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Exploring the effects of milk-enriched walnut soy sauce: Insights from GC-IMS and metagenomics approach to flavor and microbial shifts

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ABSTRACT

This study investigates the impact of milk addition on the fermentation of walnut soy sauce, using Gas Chromatography-Ion Mobility Spectrometry (GC-IMS) and metagenomics to analyze flavor profiles and microbial dynamics. GC-IMS analysis showed significant increases in volatile compounds such as esters (ethyl acetate), aldehydes (hexanal), and alcohols (isoamyl alcohol), enhancing the aroma and taste. Metagenomic analysis revealed that milk increased microbial diversity, with *Weissella* and *Lactobacillus* dominating early fermentation. The milk-enriched soy sauce (SYM) exhibited higher amino acid nitrogen (2.67 g/L), and total nitrogen (7.18 g/L) compared to the control, indicating improved nutritional quality. Protease activity peaked at 2438.5 U/g for neutral protease, supporting efficient protein hydrolysis. Relative Odor Activity Value (ROAV) analysis identified 29 key flavor compounds, including 3-methyl butanol and ethyl 2-methyl butyrate, which contributed fruity and buttery notes to SYM. These results suggest that milk enhances microbial growth and improves both flavor and nutritional quality of walnut soy sauce.

1. Introduction

Soy sauce, a traditional fermented condiment, is rich in nutrition and unique flavors, containing amino acids, organic compounds, and minerals (Diez-Simon et al., 2020). It is primarily made from plant proteins like soybeans or soybean meal, with starch as an auxiliary, and *Aspergillus oryzae* driving fermentation (Devanthi & Gkatzionis, 2019). Koji, the foundation of soy sauce production, is crucial, with high-quality koji being vital for superior soy sauce (Feng et al., 2013). The low-salt solid-state fermentation method dominates 90 % of the market, due to its high efficiency and low cost (Jin et al., 2024). Microorganisms involved in soy sauce brewing include *Aspergillus oryzae*, *Aspergillus sojae*, *Saccharomyces*, and *Lactobacillus*, with *A. oryzae* at the core due to its rapid growth, robust enzyme production, and high enzymatic activity (Devanthi & Gkatzionis, 2019; Jin et al., 2024). It secretes enzymes such as protease, amylase, cellulase, and esterase (Zhang et al., 2016), essential for enzymatic hydrolysis and Maillard reactions that define soy sauce fermentation. In China, strains like 3.863, Huyan 3.042, UE336, UE328, and Yu 3.811 are commonly used (Tan et al., 2022). The fermentation involves enzymatic hydrolysis and Maillard reactions driven by microbial metabolism in koji. In solid-state fermentation (SSF), molds produce key enzymes, protease and amylase, which break down soy proteins into peptides and amino acids, contributing to soy sauce's umami flavor (Ito & Matsuyama, 2021; Tan et al., 2022).

In recent years, using diverse plant and animal-derived raw materials to expand protein sources has gained attention. Soy sauce production

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has been extensively studied using various protein sources, including peanut cake, corn, and rice protein residues but research on combination of milk and walnut meal as raw materials remains unexplored. Walnuts, known for their nutritional richness, are a promising alternative due to their high protein content and amino acid composition (Sassi et al., 2021). Walnut kernels are highly nutritious, packed with protein, fat, minerals, and vitamins. Their fat, primarily glyceryl linoleic acid, helps lower cholesterol and supports brain health (Binici et al., 2021). Walnut protein contains 18 amino acids, including all eight essential amino acids, making it a viable alternative to soybeans for functional soy sauce production. Walnut meal, a by-product of oil extraction, contains about 40 % protein, making it a high-quality raw material for soy sauce production. Using walnut meal enhances the value of soy sauce, offering a nutrient-dense product (Xu et al., 2020).

Replacing water with milk in walnut soy sauce fermentation adds essential nutrients, including protein and fat, enriching the fermentation environment. Milk proteins metabolize efficiently, promoting microbial growth and increasing amino acid nitrogen content. It also provides essential amino acids, dietary fiber, and lactose, aiding the absorption of minerals like calcium, magnesium, and zinc (Medhammar et al., 2012). The natural sweetness of milk complements the umami flavor of walnut soy sauce, creating a more complex taste. Beneficial bacteria in milk, such as *Lactobacillus* and *Bifidobacterium*, enhance digestion, nutrient absorption, and immunity, boost nutritional value (Xu, 2012). Solid-state fermentation (SSF) uses microbial and biochemical interactions to produce nutritious fermented foods with health benefits (Giraffa, 2004). Most studies focus on amino acid composition and microbial metabolism in soy-based fermentation (Geng & Wang, 2019; Li et al., 2019). During fermentation, protease enzymes from Aspergillus oryzae break down walnut meal proteins into polypeptides, which are further broken-down into peptides and eventually into free amino acids, resulting in a flavorful and nutrient-rich product. Optimizing koji conditions to enhance *A. oryzae* enzyme activity is crucial for improving soy sauce quality and raw material utilization in SSF (Xu et al., 2020).

Soy sauce flavor is shaped by volatile aromatic compounds from microbial metabolism, with over 1315 compounds identified, influencing its aroma and taste (Diez-Simon et al., 2020). Key flavor contributors include esters, impart sweetness; aldehydes, harmonizing the aroma; and pyrazines, giving a roasted note. Compounds like 4-ethyl guaiacol enhance the smoky flavor (Syifaa et al., 2016). Alcohols such as isoamyl alcohol and esters like ethyl acetate and methyl benzoate enhance the soy sauce taste (Pu et al., 2023). Methoxyphenols, especially 2-methoxyphenol and 2,6-dimethoxyphenol, are vital for the smoky properties. Propanol, however, increases bitterness and astringency, and reducing it improves flavor. (Xu et al., 2020) used a walnut meal with *Aspergillus oryzae* (strain 3.042) to optimize amino nitrogen content, a critical soy sauce quality indicator. However, their study did not evaluate essential factors such as flavor profiling and consumer acceptability. Ly et al. (2023) investigated volatile compounds during moromi fermentation using diverse raw materials like soybeans, rice, and black beans, providing valuable data on aroma formation but did not evaluate volatile profile, leaving a gap in understanding consumer preferences and product quality. The effects of raw materials and starter cultures on microbial and metabolite dynamics were examined by (Liang et al., 2019), highlighting microbial interactions in fermentation. However, they neglected sensory evaluations.

This study aimed to explore microbial dynamics and physicochemical transformations during the fermentation of walnut soy sauce (SYW) and walnut milk soy sauce (SYM). The addition of milk to walnut soy sauce was aimed at enhancing the flavor complexity and nutritional value of the final product. The impact of animal proteins, particularly milk proteins, on flavor development was significant, as they contributed to the formation of key umami and aromatic compounds, which are crucial for the sensory attributes of the soy sauce. Multivariate statistical analysis revealed distinct differences between the milk walnut soy sauce (SYM) and walnut soy sauce (SYW), highlighting the enhancement in flavor complexity due to the milk addition. These findings underscore the importance of milk as alternative raw materials, offering new avenues for flavor improvement nutritional value. This provides a foundation for developing high-quality functional soy sauces and advancing the comprehensive utilization of animal-based proteins.

2. Materials and methods

2.1. Production of Milk walnut soy sauce

The process of milk walnut soy sauce production was carried out at Yunnan Lao Sanjian Food Co., LTD in Anning City, Yunnan Province in China. The fermentation and processing technology of the milk walnut soy sauce production was the same as traditional walnut soy sauce, but we replaced water with milk. It involves pulverizing walnut meal, mixing them with wheat flour (16 %), and hydrating with milk (80 % of walnut meal weight) for 30 min. The mixture is cooked and sterilized at 120 °C for 20 min, cooled to 37–39 °C, and inoculated with *Aspergillus oryzae* 3.042 (0.05 %) and lactic acid bacteria (0.05 %). It is incubated at 32–42 °C for 48 h, with turning every 6 h to ensure even fermentation. Successful koji preparation is identified by a loose texture and abundant spores. The koji is mixed with 18 % brine and fermented at 42 °C for 48 h, followed by 15 days of maturation to produce high-quality milk walnut soy sauce. This process optimizes hydration, sterilization, and fermentation.

2.2. Preparation of enzyme solution

During koji preparation, samples were collected at 12 h, 24 h, 36 h, 42 h, and 48 h, and during soy sauce fermentation, samples were taken on the 1st, 5th, 10th, and 15th days. A 2.5 g sample was randomly taken from each box, ground, and adjusted to 50 mL. Samples were incubated at 40 °C for 1 h with constant shaking, then filtered. The crude enzyme solution was diluted with the appropriate buffer. Milk walnut soy sauce samples were labeled SYM12H, SYM24H, SYM36H, SYM42H, and SYM48H for the koji process and SYM1D, SYM5D, SYM10D, and SYM15D for fermentation. Control walnut soy sauce samples were labeled SYW12H, SYW24H, SYW36H, SYW42H, and SYW48H for koji and SYW1D, SYW5D, SYW10D, and SYW15D for fermentation.

2.3. Determination of physicochemical indexes of milk walnut soy sauce

The activity of three proteases was determined following SB/T10317–1999 (R. Gao et al., 2017), with neutral protease measured in pH 7.2 phosphate buffer, acid protease in pH 3.0 lactic acid buffer, and alkaline protease in pH 10 borax-sodium hydroxide buffer. Saccharifying enzyme and cellulase activities were assessed using the 3,5-Dinitrosalicylic Acid (DNS) method (Anuradha Jabasingh & ValliNachiyar, 2011). pH was measured with a Sartorius PB-10 m, and reducing sugar content was analyzed via the DNS method (Nam et al., 2023). Total nitrogen was determined as per GB18186–2000 (Yang GuiLing et al., 2008), while total acid and amino acid nitrogen followed GB 5009.235–2016 (Gao et al., 2021). Soluble salt-free solids were analyzed per GB18186–2000 (Yuan et al., 2015), and the color index was evaluated by measuring OD values of 10-fold diluted soy sauce at different wavelengths using distilled water as a blank.

2.4. GC-IMS volatile flavor compound analysis

A 1 mL sample was incubated at 60 °C for 15 min in a 20 mL headspace vial and analyzed in triplicate. N-ketones (2-butanone to 2-nonanone) were analyzed using analytical-grade standards (Aladdin Corporation) and high-purity nitrogen (99.999 %) as the carrier gas with an MXT-WAX capillary column (15 m × 0.53 mm, 1.0 μm, Restek, USA). Headspace sampling involved a 500 μL injection at 110 °C. GC conditions included a column temperature of 60 °C, nitrogen carrier gas, a pressure program from 2.0 mL/min to 100.0 mL/min (30-min runtime), and an inlet temperature of 80 °C. IMS analysis employed a tritium ionization source, 98 mm migration tube at 45 °C, 500 V/cm electric field strength, and 150 mL/min drift gas flow in positive ion mode.

2.5. *Volatile flavor compound analysis (ROAV)*

The Relative Odor Activity Value (ROAV) is a measure of the contribution of volatile compounds to a sample's overall flavor profile (M. Feng et al., 2021). Compounds with ROAV values between 0.1 and 1 significantly contribute to the flavor, while those with ROAV $\geq$1 are classified as key flavor compounds. The ROAV is calculated using the formula ROAV $\approx 100 \times$ (Ci / Cmax) $\times$ (Tmax / Ti), where Ci represents the relative content of each volatile compound (%), Ti is the sensory threshold of each volatile compound (μg/L), Cmax denotes the relative content of the compound contributing most to the flavor (%), and Tmax is the sensory threshold of the compound with the greatest flavor contribution (μg/L).

2.6. *Metagenomic analysis of microbial community diversity*

High-throughput sequencing was conducted on the Illumina NovaSeq/HiSeq platform using a Whole Genome Shotgun (WGS) approach. Deoxyribonucleic Acid (DNA) or Complementary Deoxyribonucleic (cDNA) was fragmented to construct paired-end libraries, with raw data stored in FASTQ format. After quality filtering, reads were clustered into Operational Taxonomic Units (OTUs) at 97 % similarity using the Ribosomal Database Project (RDP) classifier. Alpha diversity indices (Shannon, Chao1, Simpson, ACE) were calculated with mothur, while beta diversity was analyzed using Quantitative Insights Into Microbial Ecology (QIIME) with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering. Principal Coordinates Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) visualized community similarities, and group differences were assessed using the Kruskal-Wallis test. R tools were used for Venn diagrams and microbial diversity visualizations.

2.7. *Data processing and statistical analysis*

The retention time and retention index calibration curve were established to calculate the retention index of target substances. The GC retention index database (NIST 2020) and IMS migration time database in VOCal software were used for identification and qualitative analysis of target compounds. VOCal software tools, including Reporter, Gallery Plot, and Dynamic Principal Component Analysis (PCA), were employed to generate three-dimensional, two-dimensional, difference, fingerprint spectra, and PCA diagrams for comparing volatile organic compounds across samples. Variance analysis was performed to assess differences among samples using one-way Analysis of Variance (ANOVA) with Tukey's test at a 95 % confidence level (SPSS v23, IBM, Chicago, IL, USA). Each sample was replicated three times, with results presented as mean ± standard deviation (SD). Origin 2021 software was used to create column charts of volatile compounds.

3. Results and discussions

3.1. *Physicochemical analysis*

3.1.1. *Changes in protease activity levels during koji preparation*

Protease produced by *Aspergillus oryzae* hydrolyzes walnut meal proteins into polypeptides, which are further broken-down into peptides and eventually into free amino acids, resulting in a fermented sauce with enhanced flavor and nutritional value. The final quality of soy sauce is closely linked to the hydrolase activity of microorganisms during the koji-making stage, particularly protease and saccharifying enzymes, which influence metabolic profiles and provide energy for microbial growth. The changes in enzymatic activities during koji making in the SYW and the SYM group are summarized (Table 1). In the SYW group, protease activity increased initially and then decreased, with neutral protease reaching its peak at 42 h (2,446.9 U/g), and acid protease and alkaline protease peaking at 24 h with 1054.0 U/g and 937.6 U/g, respectively. In contrast, the SYM group displayed a unique pattern of increasing, decreasing, and subsequently rising protease activity. By 48 h, the SYM group exhibited higher maximum activities of neutral protease (2438.5 U/g), acid protease (932.5 U/g), and alkaline protease (831.8 U/g) compared to the SYW group. Saccharifying enzymes further promoted starch hydrolysis, supporting microbial growth and enhancing enzymatic activity during fermentation. It has been confirmed that milk addition in the SYM group enhanced protease activity patterns and supported higher enzymatic activities, contributing to improved fermentation efficiency and flavor development.

3.1.2. *Changes in physicochemical properties of milk walnut soy sauce (SYM) and walnut soy sauce (SYW) during fermentation*

The total acid, amino acid nitrogen, and total nitrogen contents increased consistently in both the SYW and SYM group (Table 2). Total acids, primarily composed of organic acids such as citric acid, malic acid, and succinic acid (Li et al., 2023), enhance soy sauce flavor. The SYM group exhibited superior fermentation performance compared to SYW, with total acid, amino acid nitrogen, and total nitrogen levels increasing by 38.10 %, 38.89 %, and 43.21 %, respectively, by the 15th day. Lower reducing sugar and total sugar in SYM indicated enhanced microbial sugar utilization, while higher total soluble solids reflected greater metabolite production. Total nitrogen, representing all nitrogenous compounds in a sample, increased during fermentation, consistent with amino acid nitrogen trends. This increase reflects raw material utilization, which directly influences soy sauce flavor (Kuang et al., 2022). Zhao et al. (2020) reported that total nitrogen levels ranging from 1.35 to 1.57 g/100 mL in Chinese soy sauce, 0.32–1.14 g/100 mL in salty soy sauce, and 0.14–0.25 g/100 mL in sweet soy sauce. The reducing sugar content in the SYW group decreased by 50.60 % from day 1 to day 10, then increased by 151.20 % by day 15. Similarly, the SYM group showed a 46.39 % decrease on day 10 and a 36.79 % increase on day 15. Total sugar in the SYM group was 36.63 % lower than in the SYW group on day 15. The total soluble solids (TSS) decreased initially due to brine fermentation limiting microbial activity (Abdurrauf & Aceh, 2019) but increased gradually due to salt's hygroscopic effect (Zhao et al., 2020) and amylase activity (Lv et al., 2021). In SYW, TSS decreased by 16.40 % by day 5 and rose to 29.16 ± 0.45 g/100 g by day 15. In SYM, TSS dropped by 8.36 % by day 10 and increased to 31.11 ± 0.59 g/100 g by day 15. High TSS indicates accelerated substrate hydrolysis (Amar et al., 2024), reflecting soluble components like sugars, salts, and amino acids (Khasanah et al., 2022). These results

Table 1
Changes in biochemical indices of SYM and SYW during koji production.

Sample	Neutral protease activity (U/g)	Acid protease activity (U/g)	Alkaline protease activity (U/g)
SYM12H	881.3 ± 0.34a	831.1 ± 0.07c	708.4 ± 0.4b
SYM24H	786.6 ± 0.9b	934.1 ± 0.18a	813.9 ± 0.3b
SYM36H	1310.5 ± 0.2b	897.4 ± 5.51	793.0 ± 0.2b
SYM42H	2057.1 ± 0.3c	894.0 ± 0.05b	789.1 ± 0.5a
SYM48H	2438.5 ± 0.3d	932.5 ± 0.9b	831.8 ± 0.57b
SYW12H	561.4 ± 0.5a	858.2 ± 0.5a	722.7 ± 0.12c
SYW24H	1758.7 ± 0.69a	1054.0 ± 0.65b	937.6 ± 0.18a
SYW36H	1826.4 ± 0.2b	1017.5 ± 0.3c	915.8 ± 0.95b
SYW42H	2446.9 ± 0.2b	866.0 ± 0.19bc	777.2 ± 0.44c
SYW48H	1759.9 ± 0.12c	888.7 ± 0.1a	809.9 ± 0.21a

Note: SYM group: milk walnut soy sauce sample, SYW group: walnut soy sauce sample. Each sample underwent three replications. All data are presented as mean ± standard deviation ($n = 3$); Different lowercase letters of superscripts (a, b, and c $p < 0.05$) indicate significant differences among groups.

Table 2
Changes in physicochemical properties of SYM and SYW during fermentation.

Sample	Total acid (g/100 g)	Amino acid nitrogen (g/ 100 g)	Total nitrogen (g/100 g)	Reducing sugar (g/100 g)	Total sugar (g/100 g)	Sodium chloride (g/100 g)	Total soluble solids (g/ 100 g)
SYM1D	0.88 ± 0.144bc	0.29 ± 0.052 cd	0.32 ± 0.006 h	0.360 ± 0.004b	0.649 ± 0.008b	5.85 ± 0.05d	7.66 ± 0.14ef
SYM5D	0.72 ± 0.119d	0.27 ± 0.038d	0.46 ± 0.006d	0.219 ± 0.013e	0.395 ± 0.024e	5.85 ± 0.20d	7.80 ± 0.19e
SYM10D	0.72 ± 0.045d	0.35 ± 0.021c	0.47 ± 0.000c	0.193 ± 0.008f	0.347 ± 0.015f	5.02 ± 0.65bc	7.02 ± 0.77f
SYM15D	1.30 ± 0.000a	0.75 ± 0.000a	1.16 ± 0.000a	0.264 ± 0.008d	0.476 ± 0.014d	26.38 ± 0.57d	31.11 ± 0.59a
SYW1D	0.49 ± 0.078e	0.19 ± 0.028e	0.39 ± 0.000 g	0.336 ± 0.008c	0.605 ± 0.015c	4.68 ± 0.03ab	9.93 ± 0.05d
SYW5D	0.64 ± 0.052d	0.25 ± 0.043de	0.4 ± 0.000f	0.197 ± 0.005f	0.354 ± 0.010f	5.85 ± 0.18a	8.30 ± 0.07e
SYW10D	0.75 ± 0.069 cd	0.35 ± 0.050c	0.45 ± 0.006e	0.166 ± 0.002 g	0.299 ± 0.004 g	7.02 ± 0.33c	12.74 ± 0.10c
SYW15D	0.94 ± 0.000b	0.54 ± 0.000b	0.81 ± 0.000b	0.417 ± 0.022a	0.750 ± 0.040a	24.38 ± 0.21e	29.16 ± 0.45b

Note: SYM group: milk walnut soy sauce sample, SYW group: walnut soy sauce sample. Each sample underwent three replications. Values are expressed as the mean ± standard deviation (n = 3). Different lowercase letters of superscripts (a, b, c, d, e, f, g and h $p < 0.05$) indicate significant differences among groups.

demonstrate the positive impact of milk addition on fermentation efficiency and nutritional quality in milk walnut soy sauce.

3.2. *Comparative analysis of volatile organic compounds during the fermentation of milk walnut soy sauce (SYM) and walnut soy sauce (SYW)*

3.2.1. *Comparative analysis of GC-IMS volatile organic compounds*

The ion migration pattern of volatile compounds during milk walnut soy sauce fermentation is illustrated in (Fig. 1a), presents the three-dimensional GC-IMS spectrum, where the X-axis, Y-axis, and *Z*-axis represent migration time, retention time, and signal peak intensity, respectively. The addition of milk altered the composition and content of volatile organic compounds (VOCs) in milk walnut soy sauce (SYM group) over time, with noticeable differences observed. For better visualization, the three-dimensional spectrum was converted into a two-dimensional top-view map for comparative analysis of VOCs (Fig. 1b). During fermentation, the VOC ion migration time extended from 1.0 to 1.65 ms on the first day to 1.0–1.75 ms, with retention times within 0–900 s for all samples. Each point around the RIP peak represents a VOC, where color intensity indicates content levels (darker colors signify higher content). The VOC content in the SYM group was higher in region A from days 5 to 15, while region B showed higher VOC content on day 1. Signal peaks in region A on days 5, 10, and 15 showed similar color compositions, indicating comparable VOC profiles during this period. To visually compare VOC differences, the day 1 spectrum (SYM1D) was used as a reference, and spectra from days 5–15 were subtracted to generate difference maps (Fig. 1c). White areas indicate no

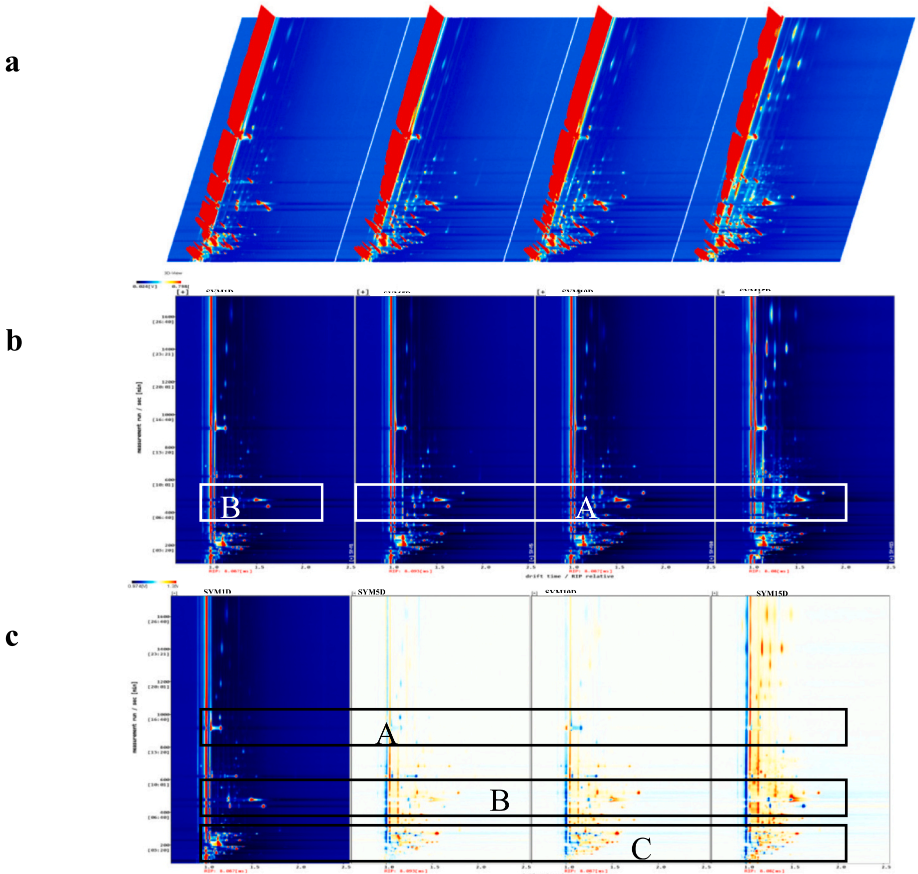

Fig. 1. Volatile organic compounds ion migration spectra during fermentation a) GC-IMS three-dimensional spectrum b) GC-IMS two-dimensional spectrum c) GC-IMS difference spectrum.

difference, red peaks indicate higher VOC content, and blue peaks indicate lower VOC content compared to the reference. Similar color patterns in regions B and C from days 5 to 15 suggest that milk influenced VOC composition and effectively shortened the fermentation time, offering guidance for optimizing fermentation duration.

3.2.2. Quantitative analysis of volatile organic compounds during fermentation of milk walnut soy sauce (SYM) and walnut soy sauce (SYW)

The GC-IMS analyzed VOCs in SYM and SYW on days 1, 5, 10, and 15 of fermentation. A total of 66 VOCs were identified across 8 samples, including 8 uncertain and 58 accurately characterized compounds. These included 5 acids, 10 alcohols, 8 aldehydes, 19 esters, 13 ketones, 1 furan, and 2 pyrazines, with some compounds existing as both monomers and dimers. Detailed volatile compounds are shown in (Table S1). The changes in peak area and relative content of volatile organic compounds (VOCs) during walnut soy sauce fermentation under different production processes are illustrated in (Fig. 2a, b). The VOCs peak area in both the milk walnut soy sauce (SYM) and walnut soy sauce (SYW) increased progressively throughout fermentation, with higher peak areas observed in the SYM group under same fermentation time. Alcohol and ketones ranked first and second in peak areas across all VOCs, while esters and acids ranked third. In the SYW, alcohol and ketones increased initially due to active microbial metabolism and sugar fermentation in early stages (Wang et al., 2023), esters showed a declining trend, and acids fluctuated. In the SYM group, alcohol and esters first increased and then decreased, ketones showed a decreasing trend, and acids decreased initially then gradually rose. The relative content of alcohol, which contributes to a mellow flavor through yeast metabolism, in the SYW group was 36 % (SYW1), 37 % (SYW5), 29 % (SYW10), and 27 % (SYW15). For the SYM group, the relative content of alcohol was 35 % (SYM1D), 37 % (SYM5D), 33 % (SYM10D), and 30 % (SYM15D). Ketones, derived from aldehyde oxidation, were dominant in the SYW group at the end of fermentation, with relative contents of 17 % (SYW1D), 19 % (SYW5D), 32 % (SYW10D), and 25 % (SYW15D), compared to 34 % (SYM1D), 25 % (SYM5D), 22 % (SYM10D), and 22 % (SYM15D) in the SYM group.

At the end of fermentation, ketones were the dominant VOCs in the SYW, while alcohols were dominant in the SYM because yeast metabolism is enhanced by the nutrient-rich milk, sustaining alcohol production throughout fermentation (Wakita et al., 2018). Esters, known for their fruity aroma that enhances the richness of soy sauce by reducing sourness, showed relative contents of 30 % (SYW1D), 24 % (SYW5D), 24 % (SYW10D), and 19 % (SYW15D) in the SYW group, compared to 11 % (SYM1D), 26 % (SYM5D), 29 % (SYM10D), and 18 % (SYM15D) in the SYM group. Acid compounds, which contribute to preservation and flavor but can cause sour or pungent odors if excessive,

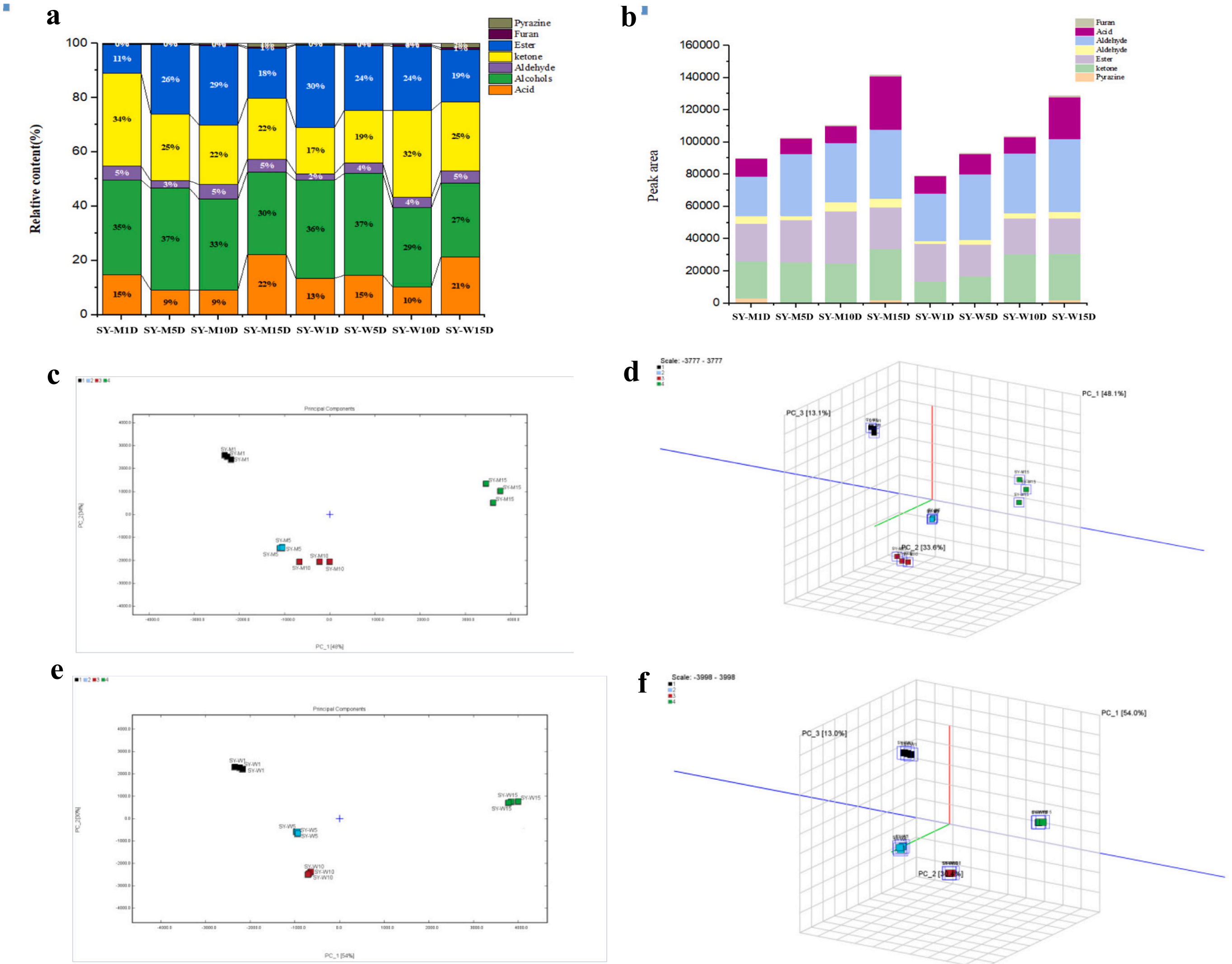

Fig. 2. Volatile organic compounds and PCA score plot during walnut soy sauce fermentation under different production processes. a) Peak areas b) relative content c) PCA score plot of SYM d) 3D PCA score plot of SYM e) PCA score plot of SYW f) 3D PCA score plot of SYW.

had relative contents of 13 % (SYW1D), 15 % (SYW5D), 10 % (SYW10D), and 21 % (SYW15D) in the SYW group, and 15 % (SYM1D), 9 % (SYM5D), 9 % (SYM10D), and 22 % (SYM15D) in the SYM group. By the end of fermentation, the relative acid contents were similar between the SYW and SYM groups. Furan compounds, derived from glucose degradation and essential for flavor, increased steadily in both groups during fermentation, reaching 1 % at the end with minimal differences between the groups. These results highlight distinct VOC profiles between the SYW and SYM groups throughout fermentation.

3.2.3. *Qualitative analysis of volatile organic compounds during fermentation of milk walnut soy sauce (SYM) and walnut soy sauce (SYW)*

The changes in volatile organic compounds (VOCs) in milk enriched soy sauce (SYM) and without milk (SYW), are compared in (Fig. 3a), which highlights the VOCs variation across samples, with each row representing a specific VOC type and each column showing content variation. A red background indicates higher content, while lighter colors signify lower content, and black or blue represent minimal levels (Ge et al., 2020). As shown in (Fig. 2a, b), regardless of the production process, alcohols, aldehydes, and ketones were dominant VOCs in the early fermentation stages, while acids and esters increased in the later stages. Significant differences in VOC composition were observed during the same fermentation period under the two processes. For instance, on the first day of fermentation (SYM1D), contained higher levels of aldehydes and ketones, including benzaldehyde, trans-2-octenal, 3-methyl-2-butenal, 2-hexanone, 2-heptanone, 2-butanone, and 2-pentanone. In contrast, SYW exhibited higher ester content, such as hexyl formate, isoamyl acetate, propyl acetate, isobutyl acetate, and ethyl propionate. These findings highlight the impact of milk addition on the VOC profiles during fermentation.

The clustering heat map (Fig. 3b), used 70 selected volatile organic compounds (VOCs), including both monomers and dimers, to differentiate walnut soy sauce samples across various fermentation times. These VOCs effectively distinguish milk walnut soy sauce samples by fermentation stage, forming a basis for identifying samples at different fermentation times. The samples were grouped into four categories: SYM1D, SYM5D, SYM10D, and SYM15D. VOC content in Region A was highest on the first day of fermentation (SYM1D), while Region B showed higher VOC content on the 15th day (SYM15D). Region C exhibited elevated VOC levels on days 5 and 10 (SYM5D and SYM10D). These findings confirm that the screened VOCs can reliably differentiate samples based on fermentation time.

3.2.4. *Principal component analysis of volatile organic compounds in Milk walnut soy sauce (SYM) and soy sauce (SYW)*

The PCA score plots were used to assess the statistical differences between sample groups and the level of parallelism within each group. The contribution rates of the first two principal components for SYM, PC1 and PC2, were 48.0 % and 34.0 %, respectively, with a cumulative contribution rate of 72.0 % (Fig. 2c). The first three principal components, PC1, PC2, and PC3, contributed 48.1 %, 33.6 %, and 13.1 %, respectively, with a cumulative contribution of 95.0 %, indicating strong variable correlation, high interpretability (Fig. 2d). The tightly clustered parallel samples confirm good parallelism. With fermentation progress samples (SYM1D, SYM5D, SYM10D, and SYM15D) showed increased separation, highlighting the significant impact of fermentation time on the flavor of milk walnut soy sauce which is in agreement with (Zhang et al., 2024). This observation aligns with fingerprint spectrum results.

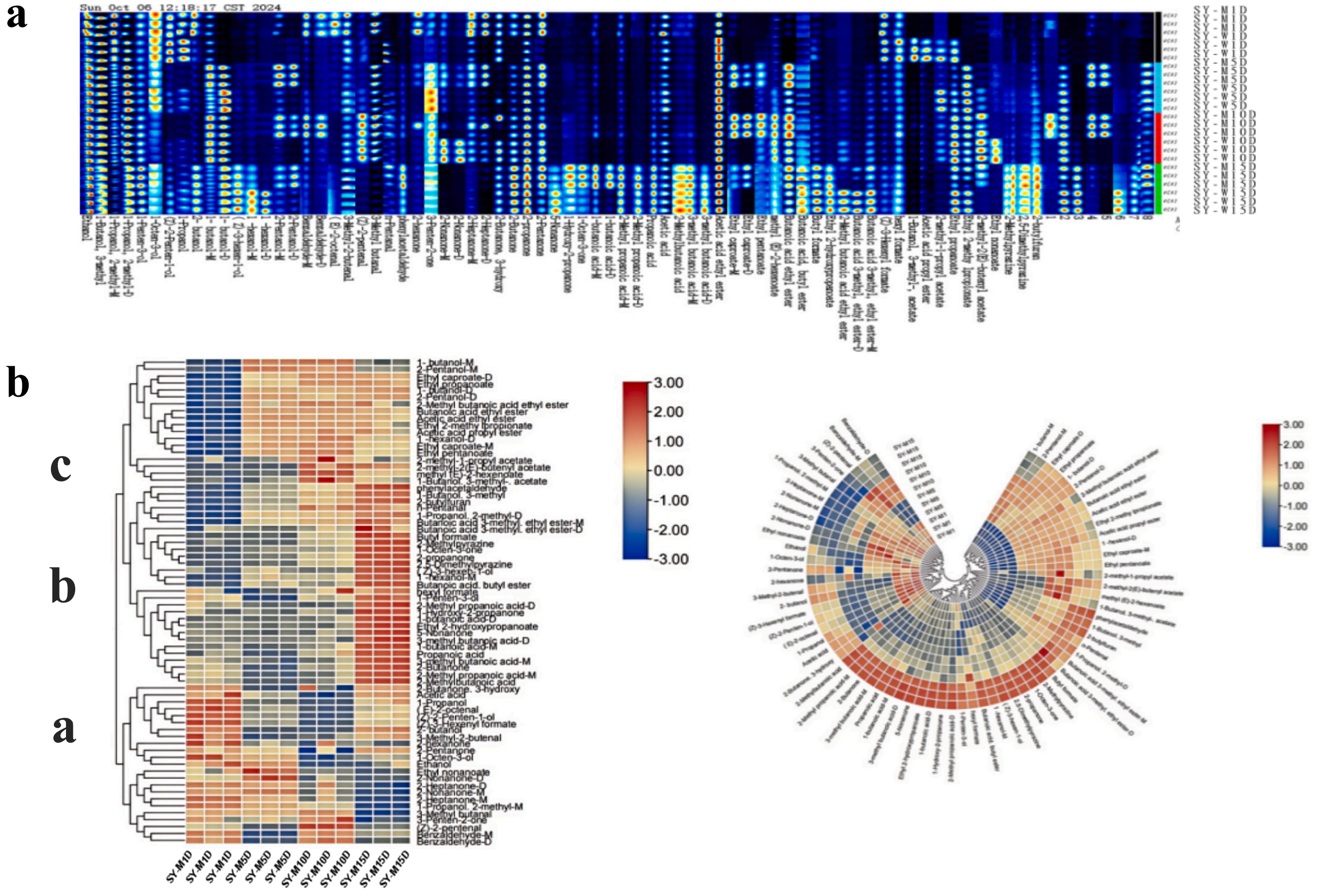

Fig. 3. a) Comparison of volatile organic compounds fingerprints during fermentation under different production processes b) Clustering heat map of volatile organic compounds during fermentation.

The first two principal components, PC1 and PC2 for SYW, accounted for 54.0 % and 30.0 % of the variance, respectively, with a cumulative contribution rate of 84.0 % (Fig. 2e). Including PC3 increased the contribution rates to 54.0 %, 30.4 %, and 13.0 %, respectively, with a total cumulative contribution of 97.4 % (Fig. 2f). Samples from the first day of fermentation (SYW1D and SYM1D) were clustered in the second quadrant, those from the fifth (SYW5D and SYM5D) and tenth days (SYW10D and SYM10D) in the third quadrant, and those from the 15th day (SYW15D and SYM15D) in the first quadrant. These results confirm that fermentation time significantly influences the VOC profiles and flavor development of walnut soy sauce.

3.2.5. *Relative contents of volatile organic compounds during fermentation*

During fermentation, milk walnut soy sauce (SYM) produced 58 volatile organic compounds (VOCs), including 5 acids, 10 alcohols, 8 aldehydes, 19 esters, 13 ketones, one furan, and two pyrazines, with some compounds exhibiting both monomeric and dimeric forms (Table SM1). Key compounds with high relative contents (>1.6 %) included ethanol, 2-acetone, 3-methyl butanol, ethyl acetate, 2-methyl propanol-D, 1-butyric acid-M, and acetic acid, but their trends varied during fermentation. Compounds such as 2-acetone, 2-methyl propanol-D, and 1-butyric acid-M showed a steady increase with increase in fermentation time, peaking on the 15th day (SYM15D) with relative contents of 11.36 ± 0.08 %, 4.55 ± 0.1 % and 6.69 ± 0.24 %, respectively, significantly higher than other samples. Ethanol exhibited an opposite trend, progressively decreasing as fermentation progressed. Its highest relative content was recorded on the first day of fermentation (SYM1D), which was higher than that of other fermentation periods. The relative contents of 3-methyl butanol and ethyl acetate exhibited an initial increase followed by a decrease as fermentation progressed. Their highest levels were observed on the 10th day (SYM10D) and 5th day (SYM5D), respectively, with 3-methyl butanol reaching 9.17 ± 0.43 % and ethyl acetate 10.03 ± 0.17 %. These values were significantly higher than those in other samples, ranging from 3.7 %–27.4 % for 3-methyl butanol and 10.0 %–49.0 % for ethyl acetate. The relative contents of acetic acid, 2-pentanone, and 2-butanone initially decreased and then increased as fermentation progressed. Their highest values were observed on the first day (SYM1D) for acetic acid (7.29 ± 1.48 %) and 2-pentanone (4.05 ± 0.34 %), and on the 15th day (SYM15D) for 2-butanone (3.6 ± 0.17 %), significantly higher than other samples by 69.5 %–165.1 %, 27.8 %–108.8 %, and 33.3 %–123.6 %, respectively. Benzaldehyde-D and ethyl butyrate exhibited low relative contents on the first day of fermentation (SYM1D, 0.64 ± 0.04 %) but showed a rapid increase, reaching their peak on the 10th day (SYM10D) at 8.72 ± 0.15 %. These values were significantly higher than other fermentation periods, ranging from 3.2 %–1262.5 %. The relative contents of butanol-D and 3-hydroxy-2-butanone were lowest on the 1st and 5th day of fermentation. Similarly, the contents of methylbutyric acid-M, 2-methylpropionic acid-M, and 1-butyric acid-D were very low during the 1st to 10th days (ranging from 0.24 ± 0.01 % to 1.58 ± 0.26 %) but increased significantly on the 15th day to 3.32 ± 0.24 %, 2.53 ± 0.13 %, and 2.25 ± 0.42 %, respectively. The relative contents of n-valeraldehyde and ethyl caproate-M were significantly higher during the 5th to 10th days of fermentation compared to other periods. The distinct changes in the main volatile organic compounds in milk walnut soy sauce throughout fermentation duration, revealing that fermentation time plays a crucial role in shaping the VOC composition and quality attributes of walnut soy sauce.

During walnut soy sauce (SYW) fermentation, 55 volatile organic compounds (VOCs) were identified, including acids, alcohols, aldehydes, esters, ketones, a furan, and pyrazines. Key VOCs, such as ethanol, 2-acetone, 3-methyl butanol, ethyl acetate, 2-methyl propanol-D, acetic acid, and 3-hydroxy-2-butanone, exhibited distinct trends. 2-Acetone and 2-butanone steadily increased, peaking on Day 15 (12.29 ± 0.08 % and 3.71 ± 0.04 %), which is due to sustained microbial metabolism and enzymatic activity (Z. Wang et al., 2022) while ethyl acetate decreased, with the highest content on Day 1 (17.3 ± 0.21 %). Acetic acid, butanol, and 2-methyl propanol-D peaked on Day 5 (8.28 ± 0.34 %, 5.51 ± 0.04 %, and 6.03 ± 0.11 %), whereas ethanol and hexyl formic acid peaked on Days 5 and 10 (15.69 ± 0.25 % and 5.29 ± 0.14 %). Compounds like 3-methyl butanol, 3-methylbutyric acid-M, 3-hydroxy-2-butanone, and 1-butyric acid-M fluctuated, peaking on Day 15. The dynamic changes in VOCs during fermentation were influenced by time and production processes.

3.3. *Relative odor activity value (ROAV) of volatile organic compounds during fermentation under different production processes*

Out of the 53 volatile flavor compounds detected in the SYM group, 29 had ROAV values ≥1, highlighting their notable impact on the flavor profile (Table SM2). A higher ROAV indicates a more significant contribution of an organic compound to the overall flavor. These compounds include 3-methyl butanol, 3-hydroxy-2-butanone, 3-methyl-n-butanal, n-valeraldehyde, ethyl 2-methyl butyrate, 1-octene-3-one, 2-butyl furan, and 1-octene-3-ol significantly contributed to the flavor, with ROAV values exceeding 125. 3-Methyl butanol imparts whiskey and banana fruit notes, 3-hydroxy-2-butanone provides butter and cream notes, 3-methyl butyral and ethyl 2-methyl butyrate contribute apple and fruit notes, while n-valeraldehyde offers grassy and slight banana notes with a mildly pungent taste. Additionally, 1-octene-3-one and 1-methyl butanone add firm soil, mushroom, and vegetable notes, while 2-butyl furan contributes mild fruit, wine, sweet, and spicy notes, and 1-octene-3-ol (mushroom alcohol) adds mushroom, lavender, rose, and hay aromas. Additionally, compounds with ROAV values >10 included 2-methylpyrazine, 2-heptanone, hexanol, 2-methylpropionate, trans-2-octenal, and benzaldehyde-D, which contributed fruity, nutty, and herbal notes. Overall, the flavor of milk walnut soy sauce during fermentation was characterized by a blend of fruit, wine, and vegetable aromas (Table SM 2).

A total of 50 volatile flavor compounds were identified in the SYW group, of which 28 had ROAV values ≥1, indicating their significant contribution to the flavor. During the fermentation of walnut soy sauce, compounds such as 3-methyl butanol, 3-methyl-n-butanal, 3-hydroxy-2-butanone, ethyl 2-methyl butyrate, 2-butyl furan, 1-octene-3-ol (mushroom alcohol), 2-methyl pyrazine, and 1-octene-3-one demonstrated notable influence, with ROAV values exceeding 107. The 3-Methyl butanol imparted whiskey and banana fruit notes; 3-hydroxy-2-butanone added buttery and creamy characteristics; 3-methyl-n-butanal and ethyl 2-methyl butyrate provided apple and fruity notes. Additionally, 2-butyl furan contributed mild fruity, wine, sweet, and spicy notes, while 1-octene-3-ol (mushroom alcohol) introduced mushroom, lavender, rose, and hay aromas. 2-Methyl pyrazine exhibited nutty, moldy, roasted, and earthy aromas, and 1-octene-3-one contributed a firm soil, mushroom, and vegetable aroma, with a subtle hint of fish and chicken. In comparison to the SYW group, the addition of milk significantly increased the ROAV values (Zhang et al., 2020) of certain compounds in the SYM group. For instance, 1-octene-3-one with earthy and mushroom aromas increased by 2060.3 %, while n-valeraldehyde with a faint banana aroma and slightly pungent taste rose by 40.5 %. However, the ROAV values of other compounds decreased by 3.5 %–60.8 %. Compounds with ROAV values above 10, such as hexanol, 2-methyl propionate, 2-heptanone, 2-nonen-M, 1-pentene-3-alcohol, and n-valeraldehyde, contributed fresh pear, banana, raspberry, green, and slight herbal notes. Overall, the flavor profile of walnut soy sauce during fermentation was characterized by fruity, green, wine, and vegetable aromas.

3.4. *Effects of milk addition on bacterial community structure*

3.4.1. *Comparative OTU profiling of microbial communities*

The bacterial community Operational Taxonomic Unit (OTU) analysis using high-throughput sequencing on walnut soy sauce fermented samples across two groups, SYW and SYM, at four fermentation stages (day 1, 5, 10, and 15) were performed. A total of 14,001 OTUs were identified, with 4760 OTUs shared between the two groups. Unique OTUs were found in the SY-M1, SY-M5, SY-M15, SY-W1, and SY-W5 groups, totaling 1852, 1367, 3515, 1634, and 873, respectively (Fig. 4a). Alpha diversity analysis, including Chao1, Shannon, Simpson, and Ace indices, revealed that microbial diversity in the SYM group was significantly higher than the SYW group during the first 5 days of fermentation (Fig. 4b, c, d,e) This was due to milk's rich nutrients (lactose, proteins, fats, and vitamins), which provided abundant resources for microorganisms, fostering the growth of yeasts and *Aspergillus oryzae*. Yeasts could utilize lactose in milk, enhancing microbial diversity (Cenci-Goga et al., 2021). However, by days 10 and 15, microbial diversity in the SYM group decreased compared to SYW, likely due to the depletion of lactose and the influence of milk's other components, such as fatty acids and peptides, which began affecting the microbial community. The accumulation of metabolic products like lactic acid (Shi & Maktabdar, 2022) and milk's antimicrobial compounds, such as lactoferrin (Niaz et al., 2019) and lactoperoxidase, likely inhibited the growth of some microorganisms, further reducing microbial diversity in the SYM group. Despite higher diversity in the early stages, the later stages saw a decrease in microbial diversity in the SYM group due to the enrichment of dominant strains and the inhibition of others. This suggests that milk components not only promote microbial growth but also selectively favor specific strains, which may contribute to improved soy sauce fermentation quality and flavor stability.

β diversity was assessed using Principal Coordinates Analysis (PCoA) (Fig. 4f) and Non-metric Multidimensional Scaling (NMDS) (Fig. 4g) to examine microbial community composition differences between groups. The results showed good clustering within each group and significant differences between them, suggesting that the microbial structure of each group changed as fermentation progressed. In the early stages, the SYM and SYW groups exhibited distinct separation, indicating a considerable difference in their microbial compositions. This difference likely resulted from the varying compositions of the soy sauces, with the SYM group enriched with nutrients like lactose, proteins, and fats, providing abundant resources for microbial growth (Aslam et al., 2020) and significantly impacting the community structure. In contrast, the SYW group, with simpler nutritional content, likely experienced slower microbial changes. As fermentation time increased, particularly in later stages, both groups began to converge, showing greater similarity. This convergence was likely due to the depletion of the nutritional components of milk, which were consumed in earlier stages. Consequently, the microbial communities of both groups stabilized, and their compositions became more similar. This phenomenon suggests that in the later fermentation stages, the dynamic changes in microbial communities balanced out, reducing the differences between the groups. The changes in microbial communities are closely tied to the composition of milk. In the early stages, milk's nutrients promote rapid microbial shifts, while in the later stages, the depletion of these components leads to stabilization and reduced differences between groups, offering new insights into microbial succession during soy sauce fermentation.

3.4.2. Effect of milk addition on milk walnut soy sauce (SYM) and soy sauce (SYW).

3.4.2.1. Effect of milk addition on milk walnut soy sauce (SYM) and soy sauce (SYW) microbiota composition. The microbial composition of the top 20 phyla, genera, and species in terms of overall abundance during fermentation is shown in (Fig. 5a, b, c). The overall microbial colony composition in both groups was similar, the relative abundance of each phylum differed. In the SYM group, the dominant phyla were Proteobacteria, Firmicutes, and Ascomycota, with their relative abundances exceeding 80 % during fermentation, due to the nutrient-rich milk promoting their growth and metabolic activity during fermentation (Amin & Mao, 2021). On day 1 (SYM1D), Firmicutes was the dominant phylum, followed by Proteobacteria and Ascomycota. By day 5 (SYM5), Ascomycota became the main dominant phylum, overtaking Firmicutes and Proteobacteria. On day 10 (SYM10D), the relative abundance of Proteobacteria increased, becoming the dominant phylum, while Firmicutes and Ascomycota declined. By day 15 (SYM15D), Ascomycota's relative abundance more than doubled, while Proteobacteria remained the dominant phylum, with Firmicutes and other phyla decreasing in relative abundance. In the SYW group, Proteobacteria and Firmicutes were the dominant phyla, with relative abundances exceeding 60 % which is consistent with result of heatmap (Fig. 5d). On days 1 and 5

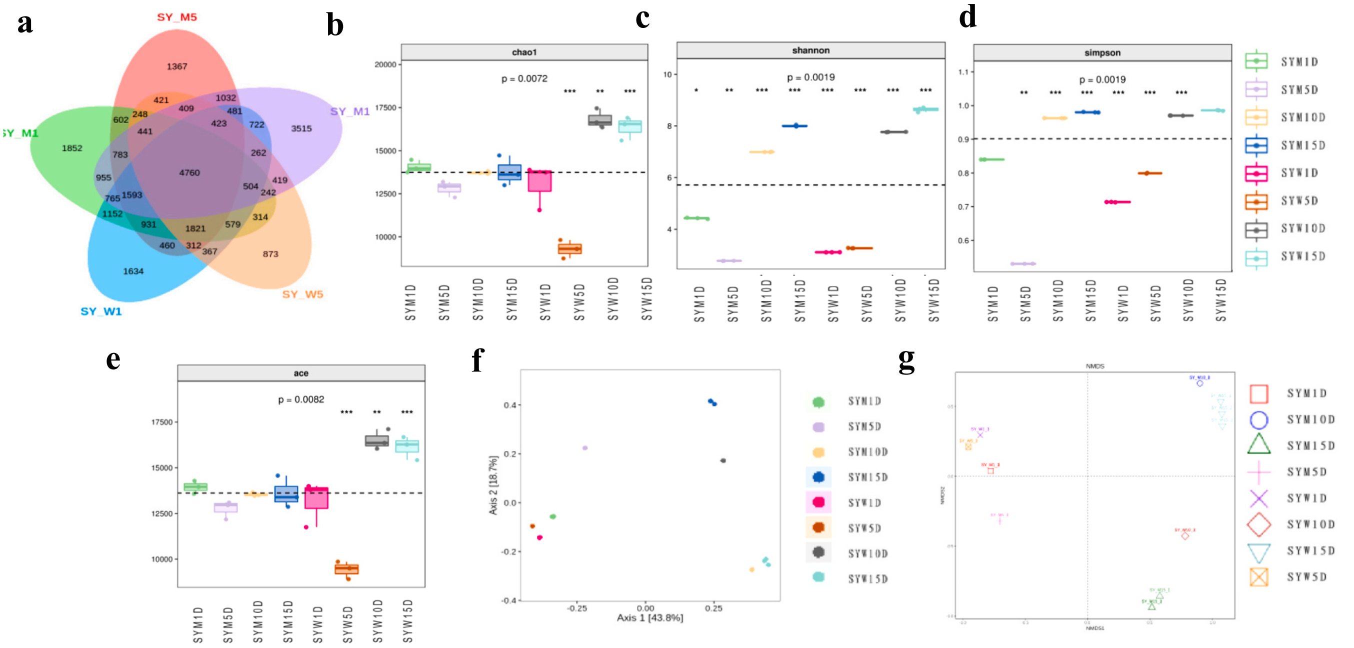

Fig. 4. Walnut milk soy sauce and microbiota diversity a) Venn diagram, b) Chao index, c) Shannon index, d) Simpson index, e) Ace Index f) PCoA, and g) NMDS of microbiota.

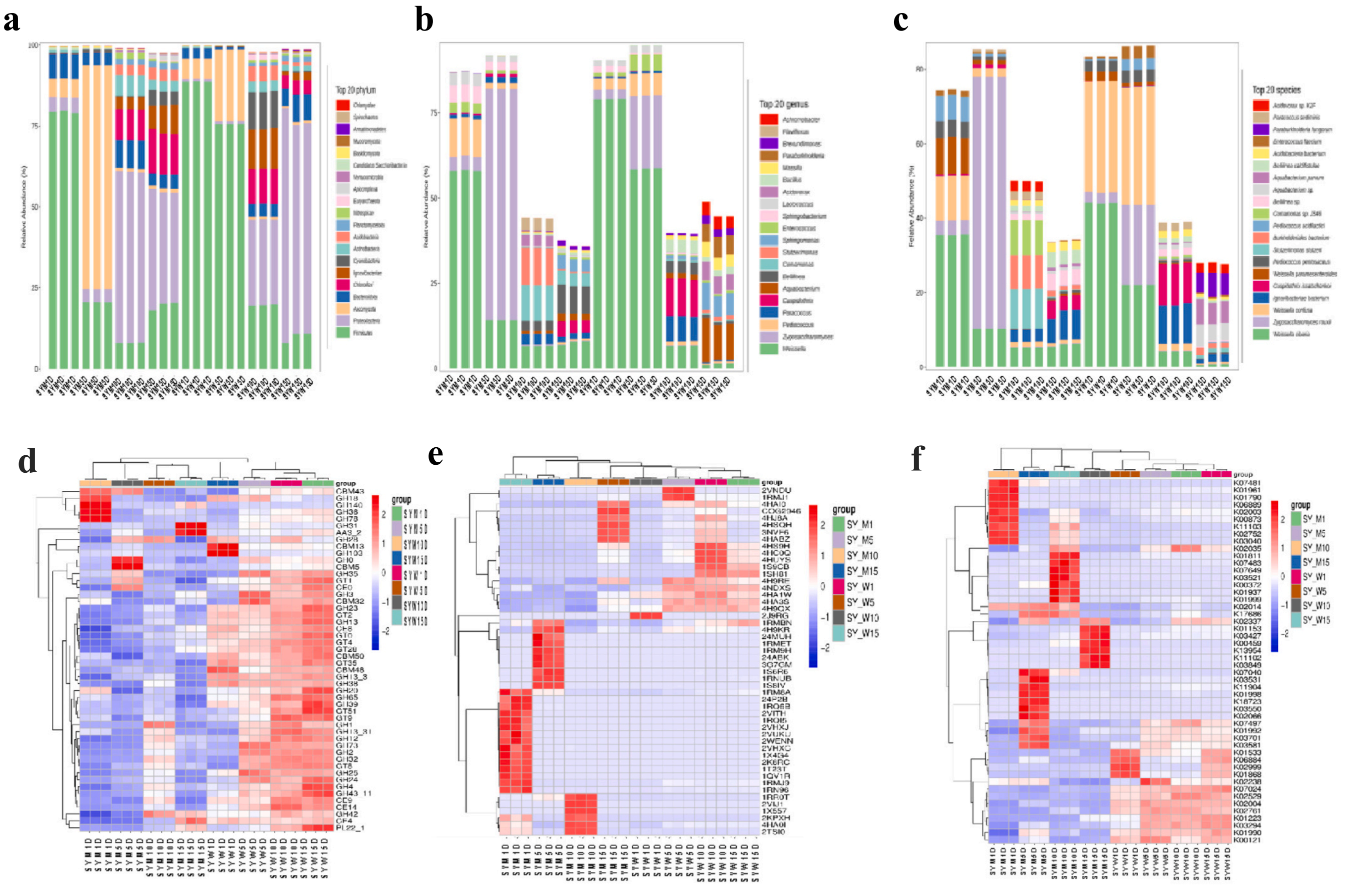

Fig. 5. (a) Microbial composition at phylum level during fermentation of milk walnut soy sauce and walnut soy sauce, (b) microbial composition at genus level (c) microbial composition at species level. The heat-map of microbiota (d) phylum level (e) genus level (f) species level.

(SYW1 and SYW5), Firmicutes was the dominant phylum, followed by Proteobacteria and Ascomycota. On day 10 (SYW10), Proteobacteria increased rapidly to become the dominant phylum, while Firmicutes decreased. Ignavibacteriae and Chloroflexi also increased significantly. By day 15 (SYW15), Proteobacteria remained the dominant phylum, and Firmicutes decreased, with other phyla declining as well. Throughout fermentation, compared to the SYW group, the SYM group showed different trends: a decrease in Firmicutes, an increase in Ascomycota, and an initial rise followed by a slight decline in Proteobacteria.

As shown in (Fig. 5b), on day 1st of fermentation, the walnut soy sauce (SYW) was dominated by the *Weissella* genus, with relative abundances exceeding 90 %, while the milk walnut soy sauce (SYM group) was also dominated by *Weissella* (over 70 %) followed by *Pediococcus*. The SYM group showed a decrease in *Pediococcus* and *Zygosaccharomyces* relative abundance compared to the SYW group. On day 5, the SYW group was dominated by *Pediococcus, Weissella*, and *Zygosaccharomyces*, each with relative abundances above 20 %, while the SYM group was dominated by *Zygosaccharomyces* (above 80 %), followed by *Weissella* and *Weissella confusa*. On day 10, the SYW group was dominated by *Paracoccus* and *Cuspidothrix*, with relative abundances above 30 %, while the SYM group showed dominance of *Weissella, Stutzerimonas, and Comamonas*, each exceeding 30 % which aligns with the result of heatmap (Fig. 5e). By day 15, the SYW group was dominated by Aquabacterium, Sphingobacterium, and Paraburkholderia, whereas the SYM group was dominated by Bellilinea and Weissella. This shift in microbial composition in the SYW group became more pronounced as fermentation progressed. Both groups showed a trend of increasing and then decreasing microbial abundance, with the SYM group exhibiting an increase during days 1–5 and a decrease from days 10–15, in contrast to the SYW group.

The microbial community composition at the species level showed significant differences between the SYW and SYM groups. The top 20 species included *Weissella cibaria*, *Zygosaccharomyces rouxii*, *Weissella confusa*, *Acidovorax* sp. *K2F*, *Paracoccus sediminis*, *Ignavibacteriae bacterium*, *Bellilinea caldifistulae*, *Cuspidothrix issatschenkoi*, *Comamonas* sp. *JS46*, *Weissella paramesenteroides*, *Pediococcus pentosaceus*, *Paraburkholderia fungorum*, *Pediococcus acidilactici*, *Aquabacterium parvum*, *Stutzerimonas stutzeri*, and *Enterococcus faecium* (Fig. 5c). On day 1st, *Weissella cibaria* and *Weissella confusa* dominated in SYW (>20 %), while SYM was dominated by *Weissella cibaria* (>20 %), *Weissella confusa*, and *Weissella paramesenteroides*. By day 5, *Weissella cibaria*, *Weissella confusa*, and *Zygosaccharomyces rouxii* dominated in SYW (>20 %), while SYM was dominated by *Zygosaccharomyces rouxii* (>80 %). On day 10, *Ignavibacteriae bacterium* and *Cuspidothrix issatschenkoi* dominated in SYW (>30 %), while *Stutzerimonas stutzeri* and *Comamonas* sp. *JS46* dominated in SYM (>20 %) which is consistent with heatmap (Fig. 5f). By day 15, *Aquabacterium* sp. and *Paraburkholderia fungorum* dominated in SYW, while *Ignavibacteriae bacterium* and *Weissella cibaria* dominated in SYM. The relative abundance of microbial species varied notably between the two groups, highlighting milk's impact on microbial composition during fermentation. In the early stages, SYM exhibited greater diversity due to milk's nutrient-rich nature. However, as fermentation progressed, both groups converged, reflecting the stabilizing effect of nutrient depletion.

3.4.3. LEfSe analysis of microbial differences

LEfSe analysis with an LDA threshold of 4 (LDA score > 4.0, $P < 0.01$) compared dominant bacteria in SYM and SYW across fermentation stages. For the SYM group, the classification levels ranged from 1 kingdom (Kingdom), 3 phyla (Phylum), 4 classes (Class), 7 orders

(Order), and 9 families (Family) (Fig. 6), with notable families such as *Sphingobacteriaceae* (LDA score = 4.19; *P* = 0.0019), *Streptococcaceae* (LDA score = 4.09; *P* = 0.0020), *Pseudomonadaceae* (LDA score = 5.11; *P* = 0.0019), *Enterobacteriaceae* (LDA score = 5.08; *P* = 0.0021), *Comamonadaceae* (LDA score = 4.51; P = 0.0021), *Moraxellaceae* (LDA score = 4.91; P = 0.0019), *Aspergillaceae* (LDA score = 4.88; P = 0.0021), *Anaerolineaceae* (LDA score = 4.32; P = 0.0021), and *Saccharomycetaceae* (LDA score = 5.43; *P* = 0.0019). A total of 12 genera, including *Enterobacter* (LDA score = 4.55; P = 0.0019), *Pediococcus* (LDA score = 4.55; *P* = 0.0021), *Sphingobacterium* (LDA score = 4.14; P = 0.0019), *Lactococcus* (LDA score = 4.05; P = 0.0019), *Klebsiella* (LDA score = 5.04; *P* = 0.0020), *Pseudomonas* (LDA score = 5.03; P = 0.0019), *Stutzerimonas* (LDA score = 4.38; P = 0.0021), *Comamonas* (LDA score = 4.35; P = 0.0019), *Acinetobacter* (LDA score = 4.91; P = 0.0020), *Aspergillus* (LDA score = 4.88; P = 0.0021), *Bellilinea* (LDA score = 4.31; P = 0.0019), *Zygosaccharomyces* (LDA score = 5.41; P = 0.0019), and 15 species, including *Weissella paramesenteroides* (LDA score = 4.48; P = 0.0019), *Pediococcus acidilactici* (LDA score = 4.31; P = 0.0021), *Enterobacter hormaechei* (LDA score = 4.22; P = 0.0019), *Klebsiella pneumoniae* (LDA score = 5.07; P = 0.0021), *Pseudomonas helleri* (LDA score = 4.94; P = 0.0021), *Stutzerimonas stutzeri* (LDA score = 4.38; P = 0.0021), Comamonas sp. JS46 (LDA score = 4.37; *P* = 0.0018), *Pseudomonas* sp. S 47 (LDA score = 4.09; P = 0.0018), *Pseudomonas putida* (LDA score = 4.84; *P* = 0.0023), *Acinetobacter johnsonii* (LDA score = 4.71; P = 0.0020), *Aspergillus oryzae* (LDA score = 4.41; P = 0.0019), *Acinetobacter lwoffii* (LDA score = 4.24; *P* = 0.0022), Bellilinea sp. (LDA score = 4.04; P = 0.0020), *Zygosaccharomyces rouxii* (LDA score = 5.43; P = 0.0019), and *Aspergillus flavus* (LDA score = 4.52; P = 0.0021).

For the SYW group, the classification levels ranged from 1 kingdom (Kingdom), 6 phyla (Phylum), 6 classes (Class), 7 orders (Order), and 9 families (Family), with notable families such as *Lactobacillaceae* (LDA score = 5.52; P = 0.0019), *Rhodobacteraceae* (LDA score = 4.49; P = 0.0021), *Bacillaceae* (LDA score = 4.35; P = 0.0019), *Malasseziaceae* (LDA score = 4.23; P = 0.0019), Sphingomonadaceae (LDA score = 4.40; P = 0.0019), Burkholderiaceae (LDA score = 4.39; P = 0.0023), Alcaligenaceae (LDA score = 4.21; P = 0.0023), *Oxalobacteraceae* (LDA score = 4.04; *P* = 0.0024), and *Enterococcaceae* (LDA score = 4.38; P = 0.0019). A total of 10 genera, including *Weissella* (LDA score = 5.45; P = 0.0019), *Paracoccus* (LDA score = 4.50; P = 0.0021), *Escherichia* (LDA score = 4.28; P = 0.0019), *Malassezia* (LDA score = 4.21; P = 0.0019), *Bacillus* (LDA score = 4.15; P = 0.0023), *Paraburkholderia* (LDA score = 4.41; *P* = 0.0029), *Sphingomonas* (LDA score = 4.35; P = 0.0021), *Acidovorax* (LDA score = 4.32; P = 0.0024), *Achromobacter* (LDA score = 4.22; P = 0.0023), and *Enterococcus* (LDA score = 4.35; P = 0.0019), along with 10 species, including *Weissella cibaria* (LDA score = 5.22; P = 0.0023), *Escherichia coli* (LDA score = 4.27; P = 0.0019), *Malassezia globosa* (LDA score = 4.20; P = 0.0020), *Paraburkholderia fungorum* (LDA score = 4.42; P = 0.0029), *Achromobacter* sp. DMS1 (LDA score = 4.20; *P* = 0.0096), *Acidovorax* sp. K2F (LDA score = 4.05; *P* = 0.0027), *Weissella confusa* (LDA score = 5.17; P = 0.0019), Weissella sagaensis (LDA score = 4.22; P = 0.0019), *Pediococcus pentosaceus* (LDA score = 4.20; P = 0.0021), and *Enterococcus faecium* (LDA score = 4.17; P = 0.0024).

3.4.4. Correlation analysis between microbial communities and flavor compounds

At the genus level, Spearman's analysis was conducted to explore the co-occurrence and exclusion patterns of microbial communities (20 bacterial genera and 20 volatile compounds) in walnut soy sauce. (Fig. 7a) shows that among the 20 bacterial genera in the bacterial network,

81 pairs of significantly correlated relationships were identified ($|r| > 0.7$, $P < 0.05$), with 41.7 % showing positive correlations and 58.3 % showing negative correlations. Among these, 2.5-Dimethylpyrazine had a positive correlation with *Sphingomonas* and a negative correlation with *Weissella*. (Z)-3-hexen-1-ol had a positive correlation with *Weissella* and *Lactobacillus* and a negative correlation with *Sphingomonas*. During the fermentation of walnut soy sauce, *Weissella* and *Lactobacillus* have been repeatedly reported to play an important role in the flavor of soy sauce. Investigating microbial interactions during the fermentation process is of great significance for the regulation of soy sauce fermentation and the development of fermentation agents. As shown in (Fig. 7b), RDA1 and RDA2 explained 63.5 % and 35.42 % of the correlation between bacteria and volatile compounds, respectively, shows that *Weissella*, *Sphingomonas*, and *Bacillus* were positively correlated with 1-Octen-3-ol. They also appeared in the SY-M1 and SY-W1 samples, suggesting that these bacteria play an important role in the early stages of fermentation. *Bacillus* was positively correlated with 2-butylfuran, Propanoic acid, and 2-Methylbutanoic acid, which appeared in the SY-M10 and SY-W10 samples, indicating that these bacteria are important in the later stages of fermentation. This concluded that *Weissella*, *Sphingomonas*, and *Bacillus* as crucial contributors to flavor development at different

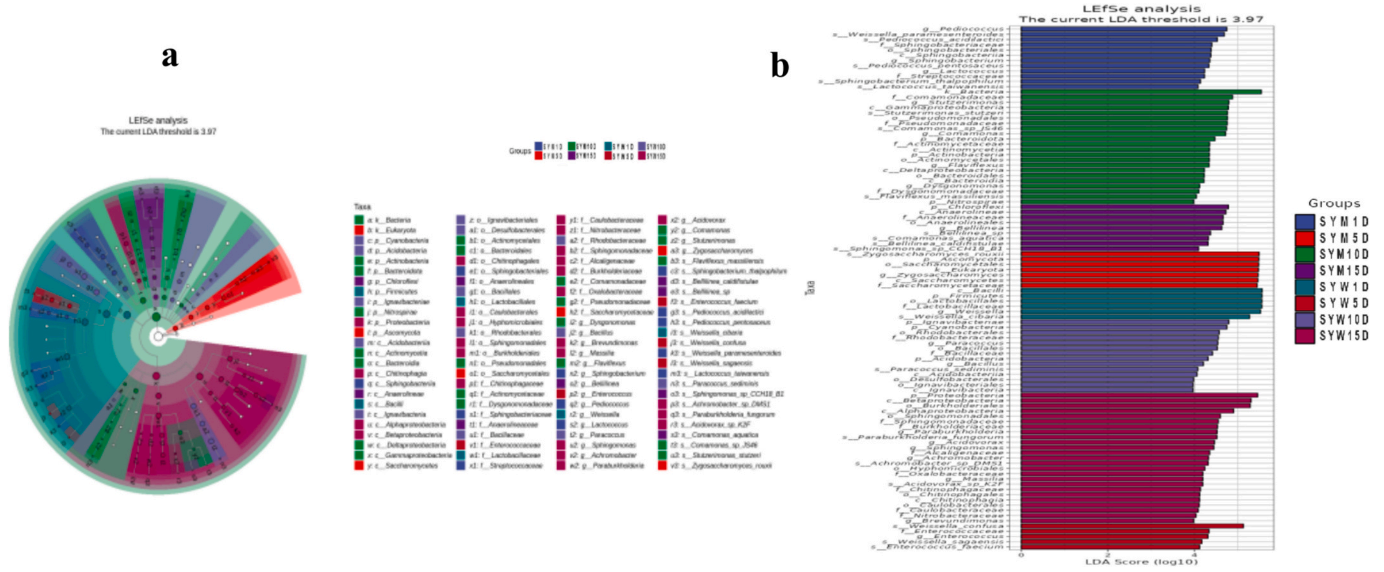

Fig. 6. LEfSe analysis of dominant microbiota in SYM and SYW (a) Taxonomic cladogram from LEfSe analysis (b) Species with significant differences with LDA score. (LDA score > 4.0).

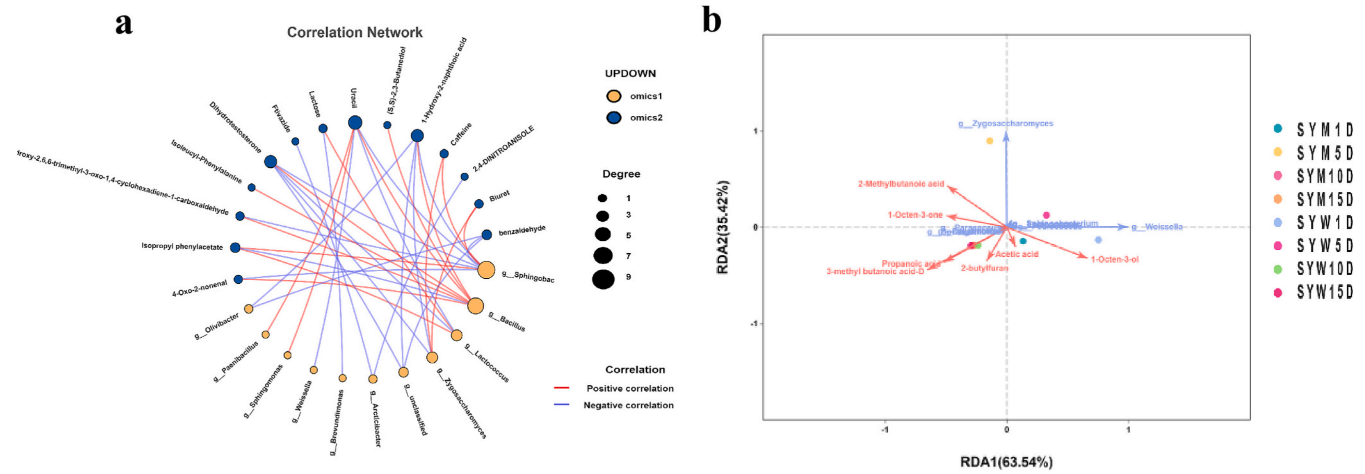

Fig. 7. (a) Correlation between microbial community and volatile compounds, (b) RDA analysis of microbial communities and volatile compounds. Note: omics1 indicates microbial community. Omics2 indicates flavor compounds.

fermentation stages.

4. Conclusion

This study explored the addition of milk to walnut soy sauce as a strategy to enhance both the flavor complexity and microbial composition of the final product. The inclusion of milk, rich in proteins, fats, and lactose, was designed to create a more nutrient-rich fermentation environment that would promote the growth of beneficial microorganisms, particularly lactic acid bacteria such as *Lactobacillus* and *Weissella*. These bacteria, known for their role in fermentation, contribute significantly to the development of the umami flavor and aroma of soy sauce. Milk proteins, including essential amino acids, played a crucial role in the formation of key flavor compounds, enhancing the overall sensory profile of the milk-enriched soy sauce (SYM). GC-IMS analysis confirmed that the milk-enriched soy sauce (SYM) exhibited a distinct volatile organic compound profile compared to traditional walnut soy sauce (SYW), with an increase in esters, aldehydes, and alcohols that contribute to a more complex and balanced flavor. Furthermore, the addition of milk to walnut soy sauce resulted in a shift in microbial community dynamics, increasing microbial diversity and improving fermentation stability. The presence of milk proteins provided not only essential nutrients but also modulated the metabolic pathways of microbial communities, contributing to a more stable and functional fermentation process. The synergistic effect of milk's nutritional components and its impact on microbial growth and metabolic activity makes it a promising ingredient for developing high-quality, functional soy sauces. This research underscores the potential of utilizing milk as alternative raw materials, advancing the comprehensive utilization of animal proteins in fermented food production.

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CRediT authorship contribution statement

Munguntsetseg Battur: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Muhammad Aaqil:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Jingchuan Zheng:** Resources, Methodology, Investigation, Data curation. **Huang Xiao Lin:** Validation, Software, Investigation, Data curation. **Bolormaa Chuluunotgon:** Validation, Formal analysis, Data curation. **Tserenkhand Zorigtbaatar:** Investigation, Formal analysis, Data curation. **Cunchao Zhao:** Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Yang Tian:** Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102364.

Data availability

Data will be made available on request.

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