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Deciphering microbial community dynamics along the fermentation course of soy sauce under different temperatures using metagenomic analysis

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Abstract

Fermented soy sauce consists of microorganisms that exert beneficial effects. However, the microbial community dynamics during the fermentation course is poorly characterized. Soy sauce production is classified into the stages of mash fermentation with koji (S0), brine addition (S1), microbial transformation (S2), flavor creation (S3), and fermentation completion (S4). In this study, microbial succession was investigated across stages at different temperatures using metagenomics analyses. During mash fermentation, *Aspergillus* dominated the fungal microbiota in all stages, while the bacterial composition was dominated by *Bacillus* at room temperature and by a diverse composition of enriched lactic acid bacteria (LAB) at a controlled temperature. Compared with a stable fungal composition, bacterial dynamics were mostly attributable to fluctuations of LAB, which break down carbohydrates into lactic acid. After adding brine, increased levels of *Enterococcus* and decreased levels of *Lactococcus* from S1 to S4 may reflect differences in salinity tolerance. *Staphylococcus*, as a fermentation starter at S0, stayed predominant throughout fermentation and hydrolyzed soybean proteins. Meanwhile, *Rhizopus* and *Penicillium* may improve the flavor. The acidification of soy sauce was likely attributable to production of organic acids by *Bacillus* and LAB under room temperature and controlled temperature conditions, respectively. Metagenomic analysis revealed that microbial succession was associated with the fermentation efficiency and flavor enhancement. Controlled temperature nurture more LAB than uncontrolled temperatures and may ensure the production of lactic acid for the development of soy sauce flavor.

Key words: *Aspergillus*, LAB, metagenomics, microbial succession, soy sauce

INTRODUCTION

Soy sauce, a popular seasoning for daily cooking, originated in ancient China (Zhou Dynasty). Traditional soy sauce is fermented through the growth of fungi and bacteria for more than six months. Specifically, soy sauce is traditionally made from fermented soybeans and salt hydrolyzed by proteolytic enzymes of the filamentous fungus *Aspergillus oryzae* or *A. sojae* [1]. Over the course of fermentation, soy sauce develops a complex microbial community of fungi and bacteria.

Soy sauce production consists of two major processes: koji fermentation and mash (moromi) fermentation. Koji fermentation, initiated by the inoculation of *A. oryzae* in steamed soybeans, is a critical step for producing high-quality soy sauce. Mash is a fermented mixture of koji, sea salt, and brine. Mash fermentation requires several months to complete. Therefore, soy sauce fermentation is a complex process in which bacteria and fungi undergo sequential changes at different time points, with dynamic changes in the dominant species. Microorganisms are critical for fermentation. By adding yeast and/or lactic acid bacteria (LAB), numerous food products can be produced via microbial activities. The metabolic differences in microbiota during fermentation yield various flavors of fermented foods. Most bacterial genera reacting in the koji at an early stage produce proteases and volatile fatty acids for soy sauce fermentation [2]. The most important function of the koji fungi in soy sauce fermentation is to provide hydrolytic enzymes, especially extracellular enzymes [3], as several studies have indicated that most hydrolysis of soy protein occurs during koji fermentation [4]. In mash fermentation, the mold is quickly destroyed, while its extracellular enzymes can continue to hydrolyze different substrates. Brine (20% salt) is added seven days later to inhibit the growth of undesirable microorganisms.

Various approaches have been employed to study the soy sauce microbial population and its roles in flavor production [5–7]. Through culture-dependent and culture-independent methods,

such as polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis, the microbiota involved in different stages of soy sauce fermentation has been explored [8]. Most studies were conducted for a single stage fermentation, such as koji making or mash fermentation [9, 10], or compared the presence/absence of bacteria between stages [11]. Moreover, different fermentation temperatures tend to generate products with different flavors [12]. Nevertheless, no studies have compared the influence of environmental temperature on the microbiota associated with fermentation of soy sauce, except for previous research [13] that addressed the environmental seasonality affecting the bacterial community. In this study, we examined the microbial succession during the fermentation process, that is, from koji to mash, and illustrated fluctuations in microbial composition from one stage to another. We aimed to elucidate the microbial differences between the soy sauces fermented under different temperature conditions, identify dominant species at each stage of the fermentation process, and observe the changes in the bacterial and fungal communities during fermentation. Investigating the microbial community in soy sauce fermentation would help us to understand the dynamic changes associated with the quality of soy sauce. In this study, metagenomic analyses verified the importance of LAB, a group of beneficial microbes profoundly applied in soy sauce fermentation [5, 14].

MATERIALS AND METHODS

Fermentation experiments and sampling

Soy sauce was produced from black soybeans (Tainan No. 5, Taiwan). Black soybeans (15 kg) were steamed in a pressure cooker for an hour, and the beans were then put on a tray to detach water, cooled to 40°C, and mixed with *Aspergillus oryzae* mold (powder, 1 g/kg soybean, Wan

Feng Sauce Farm, Yunlin, Taiwan) and fried high-gluten flour (1.5 kg). To ensure adequate growth of *Aspergillus oryzae,* moisture, temperature, and aeration were monitored. The koji-making process was controlled in a box at temperatures from 25°C to 35°C and humidity from 75% to 95%. Following spore formation after one week [15], the koji beans were mixed with 20% (w/w) sea salt and placed in a closed jar that had been sterilized. To test the effect of temperature stability, black soybean mash fermentation was conducted under different conditions, with one set-up at a controlled temperature of 24°C (CT) based on the average temperature in Tainan and another set-up at room temperature (RT). The CT experiment was conducted in a laboratory of National Cheng Kung University (Tainan, Taiwan), while the RT experiment followed factory production and was conducted indoors at a soy sauce factory in Douliu (Yunlin, Taiwan). Room temperature ranged from 32°C (September) to 26°C (March) during the daytime and from 25°C (September) to 17°C (March) during the nighttime (Supplementary Table 1).

Samples were taken from the center of each jar at five time points, day 0 (S0), day 7 (S1), day 30 (S2), day 90 (S3), and day 180 (S4), throughout the 6-month fermentation process (from September 2018 to March 2019). The jars were opened to take the samples during fermentation. At each time point, three replicates of equal amounts of mash sauce samples were collected. The mash samples (approximately 0.5 g) were finely squashed prior to DNA extraction. DNA was isolated using a Quick-DNA TM Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene of bacteria and internal transcribed spacer (ITS) of fungi were amplified using PCR with primer pairs for 16S (789-1053, 926-1392) and ITS (ITS1-ITS2, ITS3-ITS4; Supplementary Table 2).

Metagenomics sequencing and analysis

Bacterial 16S rRNA and fungal ITS amplicons were sequenced using a 300-bp paired-end MiSeq platform (Illumina, San Diego, CA, USA). Sequences with trimmed primer sequences were merged into haplotypes using the forward and reverse reads. Haplotypes were clustered into operational taxonomic units (OTUs) with 97% sequence identity by mapping to the Greengenes database (Aug. 2013 version) and UNITE database for 16S rRNA and ITS, respectively, using the "pick_closed_reference_otus.py" function implemented in QIIME package v.1.9.1 [16]. OTUs assigned to archaea, mitochondria, and chloroplasts were excluded from further analysis. To normalize the sequencing depth of the samples, we used multiple rarefactions on the OTU table at depths of 12,704 and 77,928 reads for 16S rRNA and ITS, respectively. The bacterial OTUs for RT and CT at S0, S1, S2, S3, and S4 were 378, 317, 555, 486, and 356 and 493, 585, 603, 638, and 657, respectively. Meanwhile, the fungal OTUs for RT and CT at S0, S1, S2, S3, and S4 were 26, 21, 28, 29, and 27 and 17, 27, 32, 41, and 28, respectively. A summary of the distribution of OTUs is presented in Supplementary Table 3.

To evaluate the alpha diversity of each sample, OTU richness (Chao1) and Shannon diversity indices were calculated using the diversity function in the vegan package of R version 4.0.0 [17]. Nonmetric multidimensional scaling (NMDS) analyses applying the Bray-Curtis distance were used to plot the similarity of bacterial communities based on OTU composition. The shared proportions of OTUs among the stages of fermentation were visualized using the Venn Diagrams software (http://bioinformatics.psb.ugent.be/webtools/Venn/). Statistical significance for samples under the different temperature conditions was verified using the Mann-Whitney U test. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Taxonomic assignments of the soy sauce microbiome

In total, 1,734 OTUs were identified (1,641 bacteria and 93 fungi) from 10,503,229 raw reads of the mash samples during soy sauce fermentation (Supplementary Table 3). Rarefaction analysis showed that all samples almost reached saturation (Supplementary Fig. 1). The number of bacterial OTUs under room and controlled temperatures varied across stages. Likewise, fungal OTUs changed over the course of fermentation (Supplementary Table 3).

Dynamics in bacterial and fungal communities during soy sauce fermentation

Of the bacterial microbiota, phyla Firmicutes and Proteobacteria were persistent over the whole process in both experiment set-ups, with Firmicutes comprising 87% of the bacterial composition at RT. Under the CT conditions, Firmicutes accounted for 50–66% of the bacterial microbiota from S0 to S3, and Proteobacteria dominated S4 with a relative abundance of 54% (Fig. 1a).

The top 20 abundant OTUs identified in the RT samples were mostly members of Firmicutes, including five belonging to *Bacillus* and six belonging to *Staphylococcus* (Table 1). The top 20 OTUs in the CT samples were dominated by *Enterococcus, Staphylococcus, Erwinia,* and *Lactococcus* (Table 2). In the RT samples, *Bacillus* predominated over other genera in all stages, with relative abundances ranging from 61% to 85% (74% on average), while it was rare in the CT samples, with frequencies ranging from 0.1% to 0.3% across all stages (Fig. 1b), revealing a sharp difference between the two experimental set-ups. Of the CT samples, *Enterococcus* (Firmicutes) was predominant during mash fermentation (28% on average), followed by Enterobacteriaceae (25%), *Staphylococcus* (15%), *Erwinia* (14%), and *Lactococcus* (9%). It was noted that LAB were predominant across all stages (31–47%) but remained at low abundances (0.1–0.2%) under

the RT conditions (Fig. 2). Despite the sharp contrast in abundance, LAB peaked at the S2 stage under both conditions, showing similar trends in their changes. Among the seven genera of LAB detected here, *Lactobacillus* and *Vagococcus* exclusively occurred under the CT conditions, while *Enterococcus, Tetragenococcus, Lactococcus, Streptococcus*, and *Aerococcus* were found under both conditions. Of the LAB, *Enterococcus* was the most abundant under both conditions (on average, 0.06% in the RT samples and 28% in the CT samples). *Lactococcus* was second under the CT conditions (9%) and third under the RT conditions (0.01%). *Streptococcus* was third under the CT conditions (0.07%) and second under the RT conditions (0.02%). Accordingly, the predominant LAB under both conditions were similar, and the total abundance of LAB increased drastically in the CT experiment. In addition, *Staphylococcus* was very abundant under both conditions, with relative abundances of up to 23% in the RT samples (S1 stage) and up to 21% in the CT samples (S3 stage). In the final stage, its abundance dropped to 2% in the RT samples and 11% in the CT samples. In contrast, *Erwinia* was relatively stable during mash fermentation under both conditions (3–7% for RT; 10–16% for CT).

Venn diagrams were used to visualize the numbers of persistent and unique OTUs under the different conditions. Sixty-three OTUs were shared among the microbial communities at different time points under the RT conditions (Fig. 3a). The numbers of unique OTUs varied from 6 to 38 across the stages in the RT samples. In the CT samples, 154 persistent OTUs were detected, with the number of unique OTUs varying across stages (Fig. 3b). The persistent OTUs accounted for more than 99% of the bacteria involved in RT and CT mash fermentation. In the RT samples, the most persistent OTUs were identified as *Bacillus* (75%) followed by *Staphylococcus* (12%). In the CT samples, *Enterococcus* was the most abundant (28%), followed by unidentified Enterobacteriaceae (25%), *Staphylococcus* (15%), and *Erwinia* (14%; Supplementary Fig. 2).

In the fungal microbiota, *Aspergillus* was dominant in both experimental set-ups (Fig. 4), with relative abundances ranging from 95–99% and 94–99% in the RT and CT samples, respectively. In the RT samples, *Aspergillus*, *Penicillium,* and *Rhizopus* persisted across stages (Supplementary Fig. 3). In the CT samples, the abundance of *Penicillium* ranged from 1% to 5% during fermentation. The abundance of *Rhizopus* ranged from 0.01% to 2%. *Debaryomyces* was found in the CT samples from S0 (3%) to S3 (0.1%), whereas it was absent from the RT samples (Fig. 4). In total, nine and 13 persistent fungal OTUs were detected in the RT and CT samples, respectively (Supplementary Fig. 4).

NMDS analysis indicated that the S0, S1, and S2 bacterial communities in the CT samples were clustered together, separating them from those at S3 and S4 (Fig. 5a). In contrast, the S0, S1, and S2 fungal communities clustered together, separating S3 and S4 (Fig. 5b). Principal component analysis (PCA) of the CT samples revealed that bacterial communities varied during the fermentation process (Supplementary Fig. 5). Principle component 1 (PC1) contributed 44.4% of the total variance and was negatively correlated with the samples at S2. Principle component 2 (PC2) accounted for 23.4% of the total variance, was positively correlated with samples at S3 and S4, and was negatively correlated with samples at S0 and S1. The NMDS and PCA results indicated stepwise changes of microbiota as the fermentation of soy sauce progressed.

In the RT samples, Chao1 species richness increased from S0 to S2 and decreased from S2 to S4. In the CT samples, species richness increased from S0 to S1, decreased from S1 to S3, and increased again at S4. Specifically, S2 (214.4) showed the highest richness in the RT samples, and S4 (332.9) showed the highest richness in the CT samples (Table 3). The Shannon diversity indices of the RT samples were in the range of 1.42–1.95 (1.66 in average), and those of the CT samples were in the range of 2.69–3.01 (2.83 in average). A significant difference was observed between

the two set-ups ($p < 0.001$, Levene's test). Moreover, the S3 stage displayed the largest difference between the two experimental sets (Supplementary Fig. 6).

DISCUSSION

Brewed soy sauce consists of various microorganisms that determine the sauce quality and exert beneficial health effects [10]. Agreeing with previous research [18], the phyla Firmicutes and Proteobacteria, which are known to be critical in determining fermentation quality, were prevalent in both experimental set-ups (Fig. 1a).

In the RT experiment, which mimicked industrial production, both *Aspergillus* and *Bacillus* were predominantly stable throughout the fermentation process (Figs. 1b and 4, Supplementary Figs. 4 and 5), contrasting with the microbiota of fermented brine (in Malaysia), which was dominated by *Candida* and *Weissella*, followed by *Bacillus* and *Lactobacillus* [9]. Aside from the previously detected *Staphylococcus* and *Bacillus* [2], several bacterial genera, including *Lactococcus, Erwinia, Acinetobacter, Pseudomonas, Klebsiella,* and *Streptococcus*, involved in soy sauce fermentation were observed for the first time in this study (Fig. 1b, Tables 1 and 2). The soaking step prior to the steaming of soybeans was the probable source of *Streptococcus*, which has been found in soybean soaking in Indonesian tempe [19]. *Staphylococcus* was possibly derived from the salts added during mash fermentation [20]. However, as it was detected before the addition of brine, the salts can be simply ruled out as the origin of the *Staphylococcus*. Furthermore, *Staphylococcus* and *Klebsiella* are thought to have derived from the hands of workers during production [21]. *Pseudomonas*, some members of which are able to

survive or thrive in various environments [22], was likely incorporated into the mash fermentation environment from the surrounding environment.

In the CT experiment, LAB played critical roles beginning at the initiation of fermentation (40% at S0) and stayed predominant throughout fermentation (Fig. 2). In contrast to the low abundance of LAB under the RT conditions (< 1%), the CT conditions greatly enriched LAB to 31–47% and increased their influences on the development of soy sauce flavor. Besides, all of the LAB that occurred under the RT conditions were detected under the CT conditions with greater relative abundances, suggesting that maintaining a constant temperature (24°C) universally enhanced the LAB involved in soy sauce fermentation. Among the LAB detected in this study, *Lactococcus*, *Streptococcus*, and *Lactobacillus* showed the highest abundances at the S0 stage and decreased after brine addition, indicating that their roles were more influential during Koji fermentation. *Enterococcus*, *Aerococcus*, *Vagococcus* were highest at the S2 stage and stayed dominant until the S4 stage. *Tetragenococcus* gradually increased in the late stage of the fermentation, indicating its effects on the maturation of soy sauce.

The addition of brine, specifically in S1, represents another stage of fermentation in which the microbial community was transformed. High salt concentrations tend to inhibit the growth of contaminating bacteria during fermentation [23], leading to dynamic microbial changes, which ensure better flavor and stability of the final soy sauce product [9, 24]. For instance, in the CT experiment, the relative abundance of LAB, mostly *Enterococcus* and *Lactococcus,* after the addition of brine increased from stage S1 (35%) to S2 (47%). Specifically, the relative abundance of *Lactococcus* decreased as the fermentation process progressed (16% at S0 → 3% at S4), suggesting its role was largely affected by the high salinity. In contrast, the *Enterococcus* involved here may have had better salt tolerance than *Lactococcus*, as its abundance increased

after adding brine to the mash (22% to 35%). *Enterococcus faecium* has been found to have the ability to survive in a high-salinity environment (30%) [8]. This result also implied that the role played by *Enterococcus* was more dominant in the latter stages of soy sauce fermentation.

The acidification during soy sauce fermentation is attributable to the accumulation of organic acids, particularly lactic acid. LAB are the major producers of the lactic acid in soy sauce, while other bacteria generate other types of organic acids [24]. For instance, *Bacillus* was found to mainly generate acetic acid, malic acid, and propionic acid during the fermentation of Daqu, and the accumulation of lactic acid was relatively lower [25]. As the abundance of *Bacillus* was much greater than that of LAB in the RT soy sauce, acidification was not likely to be attributable to the accumulation of lactic acid. Under the CT conditions, the boosted amount of LAB probably enhanced the accumulation of lactic acid, as lactic acid comprises more than 90% of the organic acids produced by LAB [26, 27]. Although we did not determine the level of lactic acid, the elevated abundance LAB under the CT conditions may enrich the lactic acid content (Fig. 2).

The acidification effect of LAB via the production of organic acids is well documented in fermentation [5, 9, 15]. LAB are responsible for breaking down carbohydrates into lactic acid and simple sugars, causing acidification of mash [28]. Brine is thereby acidified by halophilic LAB, which limits the growth of harmful microorganisms during fermentation [29]. In general, LAB are complex autotrophs that require sugars, vitamins, and amino acids. Although it fluctuates, the substantial growth of LAB indicates that cooked soybeans contain sufficient nutrients for the rapid growth of these bacteria. LAB-acidifying ingredients lead to tangled lactic acid flavors, frequently act on proteolytic and lipolytic activity, and produce aromatic compounds, contributing to the flavor of the final product [29]. The LAB in soy sauce fermentation help produce

2,5-dimethyl-4-hydroxy-3(2H)-furanone, which is an important aroma synthesized during mash fermentation [24].

Temperature is known to determine the microbial composition during fermentation [30]. In the present study, the diverse temperatures in the RT and CT experiments differentiated the microbiota during fermentation. In the CT experiment, the microbiota diversity was higher than that in the RT experiment (20.3–30.6°C), and 252 bacterial OTUs were observed exclusively under its conditions, indicating that a stable environment may have nurtured diverse species. The bacteria unique to the CT experiment span a diverse phylogeny across 14 orders, including Enterobacteriales, Lactobacillales, Bacillales, and Pseudomonadales. Furthermore, dominance of LAB occurred exclusively in the CT experiment (Fig. 1b). Apparently, LAB contain genera and species that are highly sensitive to temperature [31], whereas *Bacillus* can tolerate temperature fluctuations better [32]. Given their high sensitivity to temperature, the heat resistance of LAB is complex and involves proteins playing various roles in cell physiology. In addition, the timing of initiation of the stress response varies greatly depending of the species/strains [33]. Adapting to a narrow temperature range is one of the characteristics of LAB [34]. Based on the above, when the temperature shifted from daytime to nighttime, the growth of LAB was constrained.

Interestingly, previous research [11] detected seven genera missing from koji and 12 genera newly appearing in mash using metagenomic analysis. Such a "gain and loss" scenario was also present in our study, with 25 and 29 genera exclusive to the koji stage in the RT and CT experiments, respectively. One genus was exclusive to the mash stage in the CT experiment, and there were no genera exclusive to the mash stage in the RT experiment. The higher number of taxa in koji may be attributable to the black soybeans, which provide much more organic material for fermentation than the defatted regular soybeans used in the previous study [11]. Nevertheless,

many bacteria disappeared when shifting to the mash stage. In contrast, almost all bacteria in the mash stage were already present in koji. The sharp difference between our study and that of the previous research [11] may be simply due to a lower threshold for filtering taxa of a certain abundance in our study (0.002% in our study vs. 1.0% in the previous study), especially given the approximate sequencing depths.

Key microbes in the fermentation of soy sauce

The fungal species of *Aspergillus*, *Penicillium* and *Rhizopus* are often used for fermented food due to the enzymes they secrete [35]. *Rhizopus* has been detected in bean sauce mash and has functions involved in flavor improvement [36]. In the present study, *Rhizopus* and *Penicillium* were persistent in both experiment sets, indicating their critical roles in mash fermentation. Although some studies have detected *Zygosaccharomyces*, a common salt-tolerant yeast, in mash fermentation, we found *Debaryomyces* as the only yeast participating in soy sauce fermentation. Many production methods for soy sauce ensure the participation of *Zygosaccharomyces* by artificial inoculation for flavor development [37]. In the study of Harada et al. [5], both *Zygosaccharomyces* and *Tetragenococcus* were intentionally inoculated in the mash fermentation of soy sauce. In the present study, we aimed to mimic the traditional method used in Taiwan, and therefore, no microorganisms were artificially added during fermentation of the soy sauce, except for the *Aspergillus* used in koji making. In addition, Sulaiman et al. [9] also did not detect *Zygosaccharomyces* in Chinese soy sauce and suggested that the absence of the yeast was related to the low ethanol amount in Chinese soy sauce. Therefore, the absence of *Zygosaccharomyces* in our observations was not surprising. The aroma developed by yeast may

also be attributable to the halotolerant yeast *Debaryomyces*, and its function has been confirmed in a Korean soy sauce [38].

It was noted that the CT conditions could largely increase the involvement of LAB in soy sauce fermentation (Fig. 1). LAB might first be present after wheat flour and molds are added during koji preparation. Among LAB, *Lactococcus* and *Enterococcus* are homofermentative bacteria with lactic acid as the final product via the Embden-Meyerhof-Parnas pathway [39]. *Enterococcus* displayed a sharp change with temperature [40], and it was also dominant in other fermented soybean products, in which it was used to prevent food spoilage and inhibit the growth of pathogenic bacteria by producing enterocin [38]. Meanwhile, a previous study found that the enzyme activity of *Lactococcus* affected the aroma of fermented foods by producing ester and phenolic compounds [12]. *Lactococcus piscium* can tolerate a maximum NaCl concentration of 23 g/l [41]. *Lactococcus* was identified as an abundant genus during soy sauce fermentation with a salt content ranging from 19 to 20 g/100 mL [42]. In the present study, *Lactococcus* was detected throughout fermentation in the CT experiment, implying that the growth of functional microorganisms may suppress the activities of harmful bacteria [12].

In contrast to the CT experiment, which was dominated by LAB, *Bacillus* was the most dominant in the RT experiment (Fig. 1). LAB and *Bacillus* have been found in various fermented foods with effective enzymes for the hydrolysis of soybean nutrients and for food spoilage [43]. *Bacillus* is often found in the early stages of soy sauce fermentation [44]. It is known that *Bacillus* can grow in a wide temperature range, from 41–65°C [32], and can produce spores that adapt to various stresses [45]. In our study, *Bacillus* increased from S0 to S3 in the RT experiment, while it decreased at S4; however, it remained dominant across all stages. In the CT experiment, *Bacillus* was detected in all samples with very low frequencies and with the opposite trend as compared

with that in the RT experiment (Supplementary Fig. 7). *Bacillus* is a common bacterium in traditional sunbathing mash and can transform a sauce's aroma and flavor [44]. It has been widely used for fermented foods. Some *Bacillus* strains have been shown to hydrolyze proteins in soybean into active peptides by proteases and peptidases in fish sauce [46]. *Bacillus subtilis* has been found to have proteolytic and amylolytic activities that hydrolyze soybean proteins, starch, and fat in fermented soy-dawadawa, an African condiment [47]. Likewise, *Bacillus subtilis* is often used as a starter to control the fermentation quality of natto, a popular Japanese food. Moreover, most *Bacillus* species do not grow well in conditions with more than a 15% salt content [48], as observed in our experiments.

Staphylococcus has been detected in soybean mash samples with a salt concentration of more than 20% [49]. In our study, analysis of the bacterial community showed that *Staphylococcus* increased from S0 to S3 during fermentation in the CT experiment. The relative abundance of *Staphylococcus* decreased at the S2 stage in the RT experiment and in the S4 samples in the CT experiment (Supplementary Fig. 7). It has been shown that *Staphylococcus* plays an important role in protein solubilization and contributes to flavor development [50]. Therefore, *Staphylococcus* likely participated from the start of fermentation by hydrolyzing proteins, and it maintained a high abundance throughout the fermentation course in both experiments (Fig. 1b).

Erwinia was detected in all soybean mash samples [51]. We also observed this genus in all samples from both experimental set-ups, though with much a higher abundance in the CT experiment (Supplementary Fig. 7). The growth of *Erwinia* during vegetable fermentation might help sugar degradation and participate in alcoholic fermentation [52]. *Erwinia* was also found to be a dominant genus in da-jiang, a salty fermented soybean paste, suggesting its tolerance of high-salt conditions [53].

Pseudomonas was also detected in the CT experiment (Fig. 1b). Some chemical compounds generated during early fermentation may help create suitable environments for *Pseudomonas* [54], which produces glutaminase for the synthesis of glutamic acid in brewed soy sauce [55]. In our study, *Pseudomonas* appeared at S3 in the RT experiment, whereas it was detected from S0 to S4 with an increasing trend in the CT experiment, revealing differential fermentation processes between the temperature sets. *Acinetobacter, Streptococcus, Citrobacter, Pragia, Serratia,* and *Curtobacterium* were found in both experimental set-ups. These genera have different effects on the fermentation process; for example, *Acinetobacter* is a harmful microbe for soy sauce fermentation [15].

Interestingly, *Tetragenococcus* is recognized as one of the major bacteria in soy sauce fermentation [49]. However, its abundance was less than 1% in S3 samples (Supplementary Fig. 4). We found *Tetragenococcus* in the mash samples of both types. Previous studies found that *Tetragenococcus* adapted to high salt concentrations (10–18% NaCl) during mash fermentation and have an immunomodulatory effect in soy mash samples [6, 56]. It was also found that the dynamics of *Tetragenococcus halophilus* might be affected by pH values during mash fermentation [6]. *Tetragenococcus* could be inoculated as starter to ensure the production of lactic acid in soy sauce. In the present study, we did not add *Tetragenococcus* at the beginning of mash fermentation, so it was only detected at a later stage, likely due to the involvement of natural flora, and may not have played a dominant role in the production of lactic acid. Since lactic acid could be generated by other microorganisms, such as *Bacillus* spp. under the RT conditions, as well as *Lactococcus* and *Enterococcus* under the CT conditions, these bacteria may replace the role of *Tetragenococcus*, as observed in other studies [5, 6, 49, 56], in the production of lactic acid, especially when they are not artificially added.

Soy sauce fermentation is a successional process involving the dynamics of microbes that are associated with the quality of soy sauce. Controlled temperature nurture more microbes than uncontrolled temperatures and may better ensure the production of soy sauce. In our study, *Enterococcus* dominated the bacterial microbiota in samples under the controlled temperature, whereas *Bacillus* was dominant at room temperature. In contrast to the fluctuations in bacterial composition, the fungal community was stable, with *Aspergillus* as the predominant taxon under all conditions and stages. This study contributes to the knowledge of microorganisms in the fermentation process, which is valuable in controlling optimal quality.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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Data availability

The sequencing data have been submitted to the NCBI Sequence Read Archive under BioProject PRJNA719692 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA719692) with accession numbers SAMN18614620–SAMN18614639.

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Figure Legends

Fig. 1. Relative abundances of bacteria in the RT and CT samples from S0 to S4. (a) Two major phyla of the bacterial microbiota in the two experiment sets. (b) Relative abundances of the top 10 bacterial genera in the RT and CT samples from S0 to S4, namely RT0-RT4 and CT0-CT4. Each sample is grouped according to the time point of fermentation. Top 10 rankings with respect to the relative abundances of genera in each sample.

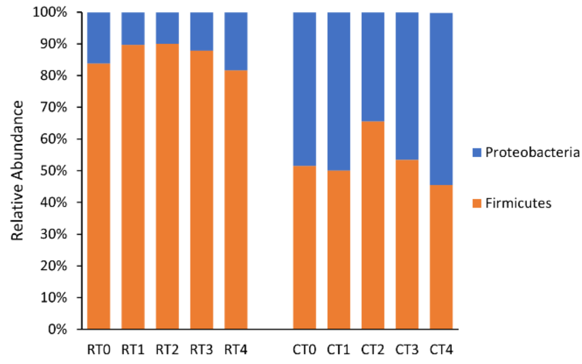
Fig. 2. Changes in the relative abundance of lactic acid bacteria (LAB) in soy sauce mash fermentation. The bars indicate the average abundance for each genus. Different time points are distinguished by colors. The LAB genera were ranked by average relative abundance. (a) LAB in the RT samples. (b) Major LAB in the CT samples. (c) Minor LAB in the CT samples.

Fig. 3. Venn diagram of the number of bacterial OTUs shared among the S0 to S4 stages. Different colors represent samples exclusively from single stages in the RT (a) and CT (b) samples. Persistent and unique OTUs were revealed.

Fig. 4. Relative abundances of the top 5 genera of fungi in the RT and CT samples. Each sample is grouped according to the time point of fermentation.

Fig. 5. NMDS plots for microbial communities of the CT soy sauce fermentation samples. (a) NMDS plot of bacterial communities. (b) NMDS plot of fungal communities. Colors represent samples taken at different time points: black for S0, red for S1, green for S2, yellow for S3, and blue for S4.

(a)



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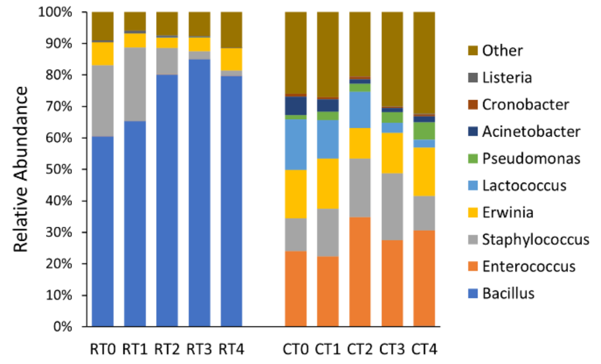
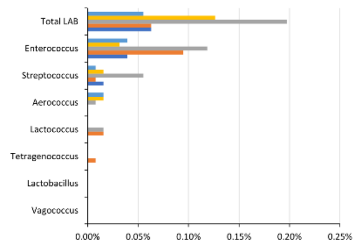


Fig. 1.

(a)



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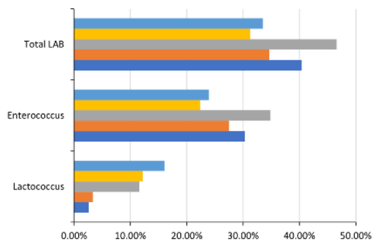

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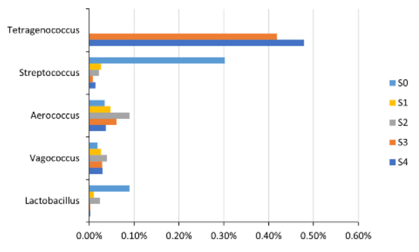
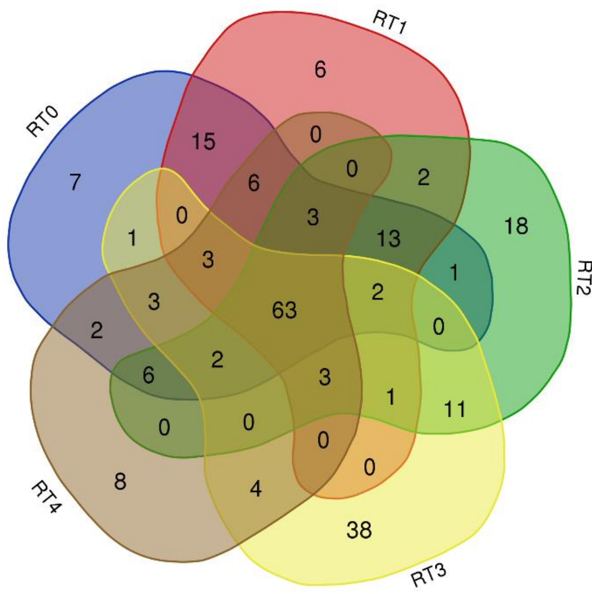


Fig. 2.

(a)



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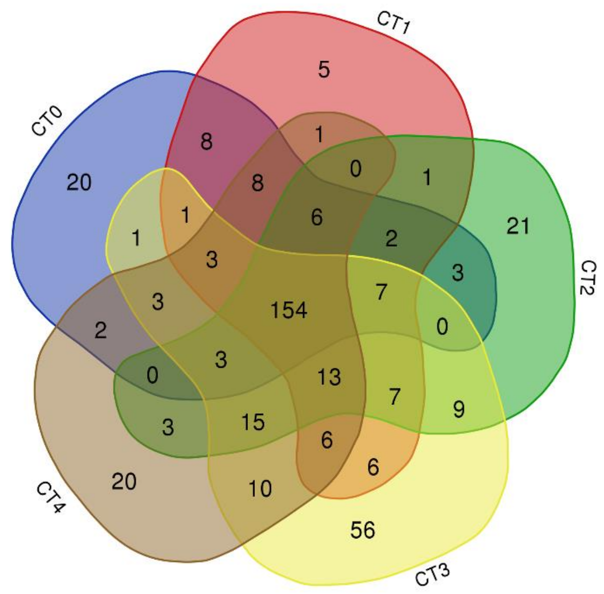


Fig. 3.

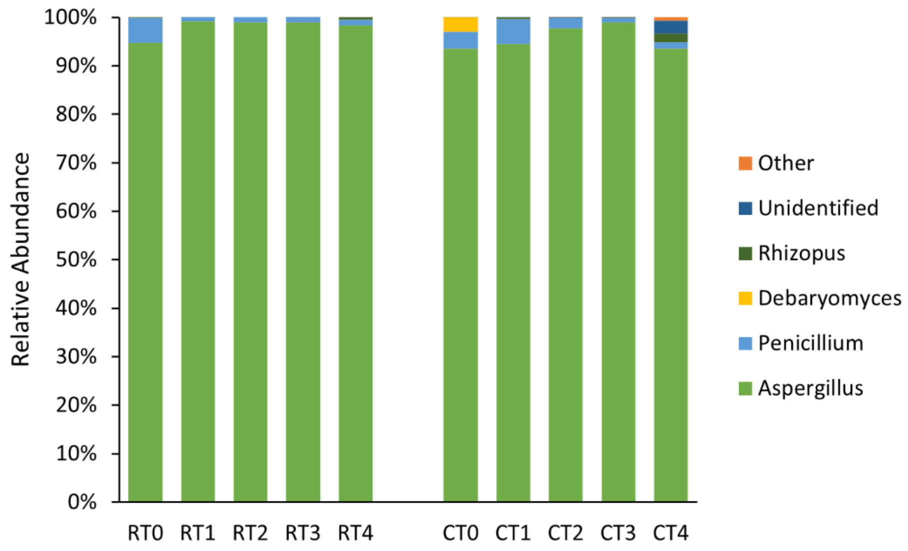
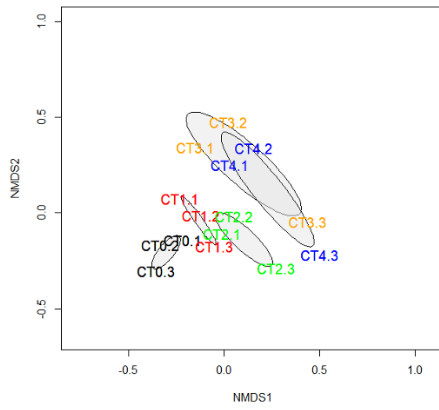


Fig. 4.

(a)



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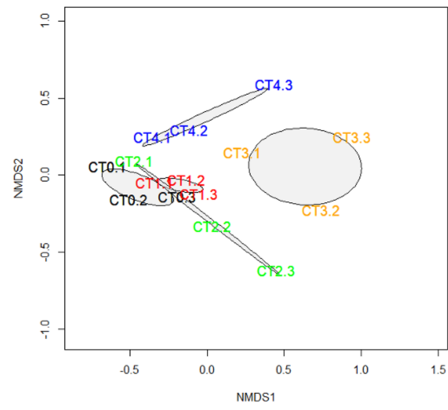


Fig. 5.

Table 1. Top 20 bacterial OTUs of samples of RT ranked by relative abundance and assigned by

Greengenes OTU ID	Phylum	Class	Order	Family	Genus	Nearest valid taxon
1050364	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	
820837	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	
1111874	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
1099674	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*S. succinus*
825033	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Erwinia*	*E. dispersa*
529219	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*S. sciuri*
895390	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	
851811	Firmicutes	Bacilli	Bacillales	Listeriaceae	*Listeria*	*L. grayi*
811219	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
1098655	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	
816420	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Delftia*	
752584	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
1081348	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	
434127	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	
811492	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
1097955	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*S. aureus*
823118	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
286880	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	
950872	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Brevibacillus*	
574051	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	

Table 2. Top 20 bacterial OTUs of samples of CT ranked by relative abundance and assigned by

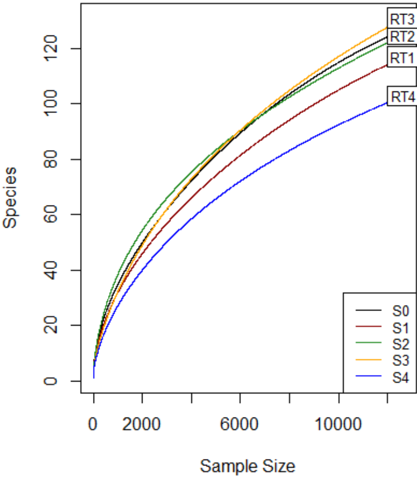
Greengenes OTU ID	Phylum	Class	Order	Family	Genus	Nearest valid taxon
1111582	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	
1111874	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
825033	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Erwinia*	*E. dispersa*
1099674	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*S. succinus*
529219	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*S. sciuri*
294254	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	
823118	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
656889	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Erwinia*	
303204	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	*L. garvieae*
811219	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
752584	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
698795	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	
928829	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	
810399	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	
593781	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*E.haemoperoxidus*
811492	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
1109844	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
829851	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	
797229	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
4352745	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		

Table 3. Alpha diversity of bacterial communities in samples of RT and CT (mean values ± SD)

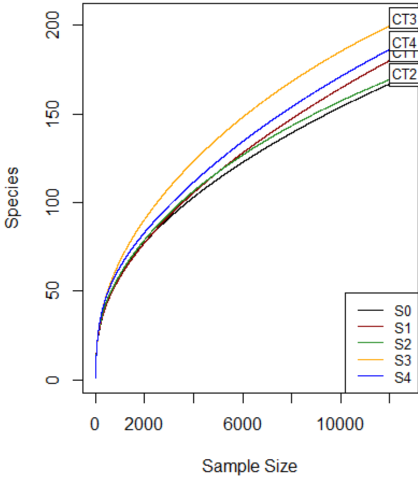
Diversity Index	Stages	RT	CT
Chao1	S0	158.78 ± 12.11	314.43 ± 24.13
	S1	164.22 ± 13.44	318.89 ± 49.79
	S2	214.44 ± 34.61	305.85 ± 63.04
	S3	214.18 ± 47.88	277.95 ± 87.49
	S4	163.88 ± 47.31	332.88 ± 78.40
Shannon-Wiener Index (H')	S0	1.95 ± 0.02	3.01 ± 0.06
	S1	1.85 ± 0.10	2.92 ± 0.12
	S2	1.64 ± 0.06	2.76 ± 0.06
	S3	1.42 ± 0.04	2.75 ± 0.03
	S4	1.45 ± 0.60	2.69 ± 0.39

Supplementary Fig. 1. Rarefaction curves of microbial diversity for 6 months of fermentation. Different colors represent RT (a) and CT (b) samples at different time points.

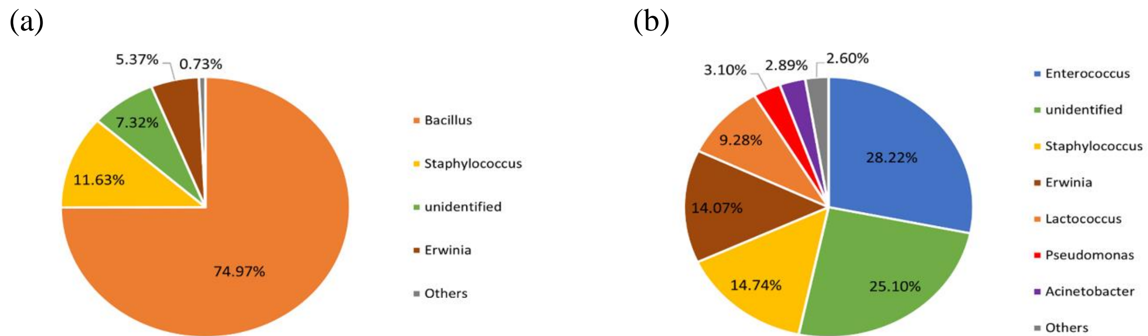
(a)



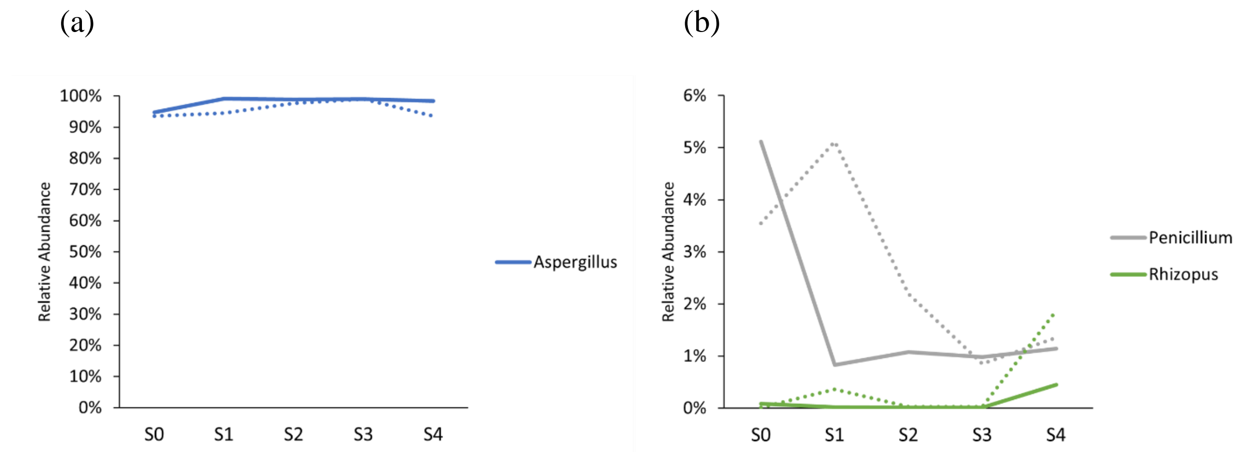
(b)



Supplementary Fig. 2. Pie chart of the persistent bacteria at the genera level for 6 months of fermentation. Persistent microbiomes of 63 OTUs in the RT samples (a) and 154 OTUs in the CT samples (b). Proportions of the persistent OTUs are shown at the genus level and visualized by pie chart. Genera with relative abundances of less than 1% were grouped as “Others.”

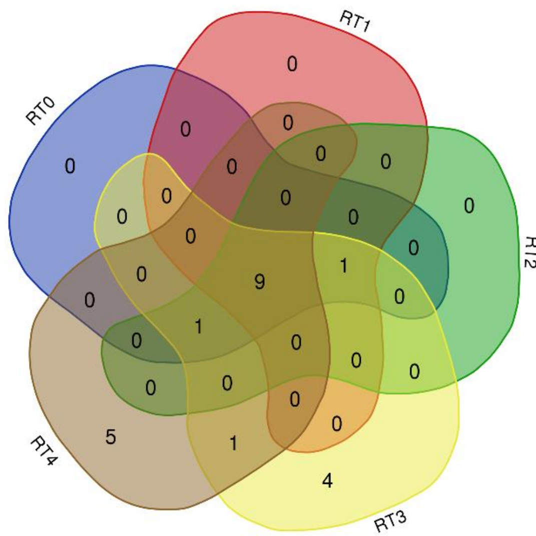


Supplementary Fig. 3. Changes in the relative abundances of persistent fungi in the RT and CT samples at the genus level from S0 to S4. Each sample is grouped according to the time point of fermentation. Straight lines represent RT samples, and dashed lines represent CT samples. The data are shown for the genera with relative abundances of more than 1%. The two vertical axes represent values with different units.

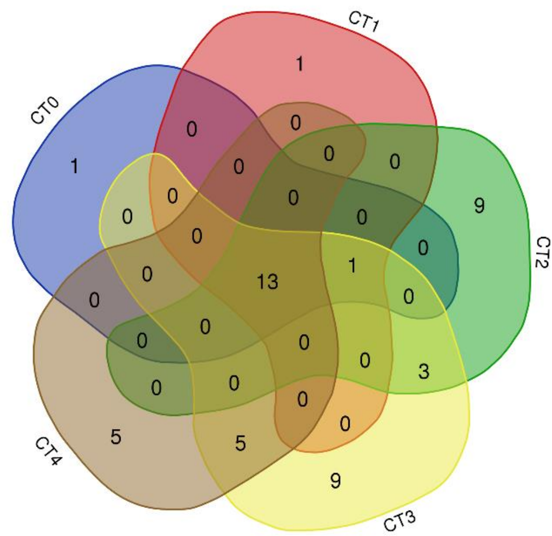


Supplementary Fig. 4. Venn diagram of fungal communities of OTUs shared among the S0 to S4 stages. Different colors represent RT (a) and CT (b) samples taken at different time points. In the RT samples, 9 OTUs shared among stages were assigned to *Aspergillus*, *Rhizopus*, and *Penicillium*. In the CT samples, 13 OTUs shared among samples at different stages were assigned to *Aspergillus*, *Penicillium*, *Pleosporales*, and *Rhizopus*.

(a)

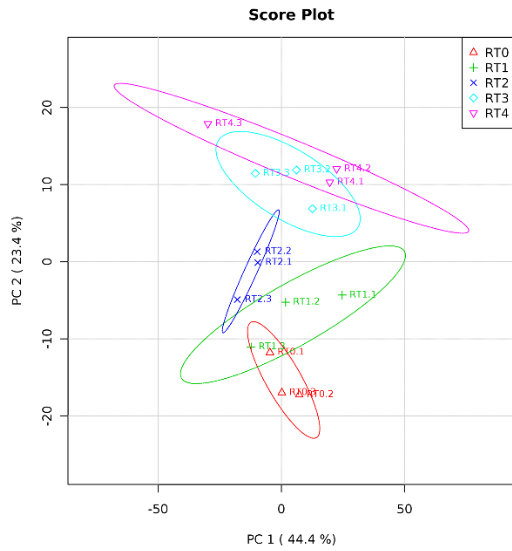


(b)

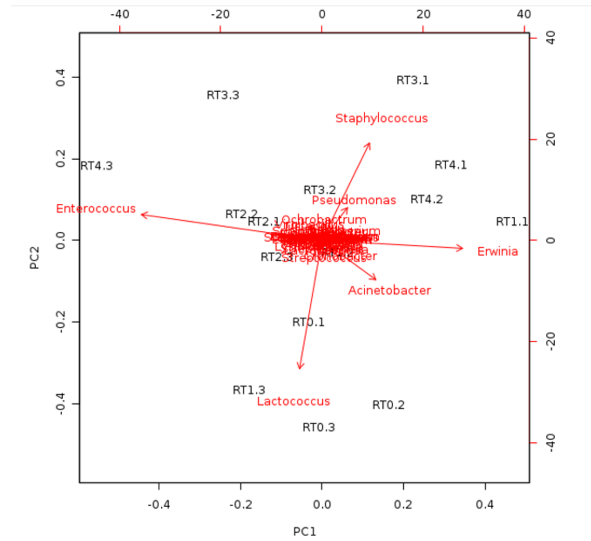


Supplementary Fig. 5. Principal component analysis of the bacterial community in the CT samples of soy sauce fermentation (a) and PCA biplot (b). Red, light blue, dark blue, pink, and green colors indicate samples taken at different time points. The ellipses indicate 95% confidence intervals for the distribution of microbiomes at each time point. Data were filtered by SD, and variables for which more than 80% of the values were zero were removed. The PCA explains 44.4% (PC1) and 23.4% (PC2) of the total variance (a). The PCA biplot shows the relative abundance data at the genus level. The PCA biplot was used to identify the specific microbes with great contributions to the differences between samples at different stages (b).

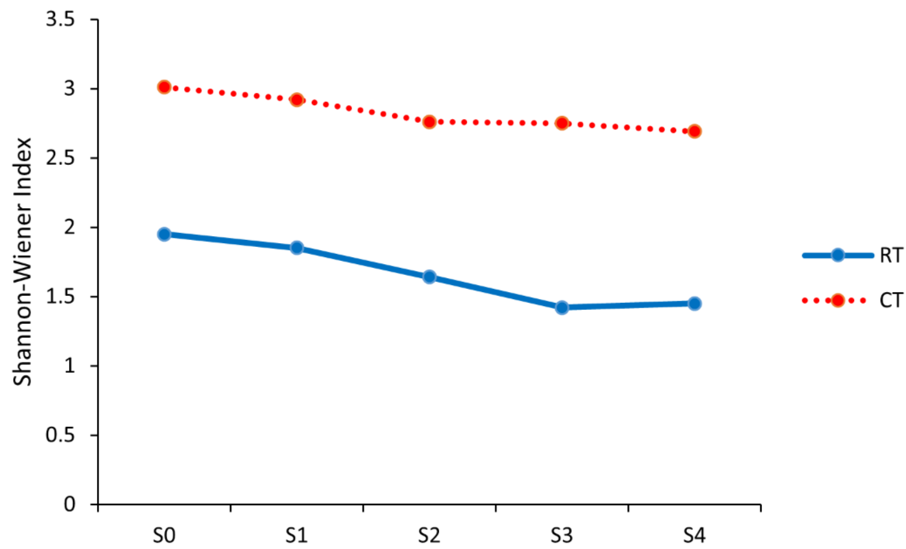
(a)



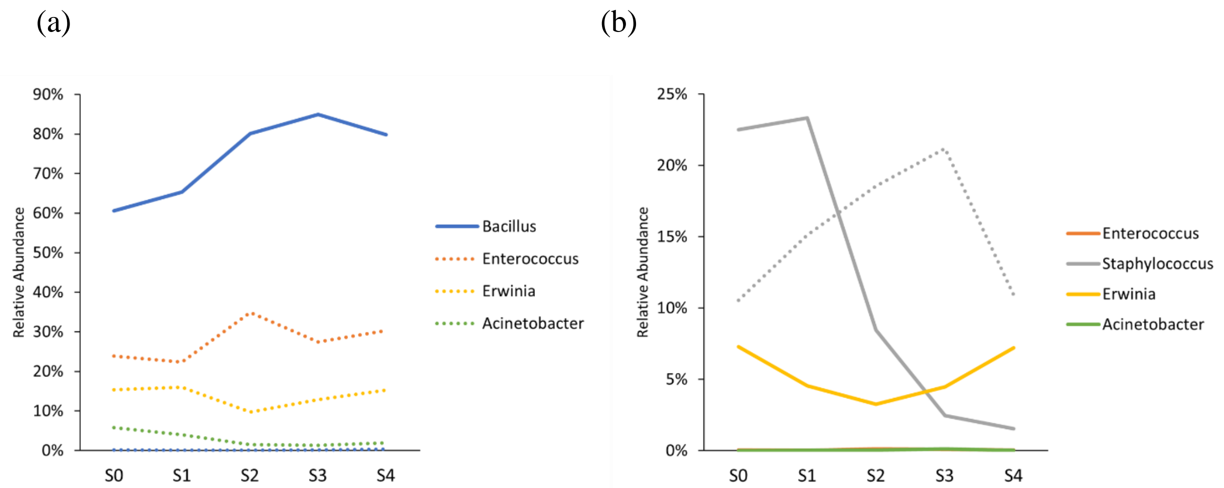
(b)



Supplementary Fig. 6. Diversity changes as fermentation progressed. The blue straight line represents the RT samples, and the red dashed line represents the CT samples. The data are shown as the averages at the different time points.



Supplementary Fig. 7. Changes in the relative abundances of persistent bacteria in the RT and CT samples at the genus level from S0 to S4. Each sample is grouped according to the time point of fermentation. Straight lines represent RT samples, and dashed lines represent CT samples. The data are shown for the genera with relative abundances of more than 1%. The two vertical axes represent values with different units.



Supplementary Table 1. Summary of RT and CT sample collection.

	Stages	RT	CT
Sample type	S0	Koji	Koji
	S1	Mash	Mash
	S2	Mash	Mash
	S3	Mash	Mash
	S4	Mash	Mash
Number of replicates	S0	3	3
	S1	3	3
	S2	3	3
	S3	3	3
	S4	3	3
Temperature	S0	30.6±0.1	24.2±0.2
(°C, mean values ± SD)	S1	26.7±0.2	24.3±0.3
	S2	27.7±0.2	24.4±0.3
	S3	25.3±0.1	24.3±0.1
	S4	26.4±0.2	24.2±0.6
Moisture	S0	99±1.2	99±1
(%, mean values ± SD)	S1	99±1.5	99±1.5
	S2	99±1.2	99±1.2
	S3	99±0.5	99±0
	S4	99±1.2	99±3.6

pH	S0	6.0±0.1	5.8±0.1
	S1	5.7±0.1	5.7±0.1
	S2	5.2±0.1	5.1±0.5
	S3	4.8±0.1	4.7±0.1
	S4	4.5±0.1	4.7±0.2

Supplementary Table 2. Primers and PCR procedure. For each sample, the PCR volume was 30 µL per tube, consisting of 9 µL *Taq* 2× Master Mix Red (Ampliqon, Odense, Denmark) and 3 µL of each primer for a final concentration of 0.2 µM. PCR products were loaded in a 1.5% agarose gel, electrophoresis was conducted, and products were purified with a Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan). The DNA concentration was quantified using a BioDrop DUO+ spectrophotometer (Biochrom Ltd., Cambridge, UK) and a Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

Primer set	Primer sequence 5' – 3'	Reference	PCR condition
789	TAG ATA CCC SSG TAG TCC	[1]	initial denaturation 94°C 3 min
1053	CTG ACG RCR GCC ATG C		
926	AAA CTY AAA KGA ATT GAC GG	[2]	(30 cycles) denaturation 94°C 50 sec
1392	ACG GGC GGT GTG TRC		annealing 55°C 40 sec
			extension 72°C 45 sec
			final extension 72°C 10 min
ITS1	CTTGGTCATTTAGAGGAAGTAA	[3]	initial denaturation 95°C 5 min
ITS2	GCTGCGTTCTTCATCGATGC		
ITS3	GCATCGATGAAGAACGCAGC	[3]	(30 cycles) denaturation 95°C 40 sec
ITS4	TCCTCCGCTTATTGATATGC		annealing 55°C 50 sec
			extension 72°C 50 sec
			final extension 72°C min

Supplementary Table 3. Summary of the reads from all samples with different primers. The number of OTUs is shown as the average of 3 replicates of 2 primer pairs. (A) Percentages for the proportions of samples under the RT and CT conditions (primer pair: 789-1053 and 926-1392).

Samples	RT					CT				
	S0	S1	S2	S3	S4	S0	S1	S2	S3	S4
Number of OTU	378	317	555	486	356	493	585	603	638	657
Phylum (%)	100	100	100	100	100	100	100	100	100	100
Class (%)	100	100	100	99.99	100	100	100	100	100	100
Order (%)	100	100	100	99.99	100	100	100	100	100	100
Family (%)	100	100	99.99	99.98	100	99.63	99.71	99.73	99.91	99.88
Genus (%)	91.45	94.47	94.47	93.43	89.10	75.71	74.37	81.94	72.92	70.69
Species (%)	28.93	7.12	8.77	26.49	11.42	29.52	31.34	26.36	31.51	26.80

(B) Percentages for the proportions of samples under the RT and CT conditions (primer pair: ITS1-ITS2 and ITS3-ITS4).

Samples	RT					CT				
	S0	S1	S2	S3	S4	S0	S1	S2	S3	S4
Number of OTU	26	21	28	29	27	17	27	32	41	28
Phylum (%)	100	100	100	100	100	100	99.99	99.99	99.95	99.99
Class (%)	100	100	100	100	100	100	99.99	99.99	99.95	99.99
Order (%)	100	100	100	100	100	100	99.99	99.99	99.95	99.99
Family (%)	100	100	100	99.99	100	100	99.99	99.96	99.93	99.79
Genus (%)	100	100	100	99.99	100	100	99.99	99.96	99.92	97.36
Species (%)	100	100	100	99.99	100	100	99.99	99.96	99.92	97.36

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