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Insights into the Biogenic Amine-Generating Microbes during Two Different Types of Soy Sauce Fermentation as Revealed by Metagenome-Assembled Genomes

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ABSTRACT: In-depth knowledge of the microbes responsible for biogenic amine (BA) production during soy sauce fermentation remains limited. Herein, the variations in the BA profiles, microbial communities, and microbes involved in BA production during the fermentation of soy sauce through Japanese-type (JP) and Cantonese-type (CP) processes were compared. BA analysis revealed that the three most abundant BA species were putrescine, tyramine, and histamine in the later three stages (1187.68, 785.16, and 193.20 mg/kg on average, respectively). The BA profiles differed significantly, with CP samples containing higher contents of putrescine, tyramine, and histamine ($P < 0.05$) at the end of fermentation. Metagenomic analysis indicated that BA-producing genes exhibited different abundance profiles, with most genes, including *speA*, *speB*, *arg*, *speE*, and *tyrDC*, having higher abundances in microbial communities during the CP process. In total, 15 high-quality metagenome-assembled genomes (MAGs) were retrieved, of which 10 encoded at least one BA production-related gene. *Enterococcus faecium* (MAG10) and *Weissella paramesenteroides* (MAG5) might be the major tyramine producers. The metabolic pathways for BA production were predicted. The high putrescine content in CP might be associated with the high abundance of *Staphylococcus gallinarum* (MAG8). This study provides a comprehensive understanding of the diversity and abundance of genes involved in BA synthesis, especially at the species level, during food fermentation.

Keywords: Soy sauce fermentation; biogenic amine; amine-producing genes; metagenome-assembled genomes; metabolic pathways

1. Introduction

The occurrence of biogenic amines (BAs) in fermented foods has attracted considerable attention due to their negative physiological effects in humans [1]. BAs are harmful organic nitrogen compounds formed through the bacterial decarboxylation of free amino acid (FAA) precursors in a variety of foods [2]. The typical BAs in fermented foods include histamine, tyramine, putrescine, cadaverine, and phenylethylamine,

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which are produced by the decarboxylation of histidine (histidine decarboxylase, HDC), tyrosine (tyrosine decarboxylase, TYDC), putrescine (ornithine decarboxylase, ODC), lysine (lysine decarboxylase, LDC), and phenylalanine (phenylalanine decarboxylase, PDC), respectively [3]. In addition to decarboxylation, the deimination of agmatine (agmatine deiminase, AgDI) and the hydrolysis of agmatine (agmatinase, AGM) also generate putrescine [4, 5]. Among these, tyramine and putrescine are often the main amines found in fermented products. Consuming foods with high BAs levels can be harmful to the body, such as headaches, flushing, and hypertension [6].

BAs can be synthesized by bacteria, yeasts, and molds [2]. Gram-negative bacteria from the *Enterobacteriaceae*, *Photobacterium* and *Pseudomonas* are known to produce a large amount of BAs. As for fermented foods, gram-positive bacteria, particularly lactic acid bacteria (LAB) such as *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, and *Tetragenococcus*, are the main producers of BAs [3]. However, the ability of bacterial FAA decarboxylation is highly variable, depending on the species, the strain and the environmental conditions [7].

Soy sauce, a traditional fermented food product, originated in China over 2500 years ago and is widely consumed in Asian countries [8]. Chinese- and Japanese-type (JP) soy sauce are the main two types of soy sauce. Chinese-type soy sauce is produced using mostly soybeans with a smaller amount of wheat flour under ambient temperatures and open environments; JP soy sauce is produced using equal amounts of soybeans and wheat flour as well as yeast, which is added as a starter under controlled-temperature and periodic stirring fermentation conditions [9]. Of Chinese-type soy sauces, Cantonese-style (CP) soy sauce is the main soy sauce type, accounting for more than 70% of soy sauce production in China [10]. The JP and CP fermentation are both composed of two fermentation stages (*koji* generation and *moromi* fermentation) [9, 11]. Several studies have shown that the BA concentration of soy sauce may exceed the limit standards, and putrescine, tyramine, histamine, and cadaverine are the main BAs [12, 13]. These BAs, together with higher contents of tryptamine and phenethylamine, are also frequently found during the soy sauce fermentation process [14-17]. Bacteria genera *Weissella*, *Staphylococcus*, *Bacillus*, *Tetragenococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Kurthia*, and *Klebsiella*, along with fungal genera *Aspergillus*, *Zygosaccharomyces*, and *Candida*, are primarily found in soy sauce communities at various fermentation stages [16, 18, 19] and some strains such as *Tetragenococcus halophilus*, have been described as BA producer [20, 21]. However, few studies have compared the BA profiles and explored the microbes responsible for BA production between the fermentation stages of CP soy sauce and JP soy sauce.

Shotgun metagenomics sequencing can comprehensively describe genetic diversity and has also been applied to reconstruct microbial genomes (metagenome-assembled genomes, MAGs) within food [22-24], unveiling the functional and taxonomic characteristics of samples or MAGs without prior knowledge of the types of microorganisms present. Recently, the microbes responsible for BA production have been investigated through shotgun metagenomic and metatranscriptomic analyses based on four decarboxylase (HDC, TYDC, ODC, and LDC) genes during soy sauce (ganjang) fermentation [14]. However, the diversity

of species or strains responsible for BA production in the soy fermentation environment remains poorly understood. The BA production mechanism revealed by strain-level MAGs remains to be further elucidated. Therefore, to uncover the differences in BA profiles and the microbial contribution to the production of BAs, this study investigated the succession and genes related to BA production as well as the BA contents by combining shotgun metagenomic and metabolomic approaches during JP and CP industrial soy sauce production. Notably, this work revealed the key microbial species responsible for BA production based on metagenome-assembled genomes. To the best of our knowledge, this is the first report to completely reveal the differences in BA concentrations and BA-production-related genes at the strain level between different soy sauce fermentation types. These findings should help to understand the BA formation mechanism and provide useful guidelines for producing high-quality soy sauce through the control of BAs.

2. Materials and methods

2.1 Analytical Standards and reagents

The standard solutions of $AgNO_3$ and NaOH were purchased from Shenzhen Bolinda Technology (Shenzhen, China). Eight standard BAs (tryptamine, β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The derivatization reagent (dansyl chloride) and other chemicals were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China). HPLC-grade acetonitrile and ammonium acetate were purchased from Merck (Whitehouse Station, NJ, USA). The oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

2.2 Sampling and chemical analyses

Soy sauce mash samples during CP and JP fermentation were collected from a local soy sauce factory in Zhongshan City, China [11]. These two different types of fermentation processes have been used for soy sauce production in this factory for a long time, and they are also the two primary soy sauce manufacturing processes in the world. The mash samples were collected on days 7, 15, 30, 60, 90, and 120. Biological triplicate mash samples were collected from three different tanks at each sampling time and placed in 50-mL centrifuge tubes, then transported on ice to the laboratory and stored at −20℃ until DNA extraction and chemical analysis.

Analyses of the pH, total acidity (TA), NaCl, and amino acid nitrogen (AAN) contents of mash were conducted as previously described [25]. BAs in mash were extracted with 0.1 mol/L HCL and were then derivatized with dansyl chloride solution (10 mg/mL in acetone). Eight BAs (tryptamine, β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) of the extracted solution were detected by ultra-performance liquid chromatography (UPLC) on an Agilent 1290 II HPLC system (Agilent Ltd., Santa Clara, CA, USA), as described in a previous study [26, 27]. Triplicates were performed in all experiments.

2.3 Total DNA extraction, library preparation and metagenomic sequencing

Total genomic DNA from mash samples (0.5 g) was extracted using an EZNA^{TM} Mag-Bind Food DNA extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. An Invitrogen Qubit 2.0 fluorometer and electrophoresis on a 1% agarose gel were used to determine DNA quantity and quality. DNA samples extracted from triplicates at the same sampling time were pooled and stored at −20℃ for subsequent metagenomic analysis. Using a Covaris M220 nucleic acid shearer (Covaris, Woburn, MA, USA), DNA was sheared into approximately 350-bp fragments. Sequencing libraries were then prepared using a NEBNext Ultra DNA Library Prep Kit (NEB, Ipswich, MA, USA) according to the manufacturer's instructions. The libraries were then sequenced on the Illumina NovaSeq 6000 platform (San Diego, CA, USA) at the Novogene Bioinformatics Technology (Beijing, China), generating 2 × 150-bp paired-end reads. Sequencing data were deposited into the NCBI BioProject under accession number PRJNA795848.

2.4 Metagenome assembly, taxonomic assignment, and BA gene annotation

Metagenome assembly, taxonomic assignment, and gene prediction were performed as described in our previous study [11]. The flow chart for metagenomics is shown in Fig. S1. In brief, after adapter sequences were removed from the generated reads (×300 bp), they were then trimmed using Trimmomatic v.0.30 [28]. All of the quality-controlled reads were combined and co-assembled to obtain contigs using IDBA-UD v.1.1.1 [29]. Genes were predicted using the MetaGeneMark program [30], and genes shorter than 300 nt were discarded. CD-HIT software [31] was used to remove redundant genes at a 95% identity threshold and ≥ 90% coverage. To identify the taxonomic profiles of microbial compositions, non-redundant genes were aligned to the National Center for Biotechnology Information (NCBI)-nr database using DIAMOND [32] with a threshold e-value $\leq 1 \times 10^{-5}$, followed by taxonomic profiling using MEGAN [33]. To determine the relative abundances of genes in each sample, the filtered reads were mapped back to the genes with Bowtie2 using default parameters [34]. For the annotation of genes involved in BA production (such as amino acid decarboxylase, amine deiminase, transferase and synthase genes) (Table S1), an open reading frame (ORF) sequence was considered to be a BA gene when its best hit showed an amino acid identity of > 30% and query coverage of no less than 95% alignment length ratio to the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology sequences involved in BA synthesis. The genes encoding the amino acid decarboxylase and other genes involved in BA production were acquired based on the numbers of ORFs that matched the corresponding KEGG orthologies (version 103.0) using the KofamScan program with an assignment threshold of e^{-5} [35]. Single-end reads from mash samples were mapped onto the BA-annotated ORFs to quantify the abundances of genes involved in BA synthesis using Bowtie2 [34] with default parameters. The transcripts per million (TPM) values for each gene abundance in different metagenomes were calculated using Salmon in mapping-based mode [36].

2.5 Genome binning, gene prediction, and annotation

After the high-quality reads were assembled, as described before, assembled-contig coverage was calculated by mapping the initial reads to the contigs larger than 1 kb using the Bowtie2 software [34].

Contigs with short length and low coverage (with length < 1 kb and average coverage < 5) were discarded before the next step of binning. For contig binning, the CONCOCT software was used based on sequence composition and coverage [37]. The completeness and contamination of all recovered MAGs were assessed with CheckM software [38]. MAGs with low-quality (80% completeness and/or 10% contamination) were removed from downstream analysis. The ORFs of all MAGs were determined using Prodigal software [39]. Then, the predicted ORFs of each MAG were checked based on sequence similarity to the NCBI-nr database using BLASTP, with a maximum allowed e-value of 1 e^{-10}. To obtain the species-level taxonomic annotation of each MAG, BLASTP results were parsed using MEGAN [33]. Taxonomic analysis of MAGs was performed using GTDB-Tk programs [40]. The average nucleotide identity (ANI) of MAGs to reference genomes was calculated using FastANI [41]. Putatively novel MAGs were assigned as potentially new species when the ANI output obtained using GTDB-Tk was < 95% [42]. MAG abundance (depth) was calculated by mapping the high-quality reads from each metagenomic dataset to the contigs from each MAG using the Bowtie2 software [34]. To generate phylogenetics tree of MAGs, all protein sequences translated in silico from these MAGs and the genomes of their closest species were input into PhyloPhlAn using default settings [43]. For BA gene annotation in each MAG, an ORF sequence with best hit (more than 30% amino acid identity and no less than 95% query coverage) to the KEGG orthology sequences was considered to be a BA gene using the KofamScan program with an assignment threshold of e^{-5} [35]. To identify virulence factor genes (VFGs) in *Klebsiella pneumoniae* (MAG13), ORF sequences were mapped against the virulence factor database (VFDB) [44] using BLASTP. Hit sequences with an identity ≥ 90% and coverage ≥ 90% were designated as VFG-like sequences.

2.6 Statistical analysis

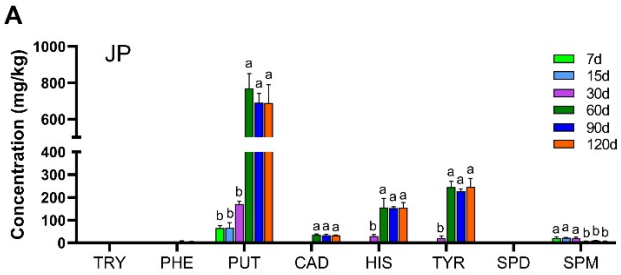
Statistical analysis was performed using the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). Differences in the BA contents during soy sauce fermentation were tested using one-way analysis of variance (ANOVA) with a post hoc least significant difference test (LSD). Differences in the BA contents at 120 d of fermentation between JP and CP samples were tested with a t-test. The correlations between physicochemical characteristics and BA gene abundances were estimated using Spearman's correlation coefficients based on values of $|\rho| > 0.7$, with statistical significance at $P < 0.05$. Heatmap visualization was created using the "vegan" package in the R environment. Cytoscape was used to visualize complex networks between BA genes and potential BA-producing microbes. A network analysis was performed to examine the relationships between BAs and the microbial community. To construct correlation matrix, all possible pairwise Spearman's correlation coefficients (ρ) among the BAs and the microbial genera were calculated. The correlation between two nodes was considered statistically significant when the P value was ≤ 0.05 and the rho coefficients $|\rho| \geq 0.6$.

3. Results and discussion

3.1 Physicochemical characteristics and BA profiles during moromi fermentation

As shown in Table S2, the change trend of basic physiochemical properties was similar during the two fermentation types. The pH dramatically decreased from 7 during 60 d of fermentation (from 6.13–4.90 in JP and from 6.11–4.11 in CP) and then stabilized at the later three stages of JP and CP fermentation, ranging from 4.90–4.82 and 4.91–4.81, respectively. In both the JP and CP fermentations, the salt contents were relatively stable, ranging from (14.11–13.88)% and (16.29–15.15)%, respectively. The salt contents were higher during the later three stages in CP (15.10% on average) than those in JP (13.75%) ($P < 0.05$). However, the TA and AAN contents increased. The increased contents of AAN were linked to the increased contents of various FAAs [11].

The BA analysis revealed that putrescine, histamine, and tyramine were the major BAs present in the two fermentation processes, but the change trend of the contents of various BAs was different (Fig. 1, Table S3). During JP fermentation, putrescine was the most abundant BA species in the later three stages (717.22 mg/kg), followed by tyramine (239.46 mg/kg) and histamine (154.69 mg/kg) (Fig. 1A). However, the contents of these three BAs remained stable in the later three stages ($P \geq 0.05$) after a dramatic increase of concentration during the initial phase (from 7 to 15 d of fermentation). In addition, cadaverine was also detected, with concentrations of (34.54 ± 1.83) mg/kg at the end of fermentation. However, the contents of spermine decreased from 21.37 to 8.19 mg/kg ($P < 0.05$). Tryptamine was not detected during fermentation. During CP fermentation, the concentrations of three BAs (putrescine, tyramine, and histamine) rapidly increased from the early fermentation period ($P < 0.05$) (Fig. 1B). The contents of putrescine, tyramine, and histamine at the end of fermentation (120 d) were (2200.48 ± 350.73), (1432.07 ± 151.20), and (327.51 ± 40.11) mg/kg, respectively. Compared to JP fermentation, higher amounts of phenethylamine were detected in the CP fermentation process. The amount of tyramine was comparable to that in a previous study, where the concentration of tyramine increased up to 2073.27 mg/kg in the third month during *moromi* fermentation [17]. Several studies have suggested limits for BA content in food products. The total BA content of the food was suggested to be 1000 mg/kg, with 100 mg/kg for histamine, (100–800) mg/kg for tyramine, and 30 mg/kg for β-phenylethylamine [45]. Furthermore, the present study compared the differences between eight BAs at the end of fermentation (120 d). Among the eight BAs detected, the contents of five BAs (putrescine, tyramine, histamine, phenethylamine, and tryptamine) in JP were apparently higher than those in CP ($P < 0.05$) (Fig. 1C). The apparent differences in BA concentrations across the two different fermentation processes can be attributed to environmental factors, especially the temperature, oxygen availability, photoperiod, and salinity, as well as the starter culture (with or without starter *Zygosaccharomyces rouxii*), which were different between the JP and CP manufacturing processes [11].



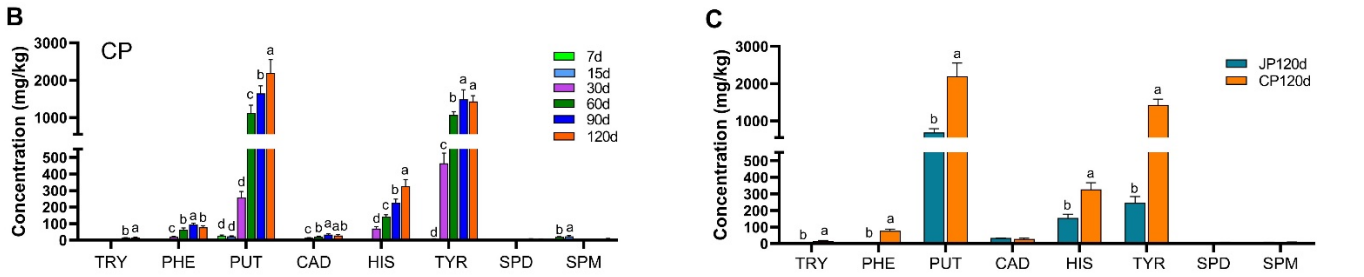


Fig. 1. Profiles of biogenic amines identified in soy sauce mash samples from Japanese-type (A) and Cantonese-type (B) soy sauce during fermentation (7, 15, 30, 60, 90, and 120 d), and comparison of biogenic amines (C) in mash samples after 120 d of fermentation in the two different fermentation processes. Data are measured in triplicate. The averages ± SD of samples in each group are expressed in each column. The different lowercase letters indicate significant differences between samples at $P < 0.05$. JP, Japanese-type; CP, Cantonese-type.

3.2 Changes in microbial community profiles across soy sauce fermentation types

The community analysis of the mash samples through metagenomic sequencing (64.5 Gbp in total, with 5.38 Gbp on average for each sample) (Table S4) revealed that the relative abundances of bacteria increased across fermentation stages (from 59.3%–98.5% in JP and from 77.2%–96.3% in CP), whereas the relative abundances of fungi dramatically decreased (from 34.3%–0.70% in JP and from 18.2%–0.70% in CP). In both JP and CP fermentation, the orders *Lactobacillales* and *Bacillales s*equences predominated (Fig. S2). In contrast, the relative abundances of fungal order *Eurotiales* declined dramatically during fermentation (from (33.26–0.13)% in JP and from (17.32–0.68)% in CP). From a genus perspective, *Tetragenococcus*, *Staphylococcus*, and *Weissella* dominated the JP and CP communities, along with fungal *Aspergillus* (Fig. 2), similar to the findings of a previous study in soy sauce mash samples [17]. Among these genera, *Tetragenococcus* increased drastically in abundance as fermentation progressed (from 0.02%–59.2% of all sequences in JP, and (0.01–36.6)% in CP), but with higher relative abundances of *Tetragenococcus* in JP samples ((60–120) d of fermentation). As a halophilic LAB, *Tetragenococcus* has been identified as the dominant microorganism in soy sauce *moromi.* In contrast, the abundance of *Staphylococcus* in CP was higher than that in JP during all fermentation stages, with average abundances of 32.6% and 8.5%, respectively (Fig. 2). *Staphylococcus* species are generally harmless to humans, and they are commonly used in traditional sauce products [46]. In CP, *Staphylococcus* abundances were higher, which might be linked to higher salt contents (Table S2), consistent with previous results where *Staphylococcus* was more abundant in soy sauce (*ganjang*) batches prepared using high salt concentrations [18]. However, *Weissella* exhibited decreasing abundances after 15 d of fermentation in both the JP and CP samples, suggesting that *Weissella* is not salt-tolerant [47]. Similarly, *Aspergillus* populations declined (from 32.1% to 0.12% in JP, and 17.1% to 0.65% in CP) throughout fermentation (Fig. 2). The higher relative abundances of *Aspergillus* at pre-fermentation stages originated from *koji* [48], in which *Aspergillus* species were used as starter culture. In addition, *Bacillus* was detected at higher abundances in CP communities (an average of 3.16%) than in JP communities (0.22%). Other fungi were found at relatively low abundances, such as yeasts *Zygosaccharomyces*, *Pichia*, *Candida*, *Kluyveromyces*, *Wickerhamiella*, and *Saccharomyces.*

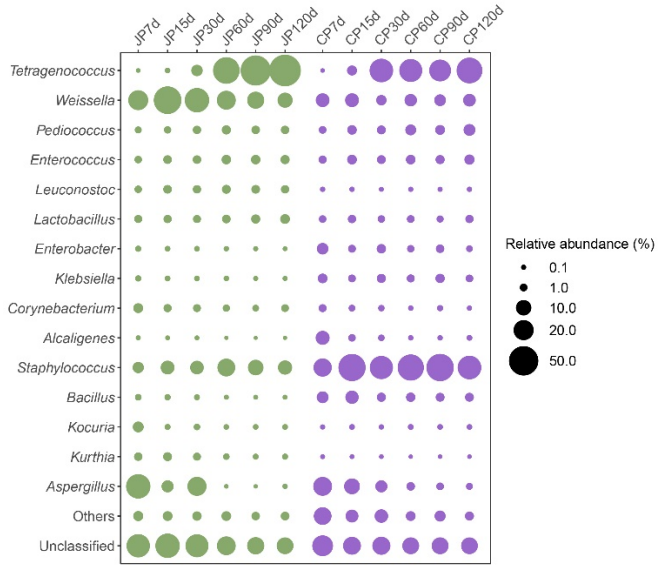


Fig. 2. Microbial taxonomic composition at the genus level for the 15 most abundant genera across the two different fermentation processes. Taxonomic profiles were obtained using the MEGAN software package through comparison against the NCBI-nr database. "Others" comprise the less-abundant genera. Sequences that could not be matched to any known taxonomic groups are designated as "unclassified." Samples are labeled according to fermentation time (7, 15, 30, 60, 90, and 120 d) and type (JP, Japanese-style fermentation; CP, Cantonese-style fermentation).

3.3 Profiles of BA genes and their relation to mash chemistry and organisms

To assess the diversity and abundance of BA-producing genes in the mash community, the shotgun metagenomic approach was applied against the corresponding KEGG orthologies (Table S1). The abundance of a particular gene was calculated based on the relative abundance (%). The detected genes involved in putrescine synthesis included two decarboxylase genes (arginine decarboxylase, *speA*; and ornithine decarboxylase, *odc*) and four non-decarboxylase genes (agmatinase, *speB*; agmatine deiminase, *aguA*; arginase, *arg*; and putrescine carbamoyl transferase, *ptcA*) (Fig. 3). These genes exhibited different abundance profiles. The genes *speB*, *arg*, and *odc* were the most abundant genes in JP, whereas the genes *speB*, *arg*, and *odc*, along with *speA*, were predominant in CP. Among them, the relative abundance of the gene *odc* in JP increased (from 2.6 e^{-5} to 4.9 e^{-4}) after 15 d of fermentation, with the highest abundance (4.9 e^{-4}) at the end of fermentation (120 d). The abundance of the gene *ptcA* increased during JP and CP fermentation, with the abundance increasing from 2.1 e^{-6} to 1.7 e^{-5}, and from 5.9 e^{-6} to 3.3 e^{-5}, respectively. However, the gene *speA* drastically decreased in abundance throughout the fermentation period (from 1.4 e^{-5} to 2.2 e^{-6} in JP and from 1.6 e^{-4} to 1.8 e^{-5} in CP). In addition, the abundances of tyrosine decarboxylase genes (*tyrDC*) involving in tyramine production increased (from 1.8 e^{-6} to 1.4 e^{-5} in JP and from 6.7 e^{-6} to 2.8 e^{-5} in CP) and was higher (average 3.3 times) in CP than in JP at each fermentation time point. Thus, it was speculated that the higher concentrations of tyramine detected in CP *mash* samples (Fig. 1) were associated with a high expression level of *tyrDC* in the tyramine-producer. Last, the genes encoding heterocyclic amine decarboxylase responsible for the production of histamine (*hdc*) and tryptamine (*tdc*) were also identified (Fig. 3). Of these, the gene *hdc* was present at relatively low abundances at an average of 1.3 e^{-6} in JP and 3.3 e^{-6} in CP. Furthermore, a lysine decarboxylase gene (*ldc*) involved in cadaverine production was detected, with an average abundance of 3.5 e^{-6} in JP and 1.7 e^{-5} in CP. The gene abundance of *speE* (encoding spermidine synthase) was higher in CP than

that in JP. However, the *sms* (encoding spermine synthase) and *pdc* (encoding phenylalanine decarboxylase for phenethylamine synthesis) genes were not detected. Although the gene *pdcA* was not identified in this study, phenethylamine was detected at an average of 6.68 mg/kg in JP and 78.38 mg/kg in CP at the 60- to 120-d fermentation periods.

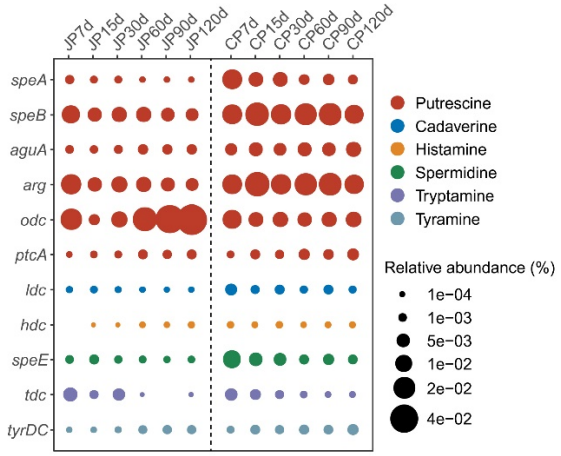


Fig. 3. Relative abundances of genes involved in biogenic amine production in the two different soy sauce fermentation processes. The relative abundances of genes encoding decarboxylase, deiminase, agmatinase, transferase, and synthase were acquired with the number of reads hit on the corresponding KEGG orthology. The colors of the bubble represent different BA products. BA, biogenic amine.

To explore the correlations between BA genes and basic physico-chemical parameters, Pearson's correlation coefficients of BA genes abundances and the pH, NaCl, TA, and ANN levels were calculated (Fig. S3). It was found that the pH was positively correlated with *tdc* genes but negatively correlated with the *ptcA* and *tyrDC* genes ($P < 0.05$). Notably, the content of NaCl had a mostly positive relationship with six BA genes ($P < 0.05$). In addition, both TA and AAN exhibited a positive correlation with *ptcA*, and a negative correlation with *tdc* ($P < 0.05$). Furthermore, the network analysis was used to reveal co-occurrence patterns between BA genes and microbial taxa (Fig. S4). The genes responsible for putrescine synthesis, such as *speA*, *speB, aguA*, *arg*, *odc*, and *ptcA*, were correlated (co-presence or mutual exclusion) with most genera, except for *Aspergillus* and *Kocuria* ($P < 0.05$). The gene *tyrDC* was positively correlated with the genera *Enterococcus*, *Pediococcus*, and *Staphylococcus* and negatively correlated with *Kurthia*, *Weissella*, and *Corynebacterium* ($P < 0.05$). Additionally, fungal *Aspergillus* showed significant co-occurrence with *tdc*. However, seven genera (*Weissella*, *Leuconostoc*, *Lactobacillus*, *Kurthia*, *Tetragenococcus*, *Kocuria*, and *Corynebacterium*) showed negative correlations with BA genes ($P < 0.05$).

3.4 Genome reconstruction and abundance of MAGs

After contig binning and genome quality control, a total of 44 MAGs from the set of mash metagenomes in JP and CP samples were reconstructed (Table S5), and 15 genomes were above 80% completeness and had less than 10% contamination (Table 1, Fig. 4). The genome sizes of these 15 MAGs varied between 1,487,942 bp and 4,833,112 bp, with an average of 2,427,150 bp; the number of contigs in each MAG varied from 60–548; and the number of ORFs ranged from 1502–4829 in each MAG (Table 1).

Table 1. Characteristics of curated metagenome-assembled genomes (MAGs) [a].

ID	Closest NCBI match [b]	Identity (%)	Genome size (bp)	Completeness (%)	Contamination (%)	N50 [c]	No. of contigs	Average contig length (bp)	Max contig length (bp)	No. of ORFs	%GC
MAG1	*Rothia kristinae*	96.6	2,168,482	94.1	0	19,166	186	11,659	104,432	2,021	72.0
MAG2	*Tetragenococcus halophilus*	98.0	2,216,259	93.2	1.2	22,532	462	4,797	81,420	2,439	35.7
MAG3	*Weissella cibaria*	98.0	3,478,498	86.8	9.9	24,655	493	7,056	120,822	3,691	44.4
MAG4	*Pediococcus acidilactici*	98.1	2,145,534	97.2	0	22,765	370	5,799	91,857	2,264	42.2
MAG5	*Weissella paramesenteroides*	98.7	1,564,222	93.3	0.6	25,238	115	13,602	90,876	1,530	38.1
MAG6	*Weissella cibaria*	98.6	1,764,468	87.8	2.0	23,348	188	9,385	88,611	1,805	44.9
MAG7	*Leuconostoc falkenbergense*	98.5	1,706,558	95.2	0	81,457	60	28,443	545,239	1,700	38.9
MAG8	*Staphylococcus gallinarum*	98.8	2,546,947	92.7	1.6	20,500	195	13,061	57,708	2,457	33.1
MAG9	*Mammaliicoccus sciuri*	96.6	1,669,979	82.9	1.3	6,352	285	5,860	25,815	1,847	32.8
MAG10	*Enterococcus faecium*	98.5	2,499,790	96.5	0.7	35,681	136	18,381	143,207	2,416	38.4
MAG11	*Leuconostoc fallax*	98.6	1,487,942	90.4	1.0	32,648	76	19,578	137,826	1,502	37.7
MAG12	*Corynebacterium* sp.	85.9	2,967,689	84.4	1.8	11,239	393	7,551	59,311	2,957	69.4
MAG13	*Klebsiella pneumoniae*	99.3	4,833,112	98.1	4.7	20,722	495	9,764	74,234	4,829	58.2
MAG14	*Bacillus velezensis*	98.2	3,488,726	83.1	9.2	8,917	548	6,366	60,024	3,676	47.8
MAG15	*Pediococcus pentosaceus*	98.9	1,869,040	85.4	3.1	7,607	311	6,010	48,137	2,042	37.7

[a] Only MAGs with completeness > 80% and contamination < 10% have been included.

[b] Based on PhyloPhlAn and MEGAN output.

[c] N50 is defined as the minimum contig length required to cover 50% of the MAG.

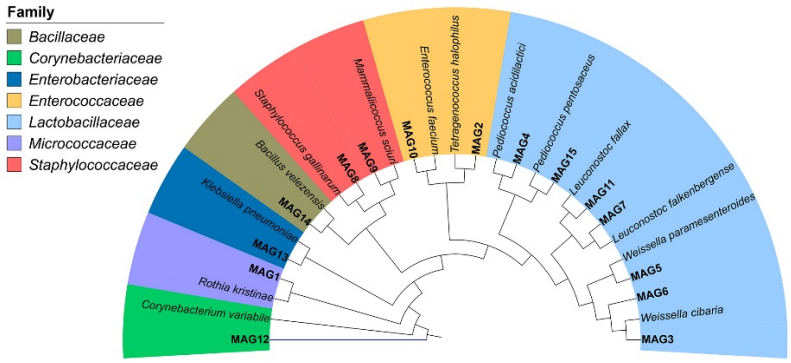


Fig. 4. Taxonomic assignment of the curated metagenome-assembled genomes (MAGs) reconstructed from the soy sauce mash metagenomes. The putatively novel species in the phylogenetic branch are labeled in blue.

The majority of MAGs (nine out of 15), accounting for 60% of MAGs, belonged to the LAB. The reconstructed MAGs were assigned to 11 different genera (including 14 different species). The most-recruited MAGs belonged to *Weissella*, *Pediococcus*, and *Leuconostoc*, which contained three, two, and two MAGs, respectively. A single MAG was recruited from the *Rothia*, *Tetragenococcus*, *Staphylococcus*, *Mammaliicoccus*, *Enterococcus*, *Corynebacterium*, *Klebsiella*, and *Bacillus* genera (Table 1). FastANI was used to assign taxonomy and assess the novelty of MAGs, revealing that one MAG had a low identity (< 95%) to known prokaryote genomes in the NCBI database. The novel species (MAG12) was identified as *Corynebacterium* sp., which was from *Corynebacteriaceae* and clustered with *Corynebacterium variabile* (85.9% identity) (Fig. 4). It should be pointed out that entire eukaryotic genomes were not retrieved in the present study, although higher-abundance *Aspergillus* species were present during fermentation, because their genomes were larger and more difficult to be reconstructed from the metagenome dataset [22]. Of the observed species, *T. halophilus* (MAG2), *Pediococcus acidilactici* (MAG4), *Weissella cibaria* (MAG6), and *Staphylococcus gallinarum* (MAG8) dominated in mash communities (Fig. 5A). In particular, *T. halophilus* gradually increased in abundance and became the most dominant species at the end of fermentation in both JP and CP. It has been reported that *T. halophilus,* a halophilic LAB used as a starter culture, may promote the flavor and taste characteristics, safety, and health-promoting effects of fermented foods, but is also a major causative agent for the generation of BAs during food fermentation [20, 49, 50]. Furthermore, *W. cibaria* was observed as a high-abundance species in the initial stage of fermentation (7–30) d in JP samples but slightly decreased in abundance in the middle to late stages (60–120) d. As a facultative hetero-lactic bacterium, *W. cibaria*, which produces acetic acid, lactic acid, CO_2, and ethanol as main end products [51], has been frequently found in soy sauce manufacturing [11, 52, 53]. In addition, *S. gallinarum* (MAG8) has also been identified during the fermentation of soy sauce [54]. The higher abundance of *S. gallinarum* (MAG8) in CP might be ascribed to higher salt contents, as reported previously [55]. *K. pneumoniae* (MAG13), a widely known opportunistic pathogen responsible for bacterial pneumonia and nosocomial infections, has also been detected during the fermentation of soy sauce and other foods [56, 57]. *K. pneumoniae* infections are difficult to treat because of the presence of certain virulence factors and antibiotic resistance. To obtain the pathogenic information of this strain, virulence factors (VFs) were identified in MAG13 (Table S6). A total of eight types of VFs, including adherence, antimicrobial activity/competitive advantage, biofilm, effector

delivery system, immune modulation, motility, nutritional/metabolic factor, and regulation, were detected in MAG affiliated with *K. pneumoniae*, indicating that the safety issues surrounding this bacterium should be of concern during soy sauce manufacturing.

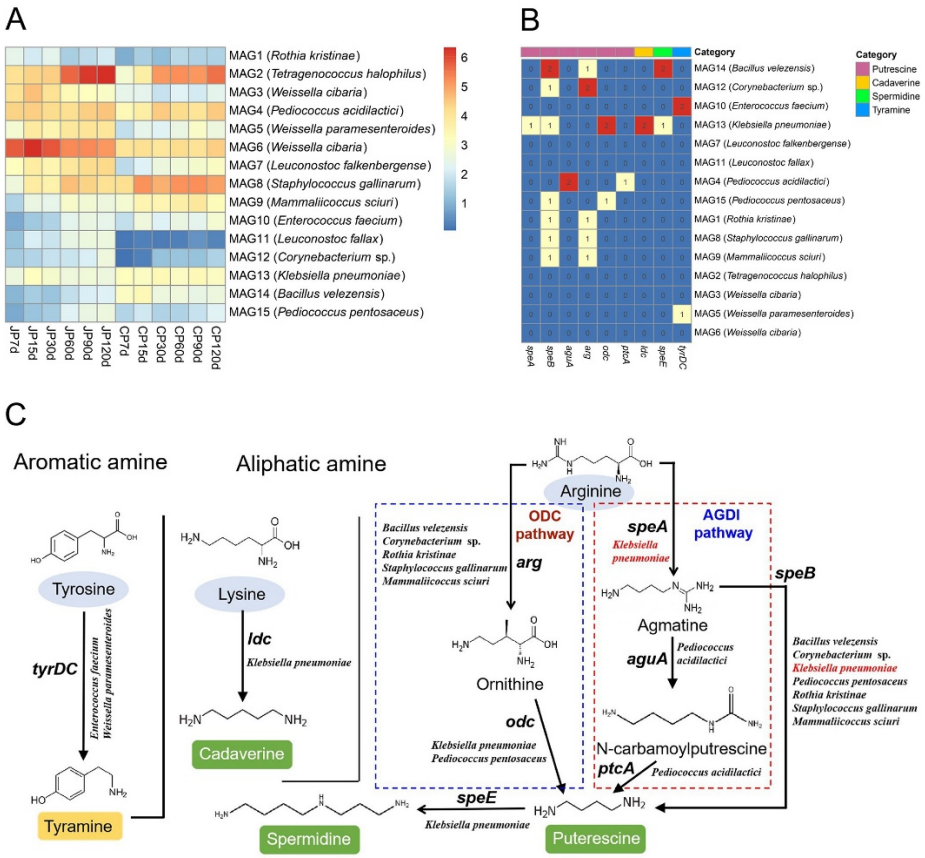


Fig. 5. Abundances of curated MAGs in each mash sample (A), BA genes in MAGs (B), and predicted metabolic pathways of key species (MAGs) involved in BA formation during soy sauce fermentation (C). The BA genes encoding decarboxylases and non-decarboxylases in each species were analyzed by mapping the metagenome reads onto the searched BA genes. The metagenome-assembled species harboring the capacity to produce putrescine without interactions with another species are labeled in red. MAG, metagenome-assembled genome; BA, biogenic amine.

3.5 Distribution pattern of BA genes in MAGs

Currently, BA-producing bacteria in fermented food are being actively researched. However, information regarding the diversity of BA-producing microbial species in soy sauce fermentation is still scarce, and the genes involved in soy sauce BA production remain to be elucidated. The assembly of the metagenome along with genome binning and annotation of this study allowed us to differentiate between the hosts harboring various BA genes in the microbiome of soy sauce fermentation. It was found that ten of the 15 observed MAGs harbored at least one gene involved in BA production (ranging from one to five genes in each MAG). These genes in MAGs are involved in four types of BA synthesis, including putrescine (*speA*, *speB*, *aguA*, *arg*, *odc*, and *ptcA*), cadaverine (*ldc*), spermidine (*speE*), and tyramine (*tyrDC*) (Fig. 5B), reflecting the BA-producing capacities in different species or strains during soy sauce fermentation. Of the four decarboxylase genes detected (*speA*, *odc*, *ldc*, and *tyrDC*), no single gene was found in all MAGs. With regard to putrescine production, seven MAGs belonging to the genera of *Bacillus* (MAG14), *Corynebacterium* (MAG12), *Klebsiella* (MAG13), *Pediococcus* (MAG15), *Rothia* (MAG1), *Staphylococcus*

(MAG8), and *Mammaliicoccus* (MAG9) carried *speB* genes (Fig. 5B). Except for two MAGs (MAG13 and MAG15), all the above MAGs also carried *arg* genes. *Klebsiella pneumoniae* (MAG13) and *Pediococcus pentosaceus* (MAG15) were found to possess *odc* genes. The genes *speA*, *aguA*, and *ptcA* were identified in the MAGs classified as *K. pneumoniae* (MAG13) and *P. acidilactici* (MAG4). These results suggested that during soy sauce fermentation, it was possible to synthesize putrescine from arginine via two different ways: the ornithine decarboxylation pathway (ODC) and the agmatine deimination pathway (AGDI). The ODC pathway involves transforming arginine to form ornithine (arginase, *arg*), then decarboxylating ornithine to form putrescine (ornithine decarboxylase, *odc*), whereas the AGDI pathway involves the decarboxylation of arginine to form agmatine (arginine decarboxylase, *speA*), the deimination of agmatine to form N-carbamoylputrescine (agmatine deiminase, *aguA*), then hydrolysisthe of N-carbamoylputrescine to form putrescine (putrescine carbamoyltransferase, *ptcA*) [4]. Surprisingly, none of these MAGs involved a complete production route of putrescine as mentioned above because they did not contain all genes associated with this specific pathway, suggesting that a synergetic interaction occurs in various MAGs for putrescine production. This is partially in accordance with previous reports [26, 58], in which some species having the ODC pathway for producing putrescine were detected, while species having the complete AGDI pathway were not found. Considering that *K. pneumoniae* (MAG13) had two genes (*speA* and *speB*), it was speculated that these species could produce putrescine through the decarboxylation of arginine (*speA*) and hydrolysis of agmatine (*speB*). It has been reported that *K. pneumoniae* is capable of producing putrescine, histamine, and cadaverine [59, 60]. Furthermore, the *ldc* and *speE* genes were found only in the MAG13 affiliated with *K. pneumoniae.* Interestingly, of all species detected, only *K. pneumoniae* harbored more than two decarboxylase genes (*speA*, *odc*, and *ldc*) (Fig. 5B), suggesting the potential to decarboxylate multiple amino acids (arginine, ornithine, and lysine). As well, two MAGs carried the decarboxylase-coding *tyrDC* gene, including *Enterococcus faecium* (MAG10) and *W. paramesenteroides* (MAG5), suggesting they might be the tyramine producers via a one-step decarboxylation reaction from the respective precursor amino acid tyrosine. *E. faecium* is frequently isolated or detected in different fermented foods. The results concerning the tyramine production of *E. faecium* species in this study are not surprising because most of the *E. faecium* strains isolated from various fermented foods have been identified as tyramine and phenylethylamine producers and have been found to possess the *tyrDC* gene [3]. The production of multiple BAs, including tyramine, by *W. paramesenteroides* strains isolated from kimchi, has been reported [61]. It was especially notable that no BA genes were found in *T. halophilus* (MAG2), which was the most dominant species during the middle to late stages in both JP and CP fermentation (Fig. 5A). This finding was similar to a previous report in which only one histidine decarboxylase gene was identified in the genome of only one strain among 14 *T. halophilus* strains [20], but was inconsistent with several studies showing that some *T. halophilus* strains harbored various BA genes, such as tyrosine and histidine decarboxylase genes [49, 62], and played a key role in the accumulation of tyramine and histamine in fermented food. Moreover, genes involved in the synthesis of histamine (*hdc*) and tryptamine (*tdc*), which were detected at the microbial

community level (Fig. 3), were not identified in any MAG, which suggests that these genes might be harbored in other unassembled microbes in this study and deeper metagenomic sequencing is required. Finally, the overview of the predicted metabolic pathways for BA production from these key species (MAGs) during soy sauce fermentation is shown in Fig. 5C. Given the higher abundance of *S. gallinarum* (MAG8) in the middle to late stages of CP fermentation (Fig. 5A), with putrescine-producing genes (*arg* and *speB*) (Fig. 5C), it was inferred that the high content of putrescine was linked to the high abundance of this species in CP samples.

4. Conclusions

In the present study, the differences in BA profiles and microbial community composition were determined and the microbes responsible for BA production along with the metabolic pathways involved were observed within and between two different types of soy sauce fermentation processes (JP and CP). The CP samples contained significantly higher levels of three BAs (putrescine, tyramine, and histamine) than the JP samples. The most abundant bacteria belonged to the *Tetragenococcus*, *Staphylococcus*, and *Bacillus* genera, but varied in percentage abundance between different fermentation types. The functional annotation of the microbial community and MAGs revealed microbial functional potential responsible for BA synthesis. In particular, microbial genome recovery from the metagenomic dataset further refined the knowledge of key members and revealed findings on various BA-producing genes involved during soy sauce fermentation. Future efforts are needed to evaluate gene expression with metatranscriptomics and investigate the metabolic interaction involved in BA production in different species occurring within soy sauce fermentation.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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