

Effect of Bacteriophages on Viability and Growth of Co-cultivated *Weissella* and *Leuconostoc* in Kimchi Fermentation

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This study aimed to understand the survival and growth patterns of bacteriophage-sensitive *Weissella* and *Leuconostoc* strains involved in kimchi fermentation. Dongchimi kimchi was prepared, and *Weissella* and *Leuconostoc* were co-cultivated in the dongchimi broth. *Weissella cibaria* KCTC 3807 growth was accompanied by rapid lysis with an increase in the bacteriophage quantity. *Leuconostoc citreum* KCCM 12030 followed the same pattern. The bacteriophage-insensitive strains *W. cibaria* KCTC 3499 and *Leuconostoc mesenteroides* KCCM 11325 survived longer under low pH as their growth was not accompanied by bacteriophages. The bacteriophage lysate of *W. cibaria* KCTC 3807 accelerated and promoted the growth of *Leuconostoc*. Overall, our results show that bacteriophages might affect the viability and population dynamics of lactic acid bacteria during kimchi fermentation.

Keywords: *Weissella*, *Leuconostoc*, bacteriophage, population dynamics, kimchi

Among the microorganisms involved in kimchi fermentation, lactic acid bacteria (LAB) play the most important role. LAB produce lactic acid, acetic acid, ethanol, carbonic acid gas, aldehydes, and so on, giving kimchi its unique flavor [1]. The LAB present in kimchi tend to vary such that those involved in initial and late fermentation sequentially grow and influence each other [2]. Different raw materials, ingredients, and fermentation conditions have been suggested to significantly affect LAB profiles during kimchi fermentation [3, 4]. In addition to LAB, several types of LAB bacteriophages have been detected and isolated during kimchi fermentation. During cheese fermentation, the bacteriophages deter fermentation by stopping milk acidification [5]. Recently, studies on the control of food production during fermentation through the control of LAB bacteriophages have been conducted [6]. In pickle fermentation, the bacteriophages of *Weissella*, *Leuconostoc*, and *Lactobacillus* were detected sequentially [7]. Therefore, it was expected that bacteriophages might affect the succession of lactic acid bacteria [8, 9].

Bacteriophages are viruses that infect specific bacterial hosts and are known to outnumber bacteria ten-fold, estimated at over 10^{31} bacteriophages globally [10]. Bacteriophages exhibit two life cycles: lytic and lysogenic.

In the lysogenic life cycle, the bacteriophage is introduced into the bacterial genome as a prophage: this prophage can later be induced by environmental stresses leading to virus production and host cell lysis [11]. Such bacterial lysates have been suggested to supply energy to the marine ecosystem food web, where bacteriophages may function as nutrient recyclers [12]. Therefore, bacteriophages might also be related to LAB lysis during kimchi fermentation, resulting in LAB succession and nutrient recycling in the microecosystem of kimchi. As such, this study investigated the effect of bacteriophages on the viability and growth of *Leuconostoc* and *Weissella* to better understand the successive population dynamics during early kimchi fermentation.

Kimchi can be generally classified in the two types, ordinary and watery kimchi. The watery kimchi is made mainly of cabbage or radish. As a watery kimchi, dongchimi was selected as a model from many types of kimchi for easy analysis. First, Dongchimi was prepared with radishes from a local market. Radishes were cut into pieces of about 3 cm in height, width, and length and placed in a container. Seven percent salt water was added to the radishes at a ratio of 1:1 (w/v). Then, green onions, garlic, and ginger were added to the mix at 3%, 1%, and 0.5% (w/w) of the radishes, respectively [13]. The Dongchimi

mixture was homogenized for 10 min by a stomacher (Stomacher Power Mixer, BNF Korea, Korea), centrifuged at 8,000×g for 10 min, and the supernatant was sterilized with a 0.22 µm filter (Millipore, USA). The sterilized solution used as the culture broth was stored at -80°C. Lactic acid bacteria (LAB) used here were *Weissella cibaria* KCTC 3807, *W. cibaria* KCTC 3499, *Leuconostoc citreum* KCCM 12030, and *L. mesenteroides* subsp. *mesenteroides* KCCM 11325. Among them, only the bacteriophages of *W. cibaria* KCTC 3807 and *L. citreum* KCCM 12030 were induced during kimchi fermentation, and however, were not instead induced by mitomycin C (Sigma-Aldrich, USA).

To analyze LAB growth and bacteriophage presence during dongchimi fermentation, *Weissella* and *Leuconostoc* were inoculated separately or simultaneously into the fresh Dongchimi broth and fermentation was carried out at 7°C for 10 days. To differentially count *Weissella* and *Leuconostoc* in the co-culture, they were spread on MRS agar (de Man-Rogosa-Sharp, Oxoid, Italy) supplemented with meropenem (2 µg/ml, Sigma-Aldrich), and count was determined by subtracting the number of *Leuconostoc* from the total number of LAB. The pH of the culture was also measured. To determine viability of LAB strains at different pH, the bacterial cells were cultured in MRS broth at 9 log CFU/ml, and the cultures were added to saline solutions of pH 3, 4, and 5 at 7 log CFU/ml. After exposure for 0, 24, and 48 h, the live bacteria were counted by plating on MRS agar [15]. The effects of bacteriophage-sensitive *W. cibaria* on the growth of *Leuconostoc* spp. in Dongchimi broth was analyzed. *W. cibaria* KCTC 3807 was cultured in broth for 5 days until the bacteriophage count reached maximum. The cultured broth was micro-filtered to remove the pre-

inoculated *W. cibaria*, and then *Leuconostoc* was cultured in the broth. Meanwhile, a *Leuconostoc* single culture was inoculated in fresh broth as a control. The cultures were incubated for 14 days and each bacteriophages of *Weissella* and *Leuconostoc* were quantified by the plaque assay using MRS agar.

W. cibaria KCTC 3807 (*W. cibaria* 3807) and *L. citreum* KCCM 12030 (*L. citreum* 12030) were inoculated simultaneously to carry out fermentation (Fig. 1A). *W. cibaria* 3807 reached about 5.6 log CFU/ml at day 3 and then died off rapidly, whereas the quantity of bacteriophages increased. *L. citreum* 12030 grew to 6 log CFU/ml after day 5, and then died off as the bacteriophage quantity increased. *W. cibaria* 3807 bacteriophages increased to about 5.6 log PFU/ml on day 3 of fermentation, rose to 7.8 log PFU/ml on day 7, and finally decreased. *L. citreum* 12030 bacteriophages increased after day 3 of fermentation and then decreased to 7.5 log PFU/ml at day 7. When the number of bacteriophages increased, the quantity of each host strain decreased rapidly. Therefore, these results showed that the decline in host bacteria might be lysis by bacteriophages.

W. cibaria 3807 and *L. mesenteroides* KCCM 11325 (*L. mesenteroides* 11325) were inoculated simultaneously to carry out fermentation (Fig. 1B). Similarly to the above results, the number of *W. cibaria* 3807 releasing bacteriophages decreased rapidly after day 5 of fermentation when the bacteriophage count reached maximum. However, *L. mesenteroides* 11325, which does not release phages, appeared to maintain bacterial numbers steadily even at low pH until fermentation ceased, as it seemed to survive for 5 days under pH 4.0.

W. cibaria KCTC 3499 (*W. cibaria* 3499) and *L. citreum* 12030 were inoculated simultaneously to carry out fermentation

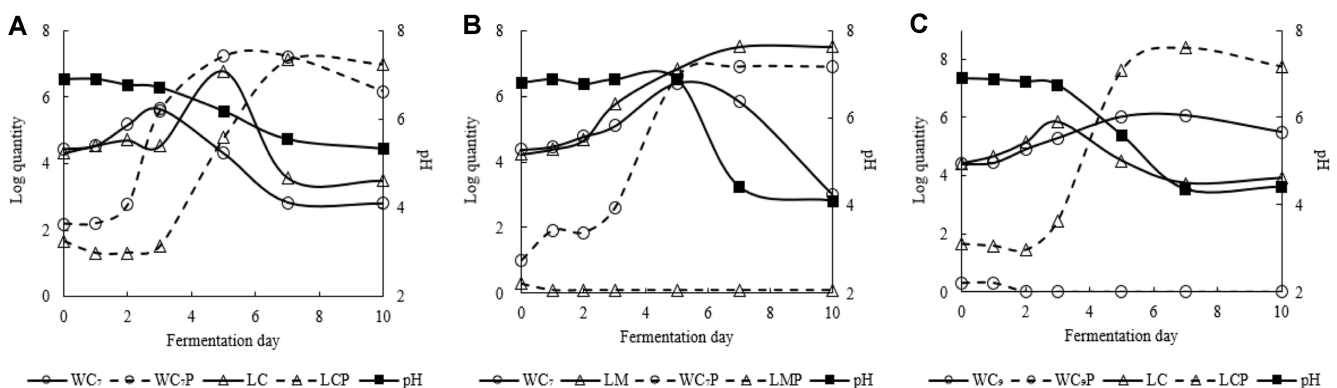


Fig. 1. Viable count profiles of *W. cibaria*, *L. citreum*, and *L. mesenteroides* with bacteriophages at co-cultures in Dongchimi kimchi at 7°C. Symbols: WC₇ and WC₇P, *W. cibaria* KCTC 3807 and its bacteriophage; WC₉ and WC₉P, *W. cibaria* KCTC 3499 and its bacteriophage; LC and LCP, *L. citreum* KCCM 12030 and its bacteriophage; LM and LMP, *L. mesenteroides* KCCM 11325 and its bacteriophage.

Table 1. Viability of lactic acid bacteria at pH 4.0 and phage detection during kimchi fermentation.

Strains	Viability at pH 4.0 (%)			Bacteriophage detection at kimchi fermentation
	0 h	24 h	48 h	
<i>W. cibaria</i> KCTC 3807	100.0	100.0	100.0	+
<i>W. cibaria</i> KCTC 3499	100.0	94.7	89.3	-
<i>L. citreum</i> KCCM 12030	100.0	99.9	96.4	+
<i>L. mesenteroides</i> KCCM 11325	100.0	97.5	93.9	-

Symbols: W, *Weissella*; L, *Leuconostoc*; +, bacteriophage induction; -, bacteriophage non-induced
Viability was determined by dividing CFU of LBA at 24 or 48 h by CFU at 0 h.

(Fig. 1C). *W. cibaria* 3499 reached about 6.1 log CFU/ml at day 5 of fermentation and maintained this level. Contrarily, *L. citreum* 12030 reached about 5.8 log CFU/mL on day 3 and then decreased rapidly. *W. cibaria* 3499 bacteriophages were not detected during fermentation. *W. cibaria* 3499 survived, maintaining its bacterial count until the end of fermentation. However, *L. citreum* 12030 bacteriophages increased to about 8.4 log PFU/ml and then decreased after day 7. *L. citreum* 12030 decreased rapidly when bacteriophages were induced. Therefore, it seemed that the number of LAB decreased when phages increased [7, 8]. The pH of the culture was reduced from 6.8–6.9 to 4.1–5.3 on the tenth day of fermentation [3]. *W. cibaria* 3499 seemed to survive when exposed to pH 4.0 for 5 days. This showed that LAB viability might be related to the presence of bacteriophages rather than the pH during the fermentation. Additionally, all the strains survived in a pH 4 solution with a survival rate of 89% or more following 48 h of exposure (Table 1). Therefore, low pH did not impact primarily bacterial

mortality, whereas the presence of bacteriophages did.

Finally, the effects of the bacteriophage-lysed *Weissella* dongchimi broth on the growth of *Leuconostoc* were analyzed. In the sterilized fresh Dongchimi broth, *L. citreum* 12030 grew to a maximum of about 7.8 log CFU/ml on day 10. Contrarily, in the Dongchimi broth containing bacteriophage-lysed *W. cibaria* 3807, *L. citreum* 12030 reached a maximum of about 7.7 log CFU/ml on day 5 (Fig. 2A), which was the fastest growth by five days. It was confirmed that *L. citreum* 12030 growth was promoted in Dongchimi broth containing bacteriophage-lysed *W. cibaria* 3807. The bacteriophage lysate appeared to act as a growth activator, accelerating *L. citreum* 12030 growth under the given conditions. Therefore, bacteriophages might contribute to recycling nutrients for the other LAB through the lysis of one LAB. As shown in Fig. 2B, in the sterilized fresh Dongchimi broth, *L. mesenteroides* 11325 grew to about 7.2 log CFU/ml at maximum on day 5. In contrary, in Dongchimi broth containing *W. cibaria* 3807 lysate, *L. mesenteroides* 11325

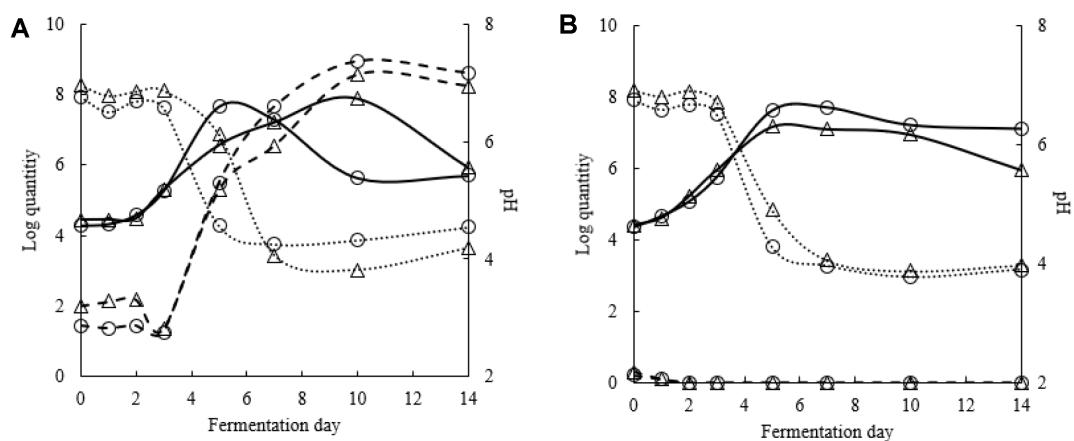


Fig. 2. *Leuconostoc* growth comparison in fresh Dongchimi broth (Δ) or bacteriophage-lyzate Dongchimi broth (\circ) at 7°C for *L. citreum* KCCM 12030 (A) and *L. mesenteroides* KCCM 11325 (B).

The bacteriophage-lyzate broth was prepared after microfiltration of pre-cultured Dongchimi by bacteriophage-sensitive *W. cibaria* KCTC 3807 to remove the live bacteria. The dashed and dotted lines indicate *Leuconostoc* phage quantities and pH values, respectively.

reached its highest bacterial count of 7.6 log CFU/ml on day 5, greater than the culture in the fresh Dongchimi broth. In the Dongchimi broth with *W. cibaria* 3807 lysate, *L. mesenteroides* 11325 growth was promoted and mortality was reduced.

In summary, bacteriophage-sensitive LAB survival was short as they died off due to bacteriophage induction or propagation [3, 7]. Contrarily, bacteriophage-insensitive LAB survived relatively longer, even at low pH. LAB death, therefore, appeared to be influenced more by bacteriophages rather than pH. Also, *Leuconostoc* growth was promoted in media supplemented with bacteriophage-lysed *W. cibaria*. Therefore, bacteriophages influenced the life span of LAB during kimchi fermentation. As expected, our results showed that LAB bacteriophages might play an important role in the viability and population dynamics of lactic acid bacteria such as succession involved in kimchi fermentation.

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Conflict of Interest

The authors have no financial conflict of interest to declare.

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