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Correlation between Changes in Microbial/Physicochemical Properties and Persistence of Human Norovirus during Cabbage Kimchi Fermentation

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Recently, cabbage kimchi has occasionally been associated with the foodborne diseases of enteric viruses such as human norovirus (HuNoV). This study aimed to evaluate the correlation between microbial/physicochemical properties and persistence of HuNoV in experimentally contaminated cabbage kimchi fermented and stored at 4°C or 10°C for 28 days. Changes in organic acid content, lactic acid bacteria (LAB), acidity, pH, and salinity were analyzed. The recovery of structurally intact HuNoV was examined for up to 28 days post-inoculation, using a NoV GII.4 monoclonal antibody-conjugated immuno-magnetic separation method combined with quantitative real-time reverse transcription polymerase chain reaction. On day 0, LAB loads were 4.70 $\log_{10}$ colony forming units/g and HuNoV GII.4 titers were 2.57 $\log_{10}$ genomic copies/μl, at both temperatures. After 28 days, intact HuNoV titers decreased to 1.58 (4°C) and 1.04 (10°C) $\log_{10}$ genomic copies/μl, whereas the LAB density increased. This correlated with a gradual increase in lactic acid and acetic acid at both temperatures. Our findings support a statistical correlation between changes in physicochemical properties and the recovery of structurally intact HuNoV GII.4. Moreover, we determined that the production of organic acid and low pH could affect HuNoV GII.4 titers in cabbage kimchi during fermentation. However, HuNoV GII.4 was not completely eliminated by microbial/physicochemical factors during fermentation, although HuNoV GII.4 was reduced. Based on this, we speculate that the persistence of HuNoV GII.4 may be affected by the continually changing conditions during kimchi fermentation.

Keywords: Cabbage kimchi, detection, fermentation, human norovirus, lactic acid bacteria, organic acid

Introduction

Human norovirus (HuNoV) is a serious foodborne virus and the main cause of acute viral gastroenteritis worldwide [1–3]. HuNoV belongs to the *Calicividae* family and *Norovirus* genus. It causes gastroenteritis, which manifests with vomit and diarrhea within 1 to 3 days after exposure to as few as 100 infectious particles [3, 4]. HuNoV leads to the hospitalization of hundreds of children and elderly people as well as approximately 20 deaths per year [5, 6]. The Korea Food and Drug Administration reported that norovirus outbreaks have taken up the largest proportion (15.22%) among foodborne pathogens over the last 10 years. This makes HuNoV a significant international public health concern, and research on noroviruses will help decrease HuNoV outbreaks of diseases.

Typical foodstuff involved in HuNoV outbreaks are raw or poorly cooked meat or shellfish, ready-to-eat foods, and commodities such as fruits and vegetables, which are typically consumed without additional thermal processing [7–13]. HuNoV accounts for more than 40% of fresh produce-associated outbreaks in vegetables, fruits, and salads [14, 15], and is occasionally caused by exposure to soil or groundwater contaminated with feces [16]. Moreover,

many vegetables and fruits have complex surfaces that prevent removal of contaminating substances, and therefore simple water washing does not sufficiently eliminate viral titers [17].

Norovirus has been recently detected in kimchi (a traditional Korean fermented cabbage), which is processed and manufactured from fresh agricultural products [18, 19]. Despite the health benefits of kimchi, there is a risk of viral contamination owing to some of its ingredients being washed with unhygienic underground water. Although kimchi is commonly believed as microbiologically safe following the fermentation process, an outbreak associated with HuNoV was reported at several schools in 2013 in Jeonju, Republic of Korea, and was traced to cabbage kimchi infected with norovirus GI.4 [19]. Five acute gastroenteritis outbreaks occurred at several schools in Gyeonggi-do, with norovirus-contaminated cabbage kimchi as the cause in May 2011, [18].

Although norovirus infection may occasionally occur, kimchi is a popular and nutritious fermented vegetable food. Many kinds of lactic acid-producing bacteria and yeasts are implicated in kimchi fermentation [20]. Fermentation by lactic acid bacteria (LAB) is well known to reduce harmful organisms and hinder the growth of foodborne pathogens [21]. Indeed, increasing acidity and decreasing pH resulting from the growth of LAB during kimchi fermentation could inactivate foodborne pathogens [22]. Temperature can control the growth of LAB in kimchi [23, 24], as well as affect its flavor. However, it is not known at present whether temperature and storage times have an effect on HuNoV in cabbage kimchi, and how non-thermal inactivation during fermentation affects specific pathogenic viruses.

In this study, we stored cabbage kimchi at two temperatures, 4°C and 10°C, to compare the persistence of HuNoV at the two conditions, and investigated the correlation between changes in microbial/physicochemical characteristics and persistence of HuNoV during kimchi fermentation for up to 28 days.

Materials and Methods

Virus Origin

HuNoV stool specimens, containing viral particles of genotype GII.4, were generously provided by Prof. Choi (Chung-Ang University). The concentration of HuNoV GII.4 in human feces was determined by quantitative reverse transcription PCR (RT-qPCR). RNase-free water (Quanta Biosciences, USA) was used to dilute the HuNoV-positive human feces, which were then

vortexed shortly, clarified by centrifugation at 550 ×*g* for 3 min to eliminate debris, and then continuously filtered through sterile Millex-HV 0.45-μm and Millex-GV 0.22-μm Teflon, low protein-binding syringe filters (Millipore, USA). Each supernatant fluid was stored in 1 ml aliquots at −80°C until use.

Sample Preparation and Virus Spiking

Cabbage kimchi samples were purchased from a local grocery store in Gwangju, Korea. Solid samples (10 g), containing leaf and stem, and juice samples (10 ml) were packaged into 50 ml conical tubes. A 210-μl aliquot of HuNoV GII.4 (approximately 3 $\log_{10}$ genomic copies/μl) was inoculated onto whole cabbage leaves and kimchi juice. The samples were then stored at 4°C and 10°C for up to 28 days. They were collected from each container at 0, 1, 2, 3, 5, 7, 10, 14, 21, and 28 days, and measured for titer of HuNoV GII.4, organic acid content, acidity, pH value, salinity, and LAB load.

Recovery of HuNoV GII.4 Particles from Kimchi Samples

For a concentration of HuNoV GII.4 viral particles, the immunomagnetic separation (IMS) method combined with RT-qPCR was performed as demonstrated previously [25]. The RT-qPCR standard curve generated using these serial dilutions of the GII.4 standard RNA transcript were linear, with a slope of -3.5163 and a coefficient of determination (R^2) >0.9945. Viral particles were eluted with 30 ml phosphate-buffered saline (PBS, pH 7.4) from each contaminated leaf, added to the 10 ml kimchi juice in the container, mixed with 50 μl of IMS bead solution, and incubated for 60 min at 25°C with continuous shaking to concentrate HuNoV GII.4. Magnet bead particles with concentrated HuNoV GII.4 were collected with a magnetic stand (PolyATtract System stand; Promega, USA) and washed with PBS. The final bead particles were resuspended in 140 μl of 1× PBS and transferred to a 50-ml sterile conical tube. A suspension of HuNoV GII.4 in 140 μl of PBS without inoculation in kimchi sample was used as a positive process control.

Viral RNA Extraction and RT-qPCR

Viral RNA extraction and RT-qPCR were performed as described previously [25]. The QIAamp MinElute Virus Spin Kit (Qiagen, Germany) was used to extract HuNoV GII.4 viral RNA from each sample following the manufacturer's protocol. The viral RNA was further purified using a QIAamp Mini Column (Qiagen) and eluted with 60 μl of elution buffer (AVE). A 5-μl aliquot of viral RNA was used promptly in a one-step RT-qPCR using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and the Quantitect Probe RT-PCR kit (Qiagen). The RT-qPCR was conducted in a final volume of 20 μl as follows: 50°C for 10 min, 95°C for 5 min, and 40 cycles at 95°C for 10 sec plus combined annealing/extension at 60°C for 30 sec. Negative controls were included in each run. The HuNoV GII.4 primer sequences (10 μM each) were COG 2F (5′-CAR GAR BCN ATG TTY AGR TGG ATG AG-3′) and COG2R (5′-TCG ACG CCA TCT

TCA TTC ACA-3'), which were used to amplify a 122-bp fragment of the HuNoV GII.4 polymerase gene [26]. The TaqMan probe (Ring2, 10 μM) was 5'-FAM TGG GAG GGC GAT CGC AAT CT BHQ-3'. The PCR products were assessed with Applied Biosystems 7500 Fast Real-Time PCR software ver. 2.0.

Organic Acid Content Analysis

Each sample (5 g of juice and 5 g of cabbage) was homogenized in a blender, 25 ml of distilled water was added to 1 g of sample, and organic acids were extracted for 30 min using a sonicator (Power Sonic 520; Hwashin Tech Co., Korea) and filtered twice, through a Toyo No. 1 and a syringe filter (RC, 0.2 μm, 25 mm). A 1260 Infinity/G4212B high-performance liquid chromatography system (Agilent Technologies, USA) with a variable wavelength detector (DAD) set at 210 nm was used. The injection volume was 10 μl. Organic acids were analyzed using an Aminex HP-87H column (300 × 7.8 mm, 9 μm) (Bio-Rad, USA) kept at 50°C. An isocratic elution was performed with 0.008 M sulfuric acid in deionized water as the mobile phase for 30 min (flow rate 0.6 ml/min). Organic acids in the samples were identified by comparing their retention times with those of lactic acid and acetic acid (Sigma, USA), which were used as standards. They were quantified using a calibration curve derived from the peak areas of the standards.

Changes of pH, Acidity, and Salinity

Each sample (5 g of juice and 5 g of cabbage) was homogenized in a blender (HR1372, 700 W; Philips, The Netherlands). The solid contents were filtered through a sterilized gauze and the filtrate was used for subsequent measurements. Acidity and pH were assessed using a pH meter (TitroLine 5000; SI Analytics, Germany). The pH was measured by immersing a pH electrode in cabbage kimchi liquid. Total acidity was determined according to the Association of Official Analytical Chemists (AOAC) method by titrating the sample to pH 8.3 with 0.1 N NaOH (Daejung,

Korea). Titratable acidity was calculated using the following formula:

Titratable acidity (%)=

$$\frac{0.1 \text{ N NaOH (ml)} \times 0.1 \text{ N NaOH factor} \times 0.009}{\text{Sample (g)}} \times 100$$

Salinity was assessed using the Mohr method (AOAC, 2000). Briefly, 1 g of each homogenized sample was serially diluted 100-fold with distilled water and filtered (Toyo No. 1). Then, 10 ml of the filtered solution was added to 1 ml of 2% K_2CrO_4 (Junsei, Japan) and adjusted with 0.02 N $AgNO_3$ (Daejung). An independent blank test with distilled water was also performed. Salinity was calculated according to the following formula:

Salinity (%)=

$$\frac{0.02 \text{ N } AgNO_3 \text{ (ml)} \times 0.00117 \times AgNO_3 \text{ factor} \times \text{dilution rate}}{\text{Sample (g)}} \times 100$$

Assessment of LAB

Each sample was diluted 10-fold with 0.2% peptone water (BD Difco, USA) and used to inoculate on MRS agar (BD Difco). LAB were numerated after each plate was sealed with a CO_2 gas pack (Bedford, USA) and incubated at 37°C for 48 h. Values were multiplied by the relevant dilution factors; results were expressed as colony-forming units per gram of sample (CFU/g).

Statistical Analysis

All experiments were carried out in triplicate and presented as the mean ± standard deviation. Duncan's multiple range test was conducted at $p < 0.05$ using SPSS software ver. 8.2 for Windows (SPSS Institute Inc., USA). A p-value < 0.05 was considered statistically significant. The RT-qPCR data are expressed as $\log_{10}$ genomic copies/μl and were obtained using SigmaPlot software ver. 7.0 (Systat Software, USA). Correlation coefficients were calculated using XLSTAT-Pro software (AddinSoft Inc., USA).

Table 1. pH, total acidity, and salinity of cabbage kimchi spiked with HuNoV GII.4 and stored at 4°C or 10°C for 28 days.

Days post-fermentation	pH		Total acidity (%)		Salinity (%)	
	4°C	10°C	4°C	10°C	4°C	10°C
0	6.02 ± 0.01[a]	6.02 ± 0.01[a]	0.35 ± 0.01[i]	0.35 ± 0.01[i]	1.58 ± 0.01[g]	1.58 ± 0.01[g]
1	5.94 ± 0.01[b]	5.89 ± 0.01[b]	0.39 ± 0.01[h]	0.43 ± 0.01[h]	1.58 ± 0.00[fg]	1.60 ± 0.00[ef]
2	5.86 ± 0.01[c]	5.42 ± 0.01[c]	0.41 ± 0.00[gh]	0.58 ± 0.02[g]	1.60 ± 0.00[de]	1.61 ± 0.00[d]
3	5.83 ± 0.01[d]	4.96 ± 0.01[d]	0.43 ± 0.00[fg]	0.67 ± 0.01[f]	1.59 ± 0.00[efg]	1.60 ± 0.01[f]
5	5.80 ± 0.01[e]	4.56 ± 0.01[e]	0.44 ± 0.01[f]	0.86 ± 0.02[e]	1.59 ± 0.01[efg]	1.61 ± 0.00[d]
7	5.73 ± 0.01[f]	4.26 ± 0.01[f]	0.47 ± 0.00[e]	1.08 ± 0.04[d]	1.60 ± 0.00[ef]	1.61 ± 0.00[de]
10	5.29 ± 0.01[g]	4.15 ± 0.02[g]	0.73 ± 0.00[d]	1.22 ± 0.04[c]	1.61 ± 0.00[d]	1.63 ± 0.01[c]
14	4.70 ± 0.01[h]	4.12 ± 0.01[h]	0.77 ± 0.01[c]	1.32 ± 0.02[b]	1.63 ± 0.01[c]	1.65 ± 0.01[b]
21	4.36 ± 0.01[i]	4.09 ± 0.01[i]	1.13 ± 0.04[b]	1.36 ± 0.01[b]	1.65 ± 0.01[b]	1.68 ± 0.01[a]
28	4.26 ± 0.01[j]	4.06 ± 0.01[j]	1.22 ± 0.01[a]	1.50 ± 0.01[a]	1.67 ± 0.01[a]	1.68 ± 0.01[a]

[a-k]Different letters within the same column indicate significant differences ($p < 0.05$).

Results and Discussion

Changes in Physicochemical Properties Following 28 Days of Storage at 4°C or 10°C

Changes in pH, total acidity, and salinity of cabbage kimchi inoculated with HuNoV GII.4 and stored for 28 days at either 4°C or 10°C are presented in Table 1. Up until day 14, the pH gradually decreased from 6.02 to 4.70 at 4°C, and from 6.02 to 4.12 at 10°C. Total acidity and pH can be used to determine the degree of cabbage kimchi fermentation. A pH value below 4.0 indicates over-fermentation [27]. After 28 days at 4°C and 10°C, the pH dropped to 4.26 and 4.06, respectively. The drop in pH was similar to that reported previously for non-inoculated cabbage kimchi [28]. The results indicate that pH tends to decrease significantly ($p < 0.05$) with length of storage.

Generally, total acidity was inversely proportional to pH. The inverse proportion between total acidity and pH is thought to depend on the buffering action of proteins and amino acids in kimchi [29]. Total acidity increased significantly ($p < 0.05$) from 0.35% on day 0 to 0.77% on day 14 at 4°C, and from 0.35% to 1.32% at 10°C. The increase was caused by lactic and acetic acids generated during fermentation. Finally, after 28 days, total acidity increased to 1.22% and 1.50% at 4°C and 10°C, respectively. Thus, storage at 10°C accelerated the kimchi fermentation process.

As reported in Table 1, the salt content at 4°C and 10°C was 1.58% and 1.67–1.68% at 0 and 28 days, respectively, suggesting that it did not change with time or temperature. This finding was in accordance with previous studies, whereby the kimchi liquid and other ingredients maintained salinity at 2% in kimchi supplemented with abalone and kelp [30].

Cabbage kimchi showed an early rapid drop in pH and an increase in total acidity, which can be explained by the production of lactic acid during the early fermentation phase (Table 2). During the first days, cabbage kimchi contained only acetic acid; specifically, 2,530.5 mg/kg at 4°C and 2,498.6 mg/kg at 10°C. The amount of acetic acid gradually increased to 3,537.7 mg/kg at 4°C and 3,705.9 mg/kg at 10°C after 28 days. The degradation of carbohydrates, a major component of Chinese cabbage, results in the formation of numerous organic acids and gives a unique flavor to kimchi [31–33]. According to Jeong *et al.* [34], lactic acid is the main organic acid at the last stage of ripening. Storage at both temperatures led to a continuous increase in lactic acid content between day 0 and day 28, reaching a maximum of 5,739.3 mg/kg at 4°C and 9,971.0 mg/kg at 10°C. The amount of lactic acid was significantly ($p < 0.05$) higher in samples kept at 10°C than at 4°C. These findings indicate that the fermentation rate of cabbage kimchi is influenced by storage temperature.

Correlation between Changes in LAB and Persistence of HuNoV during Fermentation

There has been only limited study regarding the virucidal effects and viability of kimchi fermentation against foodborne viruses, including HuNoV. Thus, we investigated structurally intact HuNoV GII.4 in kimchi stored at 4°C and 10°C for up to 28 days and evaluated the HuNoV GII.4 recovery using a HuNoV GII.4 monoclonal antibody-conjugated IMS method. This sensitive detection technique has been applied since

Table 2. Organic acids content in cabbage kimchi spiked with HuNoV GII.4 and stored at 4°C or 10°C for 28 days.
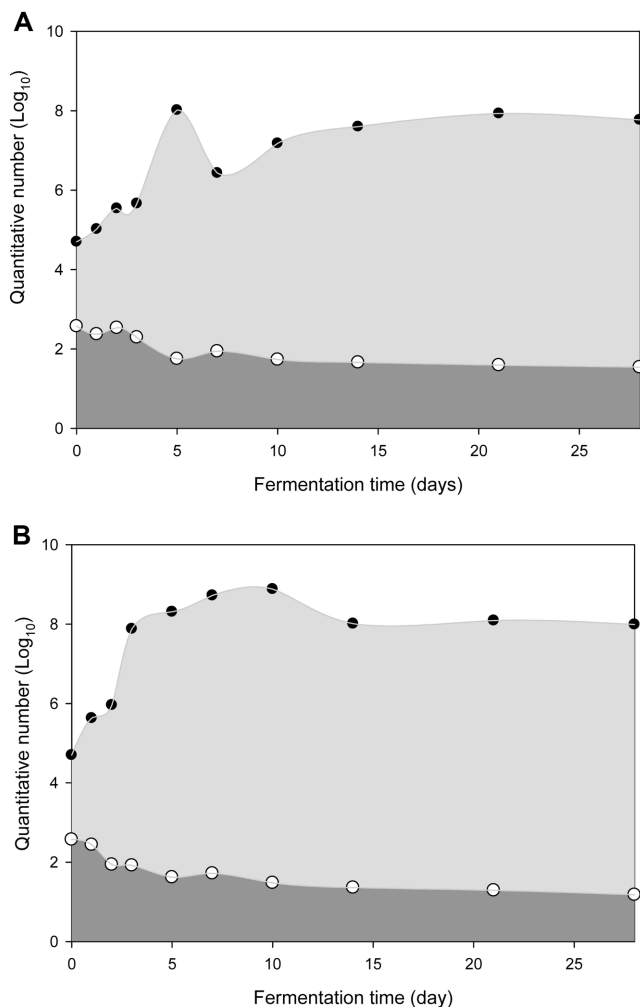
Days post-fermentation	Organic acid in kimchi (mg/kg)			
	Lactic acid		Acetic acid	
	4°C	10°C	4°C	10°C
0	0.0[f,x]	0.0[i,x]	2,530.5 ± 24.7[f,x]	2,498.6 ± 82.1[f,x]
1	0.0[f,x]	0.0[i,x]	2,642.3 ± 54.9[e,x]	2,566.8 ± 26.9[ef,x]
2	0.0[f,y]	1,298.6 ± 22.7[h,x]	2,788.3 ± 24.3[d,x]	2,607.5 ± 76.1[e,y]
3	0.0[f,y]	2,504.9 ± 10.7[g,x]	2,814.6 ± 88.4[d,x]	2,736.7 ± 90.2[d,x]
5	0.0[f,y]	6,347.8 ± 49.3[f,x]	2,823.8 ± 37.9[d,y]	2,936.4 ± 38.9[c,x]
7	463.6 ± 6.1[e,y]	7,213.1 ± 8.0[e,x]	2,795.6 ± 43.5[d,y]	2,968.5 ± 21.9[c,x]
10	1,358.0 ± 20.3[d,y]	8,496.4 ± 43.1[d,x]	2,822.8 ± 31.4[d,y]	3,569.1 ± 22.4[b,x]
14	3,132.6 ± 26.8[c,y]	8,636.1 ± 60.6[c,x]	2,963.2 ± 31.6[c,y]	3,638.7 ± 25.2[ab,x]
21	5,239.3 ± 16.9[b,y]	8,833.6 ± 80.7[b,x]	3,139.3 ± 31.6[b,y]	3,600.9 ± 31.9[b,x]
28	5,739.3 ± 16.6[a,y]	9,971.0 ± 47.9[a,x]	3,537.7 ± 38.1[a,y]	3,705.9 ± 70.1[a,x]

[a–e]Different letters within the same column indicate significant differences ($p < 0.05$).

[x–z]Different letters within the same row indicate significant differences ($p < 0.05$).

its initial development in 1973 by Tompkin [35]. The IMS method has been applied to take the place of enhancement in selective growth media [36] and, more recently, porcine gastric mucin-magnetic beads binding followed by RT-qPCR assay has been used to collect and quantify potentially infectious HuNoV strains GI.1 and GII.4 and reduce inhibitors from samples [37]. Accordingly, various studies using magnetic beads binding followed by RT-qPCR assay have shown that magnetic beads conjugated with ligand could capture infectious and structurally intact viruses and effectively eliminate RT-qPCR inhibitors [37–39]. The results of the present research demonstrate that HuNoV GII.4 was detected and recovered from all kimchi samples stored at 4°C and 10°C up to 28 days.

To assess the correlation between changes in population of LAB ($\log_{10}$ CFU/g) and persistence of HuNoV ($\log_{10}$ genomic copies/µl), cabbage kimchi was inoculated with HuNoV GII.4. As shown in Figs. 1A and 1B, the LAB density had increased continuously during fermentation and negatively correlated with the recovery of structurally intact HuNoV GII.4. The Pearson correlation coefficient between LAB and HuNoV GII.4 was -0.757 at 4°C and -0.913 at 10°C (data not shown). Cho and Rhee [40] reported a decrease in pH in relation to an increase in LAB during kimchi fermentation. The LAB population was significantly ($p < 0.05$) larger in cabbage kimchi stored at 10°C than at 4°C. Storage temperature affects the growth rate of LAB in kimchi [23, 24]. The titer of the NoV GII.4 in 140 µl of PBS solution as positive control was 2.66 $\log_{10}$ genomic copies/µl. On day 0, the LAB density was 4.70 $\log_{10}$ CFU/g and HuNoV GII.4 recovered from kimchi samples was quantified at 2.57 $\log_{10}$ genomic copies/µl, at both temperatures. The HuNoV GII.4 titers decreased significantly ($p < 0.05$) with stepwise increases in storage period at both temperatures. The minimum HuNoV GII.4 titer was 1.58 and 1.04 $\log_{10}$ genomic copies/µl at 4°C and 10°C, respectively, after 28 days (Figs. 1A and 1B). For the LAB population of samples kept at 4°C, growth of LAB was continuously observed up to 5 day (maximum 8.01 $\log_{10}$ CFU/g). Similarly, the LAB population at 10°C increased gradually up to 10 days (maximum 8.88 $\log_{10}$ CFU/g). However, the LAB population in samples stored at 4°C and 10°C differed significantly ($p < 0.05$) in all samples (data not shown). Kimchi was previously reported to over-ferment slowly at low storage temperatures (5°C), but over-ferment quickly at higher temperatures (10–30°C) [23]. These results indicate that the degree of fermentation can be controlled by temperature. Previous studies have emphasized that changes in pH, acidity, thermoacidophilic bacteria, and LAB can reduce the viability of pathogens in fermented vegetables [41, 42]. According to Gagné et al. [43], the viral particles of MNV-1 in sauerkraut with 1%, 1.5%, and 2% sodium chloride proceeded with fermentation for up to 90 days and showed a 1 $\log_{10}$ PFU reduction. Our results demonstrate that HuNoV is able to be reduced under kimchi fermentation conditions. According to Shin et al. [44], unknown secondary products related to lactic acid fermentation and antiviral compounds originating from seasoning ingredients could contribute to reduced viral infectivity. However, our findings were not able to demonstrate that an increase of LAB population specifically influences the reduction of HuNoV GII.4 titers, although LAB density negatively correlated with recovery of HuNoV GII.4. Therefore, further studies are needed to determine whether the LAB property
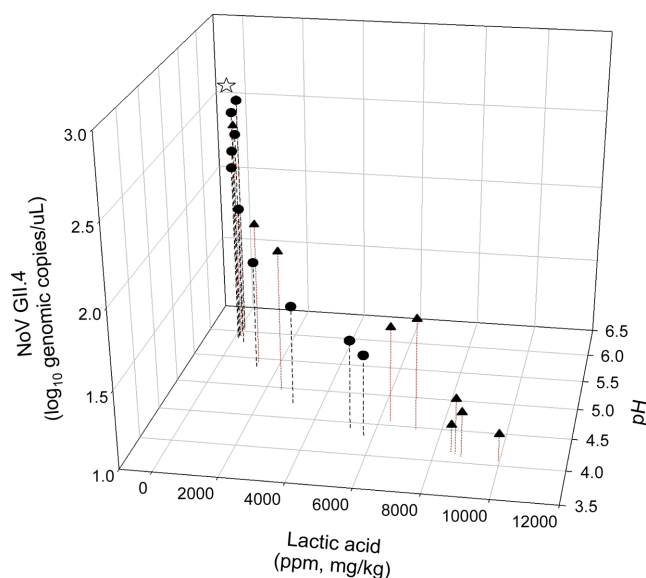

Fig. 1. Correlation between lactic acid bacteria (●) and HuNoV GII.4 (○) in cabbage kimchi during fermentation at 4°C (**A**) and 10°C (**B**).

isolated from kimchi as a single factor affects the population of HuNoV.

Correlation between Organic Acid Content, pH, and Persistence of HuNoV during Fermentation

To assess the effect of organic acids and pH on the persistence of HuNoV GII.4, cabbage kimchi was inoculated with HuNoV GII.4 and then fermented and stored at either 4°C or 10°C. Correlations between these factors are shown in Figs. 2 and 3. Acetic acid was already present at day 0 and increased gradually over the next 28 days at both storage temperatures, whereas lactic acid was not produced during the first 5 days at 4°C and 2 days at 10°C. However, it eventually reached higher levels in cabbage kimchi stored at 10°C than at 4°C ($p < 0.05$). A correlation coefficient of 0.8–1.0 indicates a very strong relationship, whereas a value of 0.6–0.8 supports a strong relationship [45]. The correlation coefficient between lactic acid and HuNoV GII.4 was -0.837 at 4°C and -0.951 at 10°C. For acetic acid, the values were -0.766 at 4°C and -0.931 at 10°C (data not shown). The reduction of HuNoV GII.4 was 0.54 $\log_{10}$ genomic copies/µl higher in kimchi fermented at 10°C than at 4°C. Moreover, the populations of HuNoV GII.4 decreased with increasing the amount of organic acid and with lowering the pH over time at both temperature (Figs. 2 and 3). These results reflect those of Shin *et al.* [44], who reported that the feline calicivirus (FCV) was reduced to 4–7 $\log_{10}$ plaque-forming units/ml during kimchi

fermentation supplemented with oysters. According to that study, the main factors responsible for reducing the virus viability were high acidity conditions and the low pH caused by organic compounds during kimchi fermentation. Substances produced by kimchi fermentation could decrease the chance of outbreak of human gastroenteritis. By analyzing saturation transfer differences using nuclear magnetic resonance, Hansman *et al.* [46] demonstrated that treatment with citrate diminished the affinity of HuNoV capsid protein for histo-blood group antigens. It is thought that changing the affinity of the capsid protein represents an antiviral mechanism elicited by organic acids. Indeed, organic acids, such as fumaric, pyroglutamic, benzoic, citric, lactic, salicylic, acetic, and malonic acids, have well-known antiviral properties against enveloped and non-enveloped viruses [47]. Poschetto *et al.* [48] have reported that a 0.5–1 h treatment with a 4–5% commercial organic acid product (composition of 7% glycoxylic acid and 55–60% formic acid) can reduce HuNoV G.II and FCV by 3 $\log_{10}$ units. Additionally, the levels of *Escherichia coli, Listeria monocytogenes, Clostridium perfringens* [49], *Enterobacter cloacae* [50], *Salmonella* spp. [50, 51], *Staphylococcus aureus* [49, 50], and *Vibrio parahaemolyticus* [51] were also reduced during kimchi fermentation. FCV, a HuNoV surrogate, is unstable under low pH and acidic environmental conditions [52]. At low pH values of 2, 3, and 4, FCV in particular was reduced by 4.4, 3.7, and 2.3 $\log_{10}$, respectively [53]. According to Park *et al.* [54], an acidic condition (0.9–6% acetic acid)

Fig. 2. Correlation between lactic acid, pH, and HuNoV GII.4 in cabbage kimchi during fermentation at 4°C (●) and 10°C (▲); positive control (☆).
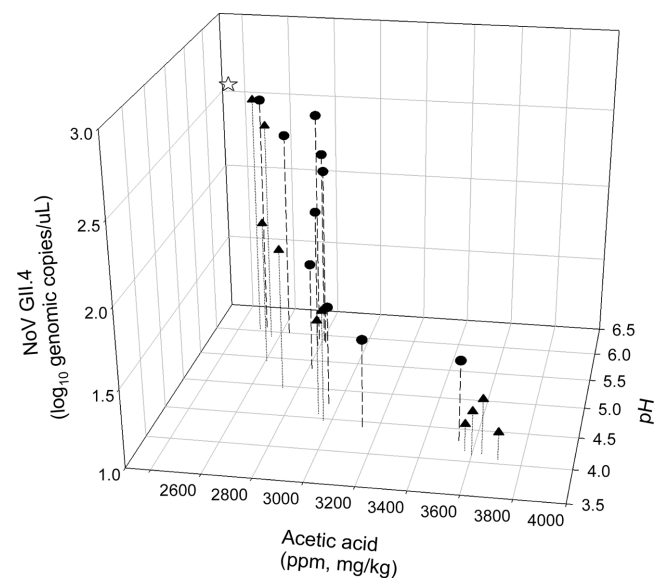
Fig. 3. Correlation between acetic acid, pH, and HuNoV GII.4 in cabbage kimchi during fermentation at 4°C (●) and 10°C (▲); positive control (☆).

resulted in an approximately 1 $\log_{10}$ PFU/ml reduction of MNV-1 for 3–5 days of storage at 4°C. This and previous studies have shown a statistical correlation between changes in physicochemical properties during kimchi fermentation and the recovery of structurally intact HuNoV GII.4. Therefore, we determined that the production of organic acids and the low pH could potentially reduce the quantity of HuNoV GII.4 during sufficient fermentation at 10°C.

In conclusion, to our best knowledge, this is the first study to demonstrate a link between changes in organic acid content, LAB, pH, acidity, and salinity and the recovery of structurally intact HuNoV GII.4 in cabbage kimchi during kimchi fermentation. Our results indicate that the changes in the physicochemical properties during kimchi fermentation could affect the reduction of HuNoV GII.4 titers. In addition, production of organic acid at 10°C was significantly greater than at 4°C. A strong correlation between microbial and physicochemical properties and reduction of HuNoV titers was observed in all kimchi samples, even though HuNoV GII.4 was not completely eliminated. Based on this, we speculate that the persistence of HuNoV GII.4 may be affected by the continually changing conditions during kimchi fermentation.

Acknowledgments

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