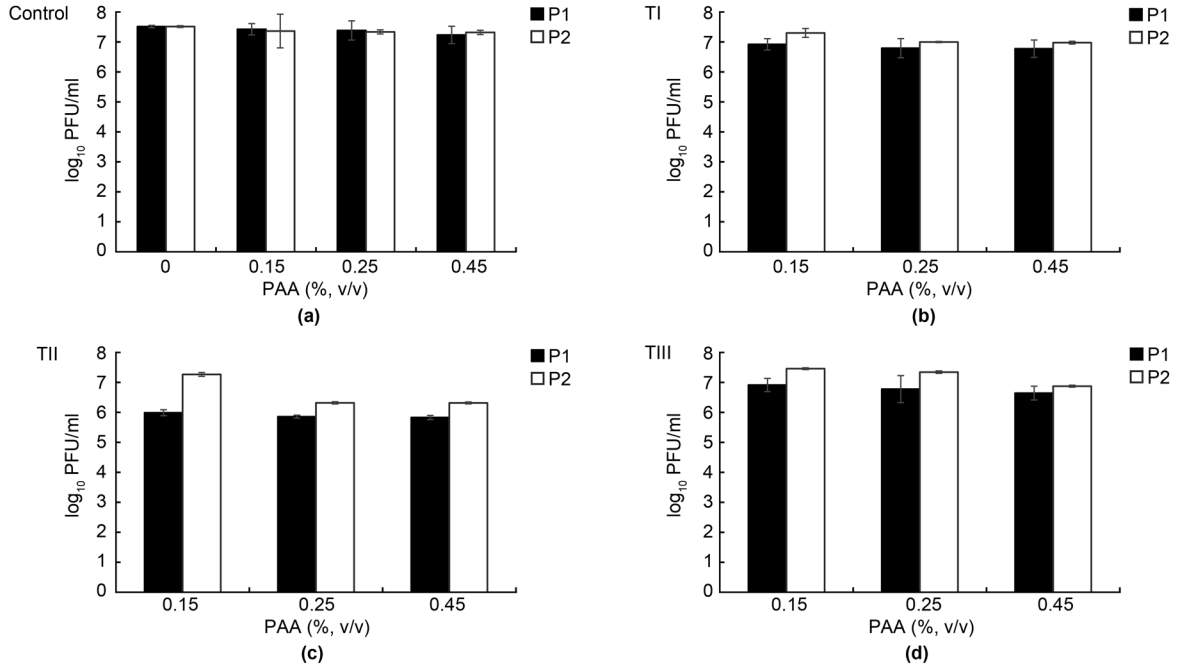


Fig. 3. Viable count of phages treated by 75% ethanol and peracetic acid (PAA).
a) Treatment with PAA for 10 min; b) treatment with 75% ethanol and PAA for 10 min; c) treatment with 75% ethanol for 5 min followed by PAA for 5 min; d) treatment with PAA for 5 min followed by 75% ethanol for 5 min.

water; the low pH of this aqueous solution (around 2) has been suggested to be responsible for phage inactivation (Mercanti et al. 2012). Nevertheless, at the same time, PAA, due to its oxidation characteristics, has a strong corrosive effect, a pungent smell, and is even dangerously explosive (Horn and Niemeyer 2022). Therefore, the use of the concentrated PAA solution has limitations.

As shown in Fig. 3, PAA was not an effective biocide against phages P1 and P2, even at a concentration of 0.45%. Phages P1 and P2 treated with the solution at a concentration of 52.6% and 63.8%, respectively, remained viable after a 10 min-treatment. Moreover, PAA did not increase the biocidal effect of ethanol (75%). As shown in Fig. 3, even the highest concentration of PAA resulted in the lowest survival rate (TII) of 2.21%. Phage P2 appeared to exhibit a higher tolerance to PAA than P1, which still exhibited a survival rate of 6.33% under the same treatment. In contrast, ethanol (75%) increased the phagocidal effect of PAA.

Peracetic acid exerts a strong oxidizing effect on proteins. It exerts a rapid bactericidal effect on various microorganisms, including spores and viruses (Yeap et al. 2015; Zonta et al. 2016). The treatment with peracetic acid (0.15%) for 60 min had little effect on the survival of P1 while increasing the concentration to 0.45% for 60 min resulted in only a 4.0 log reduction in the number of phages (Chen et al. 2017). Phage P2 demonstrated greater resistance to peracetic acid than P1. At the highest concentration evaluated (0.45%), the viability of phage P2 decreased by only 1.40 log within 60 min (Chen et al. 2018). Ethanol may accelerate the destruction of viruses by PAA, enhancing the mobility of PAA molecules in the disinfectant solutions. The combination of PAA (0.2%) and ethanol (80%) could result in a 4.0 log reduction of the poliovirus type 1 number in 1 min. A comparable virucidal effect could not be achieved with 80% ethanol, even if the exposure time was prolonged to 30 min (Wutzler and Sauerbrei 2000). However, like in the previous research results, PAA might not have a good bactericidal effect on phages P1 and P2, even when used with ethanol. In this respect, only phages with sulfur-containing amino acids (such as cysteine and methionine) in their capsid proteins show strong sensitivity to PAA (Schmitz 2021). The number and types of sulfur-containing amino acids in the capsid proteins of phages P1 and P2 must be further determined. In addition, differences in the protein configuration in the capsid brought about by secondary, tertiary, and quaternary folding may expose the different and sensitive abilities to the action of PAA (Mayer et al. 2015). The present study found that when PAA was mixed with ethanol (75%), the biocidal effect was not improved even at the highest concentration (0.45%).

In contrast, ethanol (75%) improved the biocidal effect of PAA, especially for P2. During the first 5 min of application, ethanol (75%) impacted the protein capsid allowing PAA more accessible contact with sulfur-containing groups. However, the present results also demonstrated that with the increase in PAA concentration, the biocidal effect was not increased significantly. Phages P1 and P2 may contain less PAA-sensitive target sites.

Conclusions

The dual inactivating effects of various biocides on phages P1 and P2 were evaluated. Results showed that the phages could be completely inactivated in 10 min after being treated successively with 75% ethanol and 400 ppm of sodium hypochlorite. Compared to a single biocide treatment, successive treatments could reduce the biocide's concentration and shorten the inactivation time. This study might provide some basis for controlling phage infections in laboratories and dairy plants.

Acknowledgments

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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