Bacteriophage control of *Salmonella* Typhimurium in milk

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Abstract This study was designed to evaluate the effect of bacteriophage P22 on the inhibition of growth of *Salmonella* Typhimurium. The P22 belongs to Podoviridae family consisting of a hexagonal head and short tail. The inhibitory effect of phage in milk was noticeable at the early storage period, showing more than 3 log reduction at 4 h and day 3. The pH values of milk treated with P22 were significantly decreased from 6.7 to 6.3 after 24 h incubation at 37 °C, while no significant changes in pH values were observed for the control and bacteriophage treatment throughout the storage at 4 °C for 12 days. The slight color changes were observed in the control and bacteriophage treatment throughout the storage at 4 °C for 12 days and 37 °C for 24 h. These results provide useful information for enhancing microbiological safety and quality of milk and designing effective bacteriophage-based control in food system.

Keywords Bacteriophage · Phage control · *Salmonella* · P22 · Milk

Introduction

Milk quality and safety have received a high priority in the dairy industry (Bao et al., 2015). Specifically, microbial contamination has become a great concern over raw milk and milk-based products such as cheese and yogurt (Faille et al., 2017). *Salmonella* belonging to the Enterobacteriaceae family is one of the most common foodborne pathogens found in raw milk (Mungai et al., 2015). There are many different serotypes of *Salmonella*, varying in somatic (O), flagellar (H), and capsular (Vi) antigens. The presence of *Salmonella* in dairy products is mainly due to the post-pasteurization contamination of milk (Modi et al., 2001; Olsen et al., 2004). The consumption of dairy products contaminated with *Salmonella* can cause serious foodborne illness known as salmonellosis (Eng et al., 2015). Therefore, the control of post-processing contamination of milk is a key factor in preventing the dissemination of *Salmonella* serovars to milk-based products.

In recent years, bacteriophage control has a great attention as an alternative for antibiotics (Bao et al., 2015; Kim et al., 2016). Bacteriophages are highly specific to host bacteria and have less problems associated with antibiotic-resistant bacteria (Zinno et al., 2014). The Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) approved the use of bacteriophages on ready-to-eat (RTE) meat and poultry products and affirmed as generally recognized as safe (GRAS) (Lee et al., 2017; Strydom and Witthuhn, 2015). The bacteriophage-based control can be a promising approach to enhance the microbiological safety of food (Thung et al., 2017). However, relatively few studies have investigated the effect of bacteriophages on the growth of *S. Typhimurium* in milk. Therefore, the aim of this study was to evaluate the possibility of using bacteriophage for

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improving the microbiological safety and quality during refrigerated storage.

Materials and methods

Bacterial strain and culture condition

Strain of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (KCCM 40253) were purchased from Korean Culture Center of Microorganism (KCCM, Seoul, Korea). The strain was cultured in trypticase soy broth (TSB) (BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37 °C for 20 h and then centrifuged at 3000×g for 20 min at 4 °C. The harvested cells were washed twice with phosphate-buffered saline (PBS, pH 7.2) and diluted to 10⁸ CFU/mL prior to use.

Bacteriophage propagation

Salmonella bacteriophage P22 (ATCC 97541) was propagated at 37 °C for 24 h in TSB containing recommended host strain (*S. Typhimurium* KCCM 40253). In order to eliminate host lysates, the propagated bacteriophages were centrifuged at 5000×g for 10 min and filtered through a 0.2-μm filter. The collected bacteriophages were serially (1:10) diluted and mixed with the host cells (10⁷ CFU/mL) in TSB (0.5% agar). The mixture was poured over the pre-warmed agar lawn and incubated at 37 °C for 24 h to determine the bacteriophage titers (plaque-forming unit, PFU).

Transmission electron microscopy (TEM)

The morphological property of bacteriophage P22 were evaluated by transmission electron microscope (TEM, LEO 912AB Omega; Carl Zeiss NTS GmbH, Oberkochen, Germany), located at the Korea Basic Science Institute (KBSI; Gangwon, Korea). The bacteriophage P22 was transferred to the carbon coated copper film, negatively stained with 5% aqueous uranyl acetate (pH 4.0), and then air-dried on a carbon-coated copper grid. The stained bacteriophage P22 was observed under TEM (120 kV).

Determination of multiplicity of infection (MOI)

The ability of bacteriophage P22 to lyse *S. Typhimurium* KCCM 40253 was determined at different multiplicity of infections (MOIs; 0.01, 0.1, 1, and 10). The bacteriophage P22-infected host cells were cultured at 37 °C with shaking for 6 h. After 6-h incubation, the cultured host cells were serially (1:10) diluted with PBS, spread-plated on TSA, and incubated at 37 °C for 24 h to enumerate the viable host

cells. The reduction rate ($\log N_t/N_c$) was estimated by comparing the number of the host cells in the control (N_c) with the number of the host cells treated with bacteriophage P22 (N_t) after 6 h incubation.

Storage study

The host cells were inoculated at approximately 10⁶ log CFU/mL in commercial milk (4% fat). The inoculated milk samples (10 mL each) were treated without or with bacteriophage P22 and stored at 4 °C and 37 °C. The host bacterial count and, pH and color of milk were measured at 0, 3, 6, 9, and 12 days and 0, 4, 8, 16, and 24 h, respectively, during the storage at 4 °C and 37 °C.

pH and color measurement

The pH value of each milk sample was measured by using a pH meter (Fisher Scientific, St. Louis, MO). The color changes were evaluated using a colorimeter (Chroma Meter CR-400; Minolta, Osaka, Japan). The colorimeter meter was standardized using black and white reference ceramic tiles. The L^* , a^* , and b^* coordinates indicate lightness, redness, and yellowness, respectively. The color differences (ΔE) in the treatment compared to the control were estimated as $\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$.

Statistical analysis

All analyses were performed in duplicate for three replicates. Data were analyzed using the Statistical Analysis System software (SAS Institute Inc., Cary, NC, USA). The general linear model (GLM) and least significant difference (LSD) procedures were used to compare means at the 5% of significance level.

Results and discussion

Morphological property and lytic activity of *Salmonella* bacteriophage P22

The morphological characteristic of bacteriophage P22 was examined using TEM (Fig. 1). Bacteriophage P22 had a hexagonal head (63.5 nm) and short tail sheath (10.8 nm), belonging to the Podoviridae family. The lytic activity of bacteriophage P22 against *S. Typhimurium* was observed at different MOIs ranging from 0.01 to 10 (Fig. 2). The reduction of *S. Typhimurium* increased as the MOI increased. The highest reduction was observed at the MOIs of 1 and 10, showing more than 6 log reduction. This is in good agreement with a previous report that the significant

Fig. 1 TEM image of *Salmonella* bacteriophage P22 (125,000 $\times$ magnification)

Fig. 2 Log reduction of *Salmonella* Typhimurium with different multiplicities of infection (MOIs) of bacteriophage P22. N_c and N_t represent the numbers of *S. Typhimurium* treated without and with bacteriophage P22 after 24-h incubation at 37 °C, respectively. Letters below the bars indicates significant difference at $p < 0.05$

reduction in the number of viable *Salmonella* was achieved at the higher MOI (Bao et al., 2015). The effectiveness of bacteriophages control to reduce host bacteria highly depends on the MOI, leading to the enhanced binding ability between bacteriophage and host bacteria (Guenther et al., 2012; Wong et al., 2014). The level of bacteriophage is an important factor at the initial infection process in terms of attachment and adsorption, resulting in the increase in the host specificity (Rakhuba et al., 2010). As the bacteriophage challenge study was conducted in food system, the MOI of 10 was used for storage study. Since

the whey protein in milk can interfere with bacteriophages and is responsible for the decreased interaction between bacteriophage and specific receptors on the surface of bacteria, the higher MOI allows bacteriophages to easily attach to the host bacteria in food system (García et al., 2008).

Bacteriophage-based control to reduce *Salmonella* Typhimurium in milk

The effect of bacteriophage P22 on microbial growth, pH and color change was evaluated during storage of milk at 37 °C for 24 h [Fig. 3(A)] and 4 °C for 12 days [Fig. 3(B)]. The initial number of *S. Typhimurium* at the control rapidly increased to 8.9 log CFU/mL after 8 h, whereas phage treatment effectively inhibited the growth of *S. Typhimurium* at the early stage of incubation at 37 °C, showing 4.8 log CFU/mL at 4 h [Fig. 3(A)]. *S. Typhimurium* steadily grew up to the same level of the control (9.0 log CFU/mL) after 24 h of incubation. This result might be due to the emergence of bacteriophage-insensitive *S. Typhimurium* (Bohannon and Lenski, 2000; Guenther et al., 2012). Bacteriophage resistance mechanisms include alteration in bacteriophage receptors, incomplete infection of bacteriophages, toxin-antitoxin (TA), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems (Labrie et al., 2010). In addition, the MOI used in this study was relatively low when compared to the previous studies in food system (Zinno et al., 2014). The milk components could also interrupt the phage-bacteria adsorption. This allowed bacteria to grow and develop resistance against bacteriophage. The casein micelles are able to form the complex with phage particles at pH above 4.7, leading to the decrease in bacteriophage adsorption (Erskine, 1970). The immunoglobulin agglutination in milk can result in the cell surface coating responsible for bacteriophage insensitivity and adsorption reduction (O'Flaherty et al., 2005). The regrowth of *Salmonella* cells observed during the incubation could be due to the emergences of bacteriophage-resistant mutants or the reduction in bacteriophage adsorption in milk system. Therefore, further study regarding the emergence of bacteriophage-insensitive bacteria is needed to develop effective bacteriophage-based control system. The pH values of milk treated without bacteriophage P22 (control) were significantly lower than the control between 4 and 16 h of incubation at 37 °C. Compared to the bacteriophage treatment, the color change (ΔE) was noticeable for the control during the 24 h of incubation at 37 °C [Fig. 3(A)].

The bacteriophage treatment effectively inhibited the growth of *S. Typhimurium*, showing more than 3 log reduction after 12 days of storage at 4 °C [Fig. 3(B)]. This

Fig. 3 Survival (filled circle, open circle) of *Salmonella* Typhimurium, pH change (filled triangle, open triangle), and color difference (filled square, open square) in milk samples treated without (filled circle, filled triangle, filled square) and with (open circle, open

triangle, open square) bacteriophage P22 (10 MOI) during the incubation at 37 °C for 24 h (A) and 4 °C for 12 days (B). Means with different letters within each treatment are significantly different at $p < 0.05$

confirms that the bacteriophages were still effective to inhibit the microbial growth at low temperature (Giladi et al., 1995). In general, a higher MOI can result in highest level of bacterial reduction (Viazis et al., 2011). This suggests that a proper MOI can further enhance the lytic activity at even low temperature. The pH values were maintained at around 6.7 throughout the refrigerated storage [Fig. 3(B)]. *S. Typhimurium* was unable to multiply at low temperature below 10 °C (Smadi et al., 2012). The slight color changes were observed at the control and bacteriophage treatment throughout the incubation period, showing lower than 1 of ΔE [Fig. 3(B)]. Compared to the control, the color stability of milk was enhanced by bacteriophage treatment. This implies the color change in the control is influenced by the microbial growth (Owens et al., 2001). The color of milk is mainly attributed to the size distribution and protein composition, including colloidal fat globules, casein micelles, and calcium phosphate (Bermúdez-Aguirre et al., 2009).

In conclusion, this study highlights that the bacteriophage can be used as a bioagent to enhance the microbiological safety and quality. The bacteriophage P22 effectively inhibited the growth of *S. Typhimurium* and maintained color stability in milk during the refrigerated storage at 4 °C. The MOI was an important factor for improving the lytic activity of bacteriophages in food system. However, the emergence of bacteriophage-resistant bacteria remains a question for bacteriophage control in food system. Therefore, further studies are needed to understand the mechanisms of bacteriophage resistance, which can help to develop better and effective bacteriophage-based control in food system.

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