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# Isolation and characterization of bacteriophage infecting *Lactobacillus plantarum* KCCM 12116

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**Abstract** Bacteriophages (phages) are known determinants of kimchi microbial ecology. *Lactobacillus plantarum* is related to kimchi over-acidification during the late stages of kimchi fermentation. A phage infecting *Lac. plantarum* was isolated from kimchi and characterized. The phage population for kimchi in a market was 2.3 log particles/mL, which corresponded to 32% of the bacterial population on a log scale. The isolated phage was designated as ΦLP12116. ΦLP12116 which belonged to the *Siphoviridae* family and has a very narrow host range, infecting only *Lac. plantarum*. The phage was stable at a lactic acid concentration of 1.0% and pH 4.0 at 4°C, indicating that it could survive in kimchi. In the kimchi extract broth treated by the phage, the growth of *Lac. plantarum* KCCM 12116 was inhibited by 2.2 log CFU/mL compared to the growth in non-phage-treated broth. Therefore, this study suggests that the growth of *Lac. plantarum*, which is known as an acid-producing strain during late fermentation in kimchi, may be controlled using the phage.

**Keywords:** kimchi, bacteriophage, *Lactobacillus plantarum*, biocontrol

## Introduction

Bacteriophages (phages) are viruses that infect specifically bacteria and propagate on the host. Phages are widely present in all habitats where bacterial hosts exist. Phages exist as approximately $10^{31}$ particles on Earth, about 10 times more than the number of bacteria, which may be considered to be the most abundant microbiomes (Weitz et al., 2013). Of all characterized phages, most phages of 96% belong to the order Caudovirales, known as tailed phages. Based on the classification by the International Committee on Taxonomy of Viruses (Acker HW, 1999), the order Caudovirales consists of three families depending on tail morphology: *Podoviridae* have short non-contractile tails, *Siphoviridae* have long non-contractile tails, and *Myoviridae* have long contractile tails. Phages can be classified into two types for their life cycles: lytic and lysogenic cycles. For the lytic cycle, the phages attach to the host bacteria and inject their genetic materials into the cell. The injected genetic materials are replicated and progeny phages are produced, and released from host cells. For the lysogenic cycle, the phages exist as prophages that are inserted into the chromosome of the host bacteria, and the phage is replicated along with the genome of the host cell (Wang et al., 2019). However, when the prophage is exposed to stressful environmental conditions such as UV, high temperature, or high acidity, the lysogenic cycle is switched to the lytic cycle, and the lytic cycle proceeds (Lunde et al., 2005).

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Various phages are present in foods fermented by lactic acid bacteria (LAB) and many studies have reported on phages isolated from fermented foods. Phages from cucumber pickles, fermented sausages, kimchi, and sauerkraut target *Weissella*, *Leuconostoc*, and *Lactobacillus,* respectively (Kleppen et al., 2012; Lu et al., 2003; Pringsulaka et al., 2011). Phage studies on sauerkraut and cucumber fermentation have demonstrated that the emergence of a new phage infecting LAB can affect LAB ecology (Jung et al., 2011; Lu et al., 2012). This indicates that the presence of LAB phages can influence the type and number of LAB during fermentation so that phages are closely correlated with a microbial succession of LAB (Lu et al., 2012).

Kimchi is one of the traditional fermented foods in Korea and known to be a health-promoting food. The components are cabbage, radish, ginger, green onion, and garlic, according to the regions and manufacturers, and fermented mainly by lactic acid bacteria. Among the various bacteria, LAB are the principal ones involved in kimchi fermentation. Most LAB strains are obligate or facultative heterolactic fermentative bacteria that can grow at low temperatures and in acidic environments. They make a better kimchi taste than homolactic fermentative LAB by producing acetate, lactate, ethanol, and $CO_2$ (Kim et al., 2016). Previous studies have shown that the heterolactic genera *Weissella* and *Leuconostoc* are dominant at the initial and mid-stages of fermentation; since then, the homolactic genus *Lactobacillus* is shown to be predominant at the late stage of fermentation (Park et al., 2012). In particular, *Lac. plantarum* is responsible for the increased acidity and over-acidification of kimchi (Lee and Lee, 2010). Such progression results in sour taste, off-odor, and texture softening, influencing to the quality deterioration of kimchi (Lee and Byun, 2007).

It is important to prevent the deterioration of kimchi caused by over-acidification and homolactic fermentation in order to maintain

the high quality of kimchi. The methods such as refrigeration, heat treatment, gamma irradiation, and food additives have been studied extensively (Han and Kang, 2004; Park et al., 2008; Shon and Lee, 1998). Furthermore, applications of *Leu. mesenteroides*, other lactic acid bacteria, nisin, and lytic enzymes have been used to kill *Lac. plantarum* (Chang and Chang, 2010; Lee and Lee, 2011). However, such treatments are not being commercialized because of the degradation of sensory quality and safety concerns associated with them. A biological trial using the bacterial enemy, phage, might be a way to control *Lac. plantarum* by growth inhibition for improving kimchi flavor.

Therefore, in this study, a phage infecting *Lac. plantarum* was isolated and characterized for the control of *Lac. plantarum.*

## Materials and Methods

### Kimchi samples and enumeration of microbes

Twenty kimchi samples, including cabbage kimchi, radish kimchi, green onion kimchi, *Chonggak-kimchi* with young radish, and *Nabak-kimchi* with cut radish in water were purchased from local markets in the Seoul area.

For total bacterial count, 25 g sample was mixed with 225 mL of 0.85% saline in a sampling bag (3M, St. Paul, MN, USA), homogenized using a stomacher (B&F Korea, Seoul, Korea), and diluted decimally in a saline solution. Appropriately diluted solutions were spread on plate count agar (PCA; Difco, Osi, Elancourt, France) and incubated overnight at 37°C, then counted as CFU/mL. To count lactic acid-producing bacteria, the homogenized sample was diluted and spread on bromocresol purple (BCP) late count agar (Eiken Chemical, Tokyo, Japan). The plates were incubated with a gas pack (BD, Franklin Lakes, New Jersey, USA) at 30°C for 24 h. After re-streaking on MRS agar plates (BD), yellowish colonies were counted.

To count bacteriophages in kimchi, the samples were filtered using a sampling bag (3M) and centrifuged at 8,000×*g* for 10 min. The supernatant was filtered through a 0.22-μm syringe filter (Sartorius, Goettingen, Germany). A 0.45-μm cellulose nitrate membrane filter (Whatman, GE, Germany) was placed on the aspirator and a 0.02 μm Anodisc™ 25 (Whatman) was placed on it. One milliliter of purified sample diluted properly was added to the Anodisc and filtered under vacuum. SYBR™ Gold nucleic acid gel stain (×1,000, Invitrogen, Carlsbad, CA, USA) was diluted to 1/10, and 2 μL of this solution was mixed with 78 μL of diethyl pyrocarbonate water (Bioneer, Daejeon, Korea). The filtered Anodisc was dyed with 80 μL of SYBR Gold for 15 min in a dark room. Subsequently, the Anodisc was exsiccated with Whatman paper until opaque forms were placed on the glass slide. To prevent the fluorescence from becoming blurred, Prolong™ Gold antifade reagent (Invitrogen) was added to the Anodisc, and cover glass was placed on top. Anodiscs were analyzed with a microscope (OPTIKA Srl, Ponteranica, Italy) at 1,000× magnification and at least 25 mesh out of 100 mesh were counted using a lens comprising 10×10 mesh. Three points of the Anodisc were randomly selected and counted. Phages per one mL were then calculated using the formula reported by Ortman and Suttle (2009).

### Isolation of *Lac. plantarum* phage, spot assay, and efficiency of plating (EOP)

As a host for phage isolation, *Lac. plantarum* KCCM 12116 cells were cultured and harvested. MRS soft agar (0.7%) with a culture of 2% was overlaid on an MRS agar plate (Oxoid), and then the collected sample filtrate was applied. After overnight incubation, the plaques were collected as phage isolates.

For phage propagation, the phage filtrate on MRS and bacterial culture (1:1) were combined into a mixture. After 18 h of incubation, the culture was centrifuged, and the supernatant was filtered. MRS soft agar with 2% bacterial culture was overlaid on an MRS agar plate (Oxoid), and 10 μL of the filtrate was spotted. After overnight incubation, the appearance of plaques was considered to indicate the presence of phage (Manohar et al., 2018).

The host range for the isolated phage was tested against *Weissella*, *Leuconostoc*, and *Lactobacillus*. All the strains were incubated at 30°C for 18 h. One hundred microliters of each overnight culture was inoculated into 5 mL of MRS soft agar and the mixture was overlaid on MRS plate (Oxoid). After drying the plates for a few minutes, 10 μL of phage solution was spotted on the double-layer agar plate, and the plates were incubated overnight at 30°C. The host range of phages was determined on the basis of plaque formation, and plaque clarity was assessed according to the previous study.

The efficiency of plating (EOP) was defined as the ratio of plaque forming unit (PFU)/mL on the target bacteria to the number of host bacteria (Kutter, 2009).

### Transmission electron microscopy and one-step growth curve assay

The morphological characteristics of the phage were analyzed using transmission electron microscopy (TEM). The phage solution was concentrated to 10-11 log PFU/mL by centrifuging at 26,000×*g* for 1 h. Briefly, 20 μL of phage suspension was attached to a 200 mesh carbon-coated copper grid (Ted Pella, Redding, CA, USA) for 2 min and negatively stained with 2% uranyl acetate for 1 min. The grid was then washed with sterilized distilled water and dried. The samples were examined using TEM (H-7600, Hitachi, Tokyo, Japan) at an operating voltage of 80 kV. Phages were classified according to the International Committee on the Taxonomy of Viruses.

A one-step growth curve assay was performed as described previously, with some modifications (Renata et al., 1993). Host bacteria were grown in MRS broth at 30°C for 18 h. One milliliter of the host was cultured overnight, and 1 mL of phage solution was mixed in 8 mL MRS broth at an appropriate MOI. The mixed solution was allowed to adsorb at 30°C for 10 min. After phage adsorption, the mixture was centrifuged at 8,000×*g* for 10 min. The supernatant containing free phages was discarded, and the pellet containing the adsorbed phage was re-suspended in 10 mL of fresh MRS broth and incubated at 30°C. Three sets of samples were obtained at 5 min intervals for up to 110 min and immediately titrated using the double-layer agar plate method. Through this assay, the latent period, burst size, and one cycle of the isolated

phage were calculated. All experiments were performed in triplicates.

### Stability of ΦLP12116 after exposure to temperatures, salt concentrations, pH values, and organic acids

The effect of high temperature on the phage was assessed at various temperatures using a heat block (FINEPCR, Gunpo, Korea). The phage solution (9 log PFU/mL) was exposed to 50, 60, 70, and 80°C for 30 min and the samples were taken every 5 min. Aliquots (10 μL) of the phage solution were spotted on a lawn of MRS agar. After drying and incubating at 30°C for 18 h, the phage titer was determined.

To examine phage stability with regard to salinity, the phage was inoculated into MRS broth adjusted to the desired salinity. Phages of 9 log PFU/mL were suspended in 1, 5, and 10% salinity in MRS broth and incubated at 4 or 30°C for 48 h. The number of surviving phages was counted using the top agar overlay method (Manohar et al., 2018) and the results were represented as percentage survivability.

The phage (1%) was added to MRS broth adjusted to pH 3, 4, and 5, and incubated at 4 or 30°C for 48 h. After incubation, viable phages were immediately diluted and counted using the spotting method. The results are represented as the percentage viability.

For the organic acid stability test, the phage was exposed to various organic acid conditions. Lactic acid and acetic acid were selected and used to make an organic acid environment like kimchi with MRS broth. The phage lysate (1%) was suspended in MRS broth containing organic acid and incubated at 4 or 30°C for 48 h. After 0, 24, and 48 h of incubation, the solution was serially diluted and spotted on the double-layered MRS agar by the spotting method. The number of plaques was counted, and the surviving phages were expressed as a percentage of the initial phages.

### Growth of *Lac. plantarum* with template ΦLP12116 for kimchi fermentation

To evaluate the bacterial control ability of ΦLP12116 for *Lac. plantarum* in the kimchi environment, ΦLP12116 and its host bacteria were inoculated into kimchi broth, and the number of bacteria was counted. *Lac. plantarum* KCCM 12116 were diluted to 4 log CFU/mL and mixed with ΦLP12116 at an appropriate MOI. MOI was determined based on the results of the growth inhibition assay as MOI 100. The host bacteria-phage mixture (1%) was inoculated into kimchi broth and incubated at 4°C, similar to the temperature of the refrigerator. To prepare the kimchi extract broth medium, *Nabak-kimchi* was manufactured, stomached, gauze-filtered, and sterilized (Kong et al., 2005). The number of bacteria and phages were counted by spreading on MRS agar and soft agar overlay plaque assay during the incubation. Non-phage-treated bacterial culture was used as a control. All experiments were performed in triplicate.

## Results and Discussion

### Microbe and bacteriophage populations of kimchi in a market

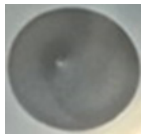
Twenty kimchi samples were collected from the local market and analyzed for total bacteria (TB), lactic acid bacteria (LAB), and bacteriophages (phages) (Table 1). The pH values ranged from 3.5 to 5.0, indicating a ripened state (Mheen and Kwon, 1984). The total bacterial counts of the samples were 5.5-8.5 log CFU/mL, and the LAB counts were 5.4-8.6 log CFU/mL with a mean value of 7.1 log CFU/mL. The numbers of TB and LAB indicated that most of the bacteria present in kimchi seemed to be almost LAB. Total phages were 1.7-3.8 log particles/mL (mean 2.3 log particles/mL) and relatively lower in quantity than the total bacteria and LAB. The counts of phage amounted to 32% of LAB population in log scale. The total number of phages in the kimchi as assessed by epifluorescence microscopy showed no correlation with the number of bacteria regardless of the type of kimchi. Phages are reported to be a major population in the environment and greatly influence microbial ecology (Lu et al., 2012; Jung et al., 2011; Weitz et al., 2013). They have been detected in kimchi

**Table 1. Distribution of total bacteria (TB), lactic acid bacteria (LAB), and phage**

| Kimchi type | Sample No. | pH | TB[1)] | LAB[2)] | Phage[3)] |
|---|---|---|---|---|---|
| Cabbage kimchi | 1 | 3.92 | 7.08 | 7.53 | 2.43 |
| | 2 | 4.08 | 7.22 | 6.78 | 2.26 |
| | 3 | 3.71 | 7.78 | 7.31 | 2.60 |
| | 4 | 4.18 | 7.45 | 7.34 | 2.31 |
| | 5 | 3.82 | 6.59 | 6.72 | 3.58 |
| | 6 | 3.74 | 6.39 | 6.26 | 3.16 |
| | 7 | 3.67 | 7.43 | 7.36 | 2.49 |
| | 8 | 3.75 | 6.30 | 6.70 | 3.75 |
| | 9 | 4.08 | 7.60 | 8.02 | 1.72 |
| | 10 | 3.88 | 6.00 | 8.36 | 2.22 |
| | 11 | 4.09 | 8.46 | 8.58 | 3.51 |
| | 12 | 3.76 | 6.88 | 6.90 | 2.18 |
| Green onion kimchi | 13 | 3.52 | 6.72 | 6.52 | 2.19 |
| | 14 | 5.00 | 5.50 | 5.42 | 2.29 |
| Young radish kimchi | 15 | 4.19 | 7.17 | 7.03 | 1.91 |
| | 16 | 4.09 | 6.15 | 6.59 | 2.49 |
| Chonggak-kimchi | 17 | 3.66 | 7.32 | 7.40 | 2.44 |
| | 18 | 3.96 | 7.31 | 7.09 | 2.30 |
| Nabak-kimchi | 19 | 3.71, | 7.74 | 7.73 | 2.47 |
| | 20 | 4.30 | 6.43 | 6.37 | 1.72 |

Symbols: [1)]TB and [2)]LAB with conventional culture method by log CFU/mL. [3)]Phage with epifluorescence microscopy by log particle/mL.

**Table 2. Host ranges of isolated ΦLP12116 by spot assay and EOP of ΦLP12116**

| Host strain | Plaque | EOP | Plaque morphology |
|---|---|---|---|
| *W. confusa* MGB 0333 | - | - | +++  |
| *Leu. citreum* K27 | - | - | |
| *Leu. mesenteroides* B6 | - | - | |
| *Leu. mesenteroides* B9 | - | - | |
| *Leu. mesenteroides* K2 | - | - | +  |
| *Lac. plantarum* KCTC 3108 | +++ | $2.00 \times 10^{-1}$ | |
| *Lac. plantarum* ATCC 8014 | + | $< 9.09 \times 10^{-7}$ | |
| *Lac.plantarum* KCCM 12116 | +++ | 1 | |

Symbols: +++, clear appearance throughout but with a faintly hazy background;
+, a few individual plaques or complete turbidity in the spot; -, no plaque.

and fermented foods, mainly as LAB phages for *Weissella*, *Pediococcus*, *Lactobacillus*, and *Leuconostoc* (Jung et al., 2011; Kleppen et al., 2012; Lu et al., 2003; Pringsulaka et al., 2011). Recently, the phage population has been enumerated by direct counting using flow cytometry and epifluorescence microscopy in kimchi. The phage showed an average count of 2.1 log particles/mL per kimchi soup and seemed to be 28% log scale to the total bacterial count (Park WJ, 2017; Kong SJ, 2019). Therefore, the phage population on a commercial kimchi market was 2.3 log particle/mL and corresponded to LAB population of 32% similarly to previous reports.

### Phage isolation for *Lac. plantarum* and determination of host range by spot assay and efficiency of plating (EOP)

The phage for *Lac. plantarum* KCCM 12116 was isolated from *Nabak-kimchi* and designated as ΦLP12116. The host range of the isolated phage was determined by confirming the formation of plaques using the spot assay and EOP (Table 2). ΦLP12116 had a narrow host range and limited only to *Lac. plantarum*, indicating that lysis occurred only within the same species. Infection with ΦLP12116 to *Lac. plantarum* KCTC 3108 and *Lac. plantarum* KCCM 12116 formed clear plaques with a faintly hazy background, but infection to *Lac. plantarum* ATCC 8014 resulted in slight turbidity on the plaque. Interestingly, plaques showed a typical bulls-eye appearance on *Lac. plantarum* KCCM 12116 via lysogenicity and greater turbidity was generally observed at the center (Jurczak-Kurek et al., 2016). The different plaques on such hosts may be due to infection resistance or mechanisms (Ali et al., 2014; da Silva Duarte et al., 2018; van Houte et al., 2016). Regarding as phage infection on LAB, two-step processes as reversible interaction through a surface carbohydrate moiety and then irreversible interaction between host receptor and phage receptor-bind protein have been suggested (Baptista et al., 2008; Mahony et al., 2017). There are a broad array of components such as carbohydrates, proteins, lipoteichoic acids, and wall teichoic acid on the surface, which may be diverse among the strains or modified to the environmental factors (Ainsworth et al., 2014). Thus, host-phage may interact strain-specifically.

There are reports that phages for *Weissella* and *Leuconostoc* show a broad host infection range on species and genera of *Weissella*, *Leuconostoc*, and *Lactobacillus* (Kong and Park, 2020; Lu et al., 2012; Pujato et al., 2017). However, the temperate *Lac. plantarum* phages ΦLP1-A, ΦLP1-B, and ΦLP2 have been reported to have a host range limited to *Lac. plantarum* strains (Caso et al., 1995). Here, ΦLP12116 seemed to have limited host infection only for *Lac. plantarum*, similar to other *Lac. plantarum* phages. EOP analysis was also carried out to identify the host with the highest infection rate (Table 2). ΦLP12116 showed different infection efficiencies against target bacteria and most infection to *Lac. plantarum* KCCM 12116. In summary, determination of host range for ΦLP12116 indicated that *Lac. plantarum* KCCM 12116 was the best host for infection.

### Morphological and culture characteristics of ΦLP12116

Morphological characteristic of ΦLP12116 was analyzed using TEM and shown in Fig. 1. ΦLP12116 had a 71.0±6.0 nm icosahedral head and a 275.1±5.3 nm long non-contractile tail. The head and tail of ΦLP12116 were relatively long. ΦLP12116 belonged to the *Siphoviridae* family, according to the International Committee on Taxonomy of Viruses. ΦLP12116 was similar to *Lac. plantarum* phage P1 that had a 71.7±3.0 nm isometric capsid and a 272±3.0 nm long non-contractile tail (Chen et al., 2016).

A one-step growth curve assay was performed and the latent period, burst size, and time required for one cycle were determined. ΦLP12116 showed that the latent period was 85 min, and the burst size was 12.9 PFU/infected cell. This explained why ΦLP12116 had a relatively long latent period and a lower burst size than the other phages (Briggiler M et al., 2012; Chen et al., 2016). The time required for one cycle of ΦLP12116 was long by 110 min. *Lac. plantarum* phage ΦLPN014, isolated from Nham (Thai fermented pork), shows a latent period of about 30 min, which is shorter than that of ΦLP12116, and 150 PFU/infected cell of the burst size, which is higher than that of ΦLP12116 (Rattanachaikunsopon P, 2014).

### Viability of ΦLP12116 after exposure to various temperatures and salt concentrations

Temperature has been reported to be related to fundamental adsorption for infection (Jonczyk et al., 2011). To investigate

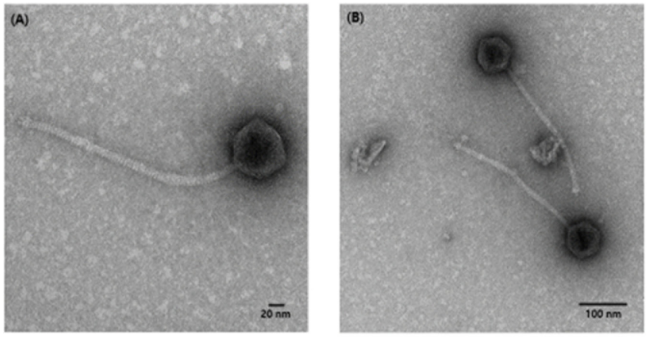



**Fig. 1. Morphology of bacteriophage ΦLP12116 by transmission electron microscopy (50,000× magnification; size bars, 20 nm and 100 nm).**

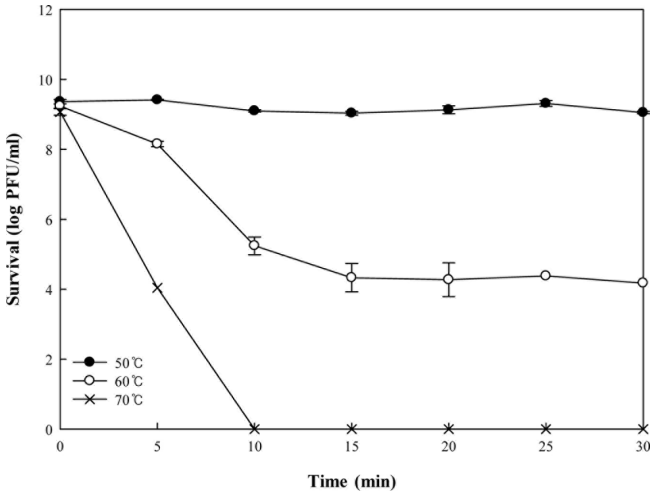



**Fig. 2. Viability of bacteriophage ΦLP12116 in log PFU/mL after exposure to 50, 60, and 70°C for 30 min.**

thermal stability, ΦLP12116 was exposed to high temperatures of 50, 60, and 70°C for 30 min (Fig. 2). ΦLP12116 was stable at 50°C, as shown by the fact that the reduction of phage was less than 0.31 log PFU/mL. Other reports on *Lac. plantarum* phages also have a viability of 95% at 50°C for 30 min (Marcó et al., 2012; Chen et al., 2016). However, the viability of ΦLP12116 was reduced from 9.1 log PFU/mL to 4.0 log PFU/mL within 5 min of heat treatment and completely inactivated out after 10 min at 70°C. Thus, ΦLP12116 would be stable below 50°C such as in the refrigerator.

Salt concentration is widely known as one of the factors affecting kimchi fermentation. It has been reported that kimchi is generally fermented at approximately 2-3% of salt concentration (Mhin and Kwon, 1984). To examine the effect of high salinity on phages, the phage was exposed to three different salinity conditions, and viability was determined. The plaque after exposure at 5% and 10% salinity was shown and the number was not significantly different compared to control exposure. ΦLP12116 showed 92% survival at 10% salinity (data not shown). Accordingly, salinity during kimchi environment might have no influence on phage inactivation.

### Viability of ΦLP12116 after exposure to various pH and organic acids

Survival under acidic conditions was investigated at pH 3, 4, and 5 for 48 h. This experiment was performed at two different temperatures of 4°C and 30°C (Fig. 3). The pH stability test indicated that the survival rate of ΦLP12116 was 99.8% after 48 h exposure at pH 5 and 4°C. The survival rate of ΦLP12116 at pH 4 and 4°C was 98.1%, which indicated that the phage was stable at pH 4 and 5 at 4°C. However, the phage was completely inactivated after 24 h of exposure to pH 3 at 4°C. The above results indicated that the phage was stable under acidic conditions above pH 3 at 4°C. However, the stabilities at 30°C were lower than those at 4°C. Therefore, ΦLP12116 might be stable in acidic condition under low temperatures.

ΦLP12116 was exposed to various lactic acid concentrations of 0.1, 0.5, and 1.0%. ΦLP12116 showed a survival rate of 96.4% at 0.1, 0.5, and 1.0% lactic acid at 4°C for 48 h (Fig. 4). No

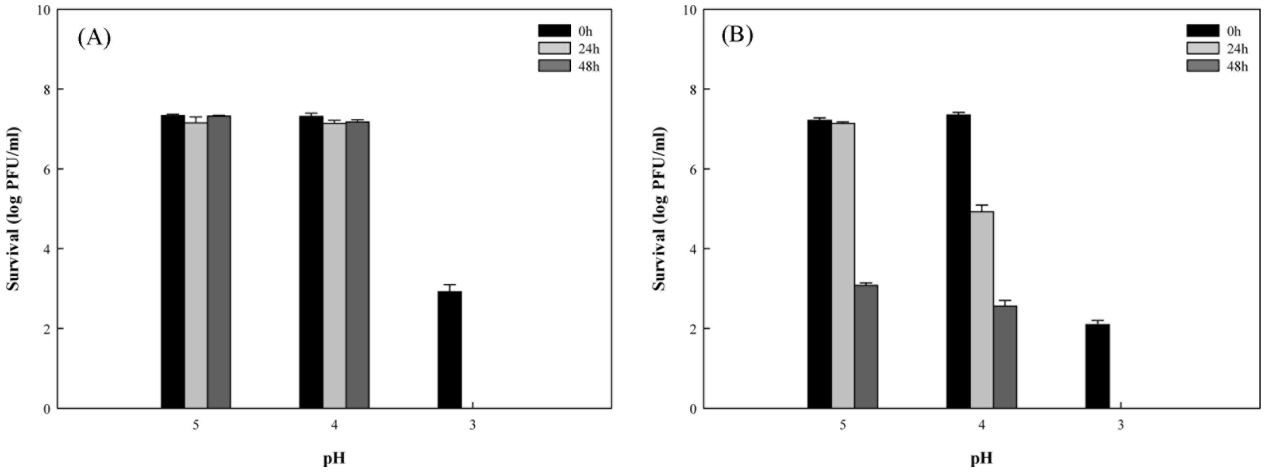



**Fig. 3. Viability of bacteriophage ΦLP12116 in log PFU/mL after exposure to pH 3, 4, and 5 at 4°C (A) and 30°C (B) for 24 and 48 h.**

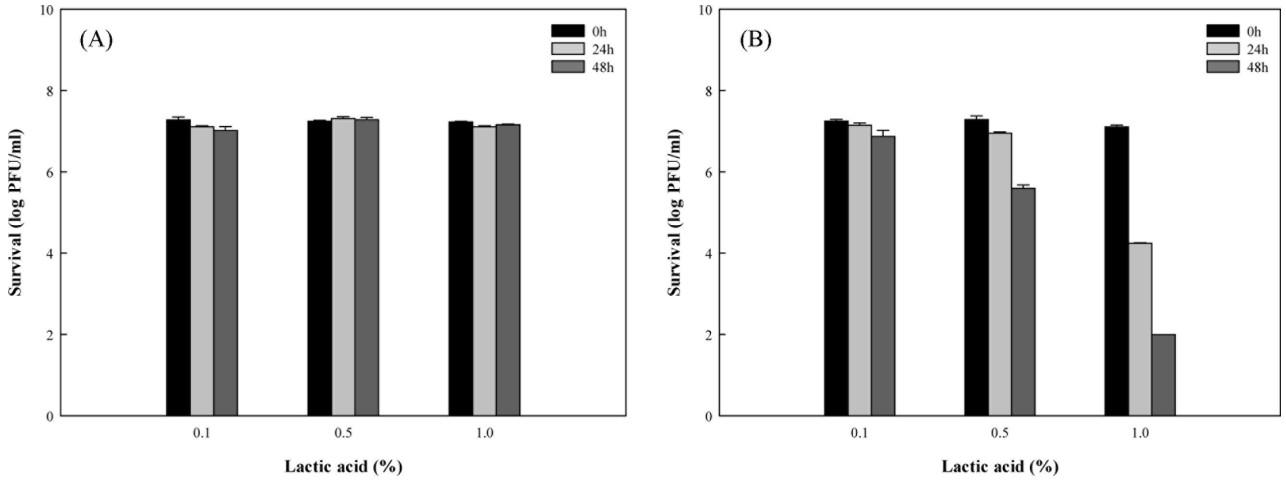



**Fig. 4. Viability of bacteriophage ΦLP12116 in log PFU/mL after exposure to lactic acid of 0.1, 0.5, and 1.0% at 4°C (A) and 30°C (B) for 24 and 48 h.**

significant reduction for the viability of ΦLP12116 occurred at these concentrations. At 30°C, ΦLP12116 exhibited a survival rate of 94.8% with 0.1% lactic acid. ΦLP12116 also showed 76.8% and 28.1% survival in lactic acids of 0.5% and 1%, respectively. These results indicated that the phage was stable at a high lactic acid concentration at 4°C for up to 48 h.

Acetic acid is present in smaller quantities than lactic acid in kimchi. During *Dongchimi* fermentation, the acetic acid content reaches approximately 0.2% after 30 days, and the range of acetic acid is 0.1-0.2% (Cho et al., 2015). The phage was exposed to acetic acid at 0.1, 0.5, and 1.0% (w/w). At any concentration of acetic acid at 4°C for 48 h, ΦLP12116 exhibited no significant difference in survival rate compared to non-treatment control. However, after 48 h at 30°C, the survival rate of ΦLP12116 decreased to 94.8% at 0.1% acetic acid, 85.5% at 0.5% acetic acid, and 59.5% at 1.0% acetic acid (data not shown). The above results indicated that ΦLP12116 was more sensitive to lactic acid than acetic acid and was inactivated more easily on lactic acid. Phage ΦT25 is also resistant to a wide range of pH; however, no survivors can be detected after incubation at pH 2 for 30 min at 37°C (Sunthornthummas et al., 2017). The viability of ΦLP12116 might be maintained at high organic acid and low temperatures similar to the other reports.

Several mechanisms have been reported on the acid tolerance for LAB, which are the neutralization processes like (a) the arginine dihydrolase system and the malolactic fermentation, (b) the biofilm formation and membrane modification, (c) the proton pump like $F_1$-$F_0$-ATPase and amino acid decarboxylation, and (d) protection and repair of cellular macromolecules (Wang et al., 2018). However, no report is shown for the different tolerance toward lactic and acetic acid, which may come from the different proton dissociation constant of lactic acid (pKa=3.86) and acetic acid (pKa=4.75). Lactic acid is a stronger acid than acetic acid to destroy the cell neutrality more easily when the organic acids come into the cell cytosol.

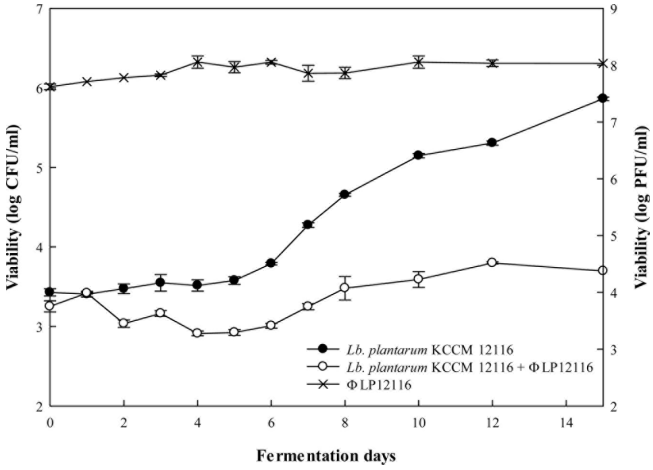



**Fig. 5. Growth inhibition of *Lac. plantarum* in kimchi broth by treating bacteriophage of ΦLP12116 at 4°C for 15 days.** Symbols; cross, phage number in PFU/mL; closed circle, *Lac. plantarum* only in CFU/mL; open circle, *Lac. plantarum* with phage in CFU/mL.

## Growth inhibition of *Lac. plantarum* by ΦLP12116 in kimchi extract

Various LAB, including *W. koreensis, Leu. mesenteroides, Lac. sakei*, and *Lac. plantarum,* are known to be involved in late kimchi fermentation (Lim et al., 1989). In particular, it has been reported that *Lac. plantarum* produces a large amount of acid, which is related to over-ripened kimchi fermentation (Lee and Lee, 2010). To control the growth of *Lac. plantarum*, ΦLP12116 and *Lac. plantarum* KCCM 12116 were co-cultivated. *Lac. plantarum* was inoculated into kimchi extract broth for 15 days at 4°C, and the population reduction of *Lac. plantarum* by phage was analyzed (Fig. 5). *Lac. plantarum* in the non-phage-treated broth grew steadily for 15 days. The number of *Lac. plantarum* increased slowly until day 5, but after that, bacteria grew rapidly and reached 5.9 log CFU/mL at day 15. However, the number of *Lac.*

*plantarum* decreased until day 4 in phage-treated broth. The number was 3.4 log CFU/mL at the start, but became 2.9 log CFU/mL at day 4. The bacteria began to grow again slowly and reached 3.7 log CFU/mL at day 15. During fermentation for 15 days, the number of phages showed no significant change from 8 log PFU particles/mL. Based on these data, it was clear that the growth of *Lac. plantarum* KCCM 12116 was inhibited by ΦLP12116 in kimchi broth, indicating growth inhibition by ΦLP12116 against *Lac. plantarum.* However, the number of *Lac. plantarum* was not reduced to zero level. As a response to phages, bacteria have developed many anti-phage mechanisms such as clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated genes (cas) systems, restriction-modification systems, superinfection exclusion (Sie) systems, and abortive infection systems (Labrie et al., 2010). Battle between bacteria and phages leads to the co-evolution between the two entities and then bacteria may be adapted and be resistant to phages.

There are many reports on reducing the over-acidification of kimchi to extend the edible period and maintain its flavor by control of *Lac. plantarum*. Physical and chemical trials, in addition to starter development and bacteriocin addition, have been conducted, but have not reached a satisfactory solution. This research suggests that growth for *Lac. plantarum* known as an acid-producing strain at the late fermentation in kimchi might be controlled by using the phage. However, the phage cocktail infecting *Lac.platarum* strains is needed because of the narrow host spectrum.

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## Conflict of Interest

The authors have no financial conflict of interest to declare.

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