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Role of jeotgal, a Korean traditional fermented fish sauce, in microbial dynamics and metabolite profiles during kimchi fermentation

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ABSTRACT

We investigated the effects of jeotgal (fermented fish sauce) on kimchi fermentation, with or without saeu-jeot and myeolchi-jeot. Bacterial community analysis showed that *Leuconostoc*, *Weissella*, *Lactobacillus*, and *Tetragenococcus* were the dominant genera; however, their succession depended on the presence of jeotgal. *Leuconostoc gasicomitatum* was the dominant species in kimchi without jeotgal, whereas *Weissella koreensis* and *Lactobacillus sakei* were the dominant species in kimchi with myeolchi-jeot and saeu-jeot, respectively. Metabolite analysis, using ^{1}H NMR, showed that the amounts of amino acids and gamma-aminobutyric acid (GABA) were higher in kimchi with jeotgal. Increases in acetate, lactate, and mannitol contents depended on fructose consumption and were more rapid in kimchi with jeotgal. Moreover, the consumption of various amino acids affected the increase in kimchi LAB. Thus, the role of jeotgal in kimchi fermentation was related to enhancement of taste, the amino acid source, and the increases in levels of functional metabolites.

1. Introduction

Kimchi is the most well-known traditional fermented food consumed in Korea and has become a popular food product worldwide because of its health benefits and nutritional properties (Park et al., 1999). The raw materials used for preparation of kimchi can be classified into three groups: major vegetables (Chinese cabbage and radishes), seasonings (red pepper, garlic, ginger, leek, and onion), and optional ingredients (e.g., jeotgal) (Cheigh & Park, 1994; Koo et al., 2016).

Jeotgal is a type of fermented fish sauce consumed in Korea and is typically produced by fermentation of highly salted (20–30% [w/w]) sea animals, such as whole fish, fish roe, internal organs of fish, and shellfish. Among the types of jeotgal used as an additive to improve the taste or flavour of kimchi, myeolchi-jeot (salted anchovy) and saeu-jeot (salted tiny shrimp) are the most popular seafoods in Korea (Koo et al., 2016). These fish sauces have various endogenous enzymes derived from the muscle and/or digestive tract of raw sea animals (Jiang, Moody, & Chen, 1991). These enzymes promote the fermentation of salted fish and increase the production of abundant amino acids, even under conditions of high salinity (Jiang et al., 1991; Kim, Sung, Han, Kang, & Jeong, 1996; Sila, Nasri, Bougatef, & Nasri, 2012; Yongsawatdigul, Rodtong, & Raksakulthai, 2007). Based on these characteristics of jeotgal, it is often consumed by itself or added to other fermented foods, such as kimchi, to improve the taste, texture, and flavour of the food and enhance the fermentation activity (Koo et al., 2016). To our knowledge, kimchi is the only fermented food in the world that has a recipe using fermented fish sauce, based on animal raw materials, for the fermentation of vegetable raw materials.

The production (and quality) of kimchi is closely related to the microbial (lactic acid bacteria [LAB]) community and metabolite activity. Previous studies have revealed the relationships between microbial succession and metabolite changes during the fermentation of kimchi and jeotgal (Jung, Lee, Chun, & Jeon, 2016; Jung et al., 2011; Lee, Jung, & Jeon, 2014). However, the microbial succession and metabolite changes occurring, following the addition of jeotgal during kimchi fermentation, have not yet been investigated.

The combined use of Illumina Miseq sequencing of the 16S rRNA gene and proton nuclear magnetic resonance (^{1}H NMR) is the most comprehensive and powerful method for monitoring of diverse microorganisms and multiple compounds, particularly in fermented foods (Jeong et al., 2013). Therefore, in this study, we applied these methods, in combination with monitoring of bacterial succession and metabolite profiles during kimchi fermentation, with or without jeotgal (myeolchi-jeot and saeu-jeot). Our results are expected to contribute to the current understanding of the role of jeotgal in kimchi fermentation and to facilitate the reliable production of high-quality kimchi.

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2. Materials and methods

2.1 Jeotgal kimchi preparation and sampling

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) was soaked in 15% (w/v) solar salt solution for 10 h, manually washed three times with water, and then drained of excess water. Two types of kimchi with jeotgal (saeu-jeot or myeolchi-jeot, with 7.3% [w/v] salt), were prepared, using the following ratios: salted cabbage:ground jeotgal:ground garlic:ground ginger:ground onion:green onion:ground radish:glutinous rice porridge:red pepper powder:water = 70:4:3.75:1.2:1.85:2.4:4.5:1.2:4.5:6.6. Two types of kimchi without jeotgal were prepared using the same ingredients in the same ratio, but water or 7.3% saline was used instead of jeotgal as a control. The four prepared kimchi samples were dispensed into three polyethylene plastic bags in 5-kg portions for triplicate analysis and fermented at 5 °C for 40 days. Kimchi soups (liquid parts of kimchi) were periodically sampled, and their large particles were filtered, using a sterile stomacher filter bag (Whirl-Pak; Nasco, WI, USA). The filtered kimchi samples were centrifuged (12,000 rpm for 10 min at 4 °C), and separated pellets and supernatants were stored at −80 °C for microbial community and metabolite analyses, respectively. The kimchi samples were labelled as "CK" for kimchi without jeotgal, "NK" for kimchi with salinity adjusted with salt instead of jeotgal, "SK" for kimchi with saeu-jeot, and "MK" for kimchi with myeolchi-jeot.

2.2. Physicochemical analysis

The pH was measured with a pH meter (Orion 3-Star; Thermo Scientific, USA). The titratable acidity was obtained by titration with 0.1 N NaOH to pH 8.3 and estimated as equivalents of lactic acid (1 ml of 0.1 N NaOH amounted to 0.009 g equivalents of lactic acid) (Ramakrishnan, Goveas, Prakash, Halami, & Narayan, 2014). NaCl concentrations in kimchi samples were measured using a PAL-SALT 4250 digital salt meter (ATAGO, Shiba-koen, Japan).

2.3. Enumeration of microorganisms

The filtered kimchi soup was serially diluted with sterilized 0.85% saline solution to determine viable bacteria counts. Bacterial counts were measured using 3M Petrifilm count plates (3M-UK; Bracknell, Berkshire, UK) as culture-based approaches targetting total aerobes, LAB, coliform, and yeast, according to the manufacturer's instructions as follows: 3M Petrifilm Rapid Aerobic Count Plates for aerobic mesophilic bacterial counts; 3M Petrifilm Lactic Acid Bacteria Count Plates for LAB; 3M Petrifilm *Escherichia coli*/Coliform Count Plates for coliform counts; and 3M Petrifilm Yeast and Mold Count Plates for yeast. Total aerobic bacteria and coliform bacteria/LAB were incubated at 30 °C for 1–2 or 2–3 days, respectively. Colonies were counted from 3M films, on which 30–300 colonies appeared, and were reported as log CFU/ml.

2.4. DNA extraction and PCR amplification for MiSeq sequencing

Total genomic DNA from the kimchi pellet was extracted using a MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) through mechanical lysis, chemical lysis, and DNA purification. The DNA quality was measured using PicoGreen and a Nanodrop instrument. Input gDNA (10 ng) was amplified by PCR. For MiSeq sequencing, bacterial genomic DNA amplification was performed using primers targetting the V3 to V4 hypervariable regions of the 16S rRNA gene. 341F (5′-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′; the underlined sequences indicate the target regions; AGATGTGTATAAGAGACAG is the adaptor sequence) (Fadrosh et al., 2014; Shin et al., 2016). The PCR products were purified using a QIA Quick PCR Purification Kit, visualized on 1% agarose gels, and adjusted to equal concentrations. Paired-end sequencing was performed by Macrogen, Inc. (Seoul, Korea), using an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

2.5. Analysis of bacterial succession during kimchi fermentation

Generated MiSeq reads were processed using CLcommunity software (v.3.46). Obtained sequencing reads underwent a quality check, and those with a low quality score (average score < 25) were trimmed by Trimmomatic 0.32 (Bolger, Lohse, & Usadel, 2014). MiSeq forward and reverse reads were then paired using PandaSeq v.2.9 (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012) with default parameters, and unjoined reads were filtered out by an in-house script. The chimeric sequences were removed by the bellerophone method, and the taxonomic classification of each read was assigned, based on the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (Kim et al., 2012). The high-quality sequences were normalized to lowest number of reads (46,826 reads) by randomly selecting reads from the sequencing of fasta files, using the Mothur programme (https://www.mothur.org/). The original and normalized sequencing reads were clustered into operational taxonomic units (OTUs), using the CD-HIT programme. The richness and diversity of samples were determined by Ace, Chao1 estimate of richness (Chao, 1987), Shannon-Weaver index (Shannon, 1997), and Simpson index (Hill, 1973) at a distance of 3%. Taxonomic assignments of the high-quality sequencing reads derived from kimchi samples were performed and visualized at the genus level, using CLcommunity software v.3.46 (ChunLab, Inc., Korea). LAB sequencing reads, assigned at the genus level by CLcommunity software, were further classified at the species level, using the local BLAST (Standalone MEGABLAST) programme, based on the nucleotide (nt) database (July 2016), as previously described (Han, Lee, Jeong, Jeon, & Hyun, 2017).

2.6. Analysis of metabolite changes during kimchi fermentation

Metabolite profiling analysis, including carbohydrates, organic acids, nitrogen compounds, and amino acids, was performed in duplicate, using ^{1}H NMR spectroscopy, as described previously (Lee, Jung, & Jeon, 2015). Briefly, five millilitres of each of the respective kimchi supernatants obtained by centrifugation were adjusted to pH 6.0 and then lyophilized. The freeze-dried powder samples were suspended in five millilitres of 99.9% D_2O (deuterium oxide; Sigma-Aldrich, USA) with 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; Sigma-Aldrich, USA). After centrifugation at 13,000 rpm for 5 min, one millilitre of supernatant was transferred into a 5 mm NMR tube. ^{1}H NMR spectra of kimchi samples were acquired at 25 °C on a Varian Inova 600-MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA), using the standard PRESAT pulse sequence. The NMR spectra were collected into 32 k data points with a spectral width of 9,615 Hz. All kimchi ^{1}H NMR spectra were manually phased and baseline-corrected, using VnmrJ 3.2 software. The NMR spectral intensities were reduced into integral bin areas (buckets) of equal width (0.04 ppm) over the range of 0.5–10.0 ppm, and the buckets were normalized to the intensity of the DSS signal at 0 ppm. Identification and quantification of targetted individual metabolites from the ^{1}H NMR spectra of kimchi samples were performed, by the Chenomx NMR suite programme (v. 8.3; Chenomx, Canada), using 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard. The programme mapped the ^{1}H NMR spectra result with the reference library 9 (600-MHz compounds) (Jung et al., 2012; Lee et al., 2009).

2.7. Statistical analysis

Total ^{1}H NMR spectra for multivariate statistical analyses were

evaluated, using Mestrenova software (ver. 6.0.2; Mestrelab Research SL, Spain), as described previously (Jung et al., 2012). Briefly, all ^{1}H NMR spectra for multivariate statistical analysis had undergone baseline correction by the MestReNova v5.2.5 software (Mestrelab Research SL, Spain). The NMR spectral data of 0.5–10.0 ppm were reduced into 0.04 ppm spectral buckets and normalized to the total spectral area. The NMR spectra were converted to ASCII format, and the region of 4.6–4.8 ppm, corresponding to water, was removed. The resulting ASCII format files were imported into the MATLAB programme and mean-centred with no scaling. Principal-component analysis (PCA) was performed using MATLAB PLS_Toolbox (v3.5; Eigenvector Research, USA) at a confidence level of 95%.

Principal component analysis (PCA) of normalized total spectral data was performed using PLS_Toolbox v4.0 (Eigenvector Research Inc., USA) in MATLAB version R2009a software (The MathWorks Inc., USA). For positive and negative correlation analysis between the bacterial community and the metabolites of kimchi, a correlation analysis, using the R programme with the Corrplot package, was performed on the basis of bacterial abundance at the kimchi LAB species level and targetted metabolite concentrations. The analysis of correlation matrices was performed using the Corrplot package (Wei & Wei, 2016) in the R programming environment (http://cran.r-project.org/), and pair-wise correlation values were visualized as heat maps and hierarchical clustering, using GENE-E (https://software.broadinstitute.org/GENE-E/index.html).

2.8. Sequence and metabolite data accession numbers

The MiSeq sequencing data of the 16S rRNA genes are publicly available in the NCBI Short Read Archive (SRA) under accession no. SRX3083401 (experiment) and PRJNA397546 (bioproject). Metabolites data are publicly available in MetaboLights (http://www.ebi.ac.uk/metabolights/) under accession no. MTBLS654.

3. Results and discussion

3.1. General features during kimchi fermentation

Four sets of kimchi samples, with or without jeotgal, were incubated at 4 °C for 40 days, and their NaCl concentrations were maintained at 2.2–2.4% (w/v) in NK, MK, and SK kimchi samples, whereas those of CK kimchi samples were maintained at 1.7–1.9% (w/v).

The initial pH values (5.2 and 5.6) of kimchi samples with jeotgal (MK and SK) were clearly higher than those (4.8) of kimchi samples without jeotgal (CK and NK; Fig. 1A). This is probably because the pH values of the added myeolchi-jeot and saeu-jeot were high, at pH 5.99 and pH 6.98, respectively. Therefore, the jeotgals served to increase the pH of initial kimchi fermentation. Subsequently, the pH values increased rapidly, up to approximately pH 6.0 in kimchi samples with jeotgal, whereas kimchi samples without jeotgal showed only a slight increase up to approximately pH 5.2 during the early fermentation period. However, pH values decreased after 8 days of fermentation and became relatively stable, at around pH 4.3, after 20 days during the late fermentation period, regardless of the kimchi type.

The total viable bacterial cells (aerobic bacteria, LAB, and coliform) in four sets of kimchi samples during the fermentation process were enumerated as colony forming units (CFU; Fig. 1B–D). Total initial aerobic bacteria were present at approximately 6.6 log CFU/ml in all kimchi samples; however, their profiles during the fermentation period were significantly different, depending on the presence or absence of jeotgal (Fig. 1B). After 8 days of fermentation, the numbers of aerobic bacteria in MK and SK samples increased rapidly up to approximately 8.1 log CFU/ml at 30 days of kimchi fermentation, whereas those in CK and NK samples showed a relatively slow increase to high values of approximately 8.0 and 7.6 log CFU/ml at 30 days, respectively. After the number of aerobic bacteria peaked, a gradual decrease was observed until late fermentation, regardless of the kimchi type. The initial number of LAB was approximately 5.1 log CFU/ml in all kimchi

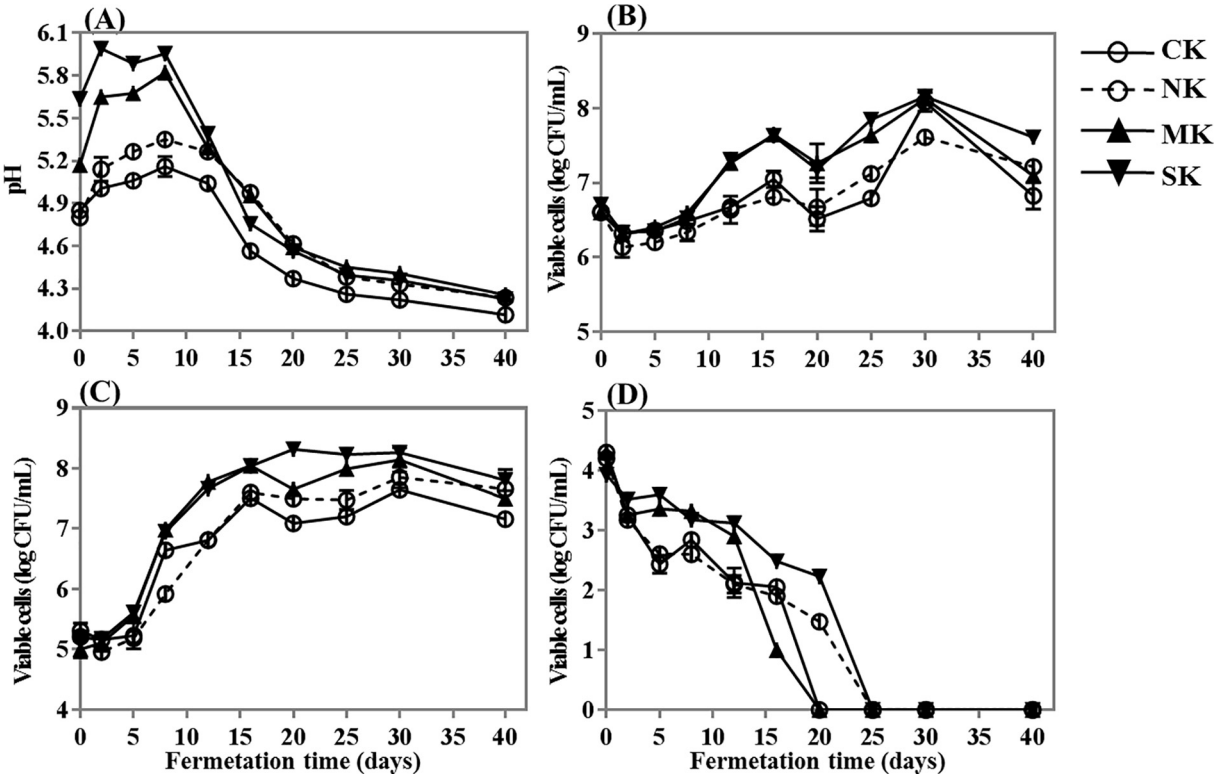

Fig. 1. Changes in pH values (A), total viable bacteria counts (B), total LAB counts (C), and coliform bacteria counts (D) during kimchi fermentation, with or without jeotgal. Bacteria were counted as CFU/g in triplicates.

samples (Fig. 1C). After the short stationary phase, the numbers of LAB in ML and SK samples rapidly increased until reaching approximately 8.1 and 8.3 log CFU/ml at 20 and 30 days, respectively, whereas those in CK and NK samples increased only to 7.6 and 7.8 log CFU/ml, respectively. The number of coliform bacteria (*E. coli*) gradually decreased in all kimchi samples; however, the time of the decrease was slightly different, depending on the type of kimchi (Fig. 1D). The initial number of coliforms was 3.9–4.3 log CFU/ml and this gradually decreased during early fermentation. However, the number of coliform bacteria in MK and CK samples began to decrease rapidly at 12 and 20 days, respectively, and could not be detected after 20 days of fermentation. Coliform bacteria in SK and NK samples also could not be detected after 25 days of fermentation. Because kimchi is a spontaneously fermented and non-sterile product, coliform bacterial growth from freshly prepared kimchi can occur, but such growth rapidly disappears and is replaced by LAB during the fermentation period (Chang & Chang, 2011). This is probably due to the extreme conditions (low pH and low temperature) and rapid growth of LAB during kimchi fermentation, which inhibits the growth of coliform bacteria. The kimchi fermented by LAB can thus be considered a safe food.

3.2. Changes in microbial diversity in kimchi, with and without jeotgal

The Miseq sequencing approach was applied to analyze bacterial diversity and communities in the four types of kimchi samples during the fermentation period. In total, 8,660,627 merging paired reads were retrieved from 40 kimchi samples. After trimming of the barcoded PCR primers and removal of low-quality chimeric sequences and plant-derived 16S rRNA gene sequences (*Streptophyta*), 3,894,730 high-quality reads with an average of approximately 97,368 sequences and an average read length of approximately 453 bp per sample were obtained; these sequence reads were used for further analysis. Statistical bacterial diversity was calculated for each sample (Supplementary Table 1). The bacterial diversity decreased in all kimchi samples as kimchi fermentation progressed. However, the bacterial diversity indices (OTUs, Ace, Chao1, Shannon, and Simpson) of initial kimchi samples varied, depending on the addition of jeotgal (Supplementary Table 1). During the initial stage, kimchi samples (MK and SK) with jeotgal showed higher bacterial diversity than did kimchi without jeotgal (CK and NK), suggesting that diverse bacteria were introduced into kimchi from jeotgal.

3.3. Changes of bacterial succession during kimchi fermentation, with and without jeotgal

To compare bacterial succession in kimchi samples, with and without jeotgal, the bacterial 16S rRNA gene sequencing reads were classified at the genus and species levels (Fig. 2I and II). At the genus level, *Bacillus*, *Pseudomonas*, *Rahnella*, *Rhizobium*, *Klebsiella*, *Pantoea*, *Enterobacter*, *Sphingomonas*, and *Pedobacter*, which are primarily derived from raw kimchi materials, were commonly identified in all early kimchi samples (Fig. 2I). Interestingly, *Tetragenococcus* was found only in early MK samples, suggesting that this bacterium was probably derived from myeolchi-jeot (Jung et al., 2016; Lee et al., 2015). The initially dominant genera were almost completely absent during the middle fermentation period. However, the abundance of the genus

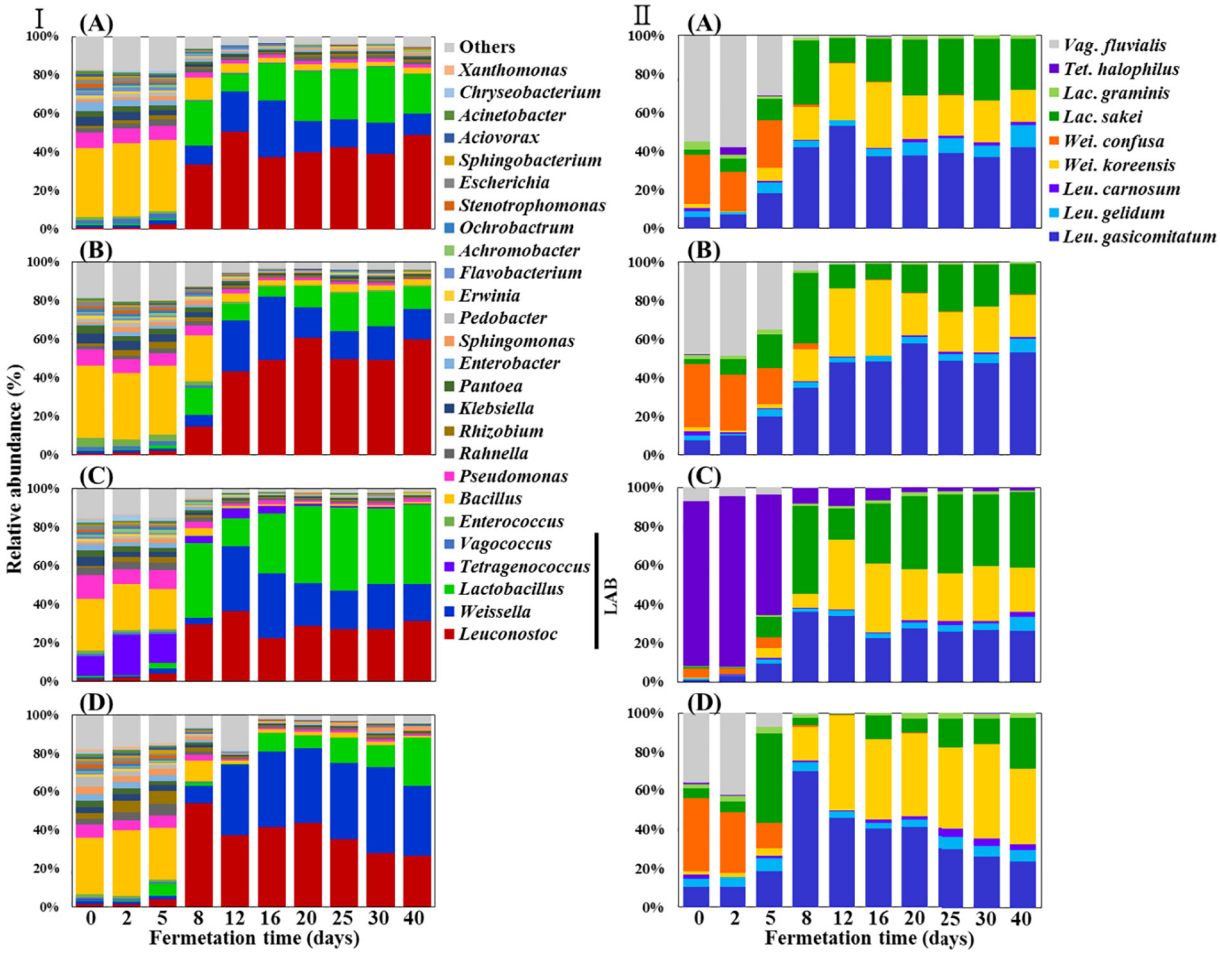

Fig. 2. Bacterial taxonomic compositions at the genus and species levels in CK (A), NK (B), MK (C), and SK (D) samples during kimchi fermentation. The "Others" category includes genera comprising less than 1.0% of the total high-quality reads in panel I.

Bacillus in the CK and NK samples decreased relatively slowly compared with those of the other two samples and persisted until the end of the fermentation period, suggesting that the addition of jeotgal may have affected the inhibition of *Bacillus*. After their initial decreases, the three LAB genera *Leuconostoc*, *Weissella*, and *Lactobacillus*, became the predominant genera until the end of fermentation, although ratios of these genera differed dramatically, depending on the kimchi type. The ratios of *Leuconostoc*, *Weissella*, and *Lactobacillus* in the CK and NK samples were similar, despite the difference in salinity of approximately 0.5%, whereas dominant genera were different, depending on the jeotgal type. The reads identified as LAB were further analyzed at the species level (Fig. 2II). Species level analysis also showed that the LAB were quite different, depending on the kimchi type. During the early fermentation period, *Vagococcus* (*Vag.*) *fluvialis* and *Wei. confusa* were dominant, and their proportions were similar in CK, NK, and SK samples during the early fermentation period. *Tet. halophillus*, which was derived from myeolchi-jeot, was predominant only in early MK samples. However, *Vag. fluvialis*, *Wei. confusa*, and *Tet. halophillus* were rapidly replaced with *Les. gasicomitatum*, *Leu. gelidum*, *Leu. carnosum*, *Wei. koreensis*, *Wei. confusa*, *Lac. sakei*, and *Lac. graminis* as the fermentation progressed, regardless of the kimchi type. These data suggest that the seven species may be more stress-tolerant or more competitive than *Vag. fluvialis*, *Wei. confusa*, and *Tet. halophillus* during the late kimchi fermentation period. In particular, *Wei. confusa* may have better growth competitiveness than *Wei. koreensis* under these kimchi fermentation conditions (e.g., low temperature and high acidity), despite belonging to the same genus. In the CK and NK samples, *Leu. gasicomitatum* became the predominant bacterium, followed by *Wei. koreensis* and *Lac. sakei* during the late stage of fermentation. In the MK sample, *Tet. halophillus*, which was the most predominant species during the initial stage of kimchi fermentation, became rapidly replaced by *Lac. sakei*, *Leu. gasicomitatum*, and *Wei. koreensis* during the middle stages of kimchi fermentation. This is presumably because *Tet. halophillus* has a slow growth rate in environments with low pH and low temperature, such as the environment encountered during kimchi fermentation (Lee et al., 2005). Among these species, *Lac. sakei* became more dominant than the other two species during the end of kimchi fermentation. In the SK sample, *Leu. gasicomitatum* was suddenly increased on day 8 of fermentation and then decreased continuously until the end of kimchi fermentation. However, *Wei. koreensis* increased steadily and dominated during the late fermentation stage. Interestingly, the proportion of *Lac. sakei* was rapidly decreased after 8 days of fermentation and was the lowest among all kimchi samples during the late stage of fermentation. The differences in the proportions of LAB species in the NK, MK, and SK samples indicated that LAB succession was more affected by the jeotgal type than the salt, despite having the same salinity.

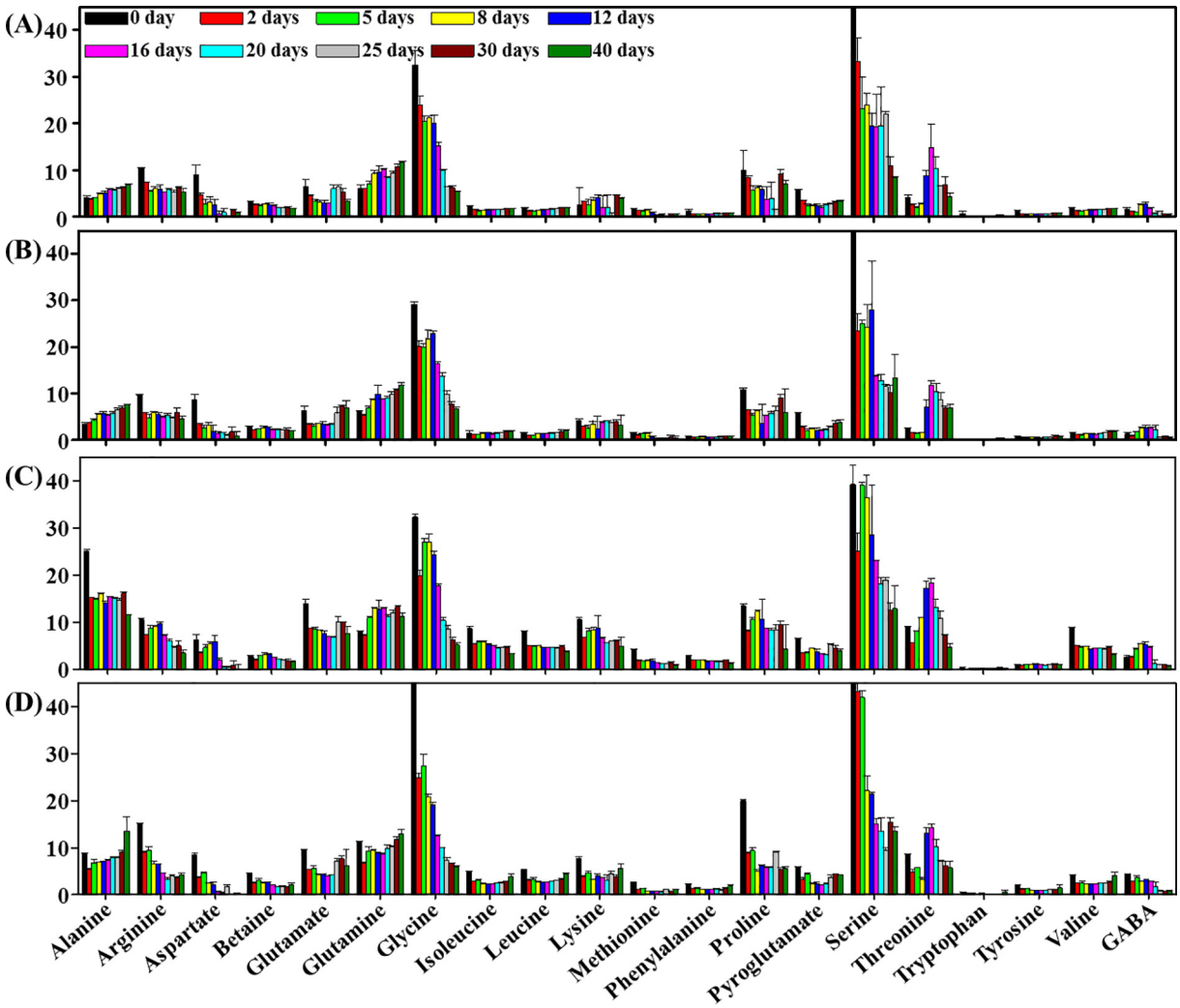

Fig. 3. Changes in major nitrogen compounds identified by ^{1}H NMR in CK (A), NK (B), MK (C), and SK (D) samples during kimchi fermentation. Data are presented as the average value ± standard deviation in triplicate. *Abbreviation*: GABA, gamma-aminobutyric acid.

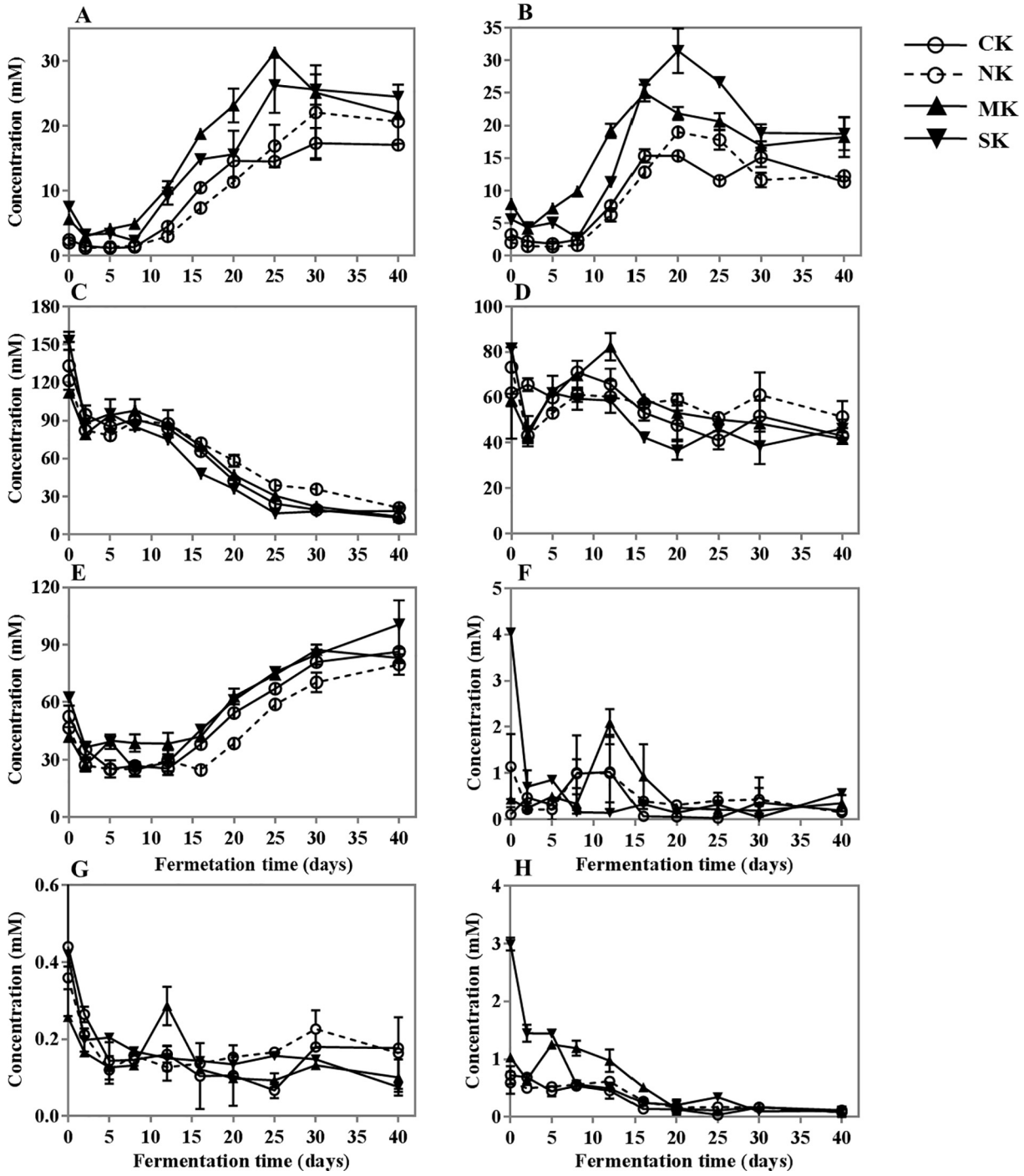

Fig. 4. Changes in organic compounds, namely, acetate (A), lactate (B), fructose (C), glucose (D), mannitol (E), trimethylamine N-oxide (F), trimethylamine (G), and dimethylamine (H), identified by ^{1}H NMR in CK, NK, MK, and SK samples during kimchi fermentation. Data are presented as the average value ± standard deviation in triplicate.

3.4. Changes of metabolites in kimchi, with and without jeotgal, during the fermentation period

To investigate the effects of jeotgal during kimchi fermentation, kimchi metabolites were analyzed by ^{1}H NMR spectra, as shown in Figs. 3 and 4. The amino acid concentrations of kimchi were determined automatically from the databases of the Chenomx NMR suite programme. Kimchi samples with jeotgal had slightly higher amino acid levels than those in kimchi samples without jeotgal, owing to the addition of amino acid-rich fermented jeotgal. In particular, alanine (PubChem CID: 5950), arginine (PubChem CID: 6322), glutamate (PubChem CID: 33032), isoleucine (PubChem CID: 6306), leucine (PubChem CID: 6106), lysine (PubChem CID: 5962), valine (PubChem CID: 6287), and gamma-aminobutyric acid (GABA, PubChem CID: 119) levels were higher in kimchi with jeotgal than in kimchi without jeotgal. During 40 days of fermentation, alanine, arginine, glutamate, and lysine concentrations in SK (8.09, 6.61, 5.84 and 4.40 mM) were slightly higher than those in CK (5.29, 5.70, 4.43 and 2.88 mM) and NK (5.49, 6.29, 4.62 and 3.42), and the values in MK (15.9, 7.21, 8.88 and 7.19 mM) were significantly higher. Isoleucine, leucine, and valine concentrations in CK and NK were less than 2 mM, whereas those in MK and SK were increased two-fold. Additionally, the gamma-aminobutyric

acid (GABA) level increased and decreased during the fermentation progress in all four kimchi conditions, but it was slightly higher in MK and SK until 16 days of fermentation (Fig. 3).

Notably, the changes in amino acid profiles during the fermentation of jeotgal increase with the decomposition of protein from fish (Jung et al., 2016; Lee et al., 2014). In contrast, in this study, most amino acid levels decreased during kimchi fermentation, owing to the decomposition of the initial amino acids (Fig. 3). Thus, amino acid catabolism increases with the growth of kimchi LAB, and utilization of these amino acids and the conversion of the peptide to free amino acids and the subsequent utilization are a major central metabolic activity of LAB (Christensen, Dudley, Pederson, & Steele, 1999). The low pH (pH 4.1–pH 4.5) during the late fermentation period, due to the production of organic acids by LAB, affects the growth of LAB. However, LAB use metabolites of glutamine, glutamate, and arginine to overcome this low pH stress (Teixeira et al., 2014). Among these metabolites, GABA production from glutamate in LAB strains with glutamate decarboxylase (GAD), such as *Lac. sakei*, *Leu. gasicomitatum, Wei. koreensis, and Leu. gelidum*, may overcome low pH, such as that encountered during kimchi fermentation (Feehily & Karatzas, 2013; Jeong, Lee, Jung, Choi, & Jeon, 2013). Indeed, the abundance of amino acids in kimchi with jeogal was expected to have helped overcome the low pH of kimchi LAB, as supported by the finding that the growth rate of LAB in kimchi with jeotgal was increased during the fermentation period (Fig. 1B). Interestingly, GABA has been shown to have health-promoting effects, such as reduction in blood pressure, and to be useful for treating neurological disorders (Gobbetti, Cagno, & De Angelis, 2010; Hayakawa et al., 2004; Okada et al., 2000). The effects of jeotgal addition were confirmed through analysis of organic acids, saccharides, and amines (Fig. 4). The concentration of fructose (PubChem CID: 5984) decreased rapidly from the initial fermentation period; however, the concentration of glucose (PubChem CID: 10954115) decreased relatively slowly (Fig. 4C and D). These data indicate that kimchi LAB may prefer fructose for fermentation (Endo, 2012). The decrease in fructose concentration was similar, regardless of the kimchi sample type; however, the concentrations of acetate (PubChem CID: 175), lactate (PubChem CID: 91435), and mannitol (PubChem CID: 6251) were higher in the MK and SK samples than in the CK and NK samples during the fermentation period (Fig. 4A, B, and E). Mannitol, a six-carbon sugar alcohol or polyol, is known as an antioxidant and as a non-metabolizable sweetener with health-promoting effects that has a sweet and cool taste (Carvalheiro, Moniz, Duarte, Esteves, & Girio, 2011; Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002). These data suggested that the addition of jeotgal affected the growth of kimchi LAB, which produce GABA, lactate, acetate, and mannitol, thereby having a significant effect on the taste and flavour of kimchi. The changes in amine concentrations with the addition of jeotgal did not differ according to sample, and the amounts of amine compounds, including trimethylamine *N*-oxide (TMAO, PubChem CID: 1145), trimethylamine (TMA, PubChem CID: 1146), and dimethylamine (DMA, PubChem CID: 674), decreased rapidly from the beginning of kimchi fermentation, showing relatively constant concentrations thereafter until the end of fermentation (Fig. 4F–H). TMA and DMA, which impart the strong fishy odour to fermented products, are produced by the reduction and/or demethylation of TMAO (daCosta, Vrbanac, & Zeisel, 1990). These data confirm that the jeotgal did not generate abnormal taste or flavour during kimchi fermentation.

3.5. Multivariate statistical analysis

Total ^{1}H NMR spectra analysis of all kimchi samples showed that the metabolite fermentation patterns of the four kinds of kimchi samples were similar during the early stages of fermentation but differed in kimchi samples with jeotgal (MK and SK) during late stages of fermentation (Fig. 5). Finally, metabolites from all kimchi samples were almost similar at the end of fermentation, and the fermentation rate of the CK sample with lower salinity was faster than those of MK and SK. Even NK samples with similar salinity to that of the jeotgal kimchi had fermentation rates faster than MK and SK. Therefore, the addition of jeotgal may inhibit over-fermentation by delaying the fermentation process.

Positive and negative correlation analyses between LAB species level succession and changes in metabolites were performed during kimchi fermentation (Fig. 6). In the two kimchi samples without jeotgal, similar heat map patterns were observed, despite the 0.5% difference in salinity (Fig. 6A and B) whereas, in the two kimchi samples with jeotgal, amino acids having a negative correlation with LAB species (*Lab. sakei*, *Leu. gasicomitatum*, *Wei. koreensis*, *Lac. graminis*, *Leu gelidum*, *Leu. carnosum*) were more diversified during the late fermentation period. The degrees of negative correlation between the amino acid and the LAB species were higher in kimchi with myeolchi-jeot than in kimchi with saeu-jeot. These changes, due to the addition of jeotgal, may be related to the consumption of amino acids by metabolism in LAB; therefore, abundant amino acids in myeolchi-jeot and saeu-jeot may contribute to the promotion of kimchi fermentation.

4. Conclusions

Jeotgal was found to be an important ingredient in kimchi fermentation, facilitating the growth of kimchi LAB and producing functional materials, such as GABA and mannitol. This study demonstrated the production principle of functional materials by kimchi LAB and

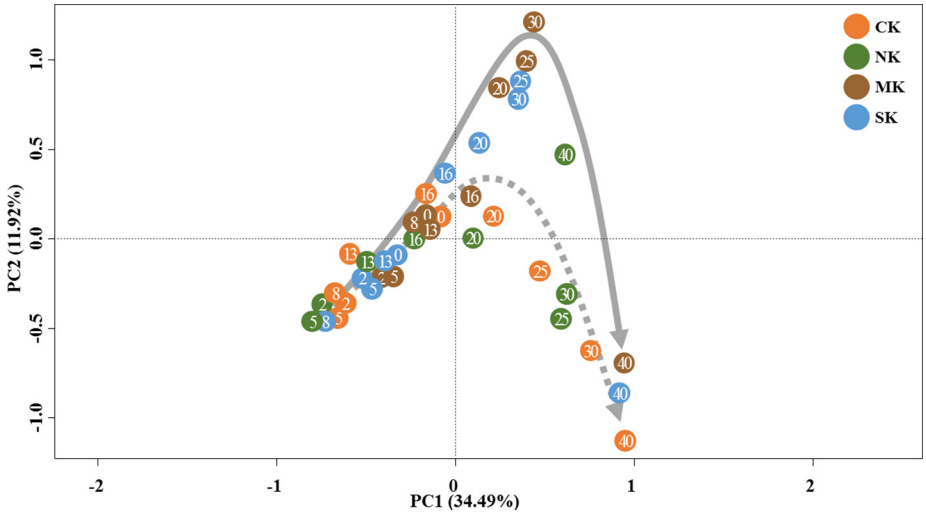

Fig. 5. Score plots of principal component analysis (PCA) based on the total ^{1}H NMR spectra of kimchi during fermentation. The orange, green, brown, and blue circles indicate CK, NK, MK, and SK, respectively. Numbers inside the circles indicate the fermentation time (days). The directions of the curved arrows indicate the route of metabolite changes on the PCA plot during kimchi fermentation. The straight line and dotted line indicate the fermentation routes of kimchi, with and without jeotgal, respectively.

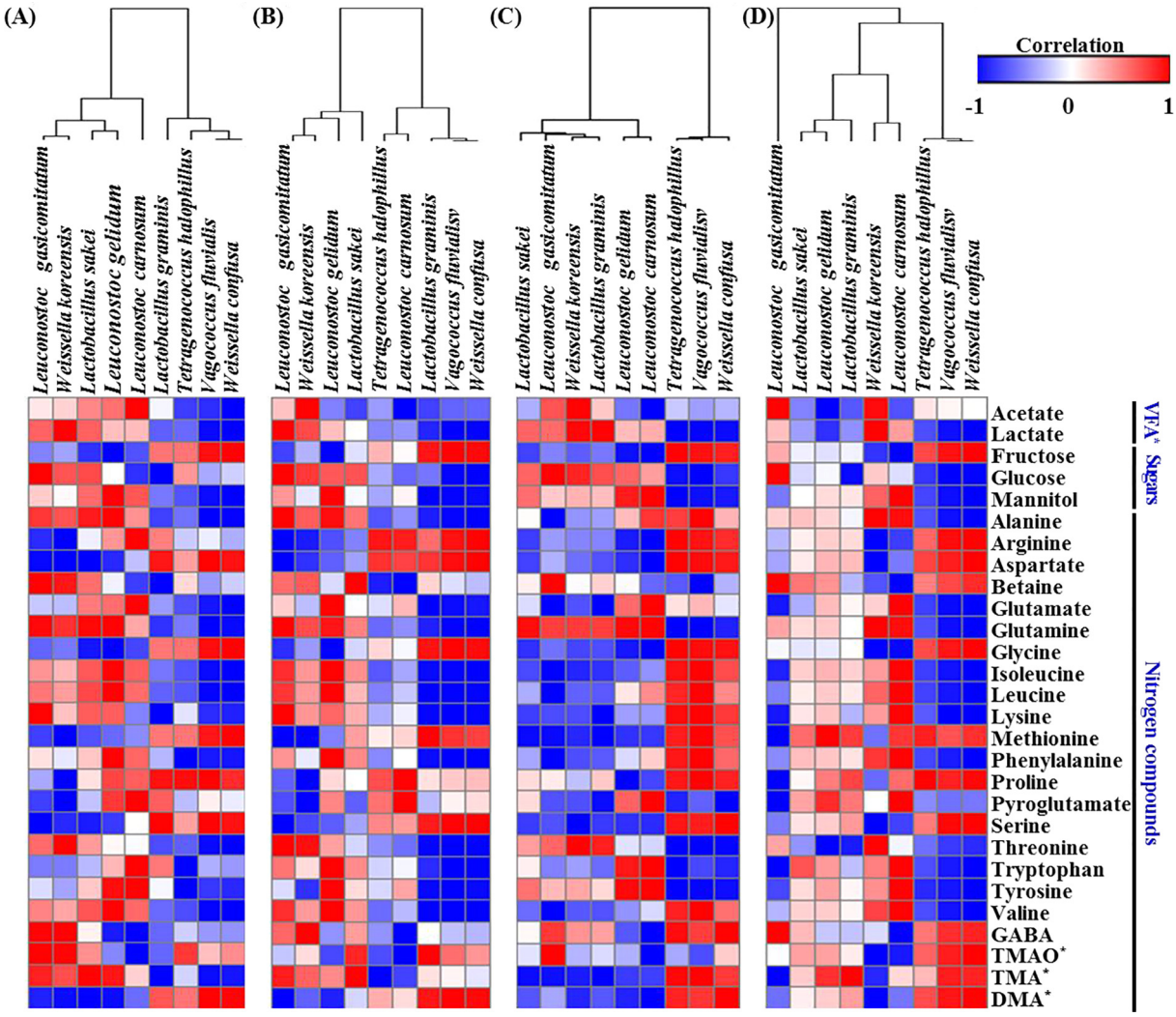

Fig. 6. Correlation analysis between the succession of kimchi LAB species and metabolite profiles in CK (A), NK (B), MK (C), and SK (D) samples during kimchi fermentation, shown as a heat map. Shades of red and blue indicate strong positive and negative correlations, respectively. *Abbreviation*: VFA, volatile fatty acid; TMAO, trimethylamine *N*-oxide; TMA, trimethylamine; DMA, dimethylamine.

confirmed the value of kimchi as a functional fermented food to produce health-beneficial substances. Further studies are needed to investigate the mechanism of kimchi fermentation through genetic and metabolic studies of individual kimchi LAB.

5. Conflict of interest

The authors declare no conflicts of interest.

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