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ORIGINAL ARTICLE

Profiling of Fermentative Metabolites in Kimchi: Volatile and Non-volatile Organic Acids

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Abstract Gas chromatograph/mass spectrometry (GC/MS) analysis was carried out to determine both the volatile and non-volatile organic acids in kimchi during 60 days of fermentation at 10°C. Principal component analysis (PCA) was applied to differentiate the pre-defined organic acids and lactic acid bacteria (LAB) during fermentation. Acetic acid was observed as dominant, which was vigorously produced until the middle of fermentation. Lactic acid was the major non-volatile organic acid in the kimchi and was produced throughout fermentation. In contrast, malic acid content decreased sharply at the initial stage of fermentation. Colony forming units of LAB in the kimchi, such as Leuconostoc, Lactobacilli, Pediococci, and Streptococci, were measured on selective media. Populations of Leuconostoc and Lactobacilli increased exponentially over 7 days of fermentation, indicating acetic acid and lactic acid were mainly produced by Leuconostoc and Lactobacilli. PCA demonstrated that acetic acid, propionic acid, lactic acid, butanoic acid, malic acid, Leuconostoc, and Lactobacilli were major components that differentiated the kimchi according to fermentation time.

Keywords gas chromatography-mass spectrometry · kimchi · lactic acid bacteria · organic acid · principal component analysis

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Introduction

Kimchi is a traditional fermented food, and has been one of the most common side dishes for hundreds of years in Korea (Mheen and Kwon, 1984). It is made from various ingredients including salted *Baechu* (also known as Chinese cabbage), radishes, *Jeot-gal* (salted fish), and spices such as garlic, ginger, green onions, and red pepper powder. Kimchi products vary according to their main vegetable ingredients and seasonings, and the most popular type of kimchi is *Baechu* kimchi (Cheigh and Park, 1994). *Baechu* kimchi is very spicy and also provides exceptionally sour, sweet, and carbonated tastes. In addition, it is rich in diverse nutrients including vitamin C, β-carotene, vitamin B complex, calcium, iron, potassium, and dietary fibers (Kim et al., 2006). Kimchi is primarily fermented by LAB such as *Leuconostoc* and *Lactobacillus* that produce various organic acids including lactic acid, acetic acid, propionic acid, benzoic acid, and sorbic acid (Kim et al., 2006). Kimchi ripens rapidly due to the formation of excessive organic acids such as lactic acid and acetic acid, resulting in sourness and texture softening, which affect consumer acceptance as well as the flavor and quality of kimchi (Beuchat and Golden, 1989). Acid formation, routinely determined by measurements of titratable acidity and pH, has been a useful criterion for evaluating the process of kimchi fermentation as well as its quality (Kim et al., 1986; Hong and Park, 2000). Lee et al. (1992) observed that the acidification of kimchi by organic acids such as lactic acid and pyruvic acid can be controlled by the buffering effects of amino acids at the latter stage of fermentation. Therefore, the pH of kimchi drops faster in the early stage of fermentation than in the latter stage, but then becomes stable due to the buffering effects of amino acids as well as neutralization by calcium. The quality of kimchi can be effectively controlled by changing its fermentation conditions including temperature, organic acid content, fermentation period, and number of LAB. In addition, the growth patterns of LAB during kimchi fermentation highly depend on storage temperature and acid production. For example, strains of Leuconostoc, Lactococcus, and Pediococcus proliferate initially and then rapidly decrease after an optimum time of ripening and between 20 and 30°C of storage temperature. The number of LAB increases with the onset of acid production, and properly fermented kimchi contains 10^7–10^9 CFU/g LAB (Lee et al., 1992). Therefore, changes in organic acids during kimchi fermentation can be considered not only as one of the most important factors for determining the quality of kimchi, but also affect the growth of LAB. However, little information has been reported on the profiles of volatile and non-volatile organic acids in kimchi during fermentation.

The objective of the present study was to profile both volatile and non-volatile organic acids in kimchi during fermentation using a gas chromatography-mass spectrometry (GC-MS) data set of pre-defined organic acids combined with a multivariate statistical method.

Materials and Methods

Sample preparation. *Baechu* kimchi samples were obtained from Chonggazip, Doosan Co., Korea. Samples were vacuum-packed (500 g per pack) and then transported in an icebox to our laboratory on the day of manufacture. The *Baechu* kimchi was made with Chinese cabbage, radishes, red pepper, garlic, ginger, green onions, anchovy sauce, shrimp paste, and sea tangle base. They were stored in a kimchi refrigerator (LG 1124 Rhythm Algorithm, LG Electronics, Korea) set at 10°C for 60 days. A 500-g portion of the *Baechu* kimchi (1 pack) was blended (Woorizip Bangatgan, Shinil Co., Korea) and then filtrated through thin cloth (Dasibak, TNC Electronics Co., Korea) to obtain the kimchi samples.

Measurements of pH and titratable acidity. The pH of the samples was measured using a pH meter (Corning pH meter 220, USA), and the titratable acidity was determined by the Association of Official Analytical Chemists (AOAC) method (AOAC, 2000). In brief, 10.0 g of test sample was adjusted to pH 8.3 with 0.1 N NaOH for titratable acidity. Total acidity was calculated as a percentage of lactic acid.

Analysis of volatile organic acids. For the extraction of volatile organic acids, a method developed by Park and Jo (1989) was used with some modifications. A 20-mL of test sample was transferred to a 60-mL vial with a silicon/teflon septum (Supelco, USA) and then maintained at 70°C for 1 h to obtain an equilibrium state. The volatile organic acids were adsorbed for 30 min onto a carboxen/polydimethylsiloxane (CAR/PDMS)-coated solid phase microextraction (SPME) fiber that was inserted into the sealed vial. GC-MS analysis was performed to identify the volatile organic acids using an HP 5890A series II GC-5972 mass selective detector (MSD) (Hewlett-Packard, USA) equipped with a DB-Wax column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness, J&W Scientific, USA). The oven temperature was held at 50°C for 8 min, and increased to 160°C at 4°C/min and then held at 160°C for 1 min. The injector and detector transfer line temperatures were 200 and 250°C, respectively. The ionization energy of 70 eV, mass scan range of 50–550, and scanning rate of 1.4 scan per sec were used. Volatile organic acids such as acetic acid, propionic acid, 2-methylpropionic acid, butanoic acid, and 2-methylbutanoic acid were positively identified by comparing their mass spectral data and retention times to those of authentic standard compounds. For the quantification of each volatile organic acid, the MS was operated in the selected ion monitoring (SIM) mode, and the scanning specific ions are presented in Table 1.

The contents of five volatile organic acids including acetic acid, propionic acid, 2-methylpropionic acid, butanoic acid, and 2-methylbutanoic acid were determined from the peak area ratios for the unlabeled and labeled compounds using stable-isotope dilution assays (SIDA). The internal standards used included [$^{13}C_1$]-acetic acid (99%), [$^2H_3$]-propanoic acid (99%), and [$^2H_7$]-butanoic acid (98%). For the examination of acetic acid, which was unresolved with its corresponding internal standard, the abundances of 45 and 46 at m/z monitored for acetic acid and [$^{13}C_1$]-acetic acid were recalculated by subtracting the contamination from each other. The quantitative data were the mean values of triplicate measurements. Table 1 shows the selective ions of the volatile organic acids in the *Baechu* kimchi and their internal standard compounds.

Analysis of non-volatile organic acids. For the extraction of non-volatile organic acids, a method described by Hope et al. (2005) was used with some modifications. *Baechu* kimchi sample (100 g) was kept at −70°C in a deep freeze dryer (Bondiro, Ilshin Lab Co., Korea) for 24 h to obtain a dried sample. Subsequently, the kimchi powder was pulverized in a blender for 1 min. Three grams of kimchi powder was added to a mixture of 14.0 mL methanol (Fisher Scientific Ltd., 99.9%) and 0.2 mL glutaric acid internal standard [10% (w/w) in methanol] and was heated at 70°C for 25

Table 1 Analytical parameters used in stable isotope dilution assays (SIDA) for volatile organic acids. (*10% water/ethanol solution containing 7 g/L glycerin at pH 3.2, Ferreira et al. (2000); Yonca et al. (2002))

Compound	Selected ion (m/z)	Threshold value (μg/L)^A	Internal stadards	Selected ion (m/z)
Acetic acid	45	200,000	[$^{13}C_1$]-acetic acid	46
Propanoic acid	74	8,100	[$^2H_3$]-propanoic acid	77
2-Methylpropanoic acid	73	2,300	[$^2H_3$]-propanoic acid	77
Butanoic acid	60	173	[$^2H_7$]-butanoic acid	63
2-Methylbutanoic acid	74	33.4	[$^2H_7$]-butanoic acid	63

min, followed by cooling to ambient temperature for 20 min. Fourteen milliliters of deionized water and 7.0 mL of chloroform (Fisher Scientific Ltd., 99.9%) were then added prior to vortexing for 1 min. The mixture was centrifuged (UNION 32R plus, Hanil Science Industrial, Korea) twice at 3000 rpm for 10 min. The aqueous layer was then transferred to a flask before being evaporated in a rotary evaporator to 0.3 mL (EYELSA, Tokyo Rikakai Co., Japan). The aqueous layer was collected and dried completely in a vacuum oven at 40°C for approximately 4 h.

N, *O*-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) is a derivatization agent for the trimethylsilylation of alcohols, alkaloids, amines, carboxylic acids, phenols, and steroids. It reacts with a range of polar organic compounds, replacing active hydrogen with the $-Si(CH_3)_3$(trimethylsily) group. BSTFA and its by-products (trimethylsilytrifluoroacetamide and trifluoroacetamide) are more volatile than other silylating reagents; thus, non-volatile organic acids were analyzed by GC-MS (Blau and King, 1994). A mixture of 200 μL BSTFA containing 1% trimethyl-chlorosilane (TMCS, Supelco) and 200 μL of acetonitrile (Fisher Scientific Ltd.) was added to the dried samples. The samples were then heated at 70°C for 10 min and allowed to cool prior to GC-MS analysis.

GC-MS analysis was performed to identify the non-volatile organic acids using an HP 6890N GC-5975 mass selective detector (MSD) (Hewlett-Packard) equipped with a DB-5MS-fused silica column (30 m length × 0.25 mm inner diameter × 0.25 ìm film thickness, J&W Scientific). The oven temperature was maintained at 5°C for 3 min, and increased to 230°C at 3°C/min, and then held at 230°C for 1 min. The temperatures of the injector and detector transfer lines were 250 and 280°C, respectively. For the sample analysis, 1 μL of derivatized sample was injected using a split ratio of 50 to 1. The organic acids in the kimchi were positively identified by comparing their retention times and mass spectra with those of authentic standards derivatized using the above method. Ionization energy of 70 eV, mass scan range of 50–550, and scanning rate of 1.4 scans per second were used. The relative contents of the non-volatile organic acids in the samples were calculated by comparing their peak areas to that of each internal standard compound on GC-MS total ion chromatograms. The quantitative data are the mean values of triplicate measurements.

Enumeration of microorganisms. In order to obtain the total lactic acid bacteria (LAB) population in the *Baechu* kimchi, four species of LAB including *Leuconostoc mesenteroides* (KCCM 35471)*, Lactobacillus plantarum* (KCCM 11322)*, Streptococcus faecalis* (KCCM 11729), and *Pediococcus pentosaceus* (KCCM 40703T) were evaluated in the present study. The LAB population in the *Baechu* kimchi was examined via counting CFUs of Leuconostocs, Lactobacilli, and Pediococci on selective media, after sample preparation according to a protocol reported in a previous study (Lee, 1996). In brief, the CFUs of Leuconostocs were estimated 2 days after incubation on PES agar medium at 26°C (Miyao and Ogawa, 1988). PES agar medium was used to obtain large colonies. CFUs of Lactobacilli were counted 3 days after incubation at 30°C on modified lactobacillus selection (LBS) agar medium containing sodium acetate and acetic acid (Miyao and Ogawa, 1988). Glacial acetic acid was added to inhibit the growth of Pediococci. CFUs of streptococci and pediococci were counted 3 and 4 days after incubation on m-Enterococcus agar medium at 37 and 35°C, respectively (Slanetz and Bartley, 1957). The red colored colonies were counted as *Streptococcus* due to their reduction activity of 2,3,5-triphenyl tetrazolium chloride (TTC). On the other hand, because *Pediococcus* could not reduce TTC, they appeared as white colored colonies.

Statistical analysis. Analysis of variance (ANOVA) was performed using the general line model procedure in SPSS (version 12.0, SPSS, USA) to evaluate significant differences (α=0.05) in pH and titratable acidity, volatile organic acids, non-volatile organic acids, and LAB in the kimchi according to fermentation time. Principal component analysis (PCA) was performed using SIMCA-P software (Umetrics, Sweden). PCA was applied to the raw data values of the relative peak areas of each organic acid and the number of LAB to clarify differences between the kimchi samples according to fermentation period.

Results and Discussion

Changes in pH values and titratable acidity. Changes in pH and titratable acidity were monitored during fermentation of the kimchi (Fig. 1). The pH was rapidly reduced from 5.88 to 4.51 (t= 7 days), and thereafter it decreased gradually to 3.84. This drop in pH was primarily due to the formation of organic acids from the degradation of sugars during fermentation. The titratable acidity expressed as lactic acid content showed an inverse relationship to the changes in pH, which was initially 0.26% and gradually increased to 1.35% according to the fermentation time. This increase in titratable acidity implied that organic acids with low dissociation constants, such as lactic acid and acetic acid, were formed during kimchi fermentation. In addition, previous studies confirmed that kimchi had the best taste when pH was about 4.2 and titratable acidity reached 0.7% (Mheen and Kwon, 1984; Kim et al., 1994). These studies also demonstrated that over-fermentation was induced below pH 4.0 by homofermentative *L. plantarum* strains that proliferated in the latter stage of fermentation. In the present study, the pH and titratable acidity values were 4.51 and 0.74%, respectively, at 7 days of fermentation, an indication that the kimchi is well-ripened. Therefore, decreases in pH and increases in titratable acidity could be indicative of the overall taste of kimchi.

Changes in volatile organic acids. Figure 2 shows the changes in the relative contents of volatile organic acids in the kimchi during 60 days of fermentation. Acetic, butanoic, 2-methylbutanoic, propionic, and 2-methylpropanoic acids were identified in the kimchi. Overall, the contents of all volatile organic acids showed increasing patterns according to the fermentation time. Acetic acid, which provides sourness to kimchi, appeared to be the most abundant. Its content increased until mid fermentation. Propionic

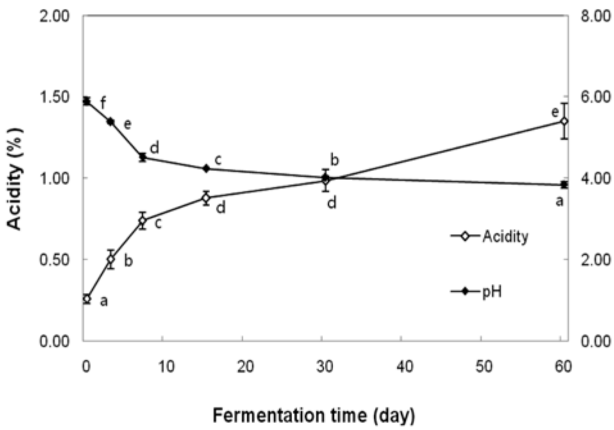

Fig. 1 pH values and titratable acidity content of kimchi during fermentation. (*different letters indicate significant differences according to fermentation time at α=0.05)

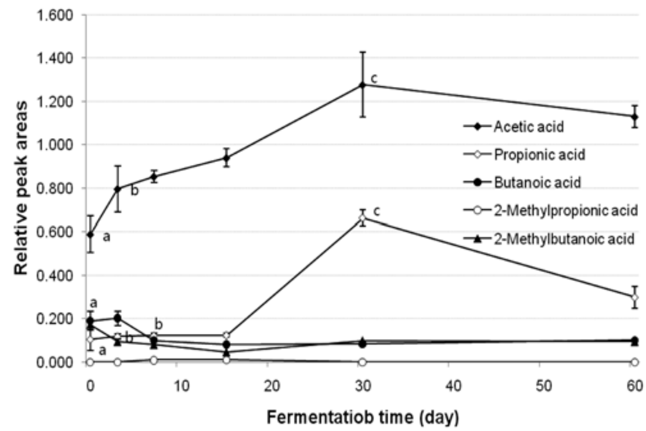

Fig. 2 Changes in contents of volatile organic acids in kimchi during fermentation. (*different letters indicate significant differences according to fermentation time at α=0.05)

acid increased sharply between 15 and 30 days of fermentation. The content of butanoic acid was reduced between 3 and 7 days of fermentation and thereafter remained unchanged. In contrast to our findings, butanoic acid content was the highest among volatile organic acids in *Baechu* kimchi stored at a low temperature (around 1°C) for one year as reported by Kim et al. (2006). It is plausible that differences in either storage temperature or fermentation period can cause different patterns in butanoic acid content. In general, branched-chained organic acids have stronger characteristic odor notes than straight-chained organic acids (Beck, 2005). For example, 2-methylbutanoic acid showed a relatively lower threshold value than butanoic acid in a water/ethanol solution (10 %, v/v) containing 7 g/L glycerin at pH 3.2 (Table 1). In our study, a trivial amount of 2-methylpropionic acid was detected between 7 and 15 days of fermentation, and 2-methylbutanoic acid content decreased throughout the fermentation period.

Branched-chained organic acids such as 2-methylbutanoic acid and 2-methylpropionic acid are mainly derived from branched-chained amino acids including valine, leucine, and isoleucine. For example, 2-methylpropionic acid is formed from valine (Beck, 2005), whereas 2-methylbutanoic acid is produced from leucine catabolism using transamination followed by oxidation steps (Thierry et al., 2004).

Changes in non-volatile organic acids. A total of seven non-volatile organic acids such as lactic, pyruvic, fumaric, succinic, malic, malonic, and tartaric acids were observed in the kimchi during fermentation (Fig. 3). Lactic acid was identified as the primary non-volatile organic acid and its content increased gradually throughout fermentation. The change in titratable acidity was similar to that of lactic acid content, indicating that the titratable acidity of kimchi is mainly influenced by lactic acid. Hence, lactic acid is a useful indicator for assessing the quality of kimchi. Malic acid was produced at the initial stage of fermentation, but its concentration was decreased to zero by the end of fermentation. Similar to our results, Ryu et al. (1984) showed that lactic acid was enhanced as a primary organic acid during fermentation, whereas malic acid was steadily reduced according to the fermentation period. Malic acid can be converted into lactic acid and acetic acid by LAB. Park and Jo (1989) observed that lactic acid and citric acid increased, whereas malic acid and succinic acid decreased during kimchi fermentation. Furthermore, only minute amounts of other non-volatile organic acids including succinic, pyruvic, fumaric, malonic, and tartaric acids remained.

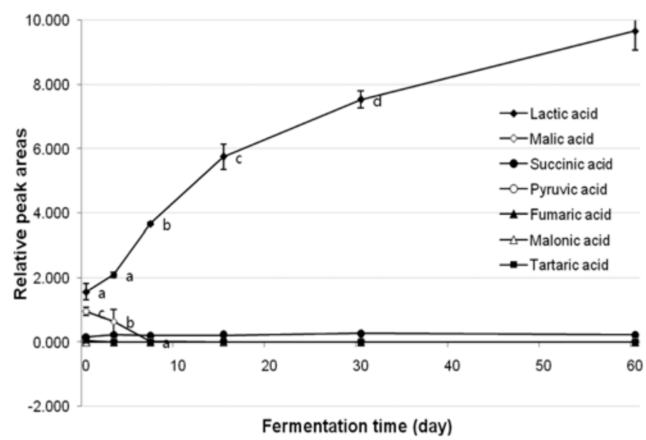

Fig. 3 Changes in contents of non-volatile organic acids in kimchi during fermentation. (*different letters indicate significant differences according to fermentation time at α=0.05)

Changes in microbial populations. Two of the major products produced during kimchi fermentation were acetic acid and lactic acid (Figs. 2 and 3). In order to elucidate how these metabolites were produced, the microbial flora in the kimchi during fermentation was investigated (Fig. 4). As fermentation proceeded, the CFU value of Leuconostocs increased exponentially and reached a maximum of 9.2×10^7 CFU/mL at 7 days of fermentation and thereafter gradually decreased. The overall profile of the Lactobacillus population was similar to that of Leuconostocs, but

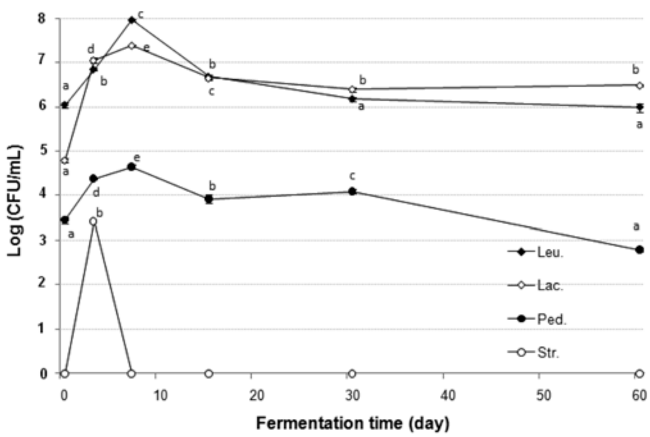

Fig. 4 Changes in the number of lactic acid bacteria in kimchi during fermentation. (*different letters indicate significant differences according to fermentation time at α=0.05)

its CFU value was better maintained until the end of fermentation. This was due to the tolerance of Lactobacilli toward acid stress. The Pediococci population remained two to three orders of magnitude lower as compared to those of Leuconostocs and Lactobacilli. Similar microbial dynamics were observed during the fermentation of *dongchimi*, watery radish kimchi (Park et al., 2008). It has been reported that Leuconostocs are hetero-lactic fermentative bacteria that produce lactic acid as well as acetic acid and ethanol during sugar fermentation (Hemme and Foucaud-Scheunemann, 2004), and Lactobacilli are homo-lactic fermentative or hetero-lactic fermentative bacteria. Therefore, it is assumed that acetic acid and lactic acid, two major products of kimchi fermentation, were mainly produced by Leuconostocs and Lactobacilli.

Principal component analysis (PCA). Original variables can be expressed as a particular linear combination of the principal components (PCs) in score plots, which account for a portion of the total variances in data sets. Plotting data in a space defined in this way provides a rapid means of visualizing similarities or differences in a data set, allowing improved discrimination among samples (Sumner et al., 2003). The kimchi samples taken at each fermentation time were clearly distinguished in the score plot using PCA, showing 64.1% of the total variance (37.4% of PC1, 26.7% of PC2) (Fig. 5). The predetermined organic acids and LAB at the initial stage of fermentation (0 day and 3 day) were separated from those of the latter stage of fermentation (30 and 60 day) mainly in the score of PC1. Fig. 6 illustrates the loading plots of organic acids and LAB contributing to changes for each fermentation period in the PCA. The major components contributing to the PC1 dimension were acetic acid (Ace), propionic acid (Pro), lactic acid (Lac), succinic acid (Suc), malonic acid (Malo), malic acid (Mali), butanoic acid (But), tartaric acid (Tar), and fumaric acid (Fum). Among them, acetic acid, propionic acid, and lactic acid were significantly responsible for the latter stage of fermentation (30 and 60 days) and malic acid and butanoic acid were mainly related to the initial stage of fermentation (0 and 3 days). The important LABs of the PC2 dimension were Leuconostoc, Lactobacilli, and Pediococci. Furthermore, they were found to be the LABs at 7 days of fermentation that mainly contributed to the differentiation of the kimchi during fermentation.

In conclusion, various changes in kimchi were shown by profiling certain pre-defined organic acids and LAB during fermentation. Overall, contents of acetic, propionic, and lactic acids increased as fermentation proceeded, whereas those of malic and butanoic acids decreased. Among the LAB, Leuconostoc, Lactobacilli, and Pediococci reached maximum populations at day 7 of fermentation. Among the volatile organic acids, acetic acid content was the highest and increased gradually throughout the fermentation period. Among the non-volatile organic acids, lactic acid was the major acid in the kimchi and was mainly produced at the beginning of fermentation and increased significantly for the entire fermentation period. Both acetic acid and lactic acid, two major products of kimchi fermentation, were primarily produced

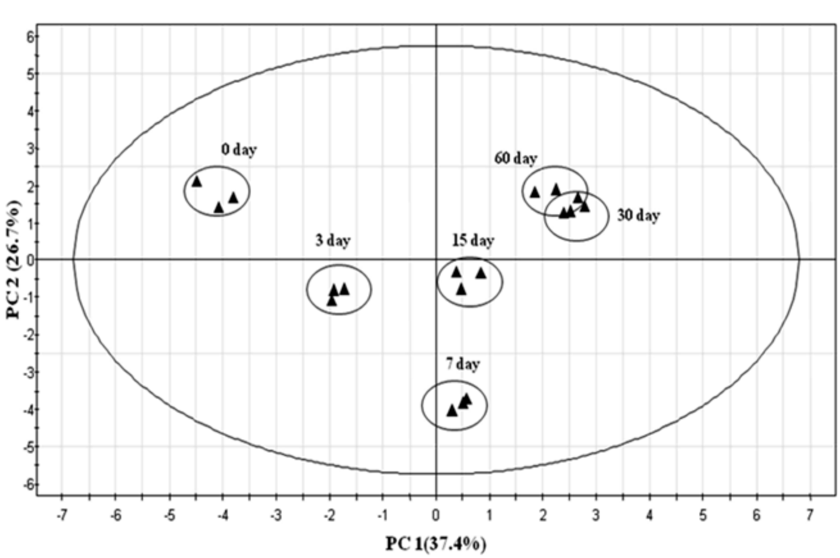

Fig. 5 Score plot of the PCA map for kimchi according to fermentation period.

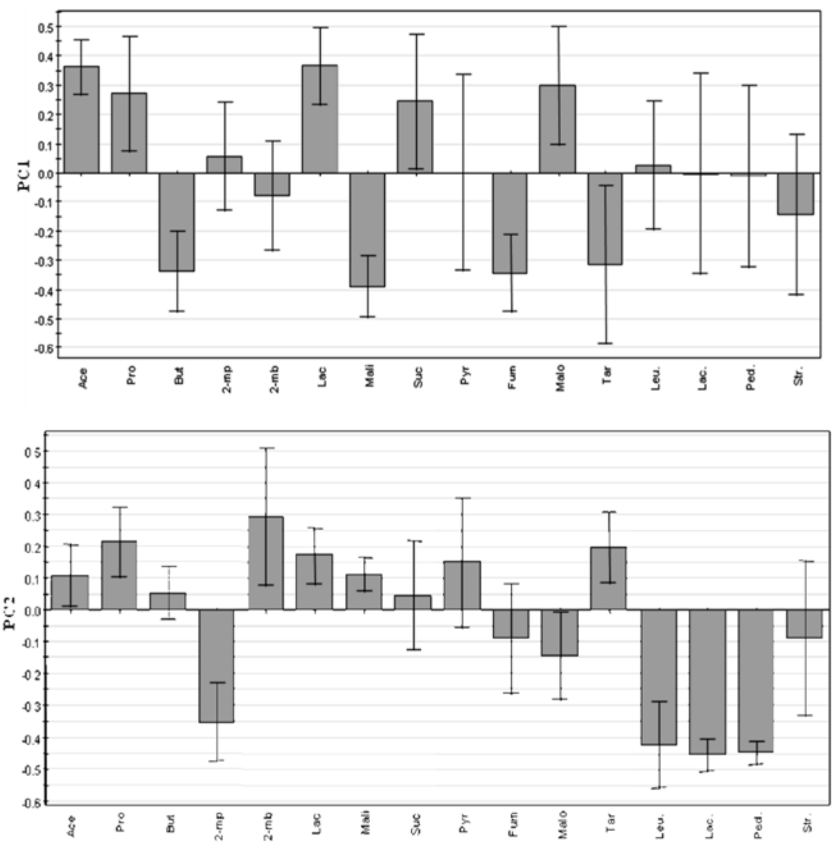

Fig. 6 Variables contributing to the PC 1 and PC 2 score plots. (Acetic acid=Ace, Propionic acid=Pro, Butanoic acid=But, 2-Methylpropionic acid=2-mp, 2-Methylbutanoic acid=2-mb, Lactic acid=Lac, Malic acid=Mali, Succinic acid=Suc, Pyruvic acid=Pyr, Malonic acid=Malo, Tartaric acid=Tar, Leuconostoc=Leu., Lactobacilli=Lac., Pediococci=Ped., Streptococci=Str.). *The column loading plot of PCA for the kimchi according to fermentation period. Bar indicates the contribution levels of individual components to the PCA score and loading plots. The error bar indicates the confidence intervals of individual components in the PCA score and loading plot.

by Leuconostocs and Lactobacilli. PCA demonstrated that acetic, propionic, lactic, butanoic, and malic acids, as well as Leuconostoc and Lactobacilli were the major components that allowed for the discrimination of the different fermentation periods of kimchi. These results can be used to control the quality of kimchi since taste and flavor are strongly related to various changes in organic acid profiles and LAB.

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