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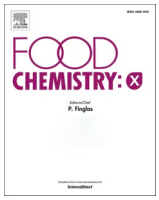

Fermentative metabolic features of doenjang-meju as revealed by genome-centered metatranscriptomics

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ABSTRACT

Fermentative features of doenjang-meju, a traditional Korean soybean brick, were investigated over 45 days via genome-centered metatranscriptomics. The pH value rapidly decreased within 10 days and successively increased after 20 days, along with an initial bacterial growth, including lactic acid bacteria, and subsequent fungal growth, suggesting their association. Polysaccharides and lipids underwent degradation, and amino acids, free sugars, and organic acids increased during the early stage. Metagenome analysis identified *Aspergillus*, *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Leuconostoc* as major microbes, which were isolated and genome-sequenced. Metatranscriptomic analysis revealed the major roles of *Bacillus* and *Enterococcus* during the early period, shifting to *Aspergillus* dominance after 10 days. Metabolic pathway reconstruction and transcriptional analysis reveal that *Aspergillus* primarily decomposed polysaccharides to free sugars; *Aspergillus* and *Bacillus* metabolized lipids, free sugars, and organic acids generated by *Enterococcus*; and *Aspergillus* and *Bacillus* were instrumental in amino acid metabolism: their contributions varied by compounds and pathways.

1. Introduction

Doenjang-meju is a key ingredient in the production of doenjang (soybean paste) and ganjang (soy sauce), both widely popular condiments in Korea. The spontaneous process of doenjang-meju fermentation, driven by microbes from the surrounding environment, results in variations in fermentative characteristics, including microbial communities and metabolites (Kim et al., 2011, Kim, Han, Baek, Chun, & Jeon, 2022; Jung, Lee, & Jeon, 2014; Song et al., 2020; Ryu et al., 2021). Doenjang-meju quality, which is influenced by its microbial community during fermentation, potentially affects subsequent food products, namely, doenjang and ganjang (Han, Chun, Feng, Kim, & Jeon, 2020; Kang, Woo, Tian, Lee, & Chun, 2023). Hence, understanding the microbial fermentative features of doenjang-meju is essential for controlling the taste and quality of the products, leading to numerous studies primarily focusing on bacterial and fungal community analyses (Jung et al., 2014; Kim et al., 2011).

Previous studies have indicated the involvement of diverse microbial groups during traditional doenjang-meju fermentation (Jung et al., 2014; Kim et al., 2011), presenting challenges in comprehending their fermentative features. While various fungi and bacteria contribute to the natural fermentation process of traditional doenjang-meju, *Aspergillus*, *Bacillus*, and lactic acid bacteria (LAB) are recognized as major contributors (Kim et al., 2022; Ryu et al., 2021; Song et al., 2020). Consequently, extensive research has sought to elucidate the fermentative features of doenjang-meju by employing these key microbes as starter cultures (Gil et al., 2021; Han, Baek, Chun, & Jeon, 2023). Additionally, efforts have been made to comprehend the metabolic characteristics of these microbes via genome and pangenome analyses (Choi, Baek, Han, Khan, & Jeon, 2024; Chun, Kim, Jeon, & Jeon, 2019; Han et al., 2024). However, solely relying on starter culture and genomic studies of individual microbes may limit our understanding of doenjang-meju's fermentative features owing to the complex microbial interactions and metabolic reactions involved during fermentation.

A fundamental yet crucial step toward understanding the microbial fermentative features of fermented foods is the simultaneous analysis of microbial communities and metabolites (Kim et al., 2022; Lu, Zuo, Cheng, & Huang, 2024; Wang et al., 2023; Wang et al., 2024). However, these approaches have limitations in fully elucidating the microbial fermentative features of fermented foods due to insufficient information on the precise metabolic activities of fermenting microbiota. On the other hand, metatranscriptome analyses have been proposed as a more accurate means of exploring in situ metabolic characteristics of microbiota in traditional fermented foods (An et al., 2021; Chun et al., 2021;

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Kim, Chun, Kim, & Jeon, 2021; Liu et al., 2019; Qin et al., 2024; Quijada, Dzieciol, Schmitz-Esser, Wagner, & Selberherr, 2022). Therefore, this study conducted a genome-centered metatranscriptomic analysis, combined with metagenome sequencing, genome sequencing of isolated key microbes, metabolic pathway reconstruction, and metatranscriptome analysis, to unravel the microbial fermentative features of doenjang-meju.

2. Materials and methods

2.1. Doenjang-meju brick preparation and sampling

Soybean bricks for doenjang-meju fermentation were prepared according to a previously described procedure, with certain modifications (Jung et al., 2014). Briefly, approximately 63 kg of dried yellow soybeans (*Glycine* max [L.] Merr.) were soaked in tap water overnight at room temperature and subsequently autoclaved at 121 °C for 30 min. Thereafter, the autoclaved soybeans were cooled to approximately 35 °C, crushed, and molded into bricks measuring approximately 22 × 14 × 9 cm, resulting in 21 doenjang-meju bricks weighing approximately 3 kg each. The bricks were slightly dried at 35 °C overnight and subsequently transferred to an incubation room where they were naturally incubated at approximately 25–30 °C for 45 days. On days 0, 2, 5, 10, 20, 30, and 45, three doenjang-meju bricks were collected and immediately homogenized using a blender (Hanil, Korea), and their pH values and bacterial and fungal cell counts were subsequently determined. The homogenized samples targeted for compound analyses were stored at −80 °C. For metagenome and metatranscriptome sequencing, 2 g samples were obtained from the three homogenized replicate doenjang-meju bricks at 2, 5, 10, 20, 30, and 45 days, mixed thoroughly, and stored at −80 °C.

2.2. Measurement of pH, enumeration of bacteria and fungi, and compound analyses

The measurement of pH and enumeration of bacteria and fungi in doenjang-meju bricks followed previously described procedures (Han et al., 2023). In brief, for pH measurement, homogenized doenjang-meju samples (2 g) were resuspended in 5 mL of distilled water (DW), and pH was determined using a pH meter (Thermo Scientific, USA). For viable cell counting, the resuspended doenjang-meju samples were serially diluted in 0.9% saline; plated on specific media: potato dextrose agar (PDA; MBcell, South Korea) for fungi, tryptic soy agar (TSA; MBcell) for total bacteria, and de Man Rogosa Sharpe (MRS; MBcell) agar for LAB; and incubated at 30 °C for 3 days. Cell counts are expressed in colony-forming units (CFU) per gram of dry weight of doenjang-meju. The cellulose, hemicellulose, starch, and pectin content of doenjang-meju bricks was assessed using the Van Soest methods (Schädel, Blöchl, Richter, & Hoch, 2010). The free sugar, organic acid, amino acid, and glycerol content of doenjang-meju bricks was analyzed using proton nuclear magnetic resonance spectroscopy, as described in a previous study (Kim et al., 2022). Total lipids were determined using Folch's method (Folch, Lees, & Stanley, 1957). The pH measurement, colony cell counting, and compound analyses were conducted using samples from three replicate doenjang-meju bricks, and the data are presented as mean values ± standard errors.

2.3. Shotgun metagenome sequencing and taxonomic classification

Genomic DNA was extracted from doenjang-meju brick samples using the FastDNA™ Spin Kit for soil (MP Biomedicals, USA), following the manufacturer's instructions. Subsequently, the extracted DNA was sequenced using an Illumina MiSeq paired-end platform (×300 bp) at Macrogen (South Korea). The Illumina sequencing reads were processed to remove low-quality sequencing reads, as described previously (Chun et al., 2021). Specifically, the sequencing reads were trimmed based on a quality threshold of 30, and those shorter than 100 nucleotides were eliminated using Sickle (Joshi & Fass, 2011). High-quality metagenome sequencing reads were taxonomically classified using Kaiju (ver. 1.9.2; Menzel, NG, & Krogh, 2016). Metagenome-assembled genomes (MAGs) were constructed by assembling and binning the high-quality metagenome sequencing reads using metaSPAdes (version 3.12.0; Nuruk, Meleshko, Korobeynikov, & Pevzner, 2017) with default settings and metaBAT2 (version 2.15; Kang et al., 2019) with a 'minS' parameter set to 95, respectively, and the constructed MAGs were taxonomically classified using Kaiju. The completeness and contamination rates of bacterial MAGs and completeness of fungal MAGs were evaluated using CheckM2 (version 1.02; Chklovski, Parks, Woodcroft, & Tyson, 2023) and BUSCO (version 5.4.4; Seppey, Manni, & Zdobnov, 2019), respectively.

2.4. Microbial isolation, genome sequencing, and functional analysis

To isolate the predominant microbes in doenjang-meju, homogenized samples were resuspended in 0.9% saline and plated on TSA, MRS agar, and PDA, followed by incubation at 30 °C for 3 days. The bacteria and fungi that grew on the agar media were taxonomically classified by sequencing their 16S rRNA and ITS genes, respectively (Han et al., 2020). Genomic DNA was extracted from bacteria and fungi using the Wizard® Genomic DNA Purification Kit (Promega, USA), following the manufacturer's instructions. Subsequently, sequencing was performed using a combination of Oxford MinION in-house and Illumina HiSeq 4000 platforms (×150 bp) at Macrogen. The sequencing reads generated via MinION and Illumina sequencing were hybrid-assembled using Unicycler (version 0.4.7), and the quality of the assembled bacterial and fungal genomes was assessed using CheckM2 and BUSCO, respectively. The genes within the bacterial and fungal genomes were identified using Prokka (version 1.14.6; Seemann, 2014) and AUGUSTUS (version 3.3.3; Hoff & Stanke, 2013), respectively, and then functionally annotated based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using BlastKOALA (Kanehisa, Sato, & Morishima, 2016). The numbers of genes assigned to each respective KEGG category in each genome were calculated.

2.5. Total RNA extraction, sequencing, processing, and metatranscriptomic analysis

Total RNA was extracted from doenjang-meju samples using the formamide/EDTA method, with certain modifications (Lee, Hong, & Kang, 2019). Briefly, homogenized samples (0.2 g) were mixed with 980 μL of formamide and 20 μL of 0.5 M EDTA (pH 8.0) solution, bead-beaten with Lysing Matrix E (MP, USA) using the FastPrep-24™ Classic Instrument (MP, USA) at 6.0 m/s for 60 s, and centrifuged for 10 min at 4 °C (16,100 ×*g*). Each supernatant was transferred to a new tube, mixed with an equal volume of RNase-free DW and two volumes of phenol/chloroform (5:1; Sigma-Aldrich, USA), and centrifuged for 10 min at 4 °C (16,100*g*). Thereafter, each upper aqueous phase was transferred to a new tube, mixed with the same volume of cold isopropanol, and recentrifuged for RNA precipitation. The resulting RNA pellets were washed with 70% ethanol and resuspended in 100 μL of RNase-free DW.

rRNA subtraction and cDNA library construction were performed using a TruSeq™ Total RNA Sample Prep Kit (Illumina), according to the manufacturer's instructions, and the resulting cDNA libraries were sequenced using an Illumina HiSeq platform (×101 bp) at Macrogen. The cDNA sequencing reads were trimmed based on a quality threshold of 30, and those shorter than 30 nucleotides were eliminated using Sickle. Structural RNA (rRNA and tRNA) reads were removed from the cDNA reads using BLASTN comparisons, with an E value ≤0.001 as the cutoff criterion against rRNA and tRNA genes. The transcriptional expression of microbes in doenjang-meju during fermentation was analyzed by mapping the putative mRNA reads to their genomes using the BWA-MEM program—based on the following best-match criteria: a

90% minimum identity and 10-bp minimum alignment—and indicated relatively based on the total numbers of mapped reads within each sample. In addition, the transcriptional expression of the functional genes in doenjang-meju microbes was calculated as the number of reads per kilobase of the coding sequence, per million mapped reads (RPKM) values within each sample. The transcriptional expression of each respective KEGG category during fermentation was calculated as the sum of the RPKM values of all the genes assigned to each KEGG category.

2.6. Metabolic pathway reconstruction of and metatranscriptomic analyses during fermentation

The carbohydrate, protein, and lipid metabolic pathways in isolated microbes were reconstructed by integrating predicted KEGG pathways with the Enzyme Commission numbers of functional genes identified in their genomes. The presence or absence of metabolic genes in each microbe was manually confirmed via BlastP analyses against their genomes, utilizing reference protein sequences from closely related microbes. Transcriptional expression of the genes involved in these metabolic pathways during fermentation was investigated by mapping putative mRNA reads to the genomes using the BWA-MEM program and visualized using pie charts based on the RPKM values within each sample.

2.7. Data availability

The metagenomic and metatranscriptomic sequencing data generated in this study are publicly available in the NCBI Sequence Read Archive under accession numbers SRR28778154 –SRR28778159 and SRR28793827–SRR28793832 (NCBI BioProject accession numbers PRJNA1103415 and PRJNA1103864), respectively. The genome sequences obtained in this study have been deposited in GenBank with the accession numbers JBDJHZ000000000, JBDJIA000000000, JBDJIB000000000, JBDJIC000000000, and JBDJID000000000 for MAGs and JBANDD000000000, JAYLVH000000000, CP143433–7, CP146481–2, and CP143438–40 for microbial genomes.

3. Results

3.1. General features of doenjang-meju fermentation

To investigate the fermentative characteristics of doenjang-meju using a metaomics approach encompassing metagenomics, metatranscriptomics, and metabolomics, doenjang-meju bricks were prepared and allowed to ferment naturally for 45 days. Considering that pH serves as a vital indicator of microbial metabolic features during food fermentation (Han et al., 2023; Kim et al., 2022), pH was measured during fermentation. The initial pH of the doenjang-meju prepared for this study was approximately 6.5. As fermentation commenced, pH rapidly decreased, reaching approximately 5.86 on day 10 (Fig. 1A). Subsequently, it promptly rose to approximately 6.67 on day 20 and remained relatively constant until the end of fermentation.

Considering the potential significant roles of fungi and bacteria, such as *Aspergillus* and *Bacillus*, alongside LAB, which have been abundantly identified in doenjang-meju fermentation (Jung et al., 2014; Kim et al., 2022), these microbes were counted on PDA, TSA, and MRS agar during fermentation. The colony counting analysis revealed that the bacteria grown on TSA and MRS agar remained considerably more abundant than the fungi grown on PDA throughout the entire doenjang-meju fermentation period, exhibiting consistency with a previous study (Jung et al., 2014). Specifically, the abundance of cells grown on MRS agar, representing LAB, rapidly increased during the early fermentation period, reaching approximately 8.7×10^6 CFU/g-doenjang-meju within just 2 days, and gradually decreased as fermentation progressed after 5 days (Fig. 1B), consistent with the rapid decrease in pH values during doenjang-meju fermentation (Fig. 1A). The abundance of bacterial cells

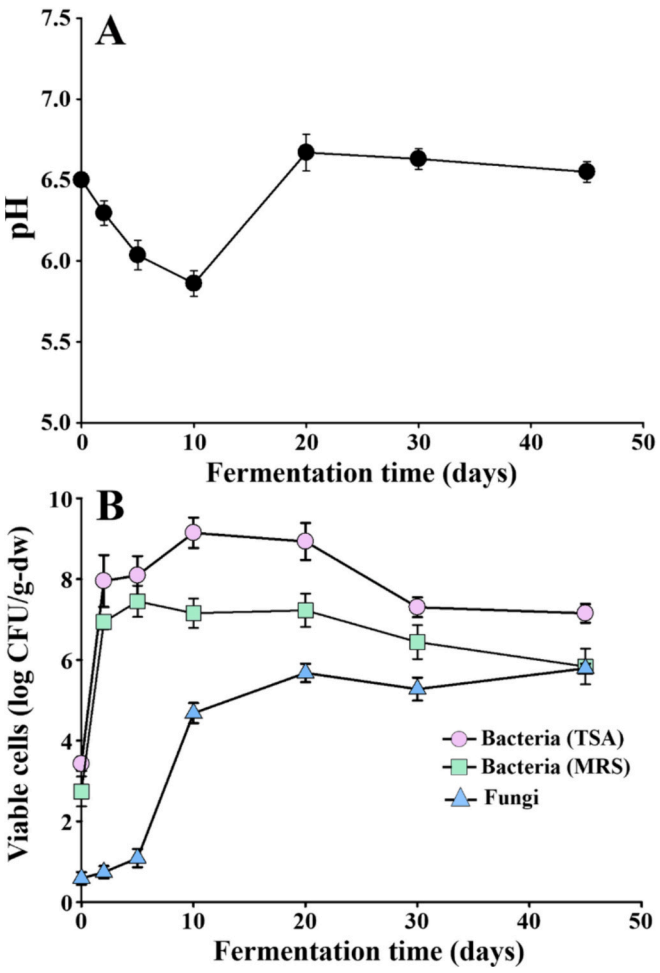

Fig. 1. Profiles of pH (A) and viable cells counted on TSA, MRS agar, and PDA (B) in doenjang-meju bricks during fermentation. Data are presented as mean values ± standard errors averaged from three doenjang-meju bricks. CFU/g-dw, colony forming unit/g-dry weight doenjang-meju.

grown on TSA also rapidly increased during the early fermentation period, reaching approximately 9.0×10^7 CFU/g-doenjang-meju within 2 days, and continued to increase until day 10, after which it gradually declined until the end of fermentation. In contrast, the abundance of fungal cells grown on PDA continued to increase until day 20, after which it remained relatively constant until the end of fermentation. These microbial abundance profiles during doenjang-meju fermentation generally aligned with those reported in previous studies (Han et al., 2023; Kim et al., 2022).

3.2. Changes in organic compounds during doenjang-meju fermentation

The primary objective behind fermenting doenjang-meju, the primary ingredient for producing soy sauce or soybean paste, is to enzymatically degrade various macromolecules, such as polysaccharides, proteins, and lipids, present in soybeans into free sugars, amino acids, fatty acids, and organic acids via fungal and bacterial metabolic activities. Hence, we analyzed these macromolecules and their metabolic products during fermentation. Starch, hemicellulose, cellulose, and pectin were the major polysaccharides initially identified in doenjang-meju, and they underwent distinctly different degrees of degradation during fermentation (Fig. 2A). Starch, initially the most abundant, decreased most rapidly throughout fermentation and remained the least at the end, suggesting its pivotal role as a primary polysaccharide in doenjang-meju fermentation. Cellulose also exhibited significant

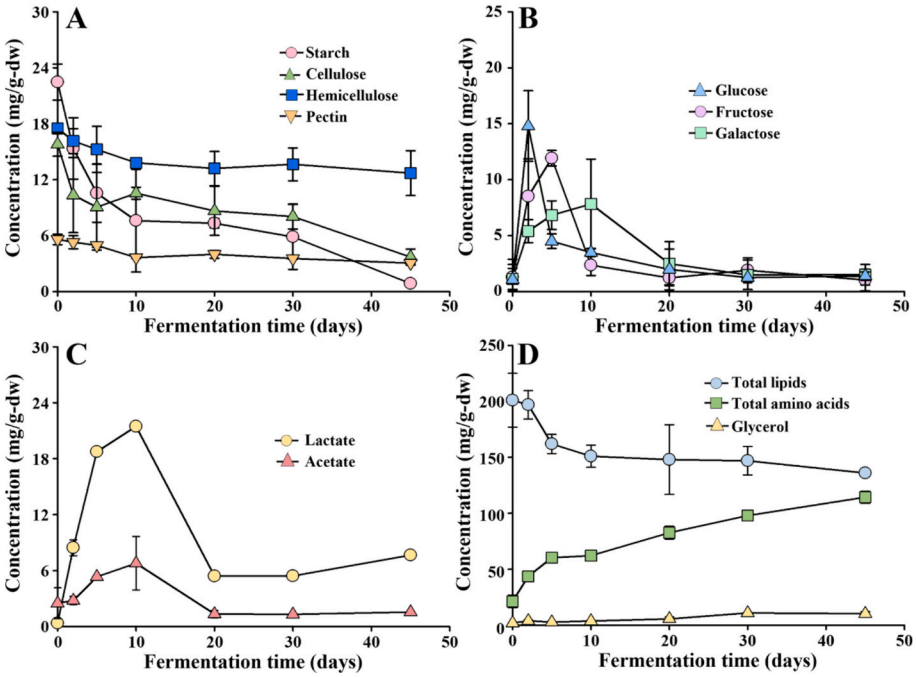

Fig. 2. Profiles of polysaccharides (A), free sugars (B), organic acids (C), and lipids and total amino acids (D) identified in doenjang-meju bricks during fermentation. The total amino acid content was determined by summing the quantities of all individual amino acids. Data are presented as mean values ± standard errors averaged from three doenjang-meju bricks. g-dw, gram-dry weight doenjang-meju.

reduction, particularly in the early fermentation period. However, hemicellulose and pectin exhibited minimal degradation during the entire doenjang-meju fermentation period.

During doenjang-meju fermentation, free sugars, which are produced via polysaccharide breakdown, were analyzed. Glucose, fructose, and galactose were identified as the major free sugars, displaying a similar pattern involving an initial increase followed by a decrease, albeit with somewhat different rates of change (Fig. 2B). Glucose exhibited the most rapid increase and subsequent decrease, followed by fructose and galactose. Notably, despite glucose being primarily produced via starch and cellulose decomposition, its observed increase was substantially smaller than the decrease in starch and cellulose. This may be attributed to the rapid consumption of produced glucose by microbes during fermentation. Furthermore, the analysis of organic acids, primarily produced through free sugar fermentation, revealed lactic acid and acetic acid as the major organic acids (Fig. 2C). These organic acids displayed a rapid increase during the early fermentation period, followed by a subsequent decrease within 20 days, exhibiting well consistence with the pH profile and the rapid increase of cells grown on MRS agar, as shown in Fig. 1A and Fig. 1B, respectively.

The concentration of amino acids, primarily derived from protein breakdown, steadily increased throughout the doenjang-meju fermentation process, continuing until the end of fermentation (Figs. 2D and S1). Noteworthily, glutamate, a crucial amino acid for enhancing taste (umami) in foods, significantly increased, with the most pronounced increase observed after 20 days (Fig. S1). By comparing these findings with those in Fig. 1B, the increase in glutamate is apparently attributed to fungal activity rather than bacterial. In contrast, the total lipid concentration decreased during doenjang-meju fermentation, particularly evident in the early fermentation period, suggesting a significant involvement of bacteria rather than fungi. Corresponding to the lipid decrease, glycerol, produced via lipid hydrolysis, steadily increased as fermentation progressed until the end of fermentation.

3.3. Metagenomic analysis, microbe isolation, and genome sequencing

Approximately 15.5–19.7 million metagenomic sequencing reads for each of the six doenjang-meju samples were obtained, and after processing, each sample yielded approximately 13.9–17.5 million high-quality sequencing reads (Table S1). Taxonomic classification of the high-quality sequencing reads revealed the abundant presence of *Aspergillus* and *Bacillus*, with their abundances varying during doenjang-meju fermentation, while *Enterococcus*, *Staphylococcus*, and *Leuconostoc* were present at lower levels (Fig. 3A). Although the abundance of *Bacillus* gradually decreased as fermentation progressed, it remained highly predominant until the end of fermentation, constituting >72.2% of the metagenomic sequencing reads. *Aspergillus* was detected at extremely low abundance levels during the early fermentation stage (days 0–10); however, its abundance began to increase after 20 days and reached 15.8% of the sequencing reads on day 45. *Enterococcus* remained almost constant at low levels of 2.5–4.1%, while *Staphylococcus* and *Leuconostoc* were also present at considerably low levels, each accounting for <0.4% of the sequencing reads during the entire fermentation period.

Metagenomic assemblies were conducted using high-quality metagenomic sequencing reads to acquire the genome sequences of the five major microbes identified in the doenjang-meju samples, resulting in the generation of their MAGs (Table S2). Although the MAGs exhibited relatively high-quality, with ≥92.7% completeness and a ≤ 0.2% contamination rate, isolation of the five major microbes from the doenjang-meju samples was attempted because the MAG qualities could potentially lead to erroneous results; moreover culturing the five major microbes was considered feasible. Ultimately, these five major microbes— *Aspergillus*, *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Leuconostoc*—were successfully isolated from the doenjang-meju samples, and their genomes were sequenced using a hybrid approach combining MinION and Illumina sequencing, resulting in the generation of their high-quality genomes (Table S3). Furthermore, the genomes of these five isolated microbes and their corresponding MAGs were found to be approximately identical. However, the smaller genome sizes and reduced gene counts in the MAGs, compared with those of the isolated microbes (Tables S2 and S3), indicate that employing microbial genomes rather than MAGs is preferable for more accurate investigations into the metabolic processes underlying doenjang-meju fermentation.

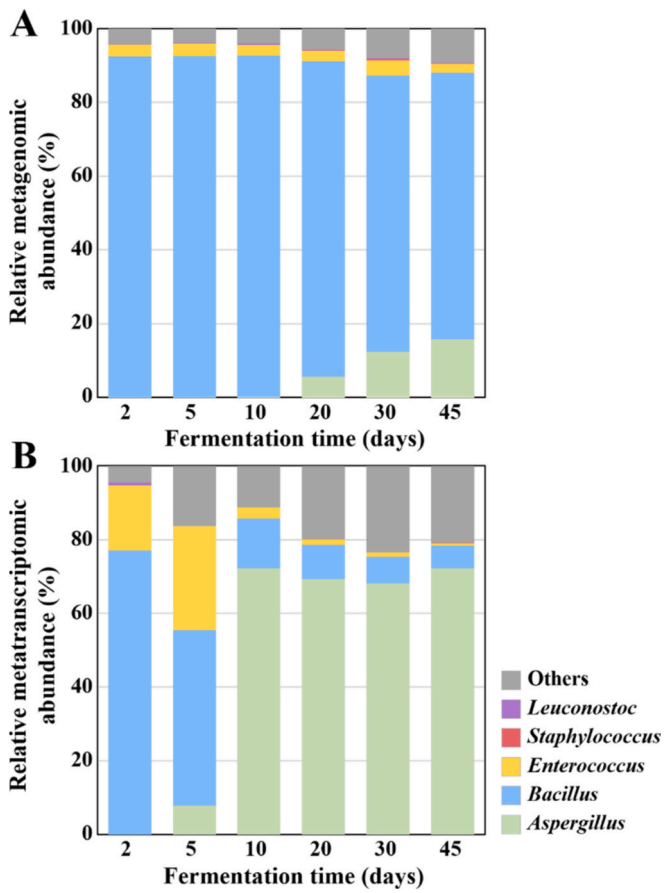

Fig. 3. Metagenomic (A) and metatranscriptomic (B) abundances of microbes in doenjang-meju during fermentation. The relative metagenomic abundances were calculated based on the taxonomic classification of metagenomic sequencing reads using Kaiju. The "Others" category represents the combined abundance of taxonomic reads ≤0.4% in all samples and taxonomically unclassified reads. Relative metatranscriptomic abundances were determined based on the proportions of metatranscriptomic sequencing reads mapped to the whole-genome sequences of the five major microbes isolated from doenjang-meju samples. The "Others" category accounts for the proportions of metatranscriptomic sequencing reads unmapped to the genomes of the five major microbes.

3.4. Metabolic characteristics of microbes during doenjang-meju fermentation

To explore the metabolic characteristics of microbes during doenjang-meju fermentation, metatranscriptomes from six doenjang-meju samples were analyzed. Approximately 10.4–54.7 million high-quality mRNA reads were obtained from approximately 63.2–84.7 million total sequencing reads and were mapped to the genomes of the five major microbes (Table S4). Thereafter, 76.6–95.4% of high-quality mRNA reads were matched to the five genomes. The transcriptional expression of doenjang-meju microbes, as revealed by mapping high-quality mRNA reads onto the five genomes, exhibited notable differences from the microbial abundance results obtained via metagenomic analysis (Figs. 3A and B). Specifically, the transcriptional expression of *Bacillus*, which predominated in metagenomic analysis throughout the fermentation process (comprising 72.2–92.5% of the metagenomic sequencing reads), was initially prominent, peaking at approximately 77% of high-quality mRNA reads on day 2. However, it rapidly declined after 10 days, dropping to only 6.1% on day 45 of fermentation (Fig. 3B). Additionally, *Enterococcus*, merely constituting approximately 3% of the metagenomic sequencing reads, exhibited relatively high transcriptional expression (accounting for 17.6% and 28.2% of high-quality mRNA reads on days 2 and 5, respectively) during the early fermentation period, consistent with the pH profile shown in Fig. 1A. However, its transcriptional expression rapidly decreased, falling to ≤3.0% after 10 days. Conversely, the transcriptional expression of *Aspergillus* was scarcely detected on day 2 but rapidly increased after 5 days of fermentation, reaching approximately 70% after 10 days. The transcriptional expression of *Staphylococcus* and *Leuconostoc*, which exhibited low abundance in metagenomic analysis, consistently remained considerably low throughout the entire fermentation period.

To explore the metabolic characteristics of microbes during doenjang-meju fermentation, the genes of each major microbe were functionally classified based on the KEGG database, and their transcriptional expression was analyzed using the metatranscriptomic data. The numbers of functional genes in *Aspergillus* in the KEGG categories generally exceeded those in other microbes (Fig. S2), primarily because of its larger total gene count than those of other microbes (Table S3). Specifically, metabolic genes related to amino acids and lipids, such as arginine, proline, tyrosine, phenylalanine, ether lipid, and sphingolipid, were evidently more abundant in *Aspergillus* than in other microbes. Interestingly, KEGG category genes responsible for metabolizing carbon compounds, including glucose, pentose, fructose, mannose, galactose, and sucrose, were more abundant in *Enterococcus* than in other microbes despite its relatively small genome size, suggesting that *Enterococcus* may more efficiently metabolize carbon sources through fermentation.

The metabolic characteristics of the five major microbes during doenjang-meju fermentation were investigated by aligning mRNA sequencing reads to functional genes categorized by KEGG categories (Fig. 4). The overall transcription of KEGG categories related to carbohydrate metabolism, such as carbohydrate metabolism, energy metabolism, and membrane transport at the secondary level, was highly expressed during the early fermentation period, particularly on days 2 and 5, but rapidly declined after 10 days (Fig. 4A). Transcriptional analyses of KEGG categories at the tertiary level, such as glycolysis/gluconeogenesis, the tricarboxylic acid (TCA) cycle, amino sugar and nucleotide sugar metabolism, and pyruvate metabolism, further corroborated the high expression of carbohydrate metabolism during the early fermentation period, particularly on days 2 and 5, followed by a rapid decrease after 10 days (Fig. 4B). Most KEGG categories related to amino acids, such as amino acid metabolism at the secondary level and glycine, serine, threonine, methionine, valine, leucine, isoleucine, and lysine metabolisms at the tertiary level, also exhibited high transcriptional expression on days 2 and 5, followed by a rapid decrease after 10 days, despite *Aspergillus* possessing a substantially more abundant set of amino acid metabolic genes than *Bacillus* and *Enterococcus* (Fig. S2C). These results suggest that the overall metabolic ability of microbes in doenjang-meju fermentation decreases as fermentation progresses. However, the overall transcription of KEGG categories related to lipid metabolism remained almost constant throughout the entire doenjang-meju fermentation process. Additionally, the overall transcription of categories related to transport and catabolism, cell growth and death, ether lipid metabolism as well as alanine, aspartic acid, and glutamic acid metabolism, was considerably elevated after 10 days.

Transcriptome analysis of each of the five microbes indicated that the transcriptional upregulation of KEGG categories related to carbohydrate and amino acid metabolism during the early fermentation period was closely associated with *Bacillus* and *Enterococcus*. Notably, transcriptional upregulation of the carbohydrate metabolism, energy metabolism, glycolysis/gluconeogenesis, fructose and mannose metabolism, and signal transduction categories on days 2 and 5, particularly on day 5, was found to be closely related to *Enterococcus*, suggesting that the pH decline during the early fermentation period was associated with fermentation by *Enterococcus*. Additionally, the low transcriptional expression of the transport and catabolism, cell growth and death, and ether lipid metabolism categories on days 2 and 5 was potentially attributable to *Bacillus* and *Enterococcus* possessing considerably fewer genes belonging to these categories (Fig. S2), resulting in their

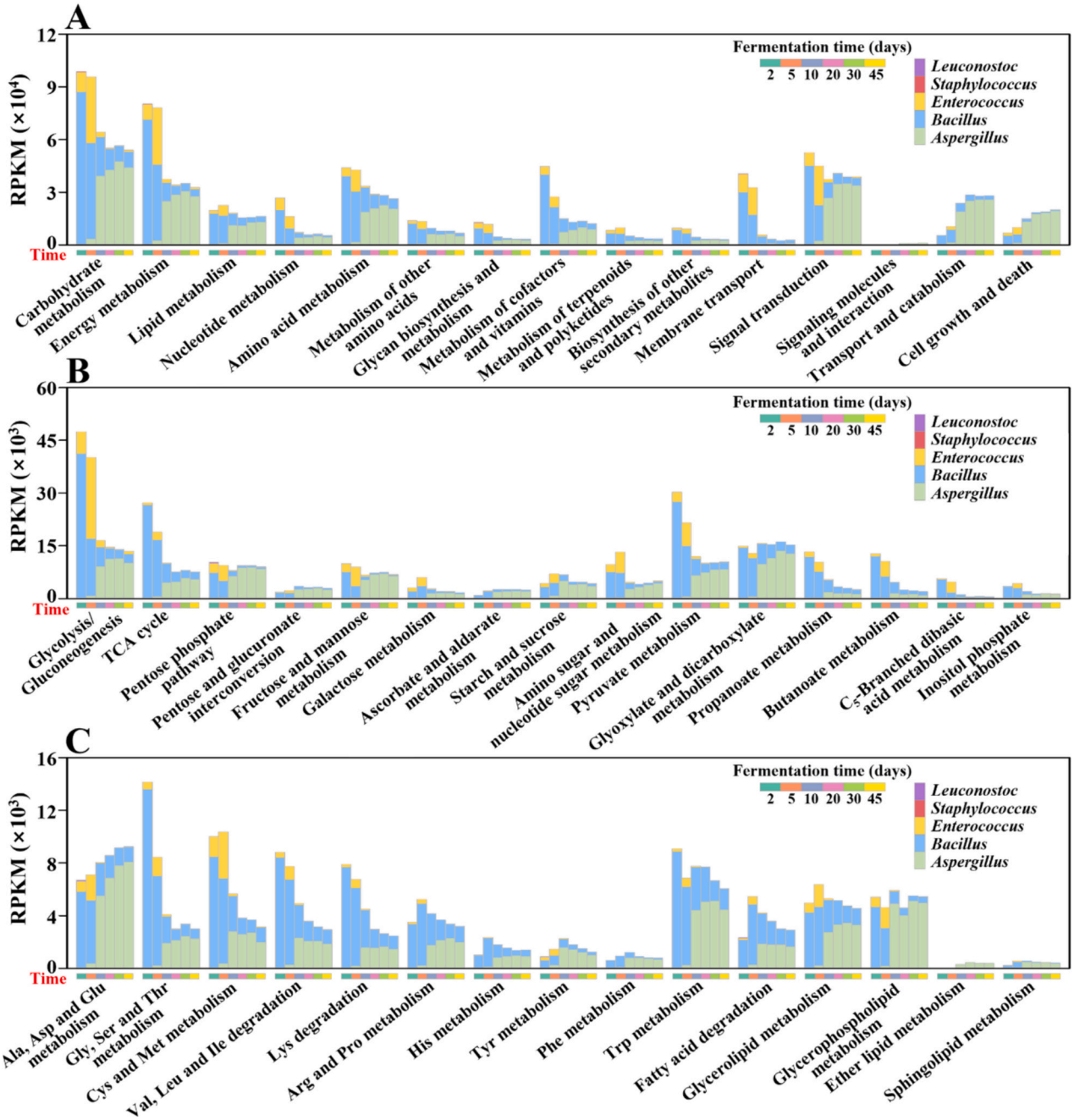

Fig. 4. Transcriptional expression of the five major microbes during doenjang-meju fermentation at the secondary level of all KEGG functional genes (A) and at the tertiary level of carbohydrate (B) and amino acid and lipid (C) metabolic genes. RPKM values on the Y axes were calculated as the sums of the RPKM values of all functional genes of the five major microbes belonging to the same KEGG categories within a sample.

transcriptional expression being substantially low during the early fermentation period, unlike those of other KEGG metabolic categories.

3.5. Metabolic pathway reconstruction and metabolic characteristics of microbes during doenjang-meju fermentation

For a more detailed investigation into the fermentative characteristics of doenjang-meju, the metabolic pathways of polysaccharides, lipids, proteins, free sugars, and amino acids were reconstructed based on the genomes of the five major microbes, and their transcriptional expression was analyzed during doenjang-meju fermentation. Transcriptional analysis of polysaccharide metabolic pathways revealed that the degradation of polysaccharides, including cellulose, starch, pectin, and hemicellulose, to produce free sugars may primarily be attributed to *Aspergillus* throughout the entire doenjang-meju fermentation process (Fig. 5A). Evidently, *Bacillus* might also have significantly contributed to the degradation of certain polysaccharides, including the production of maltose, cellobiose, unsaturated digalacturonate, and L-arabinose and D-xylose from starch, cellulose, pectate, and hemicellulose during the early fermentation period, respectively. However, *Enterococcus* might only have played a partial role in the production of D-glucose, D-galactose and D-xylose, and pectate from dextrin, hemicellulose, and pectin during the early fermentation period, respectively.

Transcriptional analysis of lipid metabolic pathways indicated that *Aspergillus* likely metabolizes lipids primarily to produce glycerol and fatty acids (Fig. 5B). However, *Bacillus* also appeared to serve a

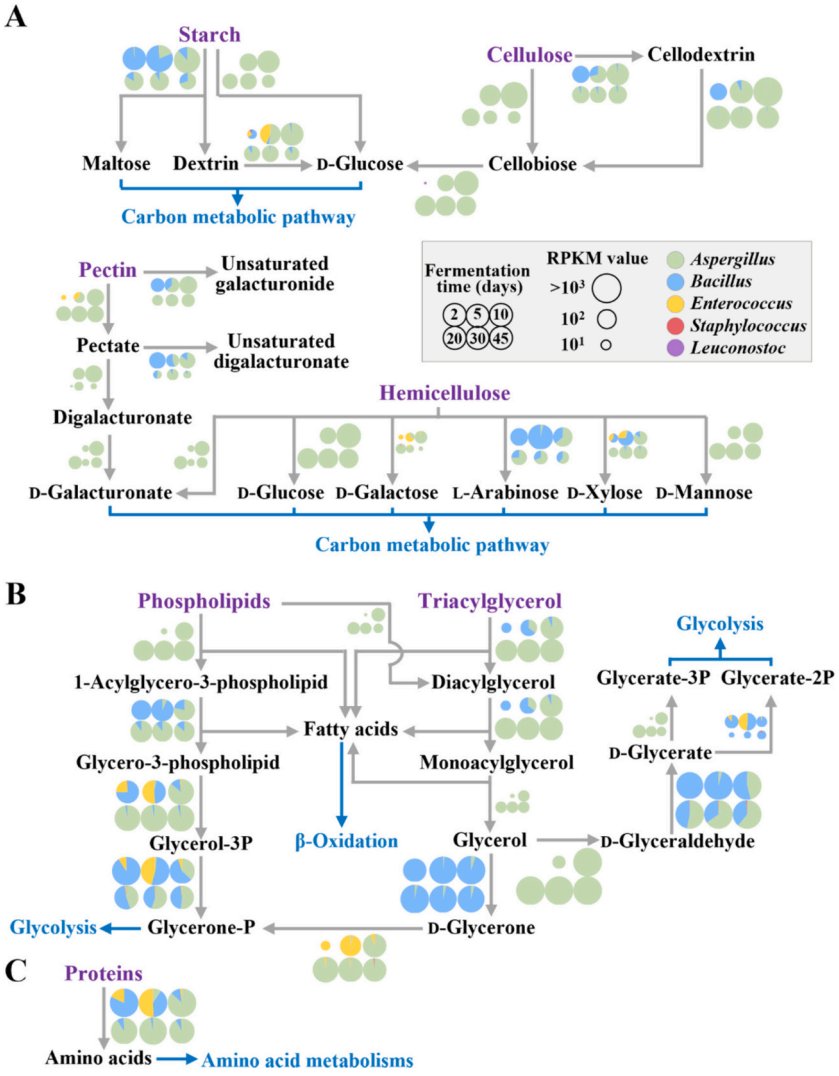

Fig. 5. Proposed metabolic pathways of the five major microbes for carbohydrates (A), lipids (B), and proteins (C) and their transcriptional expression during doenjang-meju fermentation. The transcriptional levels of metabolic genes are depicted using pie charts, representing the RPKM values for each metabolic gene across the five major microbes.

significant role in diverse metabolic pathways throughout the entire fermentation process, such as the conversions of glycerol, D-glyceraldehyde, D-glycerate, and glycerol-3P to D-glycerone, D-glycerate, glycerate-2P, and glycerone-P, respectively, throughout the entire fermentation process, underscoring its importance in lipid metabolism across the entire duration of doenjang-meju fermentation. Additionally, phospholipids appeared to be predominantly hydrolyzed by *Aspergillus*, while initially, triacylglycerol and diacylglycerol were probably hydrolyzed by *Bacillus*, with *Aspergillus* taking over after 10 days. The heightened transcriptional activity of *Bacillus* might have been associated with the rapid decrease in total lipids shown in Fig. 2D. *Enterococcus* also appeared to play an important role in specific lipid metabolic pathways, such as the conversion of D-glycerone to glycerone-P, particularly during the early stages of fermentation. Transcriptional analysis of genes encoding proteinases indicated that proteins are primarily degraded into amino acids by *Bacillus* and *Enterococcus* during the early fermentation period, shifting to *Aspergillus* dominance after 10 days (Fig. 5C), as depicted by the transcriptome profile shown in Fig. 3B.

Transcriptional analysis of the metabolic pathways involving free sugars revealed that the microbes primarily responsible for their metabolism during doenjang-meju fermentation differ depending on the free sugar. Consistent with most metabolic pathways observed during doenjang-meju fermentation, *Bacillus* and *Enterococcus* were possibly the main contributors to glucose, fructose, and sucrose metabolisms during the early fermentation period, with *Aspergillus* becoming dominant after 10 days (Fig. 6). However, galactose might have been metabolized similarly by *Bacillus*, *Enterococcus*, and *Aspergillus* throughout the entire doenjang-meju fermentation process. Moreover, mannose might have been primarily metabolized by *Enterococcus* during the early fermentation phase, with minimal involvement from *Bacillus*, and by *Aspergillus* after 10 days; furthermore, arabinose could have been exclusively metabolized by *Aspergillus* during doenjang-meju fermentation. Additionally, xylose and galacturonate might have been exclusively metabolized by two distinct specific microbes, each employing a different metabolic pathway. Specifically, xylose was potentially converted to D-xylulose and xylitol exclusively by *Bacillus* and *Aspergillus*, respectively, while galacturonate could have been converted to glycerol and tagaturonate exclusively by *Aspergillus* and *Enterococcus*, respectively. Additionally, because tagaturonate, exclusively produced by *Enterococcus*, can be converted to altonate exclusively by *Bacillus*, efficient metabolism of galacturonate requires their cooperation.

Pathway reconstruction and transcriptional analysis revealed a high expression of glycolysis; the 6-phosphogluconate/phosphoketolase pathways; and genes encoding L-lactate dehydrogenase,

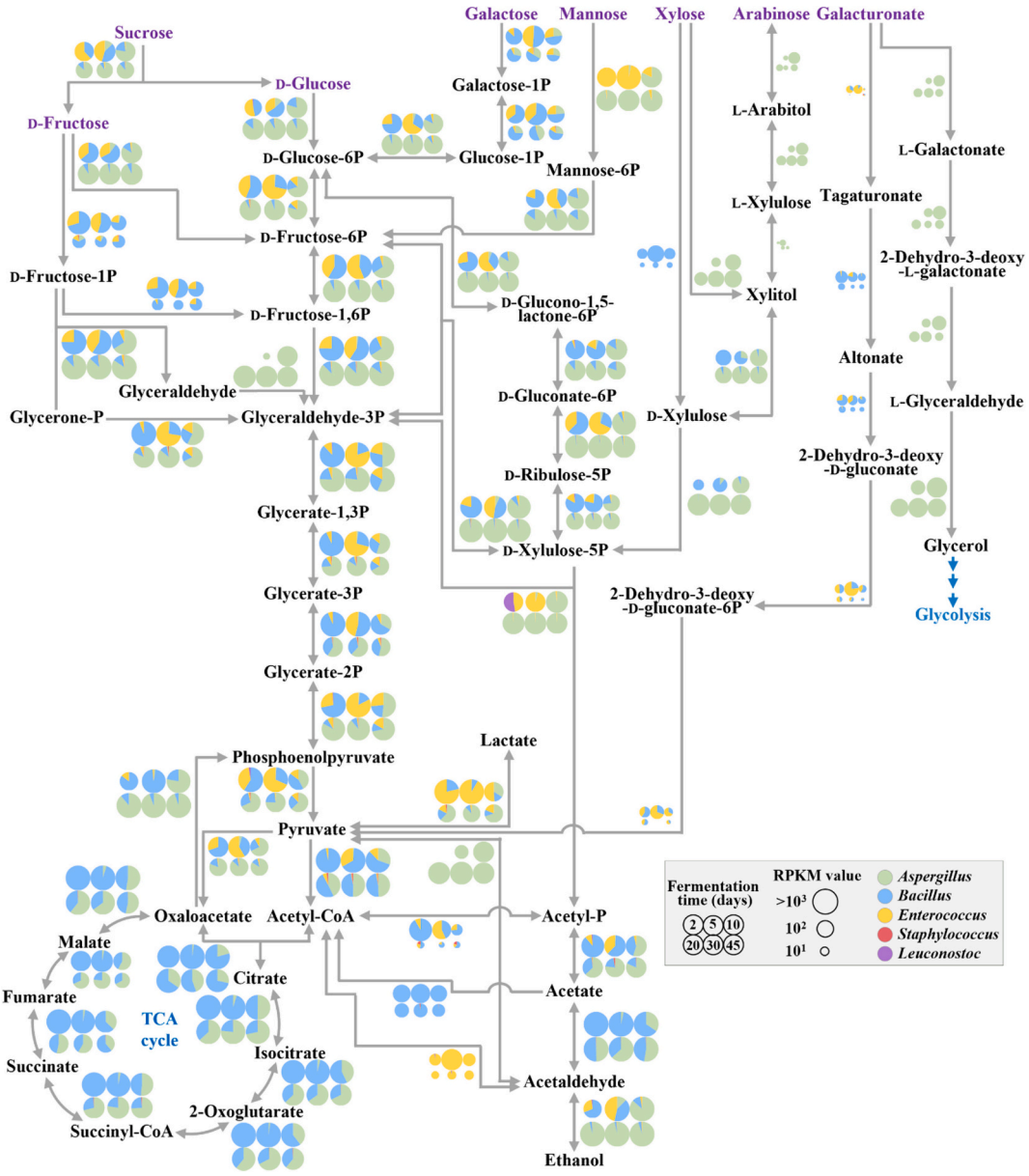

Fig. 6. Proposed metabolic pathways of the five major microbes for major free sugars and galacturonate and their transcriptional expression during doenjang-meju fermentation. The transcriptional levels of metabolic genes are depicted using pie charts, representing the RPKM values for each metabolic gene across the five major microbes.

phosphotransacetylase, acetate kinase, and acetaldehyde:NAD^+ oxidoreductase in *Enterococcus* during the early fermentation period (Fig. 6). This suggests that *Enterococcus* produces L-lactate, ethanol, acetate, and CO_2 as major products from free sugars during doenjang-meju fermentation (Choi et al., 2024). *Aspergillus* and *Bacillus* also possess metabolic pathways associated with lactate, acetate, and ethanol, as well as a complete TCA cycle representing aerobic metabolism (Chun et al., 209; Han et al., 2024), and the related metabolic genes were highly expressed throughout the entire fermentation period. These findings indicate that rather than performing fermentation, *Aspergillus* and *Bacillus* may primarily engage in aerobic metabolism, utilizing lactate, acetate, and ethanol produced by *Enterococcus* as well as directly metabolizing free sugars, exhibiting consistency with the decrease in lactate and acetate observed after 10 days, as shown in Fig. 2C. *Staphylococcus* and *Leuconostoc*, which demonstrated markedly low levels of transcriptional expression throughout the doenjang-meju fermentation process, indicated minimal contribution toward most metabolic pathways. Nonetheless, they appeared to play a substantial role in pathways associated with acetyl-P metabolism, including the production of acetyl-P from xylulose-5-phosphate and conversion of pyruvate to acetyl-CoA.

Transcriptional analysis of amino acid metabolic genes revealed that *Aspergillus*, *Bacillus*, and *Enterococcus* are primarily involved in amino acid metabolisms, with their roles varying depending on the specific amino acids and metabolic genes, even within the same pathways (Fig. 7). Generally, *Bacillus* and *Enterococcus* were found to be crucial during the early fermentation period, while *Aspergillus* became significant after 10 days according to the transcriptional analysis (Figs. 3B, 4, 5, and 6). Nevertheless, in amino acid metabolism, *Bacillus* and *Aspergillus* exclusively undertook vital roles in specific amino acid pathways and metabolic genes throughout the entire fermentation period. For instance, histidine metabolism might have been exclusively handled by *Bacillus*, while certain metabolic steps for amino acids, such as leucine and tyrosine, were potentially accomplished solely by *Aspergillus*. As shown in Figs. 4 and 7, transcriptional analysis revealed that *Bacillus*

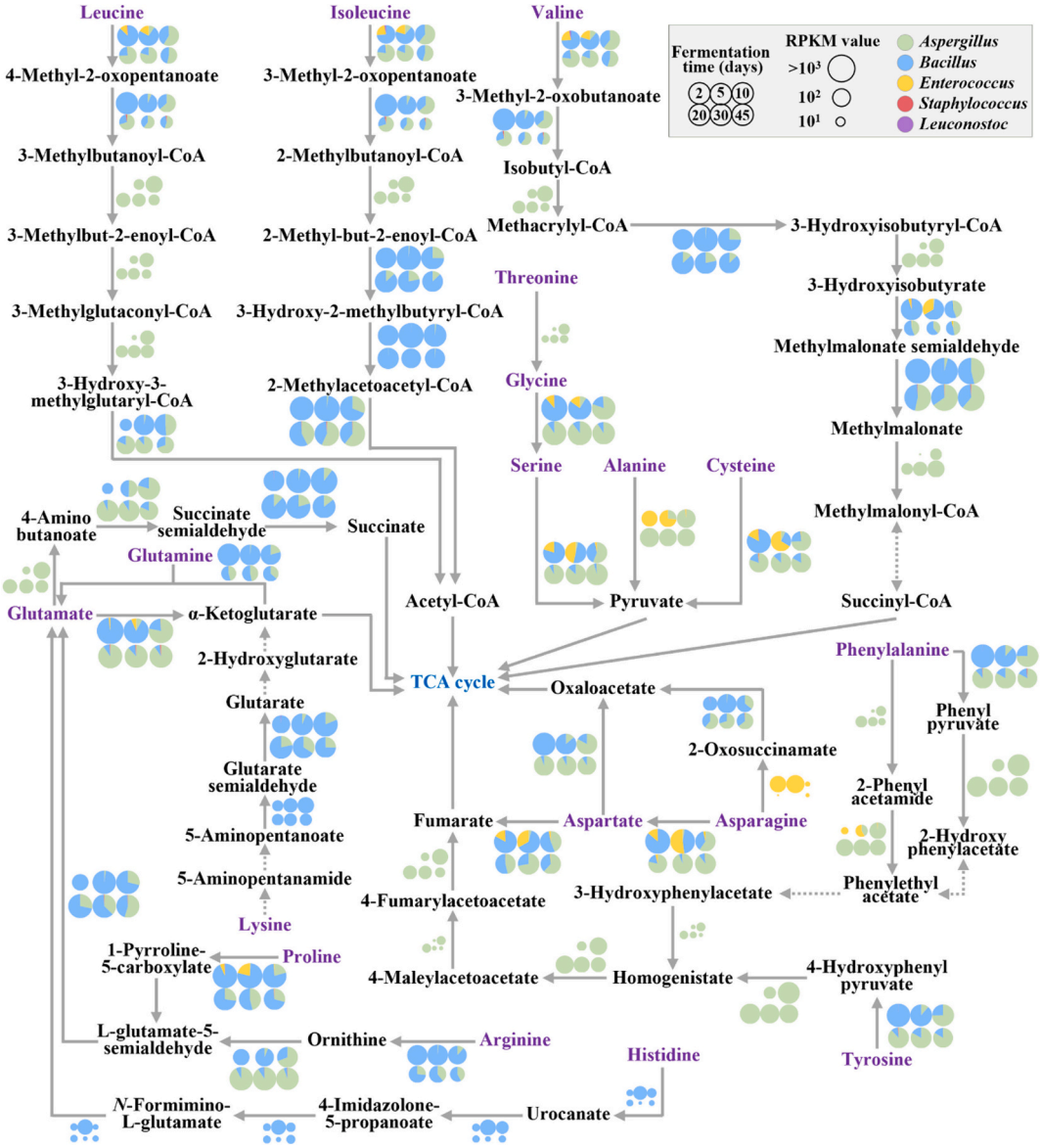

Fig. 7. Proposed metabolic pathways of the five major microbes for amino acids and their transcriptional expression during doenjang-meju fermentation. The transcriptional levels of metabolic genes are depicted using pie charts, representing the RPKM values for each metabolic gene across the five major microbes.

potentially serves a more important role in amino acid metabolism compared with other metabolic pathways; however, *Enterococcus* appeared to be chiefly responsible for only a few metabolic reactions of amino acid pathways, such as the conversion of asparagine to 2-oxosuccinamate.

4. Discussion

In Korea, traditional doenjang-meju is typically produced by molding steamed soybeans into brick shapes and allowing them to ferment naturally with microbes from the surrounding environment in a solid-state without control. Consequently, as fermentation progresses, conditions within the doenjang-meju bricks alter, leading to microbial succession during fermentation. Water content, which decreases as fermentation progresses owing to drying, has been reported as one of the most significant variables influencing microbial community alterations during doenjang-meju fermentation (Jung et al., 2014; Kim et al., 2022; Oh et al., 2024). Bacteria typically thrive in conditions with a high-water content, whereas fungi, such as *Aspergillus*, tend to outcompete bacteria in conditions with a low-water content (Troller & Christian, 2012).

Bacterial and fungal communities in doenjang-meju have extensively been analyzed in previous studies, mainly through amplicon-based community analysis (Kim et al., 2011, Kim et al., 2022; Jung et al., 2014; Song et al., 2020; Ryu et al., 2021). However, these analyses were limited by their reliance on specific PCR primers exclusively targeting either bacteria or fungi, offering relative community information within each domain but lacking the ability to directly compare their abundance (Chen et al., 2023; Liu et al., 2023). In contrast, the metagenomic analysis implemented in this study analyzes bacterial and fungal communities collectively, allowing a direct comparison of their relative abundances. Metagenome-based community analysis demonstrated that the bacterium *Bacillus* was predominant during the early fermentation period, which is characterized by a high-water content, whereas the fungus *Aspergillus* became abundant as fermentation progressed (Fig. 3A), confirming the importance of water content as a variable influencing doenjang-meju fermentation. However, metatranscriptome analysis revealed that *Enterococcus*, which yields a relatively low abundance, as well as *Bacillus*, also exhibited relatively high transcriptional expression during the early fermentation period. The high transcriptional activity of *Enterococcus* during the early fermentation period

aligned with the decrease in pH (Fig. 1A) and production of lactic acid (Fig. 2C), indicating the significant involvement of LAB during the early doenjang-meju fermentation period, as described previously (Han et al., 2023; Kim et al., 2022). Additionally, although *Bacillus* remained predominant until the end of the fermentation period in the metagenome-based community analysis, it exhibited relatively low transcriptional expression in the middle and late fermentation periods (after 10 days), with *Aspergillus* displaying considerably high transcriptional expression instead (Fig. 3B). These results suggest that solely relying on metagenome-based community analysis has limitations in identifying the key players during doenjang-meju fermentation. In addition, the metagenome and metatranscriptome analyses evidently demonstrated that bacteria such as *Bacillus* and *Enterococcus* play major roles during the early stages of doenjang-meju fermentation, which are characterized by a high moisture content, while fungi such as *Aspergillus* serve essential major roles during the later fermentation stages, which is typified by a low moisture content.

Glucose, fructose, and galactose were identified as the major free sugars during doenjang-meju fermentation (Fig. 2B), consistent with previous findings (Han et al., 2023; Kim et al., 2022). However, pathway reconstruction and transcriptional analysis revealed the potential production of a more diverse range of free sugars, such as arabinose, xylose, and mannose, alongside the major free sugars, from various polysaccharides, including starch, hemicellulose, cellulose, and pectin (Fig. 5A). These findings potentially emanate from the immediate consumption of these free sugars after their production from polysaccharides, rather than their accumulation. The major free sugars, glucose, fructose, and galactose, also exhibited a rapid increase exclusively during the early fermentation stages, followed by a decrease, and yielded extremely low levels after 20 days. Pathway reconstruction and transcriptional analysis indicated that polysaccharide decomposition may primarily be conducted by different microbes depending on polysaccharide type (Fig. 5A). Moreover, distinct microbes apparently prefer metabolizing specific free sugars and amino acids (Figs. 6 and 7). These observations imply that diverse microbial compositions potentially result in different free sugar profiles as well as free sugar and amino acid metabolic pathways, leading to a range of fermentation products with varied metabolites and flavors. Consequently, altering the microbial community within doenjang-meju has been suggested to potentially expand the range of quality and taste profiles in doenjang-meju products.

The taxonomic classification of metagenome sequencing reads in this study identified *Aspergillus*, *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Leuconostoc* as microbial members during doenjang-meju fermentation (Fig. 3). However, previous studies have reported the presence of other taxonomic groups, such as *Rhizopus*, *Penicillium*, *Mucor*, and *Lactobacillus*, in addition to the forgoing genera (Jung et al., 2014; Kim et al., 2011; Kim et al., 2022; Kim et al., 2024). These findings suggest that diverse microbes utilize various metabolic pathways to produce a range of metabolites, including free sugars, organic acids, amino acids, and volatile compounds, during doenjang-meju fermentation. Therefore, fermentative characteristics analyzed in this study merely represent a fraction of the overall fermentative features of Korean traditional doenjang-meju, and these features potentially vary in other doenjang-meju types. To comprehensively understand the fermentative features of Korean traditional doenjang-meju, further analysis of diverse doenjang-meju varieties is necessary.

5. Conclusion

In our study, we investigated the fermentative metabolic characteristics of doenjang-meju over a period of 45 days, employing a genome-centered metatranscriptomic approach. Utilizing metagenome-based community analysis, we accurately identified *Aspergillus*, *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Leuconostoc* as key microbes responsible for driving the fermentation process of doenjang-meju. These microbes were isolated and subjected to genome sequencing. Our metatranscriptomic analysis, leveraging these genomes, revealed the crucial involvement of *Bacillus* and *Enterococcus* during the initial phases of fermentation, with a subsequent shift toward dominance by *Aspergillus*. Utilizing the genomes of these microbes, we reconstructed metabolic pathways associated with carbohydrates, lipids, proteins, free sugars, and amino acids. Subsequently, we conducted transcriptional analysis of these pathways, thereby elucidating the metabolic roles of key microbes within each pathway. This study demonstrated that genome-centered metatranscriptomic analyses could offer an extensive and comprehensive understanding of the fermentative metabolic features of doenjang-meju throughout fermentation. Furthermore, this approach will help enhance our understanding of various environmental microbial processes, including those involved in other food fermentation processes. However, while metatranscriptomic analysis offers valuable insights into the fermentative metabolic features of microbiota, it alone imposes limitations on achieving a comprehensive understanding of the fermentative metabolism in doenjang-meju. Additionally, both microbial communities and metabolic compounds directly associated with the quality of doenjang-meju vary dynamically, demonstrating a close relationship during fermentation. Therefore, further studies using a combination of complementary methods, such as metabolomics or experimental validation, may be necessary to obtain a thorough understanding of the fermentative metabolic features of doenjang-meju and to overcome these challenges.

CRediT authorship contribution statement

Dong Min Han: Writing – original draft, Visualization, Methodology, Formal analysis. **Ju Hye Baek:** Methodology, Investigation. **Dae Gyu Choi:** Investigation. **Che Ok Jeon:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101658.

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