Microbial population dynamics of kimchi, a fermented cabbage product

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Abstract
Lactic acid bacteria are known to perform significant roles in the fermentation of kimchi, a fermented cabbage product. However, the microbial population dynamics inherent to kimchi fermentation remain to be clearly elucidated. In this study, we have characterized the microbial dynamics via the identification of a total of 970 bacterial isolates, representing 15 species of the genera Lactobacillus, Leuconostoc, and Weissella, all of which were primarily identified by PCR-based restriction enzyme analysis. These population dynamics appear to be influenced markedly by fermentation temperature. Distinct biphasic microbial growth was observed with preliminary 2-day incubation at 15 °C, conducted before main fermentation at 10 °C. Leuconostoc citreum, as well as Leuconostoc gasicomitatum, predominated during the first growth phase, whereas Weissella koreensis predominated during the second phase. By way of contrast, with preliminary 4-day incubation at 10 °C, only W. koreensis grew rapidly from the beginning of the process. Therefore, our findings suggest that a short incubation at 15 °C enhances the growth of the less psychrophilic Leuconostoc species, including Lc. citreum, thus delaying the growth of the predominant W. koreensis, which is a more adaptive species at −1 °C.

Introduction
Kimchi, a fermented cabbage product, is made of Chinese cabbage, which is stuffed with a host of different spices, including garlic, ginger, and hot red pepper (Cheigh & Park, 1994). The classical identification of bacterial isolates from kimchi revealed that Leuconostoc mesenteroides and Lactobacillus plantarum were the predominant species in kimchi (Lim et al., 1989; Lee et al., 1992). However, other studies exploiting molecular identification methods have reported that a variety of Leuconostoc species, including Lc. citreum, Lc. gasicomitatum/Lc. gelidum and Lb. sakei were detected in several different kimchi samples (Kim et al., 2000a; Choi et al., 2003). Two recent studies, both of which used culture-independent identification methods, have confirmed these previous findings, and indicated that the majority of bacterial cells in kimchi are culturable (Kim & Chun, 2005; Lee et al., 2005). One recently discovered change in kimchi is that the fact that Weissella koreensis is now the predominant species in the product, which was not determined before 2001 (Lee et al., 2002).

Kimchi has traditionally been produced only during the winter, because of problems with both fermentation and preservation. The preservation problem was solved, in part, by the proliferation of modern refrigerators. However, it remained difficult to control fermentation temperatures in the typical home. Therefore, special refrigerators, termed ‘kimchi refrigerators’, have recently come into markets. These refrigerators are equipped with temperature-controlling programs. Although the use of kimchi refrigerators for the control of kimchi fermentation has recently become quite popular in Korea and is generally considered satisfactory, the population dynamics and major roles of predominant species during fermentation in kimchi refrigerators still has to be clearly elucidated.

In this study, we have conducted an examination into the microbial population dynamics of kimchi that had been fermented in a kimchi refrigerator.

Materials and methods
Preparation of kimchi and fermentation in a kimchi-refrigerator
Kimchi was prepared at a kimchi factory system, using the following ingredients: Chinese cabbage (74.5%), radish (13.5%), garlic (2.0%), ginger (0.5%), onion (2.0%), green onion (1.0%), red pepper powder (3.0%), leek (0.5%), shrimp paste (1.5%), anchovy paste (0.5%), and sucrose.
The cabbages were steeped in 10–14% salt water for 16–18 h, and then washed in water three times. Kimchi fermentation and storage were conducted using a chest-type kimchi refrigerator (DC-R1566DCR, WiniaMando, Asan, Korea). This refrigerator, which features two separate chambers, is able to control chamber temperature in accordance with a program. For this study, the temperature of one chamber was set to 10°C for 4 days, after which a programmed temperature reduction to −1°C occurs over 12 h (this is referred to as the 10°C program), while the temperature in the other chamber was set to 15°C for 2 days, and then programmed to reduce the temperature to −1°C over 24 h (referred to as the 15°C program). For this study, 160 kg of kimchi was divided into 8 aliquots, and then four aliquots were put into each chamber.

Isolation of lactic acid bacteria and physiological tests

The viable cell numbers, sugar contents, and fermentation products were measured using 500 g of kimchi at days 0, 2, 4, 8, 15, 30, 45, 60, 75, and 90. The 500 g kimchi samples were squeezed with an electric juicer (DA502, Donga Co., Seoul, Korea) in order to obtain liquid juice. Viable cell numbers were measured in CFU, after the incubation of serial dilutions of juice on MRS agar plates (Difco, Franklin Lakes, NJ) at 25°C. In order to analyze microbial compositions at a given fermentation time, about 30 colonies were isolated from a suitable plate and after identification, the relative portion of each species was calculated. To avoid possible bias with regard to colony selection, we isolated almost all of the colonies from a given plate, or from a sector of the plate. All isolates were stocked in 20% glycerol solution at −70°C, pH was measured at 20°C using a pH meter (Orion 720, Thermo Electron, Waltham, MA). The fermentation substrates and products were analyzed via high performance liquid chromatography (Orom 2000Q LC, Orom, Seoul, Korea), under the following conditions: a 20 μL sample was injected into a column (Shim-pack SCR-102H, 300 × 8.0 mm; Shimadzu, Kyoto, Japan) and eluted at a flow rate of 0.8 mL min⁻¹, using 0.1% H₃PO₄ (Aldrich, St Louis, MO) as an eluent, at a pressure of 0.8 mL min⁻¹, at a temperature of 60°C. Organic acids were detected at 210 nm with an UV detector (SPD-10A, Shimadzu) and sugars were separated (Aminex HPX-87 K; Bio-Rad, Hercules, CA) at a flow rate of 0.5 mL min⁻¹ at 65°C, and detected via refractive index detection (RID-10A, Shimadzu). The sugar-usage patterns of the isolates were determined using the API 50 CHL system (BioMerieux, Marcy l’Etoile, France) at 25°C.

Identification of isolates via amplified 16S rRNA gene-based restriction enzyme assay

Chromosomal DNAs were extracted by a method described previously (Kim et al., 2000a), or by boiling bacterial cultures for 10 min in the presence of chelex-100 (Bio-Rad). The PCR amplifications targeted at a 16S rRNA gene, and the subsequent identification of species via restriction enzyme analyses were described previously for Leuconostoc species (Jang et al., 2003), Weissella species (Jang et al., 2002), and Lactobacillus sakei (Lee et al., 2004). For L. pentosus and L. plantarum, multiplex PCR assays were conducted as described by Torriani et al. (2001). The identification of some isolates (five isolates per each species) via the PCR-based restriction enzyme analysis protocol used in this study was confirmed by a polyphasic method, which included DNA–DNA hybridization and 16S rRNA gene sequencing (Kim et al., 2000a). Isolates unidentified by PCR-based restriction enzyme analyses were subjected to further identification via 16S rRNA gene sequencing.

Results

Microbial population dynamics during kimchi fermentation

The primary objective of this study was to monitor microbial population dynamics in kimchi samples fermented in a kimchi refrigerator. To do this, we first isolated a total of 970 lactic acid bacteria after 10 time intervals during kimchi fermentation at −1°C, after a short (4-day) preliminary incubation at 10°C (10°C program) or for 2 days at 15°C (15°C program). These isolates were then identified via PCR-based restriction enzyme analysis.

All isolates were found to belong to a subset of lactic acid bacteria (a total of 15 species, Table 1) which had previously been isolated from the kimchi samples (Kim et al., 2000b; Choi et al., 2003). These isolates included eight Leuconostoc species (Lc. carnosum, Lc. citreum, Lc. gasomitatum, Lc. gelidum, Lc. inhae, Lc. kimchii, Lc. lactis, and Lc. mesenteroides), three Weissella species (W. cibaria, W. confusa, and W. koreensis), and four Lactobacillus species (Lb. curvatus, Lb. plantarum, Lb. pentosus, and Lb. sakei).

In order to determine the advantages of preliminary kimchi incubation at higher temperatures before main fermentation at −1°C, we simultaneously fermented freshly prepared kimchi using either the 10°C program or the 15°C program. We discovered that, as expected, the microbial compositions of both kimchi samples were essentially similar (Table 1). However, the microbial dynamics of the two kimchi samples were slightly different. One of these differences may be attributable to the increase in total cell numbers seen with preincubation at 15°C, but not at 10°C. Leuconostoc citreum, Lc. lactis, and W. cibaria evidenced substantial growth during the first 2 days at 15°C, and the resultant increased population size resulted in competitive growth at lowered temperatures, in this case, −1°C (Fig. 1b; Table 1). On day 8 of the 15°C program, W. cibaria
exhibited the highest cell number (23%), followed by *Lc. gasicomitatum* (20%). At this point, the numbers of *Lc. citreum* and *Lc. lactis* cells evidenced a decrease, indicating slow growth at −1°C. However, *Lc. citreum* exhibited the highest cell numbers (60%) at day 30, followed by *Lc. lactis* (27%), which suggested slow but continuous growth.

Table 1. Microbial distribution of lactic acid bacterial species during kimchi fermentations

<table>
<thead>
<tr>
<th>Fermentation (days)</th>
<th>0</th>
<th>2 (120)</th>
<th>4 (120)</th>
<th>8 (30)</th>
<th>15 (30)</th>
<th>30 (30)</th>
<th>45 (30)</th>
<th>60 (30)</th>
<th>75 (30)</th>
<th>90 (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lc. carnosum</em></td>
<td>3*</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
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</tr>
<tr>
<td><em>Lc. citreum</em></td>
<td>50</td>
<td>41</td>
<td>36</td>
<td>17</td>
<td>13</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td><em>Lc. gasicomitatum</em></td>
<td>50</td>
<td>46</td>
<td>23</td>
<td>10</td>
<td>7</td>
<td>60</td>
<td>10</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td><em>Lc. gelidum</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>3</td>
<td>23</td>
<td>20</td>
<td>17</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td><em>Lc. inhae</em></td>
<td>–</td>
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</tr>
<tr>
<td><em>Lc. kimchii</em></td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>10</td>
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<tr>
<td><em>Lc. lactis</em></td>
<td>23</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>13</td>
<td>13</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td><em>Lc. mesenteroides</em></td>
<td>7</td>
<td>2</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>–</td>
<td>3</td>
<td>–</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td><em>W. cibaria</em></td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td><em>W. confusa</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td><em>W. koreensis</em></td>
<td>–</td>
<td>2</td>
<td>2</td>
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<tr>
<td><em>Lb. curvatus</em></td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
<td><em>Lb. mesenteroides</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Lb. plantarum</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Lb. sakei</em></td>
<td>–</td>
<td>50</td>
<td>41</td>
<td>17</td>
<td>10</td>
<td>–</td>
<td>7</td>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Lc.*, *Leuconostoc*; *W.*, *Weissella*; *Lb.*, *Lactobacillus*.

% composition of bacterial isolates from a kimchi sample fermented at *10°C* program or *15°C* program. –, not detected.
The number of isolates tested is indicated in parentheses.

Fig. 1. Population dynamics of predominant species in kimchi fermented using the 10°C program (a) or the 15°C program (b). Temperature decreased to −1°C (at day 4.5) from 10°C over 12 h or to −1°C (at day 3) from 15°C over 24 h. –, CFU mL⁻¹; △, pH; ●, Lactobacillus casei; ■, Lactobacillus gasicomitatum; □, Weissella koreensis; ▲, Lactobacillus sakei.
Microbial population dynamics of kimchi

Table 2. Comparison of microbial compositions in different kimchi samples

<table>
<thead>
<tr>
<th>Species</th>
<th>Microbial isolates (%)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
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<td></td>
<td></td>
<td>(n=117)</td>
<td>(30)</td>
<td>(202)</td>
<td>(30)</td>
<td>(92)</td>
<td>(36)</td>
<td>(70)</td>
<td>(106)</td>
<td>(30)</td>
<td>(120)</td>
</tr>
<tr>
<td><em>Lc. carnosum</em></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td><em>Lc. citreum</em></td>
<td></td>
<td>3*</td>
<td>63</td>
<td>55</td>
<td>–</td>
<td>16</td>
<td>11</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>68</td>
</tr>
<tr>
<td><em>Lc. gasicomitatum</em></td>
<td></td>
<td>67</td>
<td>–</td>
<td>4</td>
<td>8</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td><em>Lc. gelidum</em></td>
<td></td>
<td>14</td>
<td>13</td>
<td>–</td>
<td>7</td>
<td>–</td>
<td>12</td>
<td>–</td>
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<tr>
<td><em>L. inhae</em></td>
<td></td>
<td>5</td>
<td>–</td>
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<td>–</td>
<td>2</td>
<td>–</td>
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<tr>
<td><em>L. kimchii</em></td>
<td></td>
<td>–</td>
<td>3</td>
<td>7</td>
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<td>–</td>
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<tr>
<td><em>L. lactis</em></td>
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<td>5</td>
<td>3</td>
<td>6</td>
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<tr>
<td><em>Lc. mesenteroides</em></td>
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<td>–</td>
<td>7</td>
<td>18</td>
<td>10</td>
<td>8</td>
<td>14</td>
<td>–</td>
<td>20</td>
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<tr>
<td><em>W. cibaria</em></td>
<td></td>
<td>–</td>
<td>10</td>
<td>17</td>
<td>2</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>W. koreensis</em></td>
<td></td>
<td>4</td>
<td>–</td>
<td>60</td>
<td>7</td>
<td>3</td>
<td>–</td>
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<tr>
<td><em>W. paramesenteroides</em></td>
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<td>–</td>
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<tr>
<td><em>W. soli</em></td>
<td></td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>2</td>
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<tr>
<td><em>Lb. brevis</em></td>
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<td>3</td>
<td>10</td>
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<tr>
<td><em>Lb. curvatus</em></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>16</td>
<td>47</td>
<td>–</td>
<td>29</td>
<td>43</td>
<td>11</td>
<td>–</td>
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<tr>
<td><em>Lb. mali</em></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4</td>
<td>–</td>
<td>2</td>
<td>–</td>
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<td>3</td>
<td>–</td>
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<tr>
<td><em>Lb. paraplantarum</em></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>3</td>
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<tr>
<td><em>Lb. pentosus</em></td>
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<td>–</td>
<td>5</td>
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<tr>
<td><em>Lb. planatarum</em></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Lb. sakei</em></td>
<td></td>
<td>2</td>
<td>1</td>
<td>23</td>
<td>26</td>
<td>14</td>
<td>92</td>
<td>50</td>
<td>33</td>
<td>8</td>
<td>–</td>
</tr>
</tbody>
</table>

Lc., Leuconostoc; W., Weissella; Lb., Lactobacillus.

% composition of bacterial isolates from kimchi samples.

The number of bacterial isolates tested is indicated in parentheses. Kimchi samples are listed as sample A (8°C – fermentation temperature, November-packaged time), B (15°C, November), C (20°C, November), D (15°C, April), E (8°C, July), F (20°C, July), G (10°C, pH 3.8, August), H (15°C, September), I (15°C, October), and J (15°C, November). Only three kimchi samples (A, B, and C) were prepared in our lab and other commercial kimchi samples were purchased as freshly distributed packages. Bacterial isolates were obtained at several time points (pH 5.0–3.8) during fermentation for all kimchi samples, with the exception of sample G. Isolates from sample G were obtained at pH 3.8.

The number of bacterial isolates tested is indicated in parentheses.

In order to ascertain whether or not *W. koreensis* also predominated during the later stages of fermentation in other kimchi samples, we tested ten different kimchi samples, which were prepared during different seasons and fermented in different environments. *Weissella koreensis* was the predominant species in only one commercially prepared kimchi sample, which had been packaged in April (Table 2). Moreover, *W. koreensis* was not detected in six out of 10 kimchi samples. However, *Lb. sakei* was detected in at least seven out of 10 kimchi samples (8–92%), indicating the general emergence of *Lb. sakei* in acidic environments (pH 3.8–pH 4.0; Table 2). *Leuconostoc citreum*, however, predominated in three samples (55–66%), all of which had been prepared in November.
Kinetics of sucrose-usage and acid production during kimchi fermentation

Sucrose (about 1%) is usually added to modern-style kimchi. In order to determine the role of sucrose in the process of kimchi fermentation, we analyzed sucrose contents during fermentation via high-performance liquid chromatography. Almost all of the sucrose was consumed during the early stages of fermentation (by 15 days), eliciting a rapid increase in the total cell numbers in the 15°C program, whereas a similar amount of sucrose was consumed very slowly (by 45 days) in the 10°C program (Figs 2a and b). This difference may be induced by the predominance of Leuconostoc species during this period in the 15°C program, as another major population, W. koreensis, is incapable of fermenting sucrose, although it does utilize a small amount of sucrose, for dextran production (Lee et al., 2002). Rapid sucrose consumption was observed primarily in Lc. citreum, Lc. gascomitatum, and W. cibaria in the 15°C program, whereas slow consumption was observed, and attributed to the predominant W. koreensis, in the 10°C program. Meanwhile, higher levels of acetate were generated during the late stages of fermentation in the 10°C program (62 mM) than in the 15°C program (42 mM). This suggests different population dynamics between the two fermentation programs (Figs 2a and b).

As kimchi fermentation is accomplished solely by lactic acid bacteria, the products of fermentation are somewhat limited. Lactate, acetate, ethanol, and mannitol were detected (Figs 2a and b). The amounts of lactate and acetate were closely correlated with the growth of total populations. However, ethanol levels were not determined to have increased after the complete consumption of the sucrose, thereby indicating that fructose may have been used as an electron acceptor to produce mannitol in the later stages of fermentation (Figs 2a and b).

Discussion

This study demonstrates that kimchi fermentation is governed by the distinct population dynamics of a subset of Lactobacillus, Leuconostoc, and Weissella species. Two Leuconostoc species, namely Leuconostoc citreum and Lc. gascomitatum, as well as Weissella cibaria, predominated during the first stages of fermentation (> pH 4.6), whereas W. koreensis became predominant later, in the 15°C program (Fig. 1b; Table 1). Surprisingly, the microbial succession pattern was strikingly different in the 10°C program, even though all other conditions but temperature were identical to those in the 15°C program. Neither Leuconostoc species nor W. cibaria were dominant species, whereas only W. koreensis predominated throughout the entirety of the fermentation period. This may indicate that, unlike the majority of Leuconostoc species, W. koreensis has the capacity to grow even under very stressful conditions, e.g. −1°C and < pH 4.3, under which microbial diversity should be markedly reduced because a few well-adapted species tend to outnumber other existing species. These results also suggest that fermentation temperature is one of the primary determinants of microbial populations in kimchi, and that complex microbial succession is not crucial for kimchi fermentation. This conclusion is supported by previous results suggesting that a single Lc. citreum strain, as a starter, was sufficient to govern the entire process of kimchi fermentation (Choi et al., 2003).

It is notable that W. koreensis has only recently been detected in kimchi (Lee et al., 2002). This can be explained by the rarity of this species in most of the previously examined kimchi samples, which were fermented at temperatures above 8°C (Kim et al., 2000b; Choi et al., 2003), conditions under which this psychrophilic bacterium might...
find it difficult to compete. This notion is supported by the results of a recent study. Lee et al. (2005) demonstrated that \textit{W. koreensis} was undetectable in laboratory-prepared kimchi fermented at 10 and 20 °C, whereas \textit{Lc. citreum}, \textit{W. confusa}, and \textit{Lactobacillus sakei/Lb. curvatus} were found to predominate in these samples.

Several free sugars, including fructose, glucose, and sucrose, were also detected in the kimchi. Sucrose levels decreased rapidly during the early phases of fermentation, thereby suggesting a substantial growth of sucrose-utilizing bacteria, which would include most lactic acid bacteria, although not \textit{W. koreensis} (Figs 1b and 2b). Overall, it appears rather likely that kimchi fermentation at −1 °C occurs via a principally heterofermentative reaction, which involves the generation of large quantities of acetate (60 or 40 mM at 10 or 15 °C program, respectively) primarily via the reduction of fructose to mannitol (Figs 2a and b; Dols et al., 1997).

Finally, we attempted to determine whether our culturing method may have missed some of the species that actually existed in the kimchi samples, as some populations may have been unculturable under the experimental conditions. Two evidences support that predominant species are culturable. First, increases in the total number of viable cells are closely correlated with increases in the levels of fermentation products, including lactate and acetate (Figs 2a and b). Second, two analyses of microbial communities in kimchi by culture-independent methods revealed that the primary bacterial components include: \textit{Lc. citreum}, \textit{Lc. gascomitatum}, \textit{Lc. gelidum}, \textit{Lb. sakei}, \textit{Lb. curvatus}, \textit{W. confusa}, and \textit{W. koreensis} (Kim & Chun, 2005; Lee et al., 2005). These studies strongly supported our interpretation, that the predominant species in kimchi samples can, indeed, be cultured on MRS medium. However, these culture-independent analyses are also somewhat limited. As we showed in a previous study, PCR amplification cannot be strictly correlated with the ratio of target DNA to total DNA (Lee et al., 2000). As a result, some minor population groups may have been missed.

In conclusion, our results indicate that \textit{W. koreensis}, a psychrophilic bacterium, is probably the dominating species in kimchi produced at −1 °C and the predominance of \textit{Leuconostoc} species, including \textit{Lc. citreum}, observed after a short preliminary incubation at 15 °C, results in a delay of the rapid outgrowth of \textit{W. koreensis} at −1 °C. However, it is also apparent that population dynamics are rather sensitive to environmental conditions, including fermentation temperature. Therefore, the microbial population dynamics characterized in this study may prove applicable to the improved control of kimchi fermentation and preservation.

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References


