International Journal of Food Science +Technology

Original article

Metabolite profiling highlights the effect of microbial intervention in the soaking step of tempe

Rifqi Ahmad Riyanto,[1,2] Eiichiro Fukusaki[1,3,4] & Sastia Prama Putri[1,3]*

1 Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1, Yamadaoka, Suita, Osaka 565-0871, Japan
2 Department of Food Technology, Faculty of Agriculture, Universitas Sultan Ageng Tirtayasa, Jl. Raya Palka Km 3, Serang, Banten 42163, Indonesia
3 Industrial Biotechnology Initiative Division, Institute for Open and Transdisciplinary Research Initiatives, 2-1, Yamadaoka, Suita, Osaka 565-0871, Japan
4 Osaka University Shimadzu Omics Innovation Research Laboratories, Osaka University 2-1, Yamadaoka, Suita, Osaka, Japan

(Received 24 June 2024; Accepted in revised form 12 August 2024)

Summary Soaking of soybeans is an essential step in tempe fermentation. Owing to the uncontrolled microflora, spontaneous soaking during tempe production leads to inconsistencies in tempe quality. Common methods to control it include the addition of acids or microbial starters. Despite knowing the benefits, their impact on tempe's composition was less understood. In this study, prior to tempe fungal fermentation, soybeans were soaked with *Lactiplantibacillus plantarum* NBRC 101978 and *Pichia burtonii* NBRC 0844. Tempe samples were subjected to comprehensive analysis using a widely targeted gas chromatography–mass spectrometry (GC–MS) metabolomics approach and evaluation of physical characteristics. A total of 100 metabolites of sugars, amino acids, fatty acids, and organic acids were annotated in all samples. Principal component analysis (PCA) explaining 47.6% of the variance showed that microbial interventions led to alterations in the metabolome of all samples, including the accumulation of amino acids in lactic acid bacteria (LAB)-soaked soybean tempe and tyramine in yeast-soaked soybean tempe. Unlike chemically added soaked soybean tempe, microbial intervention significantly reduced the relative intensity levels of several sugars by more than twofold. Furthermore, microbial interventions in the tempe-soaking step significantly elevated the levels of bioactive metabolites more than twofold. The introduction of microbial interventions in the tempe-soaking step also influences the physical characteristics of the end product. These findings merit further consideration for tempe development and the food industry.

Keywords Gas chromatography–mass spectrometry, lactic acid bacteria, metabolomics, tempe, yeast.

Introduction

Tempe is the solid-state fermentation of legumes, mainly soybean, assisted by *Rhizopus* spp. mould. It is an Indonesian staple source of protein known for its functional benefits, affordability, and sustainability (Ahnan-Winarno *et al.*, 2021). Indonesia exported 533.87 tonnes of tempe in 2022, with a total value of USD 1.62 million (Badan Pusat Statistik, 2022). The increase in the volume of tempe exports is in line with the continuous growth in global consumer demand for health-promoting fermented food (Galimberti *et al.*, 2021).

*Correspondent: Fax: +81-6-6879-7416; e-mail: sastia_putri@bio.eng.osaka-u.ac.jp

The general steps involved in tempe production include soaking, dehulling, cooking, inoculating with a culture starter, packaging, and ultimately allowing fungal fermentation to occur (Rahayu *et al.*, 2015). Variations in tempe production occur primarily during the soaking and cooking steps. As a crucial step in tempe production, soaking functions to hydrate the soybean, remove anti-nutritional factors, increase protein bioavailability, inhibit harmful microorganisms, and support the tempe starter growth (Drulyte & Orlien, 2019; Romulo & Surya, 2021; Zhang *et al.*, 2021; Yarlina *et al.*, 2023). The soaking process involves microbial fermentation, which reduces the pH of both soak water and soybeans. However, owing to differences in climate and processing, acid fermentation does not occur naturally and may fail in temperate climates

doi:10.1111/ijfs.17481

(Nout *et al*., 1987; Nout & Kiers, 2005). Furthermore, the uncontrolled microflora during soaking may result in inconsistent tempe quality.

Several chemical and microbial modifications were made to the soaking step to ensure thorough acidification (Nout & Kiers, 2005). *L. plantarum* and *P. burtonii*, major species in tempe soak water (Nout *et al*., 1987), were found to have significant effects during the soaking step by accelerating acidification and changing the carbohydrate and organic acid composition of the soybean (Mulyowidarso *et al*., 1991a, 1991b). *L. plantarum* inoculation during the soaking step has been reported to produce tempe that meets Indonesian national standards for texture, odour, and colour (Magdalena *et al*., 2022). However, a thorough metabolome analysis of the effects of *L. plantarum* and *P. burtonii* during the tempe-soaking step has not yet been reported.

For the purpose of detecting and explaining compositional changes among tempe with interventions in its soaking step, a widely targeted metabolomics approach is required. Metabolomics is the comprehensive profiling of metabolites that are widely used in several fields, including the food sciences (Putri *et al*., 2022). The approach allows for a high probability of detecting both expected and unexpected metabolites, offering valuable insights into the food metabolome. Metabolite profiling using gas chromatography–mass spectrometry (GC–MS) is highly advantageous owing to its robustness, stability, and cost-effectiveness. This approach is commonly used to rapidly characterise and differentiate small hydrophilic compounds present in foods (Putri *et al*., 2019). Previous tempe metabolomics investigations (Kadar *et al*., 2018; Rahmawati *et al*., 2021; Dahlan *et al*., 2022; Prativi *et al*., 2023) were beneficial in summarising datasets, highlighting treatment-related trends, and indicating important metabolites in experimental variations. This approach could be applied to determine how microbial intervention in the tempe-soaking step affects tempe metabolomes. Therefore, this study aimed to investigate the effects of microbial intervention during the tempe-soaking step using a widely targeted GC–MS metabolomics approach.

To the best of our knowledge, this is the first report on the metabolomics of microbial intervention during the tempe-soaking step. The results of this study provide important insights for tempe manufacturers and future product development of tempe and its derivatives for the food industry.

Materials and methods

Experimental design

Metabolite profiling using GC–MS was performed on aqueous extracts obtained from each stage of tempe production, specifically raw soybeans, soaked soybeans, and tempe at final fermentation. This analysis aimed to elucidate the metabolic changes resulting from the microbial interventions of lactic acid bacteria (LAB) and yeast during the soaking step, in comparison to the soaking process with water only. We then performed a metabolite profile comparison of microbial interventions against chemical addition using lactic acid in the soaking step of tempe to highlight the differences between microbial interventions and common chemical addition. The pH of soaked water in all soaking treatments was monitored until the completion of the soaking step. By examining the metabolites significantly modulated by microbial intervention in the soaking step of tempe fermentation, we aimed to investigate deeper the effects these processes have on tempe metabolome. Lastly, the physical characteristics of tempe were analysed to ensure compliance with water-soaked soybean tempe (WST). A list of the samples is presented in Table 1.

Preparation of inoculum

L. plantarum NBRC 101978 and *P. burtonii* NBRC 0844 obtained from the Biological Resource Centre, National Institute of Technology and Evaluation (NITE) (Tokyo, Japan) were grown aerobically in MRS Vegitone medium (Sigma, Basel, Switzerland) at 30 °C and in Potato Dextrose medium (Sigma, Basel, Switzerland) at 24 °C. The growth curve of both inoculums was determined and cultivated until the optical density $(OD)_{600}$ = 1.5 for the bacteria and OD_{600} = 1.0 for the yeast to reach the exponential phase, then harvested through centrifugation at 5000×***g*** for 15 min at 4 °C. The harvested cells were decanted and sterile water was added.

Tempe production

Tempe samples were produced in triplicate at the Laboratory of Bioresource Engineering, Osaka University, Japan, according to a previously reported method by

Table 1 Sample code list

Code	Denotation
RSB	Raw soybean
WSB	Water-soaked soybean
WST	Water-soaked soybean tempe
LBSB	Lactic acid bacteria-soaked soybean
LBT	Lactic acid bacteria-soaked soybean tempe
YSB	Yeast-soaked soybean
YST	Yeast-soaked soybean tempe
LASB	Lactic acid-soaked soybean
LAT	Lactic acid-soaked soybean tempe

Prativi *et al.* (2023) with modifications. Briefly, commercial Japanese soybeans (grown in Hokkaido, Japan) were soaked for 24 h with an inoculum (5% v/v) as a microbial intervention, or lactic acid (Kenei Pharmaceutical, Japan) (0.5% v/v) as a chemical additive. After soaking, soybeans were steamed, dehulled, and dried at room with temperature of 23 °C–25 °C. Dehulled soybeans were inoculated with *Raprima* brand starter culture and incubated at 30 °C for 48 h in an incubator containing an open beaker with water to maintain humidity.

Metabolite extraction and Derivatization

Before extraction, samples were freeze-dried and homogenised using a multi-bead shocker (Yasui Kikai, Osaka, Japan). For extraction, a mixed solvent of methanol, water, and chloroform (5:2:2 v/v/v) containing 50 μg mL^{-1} ribitol as internal standard was added to 2-mL tubes containing 10 mg of homogenised samples.

Samples were then incubated at 37 °C for 30 min with agitation at 1200 rpm. After centrifugation at 11 740×***g*** for 3 min at 4 °C, the supernatant (400 μL) was transferred into a new tube. Next, 300 μL of water was added, and the tubes were centrifuged again. Aqueous phase (200 μL) was transferred to new tubes. Quality control (QC) samples were pooled by combining 200 μL of the aqueous phase from all samples. The samples and QC were centrifuged using a centrifugal concentrator (Taitec Co., Tokyo, Japan) for 2 h at room temperature.

Extracted samples were initially treated with 100 mL of methoxyamine hydrochloride in pyridine (20 mg mL^{-1}) and incubated at 30 °C for 90 min with continuous mixing at 1200 rpm. Next, 50 mL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was included in the samples, followed by incubation at 37 °C for 30 min with 1200 rpm agitation. The derivatized samples were then transferred to GC vials for GC–MS analysis.

GC–MS analysis

GC–MS analysis was conducted using a GC–MS-QP2010 Ultra instrument (Shimadzu, Kyoto, Japan) equipped with an AOC-20i/s autoinjector (Shimadzu, Kyoto, Japan) and fitted with an InertCap 5MS/NP column (GL Sciences, Tokyo, Japan). The derivatized samples (1 μL) were injected in split mode (25:1(v/v)) at an injection temperature of 270 °C and analysed in a random order. The linear velocity of the carrier gas (H_2) was 39.0 cm s^{-1}. The column temperature was maintained at 80 °C for 2 min, then increased by 10 °C min^{-1} to 330 °C, and maintained for 6 min. The interface and ion source temperatures were 310 °C and 280 °C, respectively. Ions were generated by the electron ionisation (EI) method with a filament bias voltage of 70.0 V. EI mass spectra were recorded over the mass range m/z 85–500 with an event time of 0.15 s. The retention index (RI) was determined using a standard alkene mixture.

Colour and texture analysis

The colour of the tempe samples was evaluated using a chroma meter (Konica Minolta, Tokyo, Japan) to determine the L^*, a^*, and b^* values. Texture analysis was performed using a 50 mm diameter cylindrical press jig on a Shimadzu EZ Test EZ-SX system with Shimadzu Trapezium X software ver. 1.5.4. The textural parameters analysed in this study were hardness, cohesiveness, and springiness.

Data processing and statistical analysis

The obtained GC–MS spectral data were subjected to baseline correction, peak detection, and alignment using GCMSsolution (Shimadzu) and MS-DIAL 4.9 (RIKEN, Saitama, Japan) (Lai *et al.*, 2018). The metabolites were annotated by cross-referencing the RI and MS values with an in-house GC–MS-5MP Library (RIKEN) with a minimum of 70% similarity values using MS-DIAL and then manual annotation in the GCMS solution based on NIST-11 MS Spectral Library (NIST, Maryland, USA) with a minimum of 90% similarity values. Metabolites with relative standard deviations (SD) < 30% were selected for further statistical analyses. Principal component analysis (PCA) was performed and plotted using SIMCA-P 13.0 (Umetrics, Umea, Sweden) with autoscaling and no transformation. Physical characteristics were statistically analysed using the Kruskal–Wallis test followed by Dunn's test, and metabolites were statistically assessed using analysis of variance (ANOVA) with Tukey's post hoc test on JASP Version 0.17.3 (JASP Team, Amsterdam, Netherlands). The bar graphs and volcano plots were constructed using Microsoft Excel.

Results and discussion

Microbial interventions in Tempe-soaking step resulted in metabolome alterations

The GC–MS results yielded 153 features after filtering. The tentatively annotated compounds include amino acids, sugars, fatty acids, and organic acids (Table 2 and Table S5). Compounds previously reported in tempe metabolomics, such as meglutol, genistein, and daidzein were annotated in the samples (Iman *et al.*, 2023). In total, 100 annotated and 53 unknown metabolites were subjected to PCA (Fig. 1). The score

Table 2 List of annotated metabolites

	Amino Acids	Sugars	Fatty acids	Organic acids	Others
1	Glycine	Trehalose	Palmitic acid	Malic acid	Glycerol
2	β-Alanine	Maltitol	Malonic acid	Lactic acid	Inositol
3	Tyrosine	β-Lactose	2-Hydroxyglutaric acid	Citric acid	Phosphate
4	Tryptophan	Raffinose	Stearic acid	Succinic acid	Adenosine
5	Threonine	Melibiose	3-hydroxy-3-methylglutaric acid	Glyoxylic acid	Xanthine
6	Cystathionine	Fructose	Linoleic acid	Allantoic acid	Uracil
7	Lysine	Sucrose	Glycolic acid	Fumaric acid	2-Aminoethanol
8	Asparagine	Sorbitol	Turanose	Isocitric acid	3-Hydroxy butyrate
9	2-Aminobutyric acid	Glucose	3-hydroxy-3-methylbutanoic acid	3-Phosphoglyceric acid	Genistein
10	Valine	Lyxose		Oxalic acid	3-Phenyllactic acid
11	Ornithine	Meso erythritol			Urocanic acid
12	Allothreonine	Melezitose			3-Hydroxyanthranilic acid
13	Phenylalanine	Glucono-1,5-lactone			2,6-Pyridinedicarboxylic acid
14	Isoleucine	Panose			4-Hydroxyphenylacetic acid
15	2,6-Diaminopimelic acid	Threitol			Ethyl-α-D-glucopyranoside
16	Cysteine	Galactose			Pentasiloxane
17	Methionine	Xylonic acid			Thymine
18	Leucine	Ribose			Anthranilic acid
19	Serine	Glyceric acid			Uric acid
20	Aspartic acid	Mannitol			Adenine
21	Histidine	Galactinol			Guanine
22	Alanine	Lactose			Myo-Inositol
23	Glutamic acid	3-α-Mannobiose			Daidzein
24	Proline	TDP-glucose			2-Hydroxypyridine
25	2-Aminoadipic acid	Saccharic acid			Nicotinic acid
26	4-Aminobutyric acid	Pinitol			Tyramine
27					2,3-Butanediol
28					Putrescine
29					N-acetyl-α-D-glucosamine 1-phosphate

plot of the PCA results shows the distinction between the samples based on the tempe-processing stage and microbial intervention. A clear separation was demonstrated by principal component (PC) 1 of the plot, which explained 34.8% of the variability. The metabolite profile that shifted from raw soybeans to tempe was observed along the positive axis of PC1. From the loading plot of the PCA results (Fig. 1b), raw soybeans were dominated by sugar groups before fungal fermentation; after fungal fermentation, they were dominated by amino acids, fatty acids, and others. This finding was consistent with that of a previous study (Prativi *et al*., 2023). Notably, the accumulation of amino acids at the end of the tempe processing was mainly caused by the breaking of long-chain protein molecules by proteolytic enzymes of *Rhizopus oligosporus* as the tempe starter (Witono *et al*., 2015). The microbial intervention of LAB and yeast resulted in different profiles of metabolites for the end products of tempe based on PC1. As shown in the complete list of metabolites in the loading score (Table S1), the representative metabolites contributing to the LAB-soaked soybean tempe (LBT) were amino acids, such as lysine, leucine, and phenylalanine, and other metabolites, such as genistein. According to the findings of a previous report (Aguirre *et al*., 2008), LAB strains exhibit the capacity to hydrolyze soy protein and release amino acids, such as leucine, phenylalanine, tyrosine, valine, and isoleucine from soy protein extracts. Genistein, a soy isoflavone aglycone, has several health benefits and has been reported to increase in soybeans after fermentation with LAB (Sirilun *et al*., 2017). A substantial increase in aglycones during soybean soaking has been reported to be responsible for the degradation of parent glycosides (Moa *et al*., 2013). The rise in isoflavone aglycone contents during fermentation by LAB was attributed to β--glucosidase activity toward isoflavone glycosides (Marazza *et al*., 2012).

In contrast, the representative metabolites contributing to yeast-soaked soybean tempe (YST) are amino

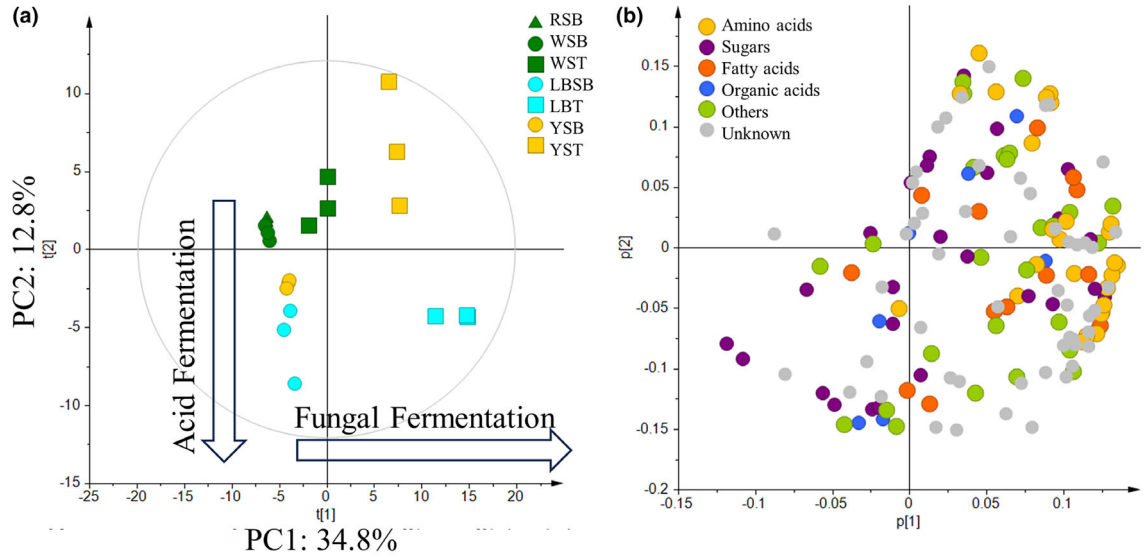

Figure 1 PCA results of soybean, soaked soybean, and tempe through GC–MS analysis. (a) Score plot of soybean, soaked soybean, and tempe. The triangle indicates raw soybean (CB), points indicate soaked soybean (LBSB, LAB-soaked soybean; WSB, water-soaked soybean; and YSB, yeast-soaked soybean), and squares indicate tempe (LBT, LAB-soaked soybean tempe; WST, water-soaked soybean tempe; and YST, yeast-soaked soybean tempe). (b) Loading plot of soybean, soaked soybean, and tempe in GC–MS analysis. Yellow indicates amino acids, purple indicates sugars, orange indicates fatty acids, blue indicates organic acids, green indicates other molecules, and grey indicates unknown molecules.

acids, such as tyramine and cysteine. Tyramine, a biogenic amine, is considered essential for the normal physiological function of the body at a reasonable level while causing adverse effects at high concentrations (Naila *et al*., 2010). Several reports have linked high amounts of tyramine in fermented foods to the action of yeast decarboxylases (Caruso *et al*., 2002; Qi *et al*., 2014). As this compound is correlated with food safety, we conducted quantification using tyramine standard compound. We observed that the concentration of tyramine in YST was 50.56 ± 14.05 mg/100 g food, which exceeds 900 times the concentration in LAB-soaked soybean tempe (Figure S3). While upper limits of tyramine in foods have been suggested to be 100–800 mg kg^{-1} food (ten Brink *et al*., 1990), and over 100 mg may cause migraine (Shalaby, 1996).

Additionally, the PCA results indicated that the data separation of PC2 was based on the acid fermentation process with microbial intervention, explained by 12.8% of the variance (Fig. 1a). Soaking altered the metabolome of soybeans, as evidenced by the negative axis of PC2. From the loading plot of the PCA results (Fig. 1b, Table S1), organic acids such as lactic acid and citric acid accumulated intensely at the end of the soaking process. Microbial intervention changed the microflora during soybean soaking. The changes in the pH of the soaked water for each treatment are shown in Figure S1. Notably, LAB intervention rapidly lowered the two degrees of acidity within 6 h of soaking. The above proposition holds the potential of LAB intervention to curtail the acid fermentation duration for tempe production, given that a pH of approximately 5 is used as the minimum threshold for tempe acid fermentation (Romulo & Surya, 2021). *L. plantarum* intervention, compared with other treatments, dominated the soaking process by producing lactic acid (Coghetto *et al*., 2016), thereby increasing the acidity of the soybeans to assist subsequent fungal fermentation. Another study noted that the introduction of LAB resulted in a significant increase in the acidification of soaked water and beans (Moa *et al*., 2013).

The association of bacteria and yeast with tempe fungal fermentation is consistent with their dominance during the soaking step of tempe production (Mulyowidarso *et al*., 1990). A previous study also observed that cooking does not necessarily eliminate dominant microorganisms (Mulyowidarso *et al*., 1989). LAB, particularly *L. plantarum*, coexist synergistically with *Rhizopus* species (Feng *et al*., 2005). Another study reported that yeast could grow together with a tempe starter (Kustyawati, 2009). Moreover, the presence of these microorganisms during tempe fungal fermentation is in agreement with previous studies (Efriwati *et al*., 2013; Radita *et al*., 2021) that reported LAB and yeast in samples of fully fermented tempe. Presumably, the surviving cells could be carried over to the soybeans and affect the fungal fermentation stage.

Microbial intervention in fermented food not only alters metabolites detected by GC–MS. Combination of diverse spectroscopic platforms is an effective approach for detecting a wider range of metabolites. A recent untargeted metabolomics study of Korean traditional fermented soy food revealed that LC–MS is able to identify glucosides and acetyl glucosides of soy isoflavones (Lee *et al*., 2012). This approach enables the comprehensive monitoring of dynamic changes in soy isoflavone levels throughout different stages of fermentation. Other research on East Asian ethnic fermented soybean products (Kwon *et al*., 2019) also reported that microbial assortments influenced the secondary metabolite contents, detected using LC–MS.

Microbial intervention modifies the metabolite profile of tempe during the soaking step in tempe production. LAB-soaked soybean tempe has been found to enhance the levels of amino acids, such as lysine and leucine. In contrast, YST had been apparently contained increasing levels of biogenic amine, tyramine. The metabolomics approach has uncovered both the benefits and drawbacks of the treatments.

Metabolite profile differences between microbial interventions and chemical addition in Tempe-soaking step

For several tempe manufacturers, chemical addition during the soaking process for tempe production decreases pH, eliminates pathogens, and increases the likelihood of successful fungal fermentation (Nout & Kiers, 2005). The following experiment aimed to compare tempes with microbial intervention and chemical addition during the soaking process using a metabolomic approach. Figure 2A shows the PCA scatter plot, which revealed a total of 55.8% variance among the tempe samples. As we have observed, PC1 separated samples according to microbial intervention and chemical addition. Interestingly, lactic acid-soaked soybean tempe (LAT) was located on the positive axis of PC1 together with water-soaked soybean tempe (WST). Based on the complete list of metabolites (Table S2), we observed that sugars and amino acids were the principal components for separating microbially soaked and chemically soaked soybean tempes. In particular, the metabolites contributing to the separation were from sugar groups, such as pinitol and raffinose, whereas the other metabolites were from amino acids such as lysine and leucine (Fig. 2B). These results are consistent with those of a previous report on the chemical addition of vinegar, which resulted in a lower accumulation of amino acid metabolites (Dahlan *et al*., 2022). Raffinose, a flatulence-contributing antinutrient compound (Gasiński *et al*., 2022), accumulated in both the water and LATs. Although raffinose has adverse effects as an antinutrient, it has been reported to have potential as a prebiotic ingredient for gut microbiota (Amorim *et al*., 2020). LAB was also reported to have the ability to utilise raffinose because of the α-galactosidase activity (Zartl *et al*., 2018), as apparently explained by the slight accumulation of raffinose in the LAB-soaked soybean tempe. Upon employing the raffinose standard compound for quantification shown in Figure S4, it was determined that LAB-soaked soybean tempe effectively reduced the raffinose content from 230.291 ± 20.26 mg/100 g food to 21.094 ± 0.55 mg/100 g food compared with LAT. Previous reports have indicated that ingestion of soy products consisting of 3.1 g raffinose and stachyose in 80 g food resulted in a significant increase in flatus frequency (Suarez *et al*., 1999), and soybean oligosaccharide extract containing raffinose had 50% effective dose for men at 0.88 g/kg body weight to induce abdominal disturbances including diarrhoea (Hata *et al*., 1991). Raffinose reduction by LAB has a positive effect on reducing the risk of flatulence. Lysine, an essential amino acid that is insufficient in most cereal flours (Meybodi *et al*., 2019), is highly accumulated in LBT tempe. Lysine is important in numerous physiological processes, such as protein synthesis, tissue regeneration, and the biosynthesis of hormones, enzymes, and antibodies (Yarlina *et al*., 2023). According to one report, soybean paste undergoes an increase in lysine content through LAB fermentation, predominantly via the conversion of peptides to free amino acids (Ng'ong'ola-Manan*i et al*., 2014). Further studies on these issues could provide interesting insights into improving the nutritional value of tempe and its application in food fortification.

In contrast to tempes made from soybeans soaked with chemical additives, tempes prepared through microbial intervention in soaked soybeans showed reduced accumulation of sugars such as raffinose.

The impact of microbial interventions in Tempe-soaking step in several bioactive metabolites

Differential analysis was employed to further observe the differences in metabolites between the tempe samples. We aimed to investigate the impact of microbial intervention in the soaking process on the tempe metabolome by examining the metabolites that were significantly modulated after tempe fermentation, as illustrated by the volcano plot (Fig. 3). When comparing raw soybean and LAB-soaked soybean tempe, 80 metabolites showed a fold change of two or more, with significant differences based on a *t*-test with $P < 0.05$; Fig. 3a. A complete list of the significantly modulated metabolites is presented in Table S3. Seven bioactive metabolites were identified, namely, meglutol, daidzein, genistein, 4-aminobutyric acid, 3-phenyllactic acid, and 2-hydroxypyridine were among this group. Some of

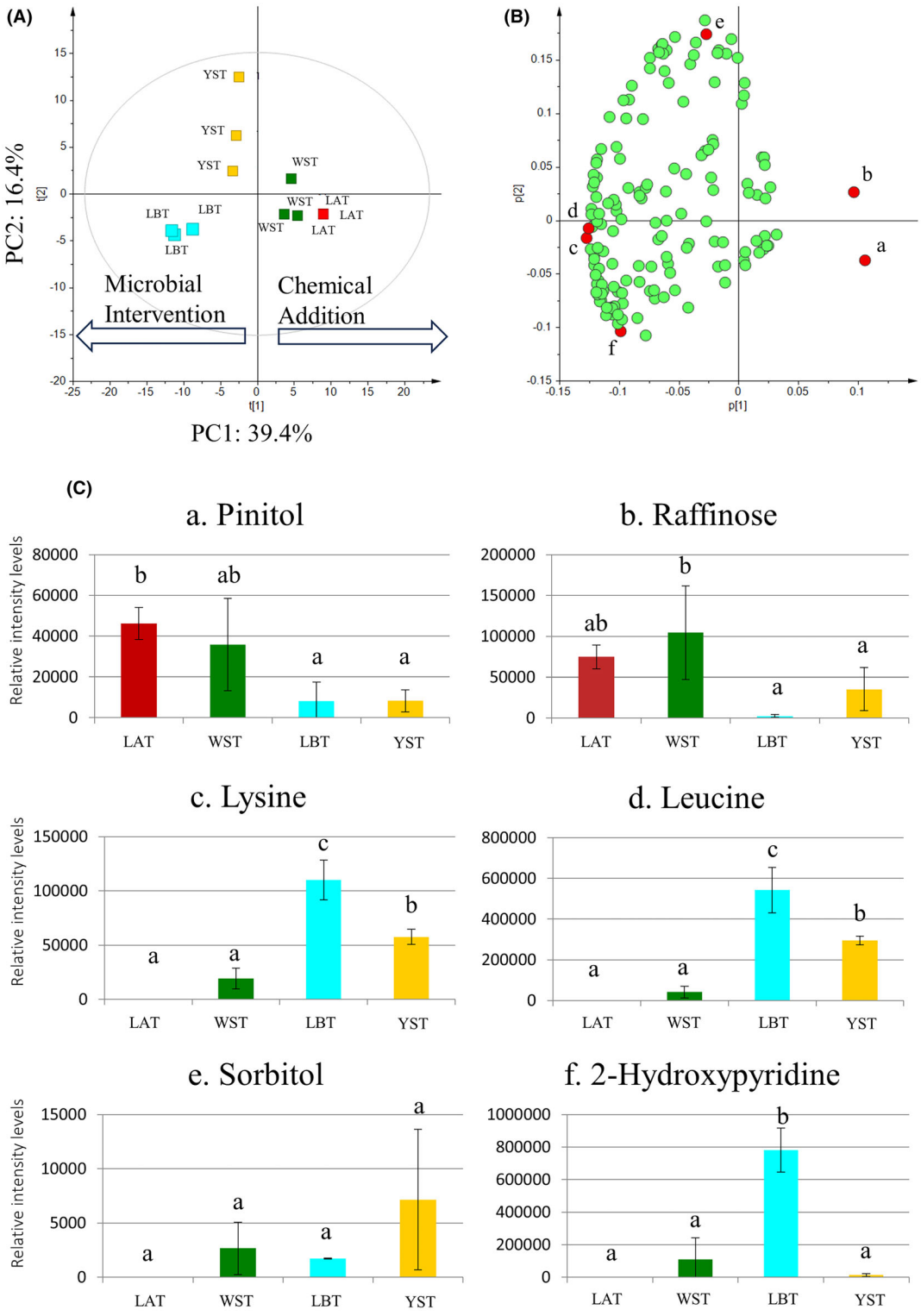

Figure 2 PCA results of different tempes through GC–MS analysis. (A) Score plot of different tempes. Squares indicate tempe (LAT, acid-soaked soybean tempe; LBT, LAB-soaked soybean tempe; WST, water-soaked soybean tempe; and YST, yeast-soaked soybean tempe). (B) Loading plot of different tempes in GC–MS analysis. Metabolites contributing to PC1 are lettered as follows: (a) pinitol, (b) raffinose, (c) lysine, and (d) leusine, and metabolites contributing to PC2 are lettered as follows: (e) sorbitol and (f) 2-hydroxypyridine. (C) Bar graph of distinct metabolites lettered in the loading plot. The vertical axis indicates relative intensity, and a horizontal axis indicates samples. The error bar shows the SD from three biological replicates. Groups labelled with different letters are significantly different, as indicated using Tukey's adjustment ($P < 0.05$).

these bioactive metabolites have been reported to be elevated after tempe fermentation in previous reports (Iman *et al.*, 2023). The LAB group has been reported to have the ability to transform plant isoflavones such as daidzin and genistin by deglycosylation into their bioactive forms, daidzein and genistein (Gaya *et al.*, 2017). In addition, other significantly modulated bioactive metabolites can be biosynthesized by numerous LABs, especially those isolated from fermented food (Jung *et al.*, 2019; Nie *et al.*, 2022; Pannerchelvan *et al.*, 2023). Interestingly, nicotinic acid, also known as vitamin B3, was significantly elevated during tempe fermentation of soybeans soaked in LAB. Previous research has demonstrated that both *Rhizopus* and bacteria are important for the formation of vitamins in tempe (Keuth & Bisping, 1993). The formation of nicotinic acid in tempe is linked to LAB activity during soaking (Denter & Bisping, 1994). This metabolite is associated with blood cholesterol level stabilisation (Bodor & Offermanns, 2008). In contrast, raffinose and pinitol levels significantly decreased by >2-fold after tempe fermentation in LAB-soaked soybean tempe, presumably because of their utilisation by LAB (Zartl *et al.*, 2018).

Following raw soybean and YST, 66 metabolites exhibited $P < 0.05$, based on the *t*-test, with a fold change of >2 (Fig. 3b). Table S4 presents a complete list of significantly modulated metabolites. The bioactive metabolites include meglutol, daidzein, genistein, 4-aminobutyric acid, and 3-phenyllactic acid. However, the bioactive metabolite 3-hydroxyanthranilic acid was significantly decreased by >2-fold after tempe fermentation in YST.

Yeasts are often present in fermented foods and play various roles in fermentation. Conventionally, yeast contributes to interactions between microorganisms, alteration in texture, and biosynthesis of flavour compounds. Because most fermentations depend on the conversion of sugars to lactic acid, LAB plays a crucial role, whereas yeasts are secondary actors (Tofalo *et al.*, 2020). *Pichia* sp. has desirable roles in olive fermentation through its enzymatic activities, such as lipase, esterase, β-glucosidase, and catalase (Arroyo-López *et al.*, 2012).

Microbial intervention during the tempe-soaking process of tempe had been found to significantly enhance the relative levels of bioactive metabolites. These compounds include meglutol, daidzein, genistein, 4-aminobutyric acid, and nicotinic acid. This highlights the potential to improve the health-promoting properties of tempe through controlled microbial processes.

The effect of soaking treatments on physical characteristics of Tempe

Colour and texture analyses were employed to compare the effects of different soaking treatments on the physical characteristics of the tempe samples. The physical characteristics of all tempe samples, listed in Table 3, showed that all tempe samples had different colours and textures, but were not significantly different from each other, except for the hardness and colour of the LAT.

The cohesiveness value indicates the ability of the product to bond and resist deformation. The compactness of tempe is characterised by its cohesiveness. As cohesiveness increased, the structural compactness of the tempe increased proportionally. The cohesiveness of tempe is affected by the ability of fungal mycelia to penetrate the seed (Wikandari *et al.*, 2020). Table 3 shows that LAB-soaked soybean tempe had the highest compactness. Springiness is defined as the speed at which a compressed material returns to its original state. A value of 1 indicates elastic behaviour, and 0 indicates viscous behaviour (Wikandari *et al.*, 2020). The highest springiness was observed for WST.

The evaluated colour was represented by L^*, a^*, and b^* values, where L^* indicates brightness, a^* corresponds to the green-red axis, and b^* indicates the blue-yellow axis. The final fermentation temperatures are shown in Figure S2. The results showed that the LAT had the highest L^* value, indicating that it appeared brighter than the others. When comparing the microbial intervention with the control tempe, the LAB-soaked soybean tempe exhibited a greater degree of brightness. Additionally, neither tempe nor microbial interventions in the soaking step significantly differed in terms of colour. The tempe colour intensity was within the range of colour coordinates reported in other studies on tempes (Handoyo & Morita, 2006; Wikandari *et al.*, 2020).

The textural characteristics of tempe, including cohesiveness, springiness, and hardness were found to differ as a result of microbial intervention during the soaking step. These differences also extend to the colour of the tempe.

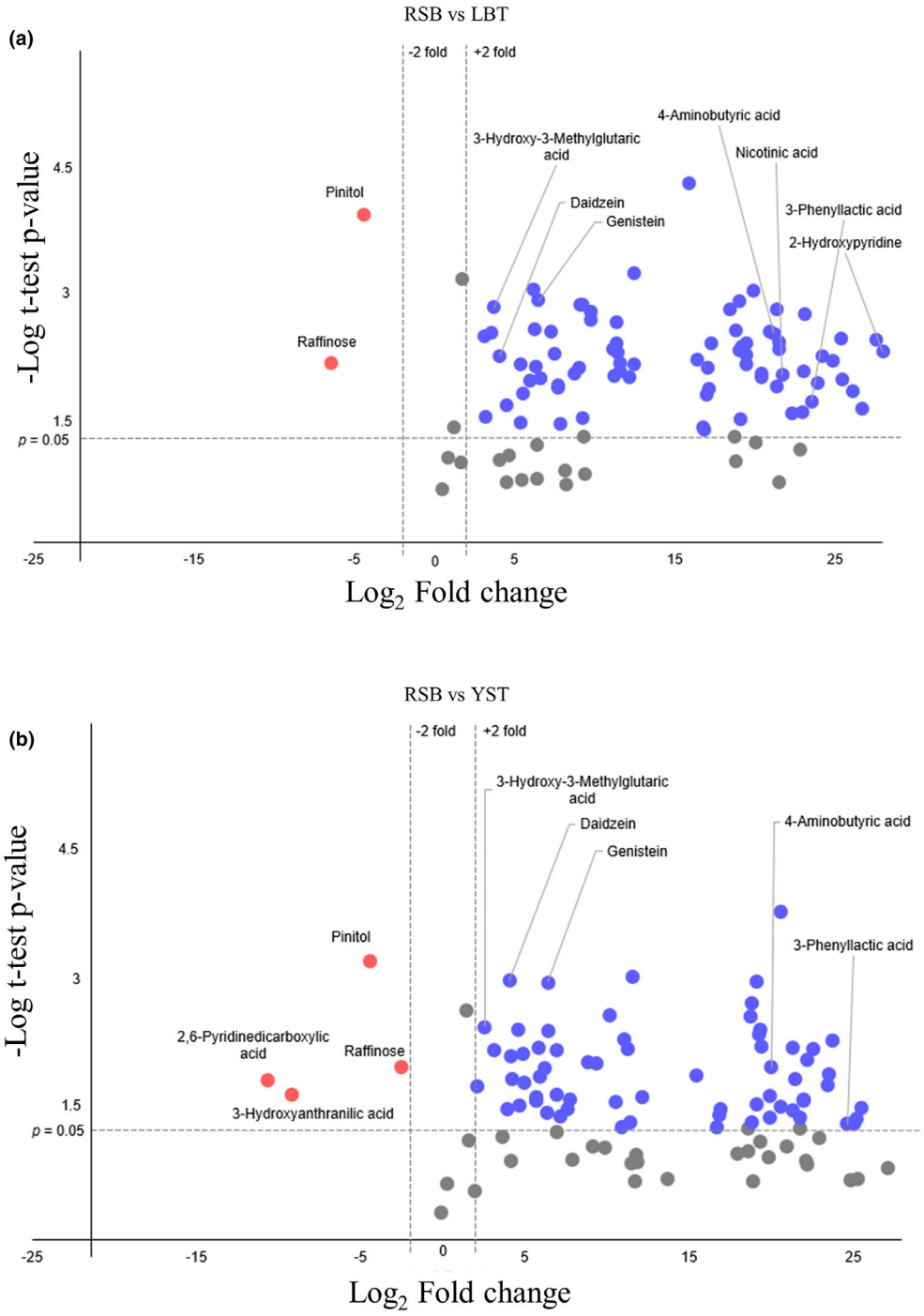

Figure 3 Significantly modulated metabolites identified the following raw soybeans: (a) LAB-soaked soybean tempe and (b) yeast-soaked tempe fermentation. Blue circles indicate metabolites significantly increased ($P < 0.05$) with at least a twofold change, red circles indicate metabolites significantly decreased ($P < 0.05$) with at least a twofold change, and grey circles indicate metabolites with a non-significant change ($P \geq 0.05$) and/or metabolites with <twofold change.

Table 3 Physical characteristics of tempe samples

Samples	Colour			Texture		
	*L**	*a**	*b**	Cohesiveness	Springiness	Hardness (N)
WST	59.7 ± 9.12[a]	1.49 ± 1.27[a]	8.6 ± 2.96[a]	0.545 ± 0.07[a]	0.724 ± 0.14[a]	19.993 ± 7.04[ab]
LAT	70.14 ± 5.97[a]	3.67 ± 1.95[a]	20.54 ± 5.26[b]	0.536 ± 0.03[a]	0.551 ± 0.08[a]	25.732 ± 10.47[b]
LBT	68.62 ± 13.06[a]	1.97 ± 2.25[a]	14.3 ± 5.78[ab]	0.672 ± 0.06[a]	0.715 ± 0.11[a]	14.318 ± 4.74[ab]
YST	67.10 ± 8.97[a]	2.44 ± 1.92[a]	17.48 ± 5.76[ab]	0.540 ± 0.02[a]	0.634 ± 0.07[a]	4.768 ± 1.60[a]

Different superscripts indicate significant differences ($P < 0.05$) using Kruskal–Wallis test followed by Dunn's test.

Conclusion

The incorporation of microbial intervention into the process of tempe-soaking has been found to alter the metabolite profile of tempe, specifically amino acids (lysine and leucine) in LAB-soaked soybean tempe and tyramine in YST, demonstrating both the benefits and drawbacks of the treatments. These alterations in metabolites are likely attributed to the activity of the microorganisms employed, for instance, the hydrolysis of soy protein by LAB (Sirilun *et al.*, 2017) and the activity of yeast decarboxylase (Qi *et al.*, 2014). Compared with chemically soaked soybean tempe, microbial intervention with soaked soybean tempe resulted in a lower accumulation of sugars such as raffinose. Furthermore, the differential analysis showed that microbial intervention in the soaking step of tempe significantly modulated bioactive metabolites, such as daidzein, genistein, nicotinic acid, and meglutol. The introduction of microbial interventions during the soaking step of tempe also influences its physical attributes at the final fermentation stage. Overall, this study offers valuable insights that can be utilised by tempe manufacturers to facilitate further product development in the food industry. The present study has certain limitations as it only pertains to the relative abundance of compounds that can be detected by GC–MS. We recommend conducting further research on the absolute abundance of target compounds of interest and also the expansion of metabolite classes through different platforms to better understand the functional effects of microbial interventions on tempe.

Acknowledgments

The authors sincerely thank the Education Financing Service Centre (PUSLAPDIK) through the Higher Education Funding Centre (BPPT), Indonesian Ministry of Education and Culture, and Indonesia Endowment Fund for Education (LPDP) for sponsoring this research. This study represents a portion of a dissertation submitted by Rifqi Ahmad Riyanto to Osaka University in partial fulfilment of the requirements for his doctoral studies.

Author contributions

Rifqi Ahmad Riyanto: Writing – original draft; writing – review and editing; visualization; methodology; investigation; conceptualization. **Eiichiro Fukusaki:** Writing – review and editing; supervision; resources; methodology; conceptualization. **Sastia Prama Putri:** Writing – review and editing; writing – original draft; supervision; resources; methodology; conceptualization.

Conflict of interest statement

None of the authors have a conflict of interest to disclose.

Ethical guidelines

Ethics approval was not required for this research.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ijfs.17481.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Aguirre, L., Garro, M.S. & Savoy de Giori, G. (2008). Enzymatic hydrolysis of soybean protein using lactic acid bacteria. *Food Chemistry*, **111**, 976–982.

Ahnan-Winarno, A.D., Cordeiro, L., Winarno, F.G., Gibbons, J. & Xiao, H. (2021). Tempeh: a semicentennial review on its health benefits, fermentation, safety, processing, sustainability, and affordability. *Comprehensive Reviews in Food Science and Food Safety*, **20**, 1717–1767.

The review provides updated information and research on Tempe, which is crucial for understanding the current knowledge of Tempe and its future perspectives. This review provided an important summary for our tempe research and further readings.

Amorim, C., Silvério, S.C., Cardoso, B.B., Alves, J.I., Pereira, M.A. & Rodrigues, L.R. (2020). In vitro fermentation of raffinose to unravel its potential as prebiotic ingredient. *LWT*, **126**, 109322.

Arroyo-López, F.N., Romero-Gil, V., Bautista-Gallego, J. *et al.* (2012). Yeasts in table olive processing: desirable or spoilage microorganisms? *International Journal of Food Microbiology*, **160**, 42–49.

Badan Pusat Statistik. (2022). *Statistik Indonesia 2022*. Available from: https://www.bps.go.id/id/publication/2022/02/25/0a2afea4fab72a5d052cb315/statistik-indonesia-2022.html [Accessed 17 April 2024].

Bodor, E.T. & Offermanns, S. (2008). Nicotinic acid: an old drug with a promising future. *British Journal of Pharmacology*, **153**, 68–75.

ten Brink, B., Damink, C., Joosten, H.M.L.J. & In't Veld, J.H. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology*, **11**, 73–84.

Caruso, M., Fiore, C., Contursi, M., Salzano, G., Paparella, A. & Romano, P. (2002). Formation of biogenic amines as criteria for the selection of wine yeasts. *World Journal of Microbiology and Biotechnology*, **18**, 159–163.

Coghetto, C.C., Vasconcelos, C.B., Brinques, G.B. & Ayub, M.A.Z. (2016). *Lactobacillus plantarum* BL011 cultivation in industrial isolated soybean protein acid residue. *Brazilian Journal of Microbiology*, **47**, 941–948.

Dahlan, H.A., Nambu, Y., Putri, S.P. & Fukusaki, E. (2022). Effects of soaking Tempe in vinegar on metabolome and sensory profiles. *Metabolites*, **12**, 30.

The paper describes the significant effect of using chemicals to soak tempe. The methods and knowledge provide fundamental aspects of the study on tempe soaking treatments. We applied the similar methodology with modifications to analyze the tempe sample based on this report.

Denter, J. & Bisping, B. (1994). Formation of B-vitamins by bacteria during the soaking process of soybeans for tempe fermentation. *International Journal of Food Microbiology*, **22**, 23–31.

Drulyte, D. & Orlien, V. (2019). The effect of processing on digestion of legume protein. *Food*, **8**, 1–9.

Efriwati, S., Rahayu, G.A. & Nuraida, L. (2013). Population dynamics of yeasts and lactic acid bacteria (LAB) during tempeh production. *HAYATI Journal of Biosciences*, **20**, 57–64.

Feng, X.M., Eriksson, A.R.B. & Schnürer, J. (2005). Growth of lactic acid bacteria and *Rhizopus oligosporus* during barley tempeh fermentation. *International Journal of Food Microbiology*, **104**, 249–256.

Galimberti, A., Bruno, A., Agostinetto, G., Casiraghi, M., Guzzetti, L. & Labra, M. (2021). Fermented food products in the era of globalization: tradition meets biotechnology innovations. *Current Opinion in Biotechnology*, **70**, 36–41.

Gasiński, A., Kawa-Rygielska, J., Mikulski, D. & Kłosowski, G. (2022). Changes in the raffinose family oligosaccharides content in the lentil and common bean seeds during malting and mashing processes. *Scientific Reports*, **12**, 1–8.

Gaya, P., Peirotén, Á. & Landete, J.M. (2017). Transformation of plant isoflavones into bioactive isoflavones by lactic acid bacteria and bifidobacteria. *Journal of Functional Foods*, **39**, 198–205.

Handoyo, T. & Morita, N. (2006). Structural and functional properties of fermented soybean (tempeh) by using *Rhizopus oligosporus*. *International Journal of Food Properties*, **9**, 347–355.

Hata, Y., Yamamoto, M. & Nakajima, K. (1991). Effects of soybean oligosaccharides on human digestive organs: estimation of fifty percent effective dose and maximum non-effective dose based on diarrhea. *Journal of Clinical Biochemistry and Nutrition*, **10**, 135–144.

Iman, M.N., Irdiani, R., Rahmawati, D., Fukusaki, E. & Putri, S.P. (2023). Improvement of the functional value of green soybean (edamame) using germination and tempe fermentation: a comparative metabolomics study. *Journal of Bioscience and Bioengineering*, **136**, 205–212.

Jung, S., Hwang, H. & Lee, J.H. (2019). Effect of lactic acid bacteria on phenyllactic acid production in kimchi. *Food Control*, **106**, 106701.

Kadar, A.D., Aditiawati, P., Astawan, M., Putri, S.P. & Fukusaki, E. (2018). Gas chromatography coupled with mass spectrometry-based metabolomics for the classification of tempe from different regions and production processes in Indonesia. *Journal of Bioscience and Bioengineering*, **126**, 411–416.

Keuth, S. & Bisping, B. (1993). Formation of vitamins by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation. *Journal of Applied Bacteriology*, **75**, 427–434.

Kustyawati, M.E. (2009). Kajian Peran Yeast dalam Pembuatan Tempe. *Agritech: Jurnal Fakultas Teknologi Pertanian UGM*, **29**, 64–70.

Kwon, Y.S., Lee, S., Lee, S.H., Kim, H.J. & Lee, C.H. (2019). Comparative evaluation of six traditional fermented soybean products in east asia: a metabolomics approach. *Metabolites*, **9**, 1–14.

Lai, Z., Tsugawa, H., Wohlgemuth, G. *et al.* (2018). Identifying metabolites by integrating metabolome databases with mass spectrometry cheminformatics. *Nature Methods*, **15**, 53–56.

Lee, S.Y., Kim, H.Y., Lee, S. *et al.* (2012). Mass spectrometry-based metabolite profiling and bacterial diversity characterization of Korean traditional meju during fermentation. *Journal of Microbiology and Biotechnology*, **22**, 1523–1531.

Magdalena, S., Hogaputri, J.E., Yulandi, A. & Yogiara, Y. (2022). The addition of lactic acid bacteria in the soybean soaking process of tempeh. *Food Research*, **6**, 27–33.

The paper utilised microbial intervention during the soaking process by using LAB, resulting in tempe that complies with the Indonesian standard. This information is important because it proves that variations in microbial treatment are able to produce standard tempe.

Marazza, J.A., Nazareno, M.A., de Giori, G.S. & Garro, M.S. (2012). Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. *Journal of Functional Foods*, **4**, 594–601.

Meybodi, N.M., Mortazavian, A.M., Mirmoghtadaie, L. *et al.* (2019). Effects of microbial transglutaminase and fermentation type on improvement of lysine availability in wheat bread: a response surface methodology. *Applied Food Biotechnology*, **6**, 151–164.

Moa, H., Kariluoto, S., Piironen, V. *et al.* (2013). Effect of soybean processing on content and bioaccessibility of folate, vitamin B12 and isoflavones in tofu and tempe. *Food Chemistry*, **141**, 2418–2425.

Mulyowidarso, R.K., Fleet, G.H. & Buckle, K.A. (1989). The microbial ecology of soybean soaking for tempe production. *International Journal of Food Microbiology*, **8**, 35–46.

Mulyowidarso, R.K., Fleet, G.H. & Buckle, K.A. (1990). Association of bacteria with the fungal fermentation of soybean tempe. *Journal of Applied Bacteriology*, **68**, 43–47.

Mulyowidarso, R.K., Fleet, G.H. & Buckle, K.A. (1991a). Changes in the concentration of organic acids during the soaking of soybeans for tempe production. *International Journal of Food Science & Technology*, **26**, 607–614.

This paper is crucial to the research on tempe soaking as it demonstrates that various known microorganisms in soybean soaking can impact the tempe product. This research is one of the foundational reasons for our selection of the microbial strains employed.

Mulyowidarso, R.K., Fleet, G.H. & Buckle, K.A. (1991b). Changes in the concentration of carbohydrates during the soaking of soybeans for tempe production. *International Journal of Food Science & Technology*, **26**, 595–606.

Naila, A., Flint, S., Fletcher, G., Bremer, P. & Meerdink, G. (2010). Control of biogenic amines in food - existing and emerging approaches. *Journal of Food Science*, **75**, R139–R150.

Ng'ong'ola-Manani, T.A., Østlie, H.M., Mwangwela, A.M. & Wicklund, T. (2014). Metabolite changes during natural and lactic acid bacteria fermentations in pastes of soybeans and soybean–maize blends. *Food Science & Nutrition*, **2**, 768–785.

Nie, X., Chen, H., Xiang, L., Zhang, Y., Liu, D. & Zhao, Z. (2022). GC-TOF-MS-based non-targeted Metabolomic analysis of differential metabolites in Chinese ultra-long-term industrially fermented kohlrabi and their associated metabolic pathways. *Metabolites*, **12**, 991.

Nout, M.J.R. & Kiers, J.L. (2005). Tempe fermentation, innovation and functionality: update into the third millenium. *Journal of Applied Microbiology*, **98**, 789–805.The manuscript highlights a critical issue in tempe soaking: the failure of soaking due to uncontrolled microflora and the failure of acidification because of climatic conditions, especially in non-tropical areas. This report is important to my research as it strengthens why the intervention in tempe soaking step is needed.

Nout, M.J.R., de Dreu, M.A., Zuurbier, A.M. & Bonants-van Laarhoven, T.M.G. (1987). Ecology of controlled soyabean acidification for tempe manufacture. *Food Microbiology*, **4**, 165–172.

Pannerchelvan, S., Rios-Solis, L., Faizal Wong, F.W. *et al.* (2023). Strategies for improvement of gamma-aminobutyric acid (GABA) biosynthesis via lactic acid bacteria (LAB) fermentation. *Food & Function*, **14**, 3929–3948.

Prativi, M.B.N., Astuti, D.I., Putri, S.P., Laviña, W.A., Fukusaki, E. & Aditiawati, P. (2023). Metabolite changes in Indonesian Tempe production from raw soybeans to over-fermented Tempe. *Metabolites*, **13**, 1–16.

Putri, S.P., Irifune, T. & Yusianto & Fukusaki, E. (2019). GC/MS based metabolite profiling of Indonesian specialty coffee from different species and geographical origin. *Metabolomics*, **15**, 1–11.

Putri, S.P., Ikram, M.M.M., Sato, A. *et al.* (2022). Application of gas chromatography-mass spectrometry-based metabolomics in food science and technology. *Journal of Bioscience and Bioengineering*, **133**, 425–435.

Qi, W., Hou, L.H., Guo, H.L. *et al.* (2014). Effect of salt-tolerant yeast of Candida versatilis and *Zygosaccharomyces rouxii* on the production of biogenic amines during soy sauce fermentation. *Journal of the Science of Food and Agriculture*, **94**, 1537–1542.

Radita, R., Suwanto, A., Kurosawa, N., Wahyudi, A.T. & Rusmana, I. (2021). Dynamics of microbial community during tempeh fermentation. *Biotropia*, **28**, 11–20.

Rahayu, W.P., Pambayun, R., Santoso, U., Nuraida, L. & Ardiansyah, A. (2015). *Tinjauan ilmiah teknologi pengolahan tempe kedelai*. Jakarta: Perhimpunan Ahli Teknologi Pangan Indonesia (PATPI).

Rahmawati, D., Astawan, M., Putri, S.P. & Fukusaki, E. (2021). Gas chromatography-mass spectrometry-based metabolite profiling and sensory profile of Indonesian fermented food (tempe) from various legumes. *Journal of Bioscience and Bioengineering*, **132**, 487–495.

Romulo, A. & Surya, R. (2021). Tempe: A traditional fermented food of Indonesia and its health benefits. *International Journal of Gastronomy and Food Science*, **26**, 100413.

Shalaby, A.R. (1996). Significance of biogenic amines to food safety and human health. *Food Research International*, **29**, 675–690.

Sirilun, S., Sivamaruthi, B.S., Kesika, P., Peerajan, S. & Chaiyasut, C. (2017). Lactic acid bacteria mediated fermented soybean as a potent nutraceutical candidate. *Asian Pacific Journal of Tropical Biomedicine*, **7**, 930–936.

Suarez, F.L., Springfield, J., Furne, J.K., Lohrmann, T.T., Kerr, P.S. & Levitt, M.D. (1999). Gas production in humans ingesting a soybean flour derived from beans naturally low in oligosaccharides. *American Journal of Clinical Nutrition*, **69**, 135–139.

Tofalo, R., Fusco, V., Böhnlein, C. *et al.* (2020). The life and times of yeasts in traditional food fermentations. *Critical Reviews in Food Science and Nutrition*, **60**, 3103–3132.

Wikandari, R., Utami, T.A.N. & Hasniah, N.S. (2020). Chemical, nutritional, physical and sensory characterization of Tempe made from various underutilized legumes. *Pakistan Journal of Nutrition*, **19**, 179–190.

Witono, Y., Widjanarko, S.B., Mujianto & Rachmawati, D.T. (2015). Amino acids identification of over fermented tempeh, the hydrolysate and the seasoning product hydrolysed by calotropin from crown flower (*Calotropis gigantea*). *International Journal on Advanced Science, Engineering and Information Technology*, **5**, 103–106.

Yarlina, V.P., Djali, M., Andoyo, R., Lani, M.N. & Rifqi, M. (2023). Effect of soaking and proteolytic microorganisms growth on the protein and amino acid content of Jack bean tempeh (*Canavalia ensiformis*). *PRO*, **11**, 1–14.

Zartl, B., Silberbauer, K., Loeppert, R., Viernstein, H., Praznik, W. & Mueller, M. (2018). Fermentation of non-digestible raffinose family oligosaccharides and galactomannans by probiotics. *Food & Function*, **9**, 1638–1646.

Zhang, L., Hu, Y., Wang, X. *et al.* (2021). Improving soaking efficiency of soybeans through sweeping frequency ultrasound assisted by parameters optimization. *Ultrasonics Sonochemistry*, **79**, 105794.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Changes in pH during soybean soaking. WSB, water-soaked soybean; LASB, acid-soaked soybean; LBSB, LAB-soaked soybean; and YSB, yeast-soaked soybean.

Figure S2. Tempe at final fermentation. WST, water-soaked soybean tempe; LAT, acid-soaked soybean tempe; LBT, LAB-soaked soybean tempe; and YST, yeast-soaked soybean tempe.

Figure S3. Concentration of tyramine in lactic acid bacteria-soaked soybean tempe (LBT) and yeast-soaked soybean tempe (YST) samples. Quantification of tyramine concentration was performed using GC–MS with a tyramine standard compound.

Figure S4. Concentration of raffinose in acid-soaked soybean tempe (LAT) and lactic acid bacteria-soaked soybean tempe (LBT) samples. Quantification of raffinose concentration was performed using GC–MS with a raffinose standard compound.

Table S1. Loading Score of PCA results of soybean, soaked soybean, and tempe based on PC1 and PC2.

Table S2. Loading score of PCA results of different tempes based on PC1 and PC2.

Table S3. List of metabolites in volcano plot comparing raw soybean and LAB-soaked soybean tempe, which differed significantly based on the *t*-test ($P < 0.05$).

Table S4. List of metabolites in volcano plot comparing raw soybean and yeast-soaked soybean tempe, which differed significantly based on the *t*-test ($P < 0.05$).

Table S5. Annotation of metabolites in soybean tempe extracts by GC–MS metabolomics, followed by the t_R, RI, quant mass, similarity score, and library. [a]Retention time in minute(s). [b]Retention indices (RI) are calculated using a standard alkane mixture (C9–C40). [c]In-house library available online as Osaka Univ library (Hydrogen carrier gas, InertCap 5MS Metabolomics, Kovats RI) in this link. https://zenodo.org/records/11649994/files/GCMS_H2_Library.msp?download=1