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Doctoral Dissertation

Metabolomics-based Study for Investigating the Effect of Microbial Interventions in Tempe Soaking Step

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List of Abbreviations

ANOVA	Analysis of variance
LAB	Lactic acid bacteria
EI	Electron ionization
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
MS	Mass spectrometry
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
PC	Principal component
PCA	Principal component analysis
QC	Quality control
SD	Standard deviations

Chapter 1

General Introduction

1.1 Tempe

Tempe is the solid-state fermentation of legumes, mainly soybean, assisted by *Rhizopus* spp. mold (Astuti et al., 2000). During the fermentation process, the mold develops mycelium that encases the soybeans. This process is considered complete when the mycelium achieves a firm texture and fully surrounds the soybeans (Tamang et al., 2022). Tempe is a high-protein, plant-based food characterized by its firm texture, which results from the mycelium formation. It is an Indonesian staple source of protein known for its functional benefits, affordability, and sustainability (Ahnan-Winarno et al., 2021).

Tempe is a commonly consumed food in Indonesia, enjoyed in various recipes. According to data from the Indonesian Central Bureau of Statistics, the average per capita consumption of tempe has shown a positive trend from 2019 to 2023, reaching 106 grams per week per person in 2023 (Fig. 1.1). This figure is significantly higher than the average per capita consumption of meat in Indonesia, which is only 10 grams per week. This indicates a strong demand for tempe in the country. Indonesia is home to over 100,000 tempe producers, located across all provinces of the country (Rahayu et al., 2015).

Over time, tempe has gained popularity among diverse groups of individuals across various regions of the world, particularly in Western countries. Globally, Indonesia exported 533.87 tonnes of tempe in 2022, with a total value of USD 1.62 million (Badan

Pusat Statistik, 2022). The rise in tempe export volume aligns with growing global consumer demand for health-promoting fermented foods. (Shah et al., 2023).

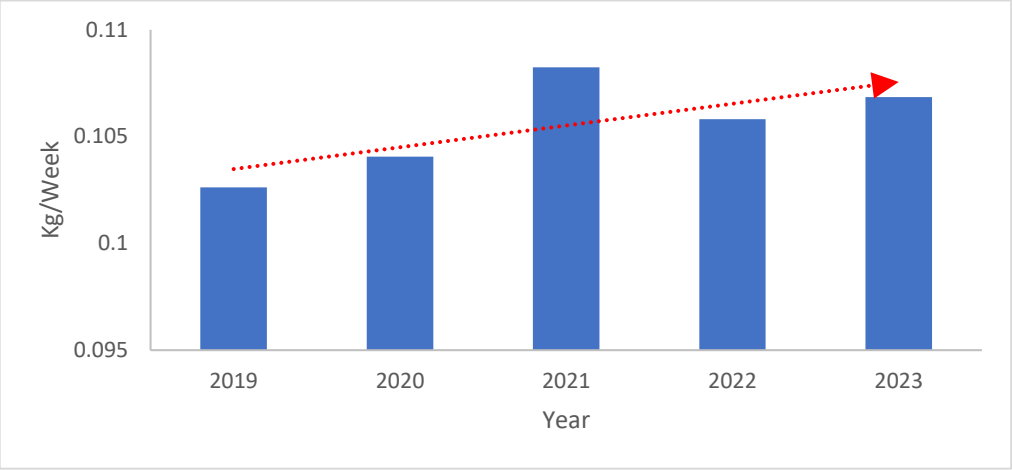


Figure 1.1 Average per capita consumption of tempe in Indonesia (Data from Indonesia Central Bureau of Statistics)

1.2 Production step of tempe

The methods used in tempe production can vary significantly by region and among individual producers, each employing distinct techniques that contribute to the uniqueness of the product (Kadar et al., 2018). The production process generally involves several key steps, beginning with the soaking of soybeans to adequately hydrate them. This initial phase is essential for preparing the beans for subsequent processing. Following the soaking, the soybeans undergo dehulling to remove their outer skins, which enhances both the texture and flavor of the final product. After dehulling, the beans are cooked to ensure they reach the proper tenderness, facilitating easier fermentation. The next step involves inoculating the cooked beans with starter culture. Several molds are utilized in the manufacturing process of tempe in Indonesia including *Rhizopus oligosporus, R. oryzae, R. arrhizus, R. stolonifer, R. microsporus, R. rhizopodiformis, R. chinensis*, and *Mucor* sp. (Tamam et al.,

2019). Once inoculated, the mixture is packaged and allowed to ferment, enabling the fungi to proliferate and transform the soybeans into tempe. This process not only highlights the craftsmanship involved in tempe production but also reflects the rich cultural heritage associated with this traditional food (Rahayu et al., 2015).

Differences in tempe production primarily arise during the soaking and cooking stages. Soaking is a vital process in the production of tempe, as it serves multiple key purposes. First, it hydrates the soybeans, enabling them to swell and soften in preparation for fermentation (Drulyte and Orlien, 2019). Additionally, soaking helps eliminate anti-nutritional factors that can inhibit nutrient absorption (Romulo and Surya, 2021). Examples of reduced antinutrients in soybeans include tannin, phytic acid, and raffinose, which are reduced due to leaching into the soaking water (Kumari et al., 2015) as well as the activity of enzymes from microorganisms during soaking (Zhang et al., 2017). This process also enhances protein bioavailability, making the nutrients more accessible to the body. Soaking is a vital process in mitigating the growth of harmful microorganisms. It fosters an environment conducive to the proliferation of beneficial microorganisms, which effectively inhibit harmful ones through mechanisms such as competitive exclusion and the production of antimicrobial compounds (Lambo et al., 2024), ensuring a safer fermentation environment (Zhang et al., 2021). Finally, it provides a supportive environment for the growth of the tempeh starter culture, which is essential for the fermentation process (Yarlina et al., 2023).

1.3 Modification in tempe soaking step

The soaking process plays a crucial role in the preparation of soybeans, as it involves microbial fermentation that significantly lowers the pH levels of both the soak water and

the soybeans themselves (Romulo and Surya, 2021). This reduction in pH is essential for promoting the development of beneficial microorganisms. However, the success of acid fermentation can be influenced by various factors such as climate and processing techniques. In temperate regions, the conditions may not be favorable for natural acid fermentation to occur, which can hinder the overall effectiveness of the soaking process (Nout and 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. Lactic or acetic acid may be incorporated during hydration to control microbial spoilage, although acetic acid was demonstrated to have a strong inhibitory effect on the fungal growth (de Reu et al., 1995). Several tempe manufacturers prefer fermentative soaking using lactic acid bacteria, in order to improve the microbiological composition of the final product (Nout and Kiers, 2005). In Canada, the government authority also published the safety practice of producing tempe by adding lactic acid bacteria, specifically *Lactobacillus plantarum* in the soaking step (Nelson et al., 2023).

L. plantarum and *Pichia burtonii* are the dominant microbial species found in the soaking water used for tempe production (Nout et al., 1987). These microorganisms play a crucial role during the soaking process by not only accelerating the acidification of the soybeans but also transforming their carbohydrate and organic acid profiles (Mulyowidarso et al., 1991a, 1991b). The addition of soybeans with *L. plantarum* during this initial soaking stage has been shown to result in tempe that meets the Indonesian national standards for desirable qualities such as texture, aroma, and color (Magdalena et al., 2022).

1.4 Food metabolomics

Metabolomics is a powerful analytical technique that involves the comprehensive profiling of metabolites, the small molecules produced during metabolic processes. This technique is increasingly utilized across various fields, particularly in food science (Putri et al., 2022). By employing metabolomics, researchers can significantly enhance their ability to identify both anticipated and unanticipated metabolites, which yields critical insights into the intricate food metabolome.

To provide a comprehensive understanding of low molecular weight hydrophilic compounds present in food products, gas chromatography-mass spectrometry (GC-MS) is employed as the analytical method of choice. This technique is widely esteemed for its robustness, stability, and cost-effectiveness. GC-MS facilitates the rapid characterization and differentiation of small hydrophilic compounds found in food matrices, thereby enhancing our ability to analyze complex food compositions (Putri et al., 2019), providing a detailed understanding of their chemical composition.

Several previous studies focusing on tempe metabolomics (Dahlan et al., 2022; Kadar et al., 2018; Prativi et al., 2023; Rahmawati et al., 2021) have proven invaluable. Previous research highlighted the differences in the tempe metabolome across various tempe manufacturers, explaining how distinct production processes influence the tempe metabolome (Kadar et al., 2018). Another study explained the metabolite profile at each step of tempe processing, highlighting the changes involved in each stage (Prativi et al., 2023). Metabolomics research involving the soaking step of tempeh has shown that adding vinegar results in a lower accumulation of nutritional metabolites. (Dahlan et al., 2022). These investigations not only summarize extensive datasets but also reveal treatment-

related trends and spotlight important metabolites across various experimental conditions. While metabolomics study of microbial interventions in tempe soaking is still underexplored. Such insights pave the way for deeper inquiries into how specific factors influence the metabolomic landscape.

1.5 Objective and strategy

By utilizing metabolomics approach, the present study aims to comprehensively investigate the effects of microbial interventions in soaking step of tempe, thereby contributing to improved understanding and potential enhancements in tempe processing and quality.

1.6 Dissertation outline

This dissertation is divided into four chapters. Chapter 1 serves as a general introduction, covering explanation about tempe, its production step, soaking modification in tempe production, and the application of food metabolomics for tempe research. It emphasizes the existing research gap that food metabolomics is able to fill in terms of microbial intervention in the tempe soaking step.

In Chapter 2, the effects of various microbial interventions utilizing lactic acid bacteria and yeast in soaking step of tempe were comprehensively investigated using metabolomics approach. Comparison between microbial interventions and chemical addition in tempe soaking step also comprehensively investigated using metabolomics approach as well. This chapter elucidates the alterations observed in the tempe metabolome as a result of these interventions during the soaking step.

Building on the findings presented in Chapter 2, Chapter 3 addresses the alterations of biochemical compounds in tempe through the intervention of different lactic acid bacteria species and inoculum sizes during the soaking process. The results show notable changes in tempe metabolites due to different lactic acid bacteria species; however, variations in inoculum sizes during the soaking step did not significantly affect the metabolome.

In the final chapter, Chapter 4 concludes with a summary of the research findings, reflecting on their implications and significance. It also explores potential future research, highlighting areas where further investigation could yield valuable insights.

Chapter 2

Impact of microbial intervention during Tempe soaking step on its metabolite profile

2.1 Introduction

Tempe is typically classified as a fungal fermented food; however, research indicates that it also contains a diverse array of accompanying microflora, including both bacteria and yeasts (Samson et al., 1987). Numerous studies have reported on the microbial communities that develop during the soaking process of soybeans. Report (Nout et al., 1987) has indicated that species of lactic acid bacteria, Enterobacteriaceae, and yeasts are predominant in the water of soaking beans at the end of the process, thereby suggesting their involvement in the fermentation.

By using conventional microbiological methods to isolate and identify microorganisms, it was reported that several species of microorganisms existed in the soaking process of soybean. Such microorganisms are *Lactobacillus casei, Lactobacillus plantarum, Streptococcus faecium, Staphylococcus epidermidis, Bacillus brevis, Klebsiella pneumoniae, Pichia burtonii*, and *Pediococcus* spp (Ashenafi and Busse, 1991; Mulyowidarso et al., 1989). The metagenomics approach was also used to elucidate the microbial consortium present during the soaking step of tempe production. The Firmicutes phylum identified in tempe were found to be closely related to those present in the soaking water, which was predominantly populated by *Lactobacillus* (Radita et al., 2017). Another report using High-Throughput Sequencing (HTS) and cloned 16s rRNA genes indicated

that both fresh tempe and soaked water of soybean were dominated by the same species of *Lactobacillus*, i.e., *L. delbreuckii* and *L. mucosae*, indicating that the soaking step was probably the source of bacterial community that was established in the final fermentation product (Radita et al., 2021).

The application of microbial intervention during the soaking step of tempe has been conducted by several researchers. Mulyowidarso (Mulyowidarso et al., 1991a, 1991b) reported that the inoculation of *Lactobacillus casei, Streptococcus faecium, Staphylococcus epidermidis, Klebsiella pneumoniae* or *Pichia burtonii* in surface decontaminated soybean soaked with sterile water resulted in changes in its organic acids and carbohydrates. Magdalena (Magdalena et al., 2022) investigated the effect of adding *Lactobacillus plantarum* and *L. fermentum* during the soaking of soybeans. The resulting tempe met the Indonesian national standard requirement in texture, odour, and color.

Previous studies have largely focused on the physical and macromolecular characteristics of tempe, particularly through microbial interventions during the soaking step. However, they have not undertaken a comprehensive examination of the metabolome associated with this fermented food. While the identification of these metabolites in tempe is both informative and valuable, a thorough analysis of its metabolome would yield substantial benefits by ensuring that no potential compounds are overlooked.

Recent research has highlighted the significance of incorporating metabolome information through the utilization of metabolomics in tempe research. Particularly noteworthy are the successful applications of metabolomics in differentiating tempe from various production place (Kadar et al., 2018), utilization of other legumes for tempe

substrate (Rahmawati et al., 2021), chemical addition in tempe soaking (Dahlan et al., 2022), as well as combination with epidemiology (Iman et al., 2024).

This study seeks to bridge the current research gap by employing metabolomics techniques to conduct a comprehensive investigation of the tempe metabolome. This investigation will focus on modifications made in the production process, particularly the microbial intervention during the soaking step. The findings from this research will provide valuable insights into the functional characteristics of tempe, illuminating both its beneficial and detrimental aspects.

2.2 Materials and methods

2.2.1 Preparation of inoculum

Lactiplantibacillus plantarum NBRC 101978 and *Pichia burtonii* NBRC 0844 obtained from the Biological Resource Center, 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.

2.2.2 Tempe production

Tempe samples were produced in triplicate at the Laboratory of Bioresource Engineering, Osaka University, Japan, according to a previously reported method by Prativi (Prativi et al., 2023) with modifications. Briefly, commercial Japanese soybeans (grown in

Hokkaido, Japan) without surface sterilization were soaked in incubator at 30°C 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 23-25°C. Dehulled soybeans were inoculated with Raprima brand starter culture and incubated at 30°C for 48 h in an incubator containing open beaker with water to maintain humidity. A list of the samples is presented in Table 2.1.

Table 2.1. 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

2.2.3 Metabolite Extraction and Derivatization

Before extraction, samples were freeze-dried and homogenized 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 ribitol as internal standard was added to 2-mL tubes containing 10 mg of homogenized samples.

Samples were then incubated at 37 °C for 30 min with agitation at 1200 rpm. After centrifugation at 10,000 rpm 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) 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.

2.2.4 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) 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 analyzed in a random order. The linear velocity of the carrier gas (H_2) was 39.0 cm/s. The column temperature was maintained at 80 °C for 2 min, then increased by 10 °C/min to 330 °C, and maintained for 6 min. The interface and ion source temperatures were 310 and 280°C, respectively. Ions were generated by the electron ionization (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.

2.2.5 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 GCMSsolution 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. 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.

2.3 Results and discussion

2.3.1 Metabolite profile of soybean, microbial intervention-soaked soybean, and tempe

The results from the GC-MS analysis produced 153 features following the filtering process. The compounds that were tentatively annotated encompass amino acids, sugars, fatty acids, and organic acids, as detailed in Table 2.2. Additionally, compounds that have been previously reported in the metabolomics of tempeh, including meglutol, genistein, and daidzein, were identified in the samples analyzed. (Iman et al., 2023). In total, 100 annotated and 53 unknown metabolites were subjected to PCA (Figure 2.1). The score plot

of the PCA results shows the distinction between the samples based on the tempe-processing stage and microbial intervention.

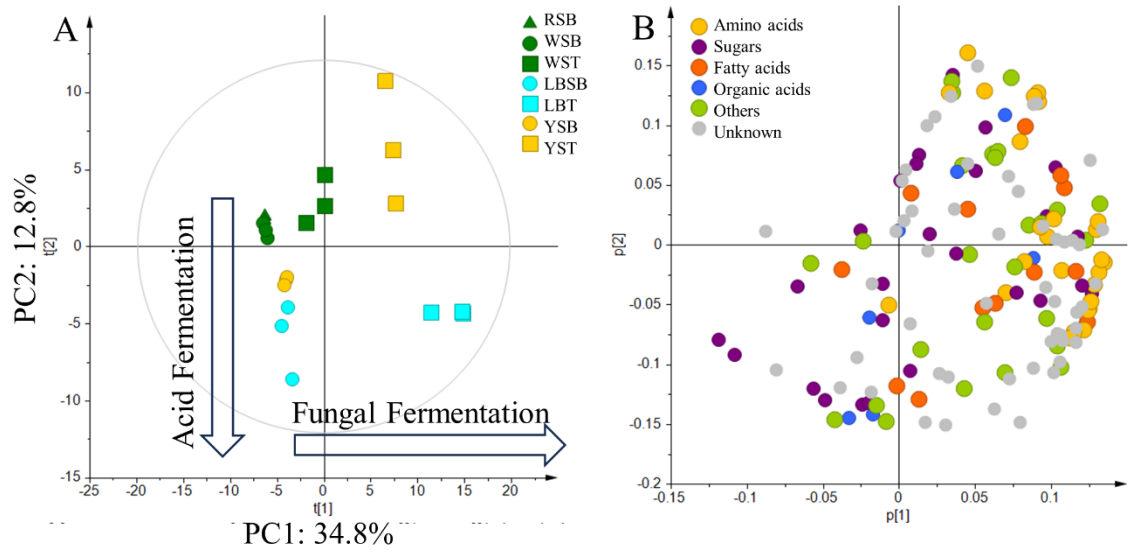


Figure 2.1. PCA results of soybean, soaked soybean, and tempe through GC-MS analysis. (A) Score plot of soybean, soaked soybean, and tempe. The triangle indicates raw soybean (CB), points indicate soaked soybean (WSB, water-soaked soybean; LBSB, LAB-soaked soybean; and YSB, yeast-soaked soybean), and squares indicate tempe (WST, water-soaked soybean tempe; LBT, LAB-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 moleculess, and grey indicates unknown molecules.

A distinct separation was illustrated by principal component (PC) 1 in the plot, which accounted for 34.8% of the variability observed. The metabolite profile transitioned from raw soybeans to tempe along the positive axis of PC1. The loading plot of the PCA results revealed that raw soybeans were predominantly characterized by sugar groups prior to fungal fermentation. Following fermentation, the profile shifted to being primarily dominated by amino acids, fatty acids, and other compounds. This finding was consistent

with that of a previous study (Prativi et al., 2023) where tempe fungal fermentation demonstrated more accumulation of amino acids. 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 lactic acid bacteria (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 (Supplementary 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 possess the capability to hydrolyze soy protein, resulting in the release of essential amino acids, including leucine, phenylalanine, tyrosine, valine, and isoleucine, from soy protein extracts. Genistein, a soy isoflavone aglycone, offers 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 acids, such as cysteine, and biogenic amine, tyramine. Research has indicated that elevated levels of tyramine present in fermented foods may be associated with negative health effects (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).

Table 2.2. List of annotated metabolites

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

Given the compound's correlation with food safety, we quantified it using a tyramine standard. We observed that the concentration of tyramine in yeast-soaked soybean tempe was 50 ± 14 mg/100 g food, which exceeds 900 times the concentration in LAB-soaked soybean tempe (Figure 2.2). While upper limits of tyramine in foods have been suggested to be 100–800 mg/kg food (Brink et al., 1990) and over 100 mg/day may cause migraine (Shalaby, 1996).

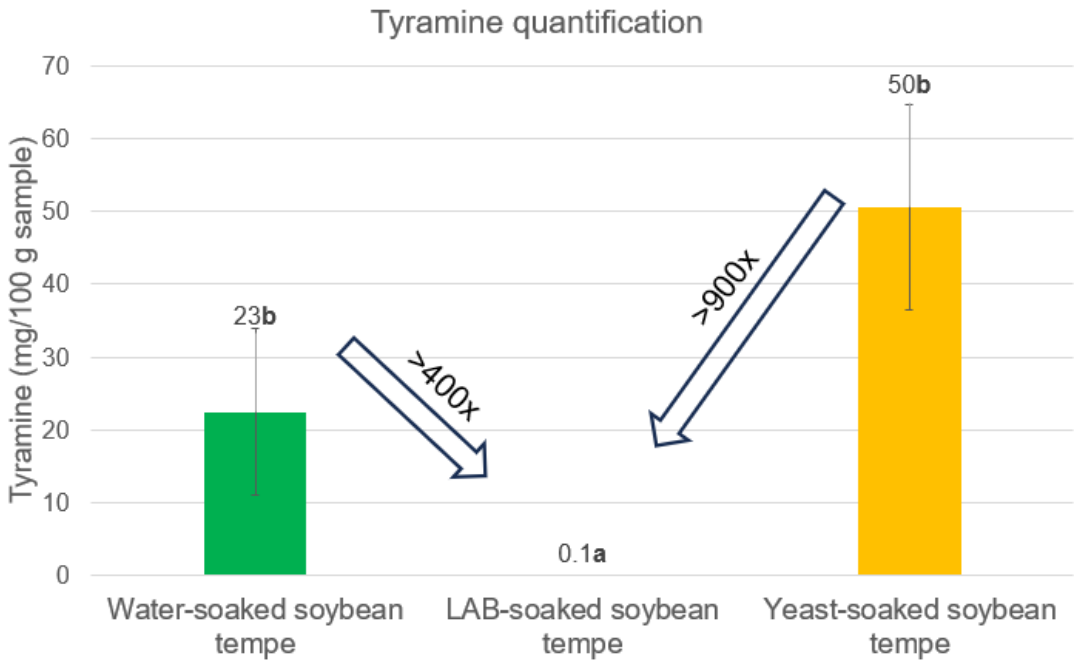


Figure 2.2. Tyramine quantification in tempe. The error bar shows the standard deviation from three biological replicates. Groups labeled with different letters are significantly different, as indicated using Tukey's adjustment ($P < 0.05$)

The PCA results revealed that the separation observed along Principal Component 2 (PC2) was influenced by the acid fermentation process with microbial intervention, accounting for 12.8% of the variance. Soaking notably altered the metabolome of the soybeans, as indicated by the negative direction of PC2. The loading plot from the PCA results demonstrated a significant accumulation of organic acids, such as lactic acid and

citric acid, by the end of the soaking process. The changes in the pH of the soaked water for each treatment are shown in Supplementary Figure S1. Notably, LAB intervention rapidly lowered the two degrees of acidity within 6 h of soaking. The proposition highlights the potential of LAB intervention to reduce the duration of acid fermentation in tempe production, considering that a pH of approximately 5 is the minimum threshold for this process (Romulo and Surya, 2021). *L. plantarum* intervention, compared to 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 of soybean before tempe fermentation 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. It is possible that the surviving cells may be transferred to the soybeans, which could, in turn, impact the fungal fermentation stage.

Microbial intervention alters the metabolite profile of tempe during the soaking step of its production. Soybean tempe soaked in LAB has been shown to enhance levels of

important amino acids, such as lysine and leucine. In contrast, tempe soaked with yeast has demonstrated increased levels of the biogenic amine tyramine. A metabolomics approach has highlighted both the advantages and disadvantages of these different soaking methods.

2.3.2 Comparative tempe metabolome between microbial interventions and chemical addition in Tempe-soaking step

For many tempe manufacturers, incorporating chemical additives during the soaking process plays a crucial role in optimizing tempe production. This practice effectively lowers the pH levels of the soaking medium, which not only helps to eliminate harmful pathogens but also creates a more favorable environment for the growth of beneficial fungi. As a result, there is a significantly increased chance of achieving successful fungal fermentation, leading to a higher quality end product (Nout and Kiers, 2005). The following experiment aimed to compare tempes with microbial intervention and chemical addition during the soaking process using a metabolomic approach. Figure 2.3 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).

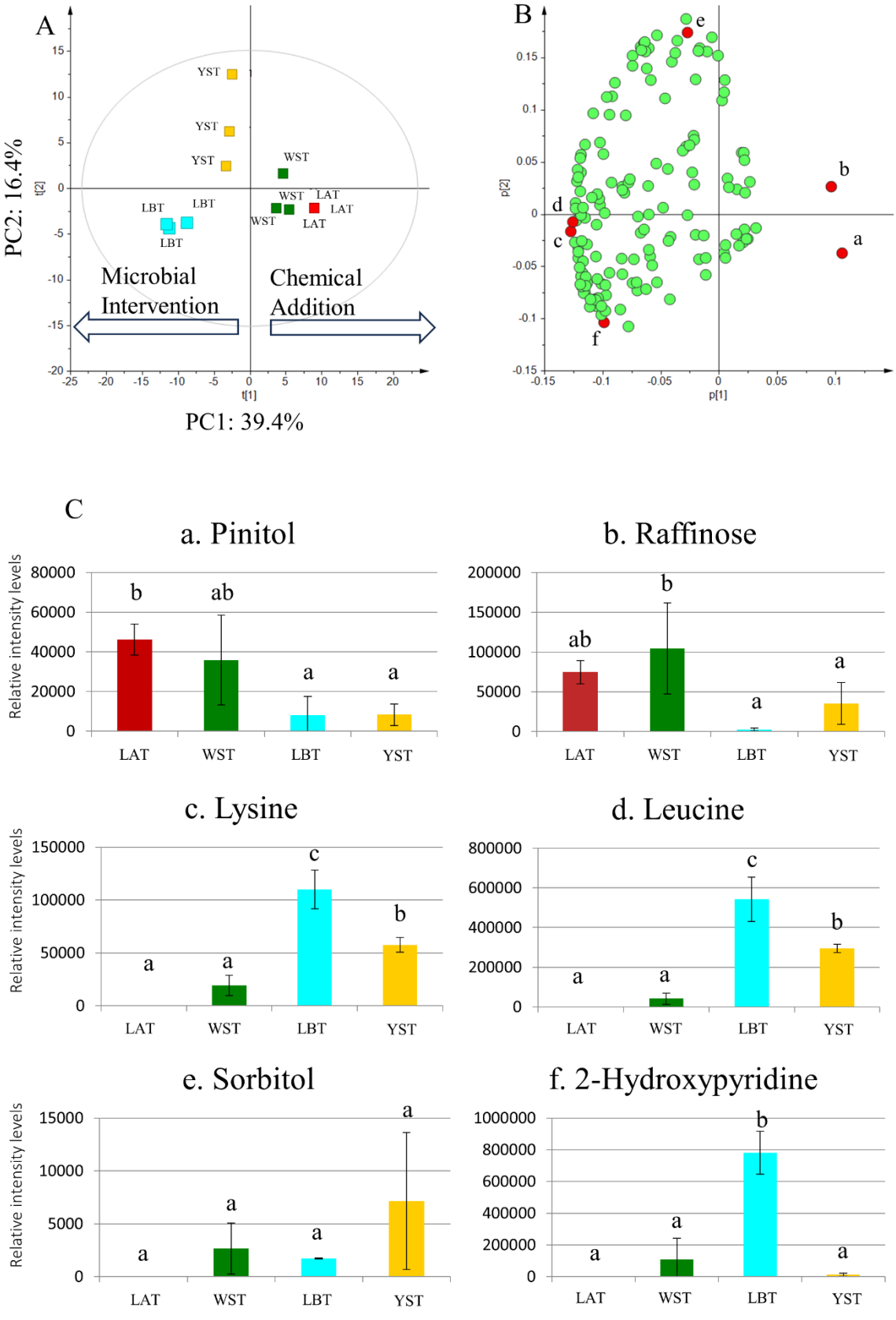

A
PC2: 16.4%
PC1: 39.4%
Microbial Intervention
Chemical Addition
YST
WST
LAT
LBT
B
C
a. Pinitol
b. Raffinose
c. Lysine
d. Leucine
e. Sorbitol
f. 2-Hydroxypyridine
Relative intensity levels
LAT
WST
LBT
YST

Figure 2.3. PCA results of different tempes through GC-MS analysis. (A) Score plot of different tempes. Squares indicate tempe (WST, water-soaked soybean tempe; LAT, acid-soaked soybean tempe; LBT, LAB-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 horizontal axis indicates samples. The error bar shows the SD from three biological replicates. Groups labeled with different letters are significantly different, as indicated using Tukey's adjustment ($p < 0.05$).

Based on the detailed list of metabolites (refer to Supplementary Table S2), we discovered that sugars and amino acids were the primary components differentiating microbially soaked soybean tempes from those that were chemically soaked. Notably, the metabolites contributing to this distinction included sugars such as pinitol and raffinose, alongside amino acids like lysine and leucine. 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 lactic acid-soaked soybean tempes. Although raffinose is identified as an antinutrient, it has been recognized for its potential benefits as a prebiotic ingredient that may positively influence gut microbiota (Amorim et al., 2020). LAB was also reported to have the ability to utilize 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 raffinose standard compound for quantification shown in Figure 2.4, it was determined that

LAB-soaked soybean tempe effectively reduced the raffinose content from 230 ± 20 mg/100 g food to 21 ± 1 mg/100 g food compared to lactic acid-soaked soybean tempe.

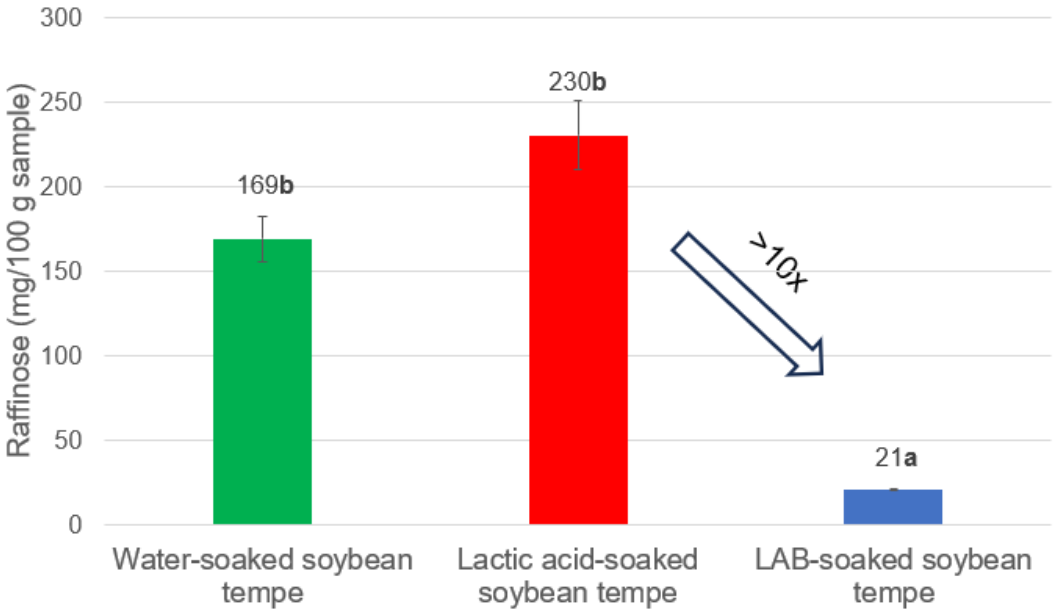


Figure 2.4. Raffinose quantification in tempe. The error bar shows the standard deviation from three biological replicates. Groups labeled with different letters are significantly different, as indicated using Tukey's adjustment ($P < 0.05$)

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 diarrhea (Hata et al., 1991). The reduction of raffinose by LAB positively impacts the reduction of flatulence risk. Lysine, an essential amino acid that is insufficient in most cereal flours (Meybodi et al., 2019), is highly accumulated in LAB-soaked soybean (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

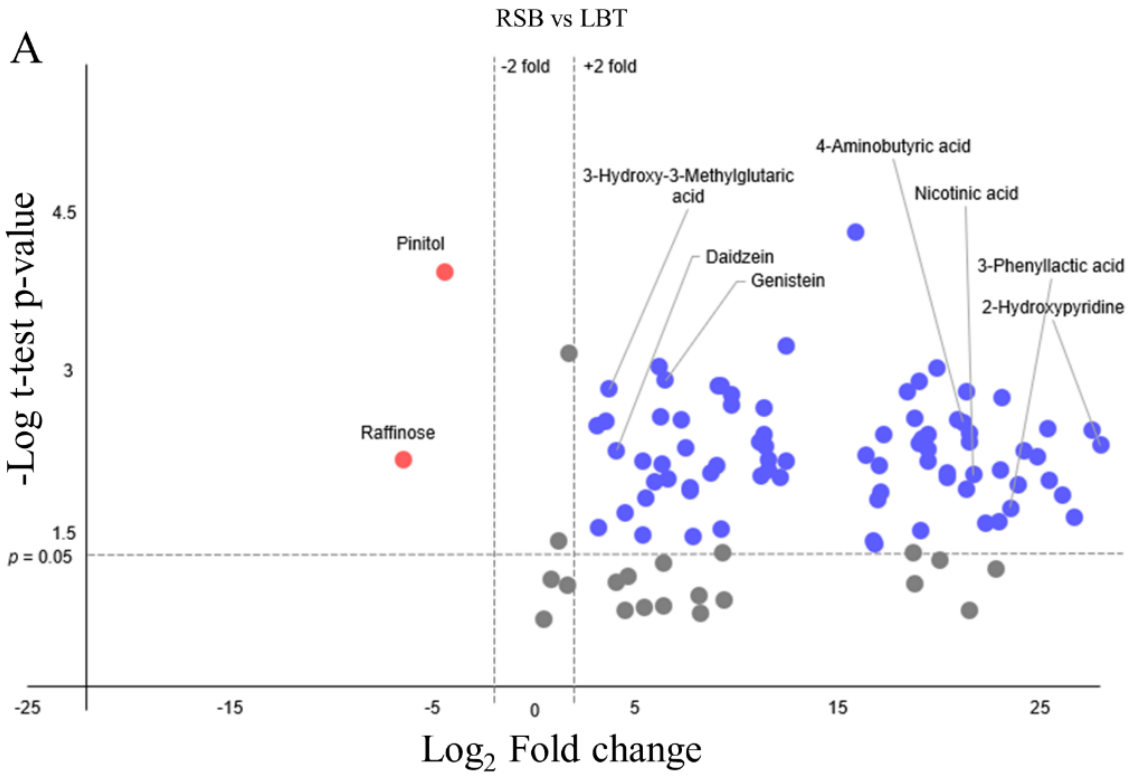
al., 2023). One report indicates that soybean paste increases its lysine content through LAB fermentation, primarily by converting peptides into free amino acids (Ng'ong'ola-Manani et al., 2014). Additional research on these topics could yield valuable insights into enhancing the nutritional value of tempeh and its use 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.

2.3.3 Soybean metabolites significantly modulated by microbial interventions in tempe soaking step

Differential analysis was utilized to explore the variations in metabolites among the tempe samples. Our objective was to examine the influence of microbial intervention during the soaking process on the tempe metabolome by analyzing the metabolites that exhibited significant modulation following fermentation, as depicted in the volcano plot (Figure 2.5). 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 $p < 0.05$). A complete list of the significantly modulated metabolites is presented in Supplementary 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 these bioactive metabolites have been reported to be elevated after tempe fermentation in previous report (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 B_3, was significantly increased 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 and Bisping, 1993). The formation of nicotinic acid in tempe is linked to LAB activity during soaking (Denter and Bisping, 1994). This metabolite is associated with blood cholesterol level stabilization (Bodor and Offermanns, 2008). Putrescine, polyamine essential for cell growth and various biological processes, but have dose-dependent toxic effect to human (Smith, 1990), is found to be significantly increased in LAB-soaked soybean tempe. In contrast, raffinose and pinitol levels significantly decreased by > 2-fold after tempe fermentation in LAB-soaked soybean tempe, presumably because of their utilization by LAB (Zartl et al., 2018).



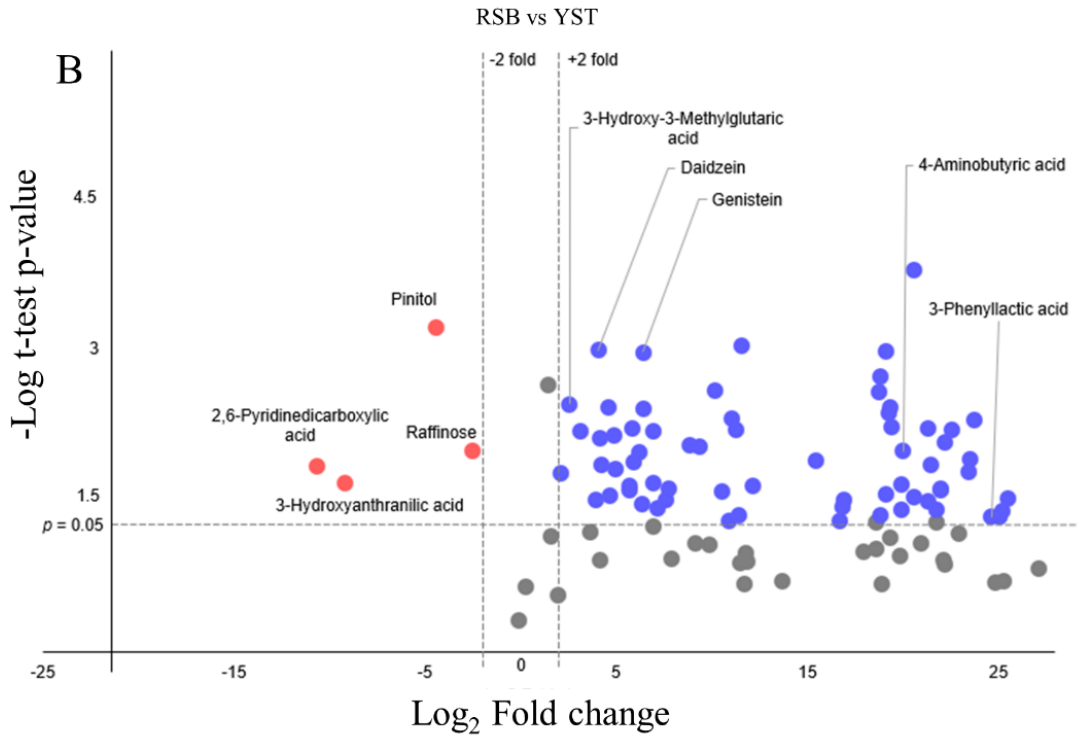


Figure 2.5. Significantly modulated metabolites identified the following raw soybeans: (A) LAB-soaked soybean tempe and (B) yeast-soaked tempe fermentation. Blue circles indicate metabolites significantly increased ($p < 0.05$) with at least a 2-fold change, red circles indicate metabolites significantly decreased ($p < 0.05$) with at least a 2-fold change, and grey circles indicate metabolites with a non-significant change ($p \geq 0.05$) and/or metabolites with < 2-fold change.

Following raw soybean and yeast-soaked soybean tempe, 66 metabolites exhibited $p < 0.05$, based on the t-test, with a fold change of > 2. Supplementary 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 yeast-soaked soybean tempe.

Yeasts are frequently present in fermented foods and fulfill various functions in the fermentation process. Traditionally, they facilitate interactions among microorganisms, modify texture, and contribute to the biosynthesis of flavor compounds. Although many fermentation processes primarily depend on the conversion of sugars to lactic acid, LAB are crucial, with yeasts acting as secondary contributors (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).

This research has revealed that microbial intervention during the soaking process of tempe significantly increases the relative levels of several bioactive metabolites. These bioactive compounds include meglutol, daidzein, genistein, 4-aminobutyric acid, and nicotinic acid. In addition, polyamines that may adversely affect humans in large doses have also been found to increase significantly. This highlights the potential to modulate properties of tempe through managed microbial processes.

2.4 Conclusion

The incorporation of microbial intervention into the process of tempe soaking has been demonstrated to alter the metabolite profile of tempe, specifically amino acids (lysine and leucine) in LAB-soaked soybean tempe and tyramine in yeast-soaked soybean tempe, highlighting 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 to chemically soaked soybean tempe, microbial intervention with soaked-soybean tempe resulted in a lower accumulation of sugars such as raffinose, an antinutrient. Furthermore, the differential analysis demonstrated that

microbial intervention during the soaking stage of tempe significantly affected the bioactive metabolites, including daidzein, genistein, nicotinic acid, and meglutol, as well as polyamines like putrescine. Overall, this study provides valuable insights about biochemical components that have not yet been discussed previously in relation to microbial interventions in tempe soaking step. This may assist tempe manufacturers in advancing product development within the food industry.

Chapter 3

Lactic acid bacteria species and inoculum size in tempe soaking step affect the metabolome of tempe

3.1 Introduction

Different lactic acid bacteria strains in several fermented foods play a significant role in shaping the nutritional value and health benefits of the final product. One example is the selection of a lactic acid bacterial strain that significantly influences the final characteristics of cheese, affecting both its sensory qualities and functional attributes, such as the inhibition of undesirable microorganisms and the promotion of health benefits (Thierry et al., 2015). Also numerous soy fermented foods utilize lactic acid bacteria in their fermentation, such as fermented soy milk, soy milk kefir, as well as tofu (Huo et al., 2023). Choice of lactic acid bacteria strains used in tofu was reported to have preferable results such as greater capability of acid production and could be used as tofu-coagulant (Li et al., 2017). In terms of the effect of lactic acid bacteria in fermented food on its metabolites, Chen et al. (2024) highlighted the alteration of the yogurt metabolome from different lactic acid bacteria strains used as the starter.

Varying the sizes of inoculum used in starter cultures can significantly influence the fermentation process of food. This variation in inoculum size leads to changes in the microbial population present during fermentation, which in turn affects the characteristics and quality of the final food product. As the microbial community shifts, it can alter nutritional profiles. Example is the different inoculum sizes applied in soy fermented food, moromi (Pramanda et al., 2023).

Our prior research into microbial intervention during the soaking process of tempe revealed a significant influence on the metabolome during final fermentation, particularly highlighting the preferable results through the application of LAB. Building on these findings, this chapter seeks to investigate the effect of different lactic acid bacteria species and inoculum size applied in tempe soaking to the metabolome of tempe. Specifically, we aim to investigate the effects of different LAB species and varying inoculum sizes during the soaking process on the metabolite profile of tempe.

3.2 Materials and methods

3.2.1 Tempe production

Lactiplantibacillus plantarum NBRC 101978 obtained from the Biological Resource Center, National Institute of Technology and Evaluation (NITE) (Tokyo, Japan) and *Limosilactobacillus fermentum* JCM 1173 obtained from Japan Collection of Microorganisms (JCM) (Ibaraki, Japan) were grown aerobically in MRS Vegitone medium (Sigma, Basel, Switzerland) at 30°C and 37°C respectively. The growth curve of both inoculums was determined and cultivated until the optical density $(OD)_{600}$ = 1.5 to reach the logarithmic 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 samples were prepared in three biological replicates at the Laboratory of Bioresource Engineering, Osaka University, Japan, according to a previously reported method by Prativi (Prativi et al., 2023) with modifications. Briefly, commercial Japanese soybeans (grown in Hokkaido, Japan) were soaked in incubator at 30°C for 24 h with inoculum size (v/v, inoculum volume per soak water volume) of 2.5%, 5%, and 10% as a microbial interventions. After soaking, soybeans were steamed, dehulled, and dried at room

with temperature of 23-25°C. Dehulled soybeans were inoculated with *Raprima* brand starter culture and incubated at 30 °C for 48 h in an incubator containing open beaker with water to maintain humidity.

3.2.2 Reagents

Ultrapure water was obtained from Genpure (Thermo Scientific, Osaka, Japan). Ribitol and pure pyridine were purchased from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol for GC-MS was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Chloroform for GC-MS was purchased from Kishida Chemical Co., Ltd. Methoxyamine hydrochloride was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Trifluoroacetamide (MSTFA) and an alkene mix (C9-C40) were purchased from GL Sciences (Tokyo, Japan).

3.2.3 Extraction and derivatization of hydrophilic, low-molecular-weight compounds for GC-MS Analysis

Prior to extraction, samples underwent freeze-drying and were homogenized using a multi-bead shocker (Yasui Kikai, Osaka, Japan). For the extraction, a solvent mixture consisting of methanol, water, and chloroform in a 5:2:2 (v/v/v) ratio—supplemented with 50 μg/mL ribitol as an internal standard—was added to 2-mL tubes containing 10 mg of the homogenized samples.

The samples were then incubated at 37 °C for 30 minutes while being agitated at 1200 rpm. After this, they were centrifuged at 10,000 rpm for 3 minutes at 4 °C, and 400 μL of the supernatant was transferred to new tubes. Another 400 μL of water was added to each tube, which were then centrifuged again. The aqueous phase (200 μL) was collected in new

tubes. Quality control (QC) samples were created by combining 200 μL of the aqueous phase from all samples. Both the samples and QC were then subjected to a centrifugal concentrator (Taitec Co., Tokyo, Japan) for 2 hours at room temperature.

The extracted samples were treated initially with 100 mL of methoxyamine hydrochloride in pyridine at a concentration of 20 mg/mL. This mixture was incubated at 30 °C for 90 minutes while continuously mixed at 1200 rpm. Afterward, 50 mL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was added, followed by a 30-minute incubation at 37 °C with agitation at 1200 rpm. The resulting derivatized samples were then transferred to gas chromatography (GC) vials for GC-MS analysis.

3.2.4 GC-MS Conditions

GC-MS analysis was conducted using a GC-MS-TQ8030 instrument (Shimadzu, Kyoto, Japan) equipped with an AOC-20i/s autoinjector (Shimadzu) 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 analyzed in a random order. The linear velocity of the carrier gas (H_2) was 39.0 cm/s. The column temperature was maintained at 80 °C for 2 min, then increased by 10 °C/min to 330 °C, and maintained for 6 min. The interface and ion source temperatures were 310 and 280°C, respectively. Ions were generated by the electron ionization (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.

3.2.5 GC-MS data 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. 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. 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).

3.3 Results and discussion

3.3.1 Metabolite profile of tempe with different bacterial species in soaking step

In this study, widely targeted metabolomic profiling of tempe using the GC-MS system resulted in annotation of 86 metabolites following filtering process. The tentatively annotated compounds comprise amino acids, fatty acids, sugars, organic acids, and others (Table 3.1 and Table S6). Apart from the previously reported metabolites in tempe metabolomics, such as phenylalanine, meglutol, and 4-aminobutyric acid (Iman et al., 2023), we were also able to detect vitamin B groups such as nicotinic acid and riboflavin. Subsequently, we then employed Principal Component Analysis (PCA) to analyze which of the 86 annotated metabolites exhibited the most significant difference between the two treatments. PCA is a widely recognized statistical technique to analyze variation and identify significant patterns within a data set. This multivariate analysis facilitates the visualization of correlations between observations and their respective variables, enhancing

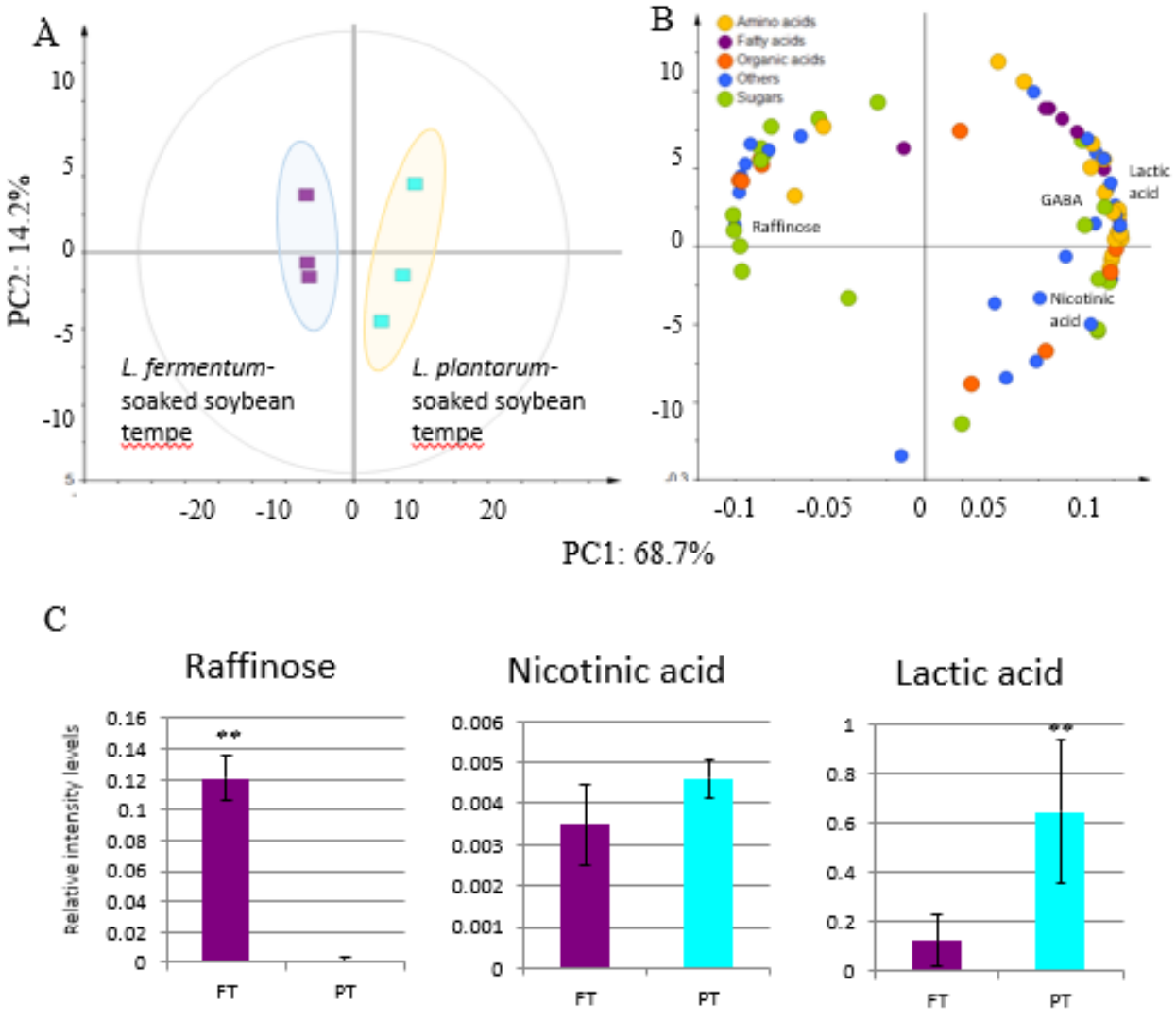
our understanding of the underlying data structure (Fathima et al., 2018). From the PCA score plot (Fig. 3.1), a distinct separation of *L. fermentum* and *L. plantarum*-soaked soybean tempe along the first principal component (PC1), accounted for 68.7% of the total variance observed in the samples, was observed while PC2 does not appear to contribute to the clusterization. Furthermore, PCA loading plot was examined in order to evaluate the factors contributing to the clustering seen on the score plots. The PCA loading plot showed that sugars were more abundant in *L. fermentum* treatment while *L. plantarum* treatment was dominated by amino acids and others. The relative intensity of several important metabolites was then investigated to get a better illustration of the distinction between the two treatments. Results indicated that raffinose was significantly higher in *L. fermentum* treatment while nicotinic acid was higher in *L. plantarum* treatment.

LAB, particularly *L. plantarum* and *L. fermentum*, synergistically coexist with *Rhizopus* species and are incorporated in tempe final product (Feng et al., 2005). Although the accumulation of amino acids at the end of the tempe processing was primarily attributed to the breaking of long-chain protein molecules by proteolytic enzymes of *Rhizopus oligosporus* as the tempe starter (Witono et al., 2015), higher abundance of amino acid groups in *L. plantarum* treatment possibly occurred due to its pronounced proteolytic activity compared to other LABs (Aguirre et al., 2014; Cao et al., 2019; Genet et al., 2023; Satılmış et al., 2023). Significant decrease of raffinose, trisaccharide associated with flatulence in human (Lacy et al., 2011), occurred in *L. plantarum*-soaked soybean tempe. This reduction is attributed to α-galactosidase enzyme which catalyzes its hydrolysis (Roopashri and Varadaraj, 2014) found in several LABs.

Table 3.1. 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	Riboflavin
5	Threonine	Melibiose	3-hydroxy-3-methylglutaric acid	Fumaric acid	Xanthine
6	Lysine	Sucrose	Linoleic acid	Isocitric acid	Uracil
7	2-Aminobutyric acid	Glucose	Glycolic acid	3-Phosphoglyceric acid	2-Aminoethanol
8	Valine	Lyxose	3-hydroxy-3-methylbutanoic acid	Oxalic acid	3-Hydroxy butyrate
9	Ornithine	Meso erythritol	Oleic acid	cis-Aconitic acid	Genistein
10	Phenylalanine	Melezitose			3-Phenyllactic acid
11	Isoleucine	Glucono-1,5-lactone			Urocanic acid
12	Cysteine	Panose			3-Hydroxyanthranilic acid
13	Leucine	Threitol			2,6-Pyridinedicarboxylic acid
14	Serine	Galactose			4-Hydroxyphenylacetic acid
15	Aspartic acid	Xylonic acid			Thymine
16	Alanine	Glyceric acid			Anthranilic acid
17	Glutamic acid	Mannitol			Uric acid
18	2-Aminoadipic acid	Galactinol			Urea
19	4-Aminobutyric acid	Saccharic acid			Daidzein
20		Galactitol			2-Hydroxypyridine
21		Turanose			Nicotinic acid
22					Tyramine
23					Putrescine
24					N-acetyl-α-D-glucosamine 1-phosphate
25					Hypoxanthine
26					Isobutylamine
27					Pyruvic acid

The results indicated that this particular strain of *L. fermentum* may not possess the capability to metabolize raffinose, a finding consistent with reports on another strain of *L. fermentum* that similarly lacks this metabolic ability (Hossain, 2022). Both *L. fermentum* and *L. plantarum* were reported to have genes required for biosynthesis of vitamin B_3 (Lehri et al., 2017; Li et al., 2016).



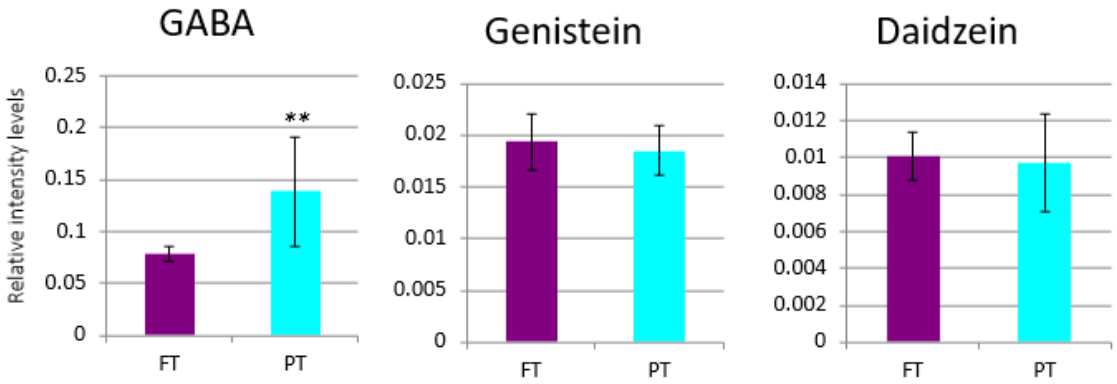


Figure 3.1. Principal component analysis (PCA) results of tempe with bacterial species variation in soaking step. (A) Score plot of tempe. Purple squares indicate *L. fermentum*-soaked tempe (FT) and turquoise squares indicate *L. plantarum*-soaked tempe (PT). (B) Loading plot of tempe in GC-MS analysis. Yellow color indicate amino acids, purple color indicate fatty acids, orange color indicate organic acids, blue color indicate others, and green color indicate sugars. (C) Bar graphs of important metabolites. The vertical axis indicates relative intensity. The horizontal axis indicates samples. The error bar shows the standard deviation from three biological replicates. Asterisks indicate significant differences (**$p \leq 0.01$).

4-Aminobutyric acid or known as GABA, metabolite that displays variety of physiological activites in human including relaxation and anti-depression (Ting Wong et al., 2003) was found significantly higher in *L. plantarum*-treatment. It has been observed that several strains of *L. plantarum* are capable of producing significant quantities of GABA in various fermented foods, including kimchi, cheese, and yogurt (Park et al., 2021). Significantly higher relative levels of lactic acid in *L. plantarum*-treatment might be attributed to its higher productivity levels of lactic acid as different lactic acid bacteria species have different productivity (Tian et al., 2021).

The composition of the microbial community involved in tempe production differs at each process stage. According to the previous report (Radita et al., 2017), the phylum Firmicutes at the soaking step significantly influence the microbial community present in tempe during the final stage of fermentation. By implementing an intervention during the soaking step through the addition of various lactic acid bacteria, it is hypothesized that the microbial community involved in the fungal fermentation process of tempe may undergo significant changes. Consequently, this alteration may lead to modulation of quality in tempe, as evidenced in other soy-based fermented food (Elhalis et al., 2023).

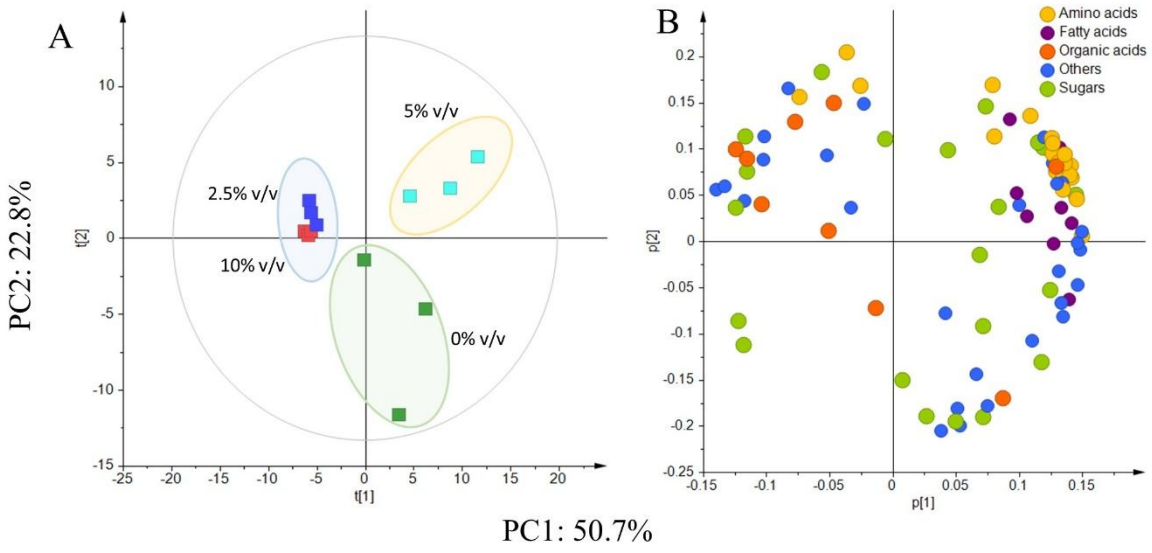
Microbial intervention using different species of LAB in soaking step of tempe modifies the metabolite profile of tempe in final fermentation. *L. plantarum*-soaked soybean tempe is preferable due to its ability to significantly reduce antinutrient raffinose and higher abundance of several bioactive compounds.

3.3.2 Metabolite profile of tempe with various inoculum sizes in soaking step

Inoculum size affects the rate of nutrient consumption and metabolite production, which can lead to alterations in the structure of microbial populations (Carrau et al., 2010). The following experiment aimed to compare tempe with variations in inoculum size of *L. plantarum* during the soaking process using a metabolomic approach. To determine the metabolomic difference of various inoculum sizes, a PCA analysis was conducted on 86 metabolites. The PCA score plot (Fig. 3.2) showed a distinct metabolite profile of tempe, with 0% and 5% located on the positive axis of PC1, while 2.5% and 10% were positioned on the negative axis of PC1. The first two principal components explained almost 72% of the total variance in data. In particular, the metabolites contributing to the separation were from organic acid, amino acid, and sugar groups. Notably, the only water-soaked soybean

tempe exhibited large variation within its cluster, suggesting an inconsistent tempe quality without microbial intervention in soaking step. From the bar graphs of several important metabolites, it was demonstrated that different inoculum sizes affect the abundance of previously reported bioactive metabolites in tempe such as daidzein, and genistein (Iman et al., 2023).

Interestingly, all interventions of *L. plantarum* during tempe soaking led to a significantly reduced abundance of tyramine, a biogenic amine responsible for the toxicological effects on human (Gillman, 2018). This reduction is presumably due to the lack of enzyme amino acid decarboxylase activity in *L. plantarum* compared to other LAB (Deepika Priyadarshani and Rakshit, 2011). Aspartic acid, a precursor in the biosynthesis of nicotinic acid within lactic acid bacteria (LAB), was found to be significantly decreased. This reduction is presumably attributed to its utilization in the production of vitamin B3, as reported during chickpea milk fermentation by *L. plantarum* (Fan et al., 2025)



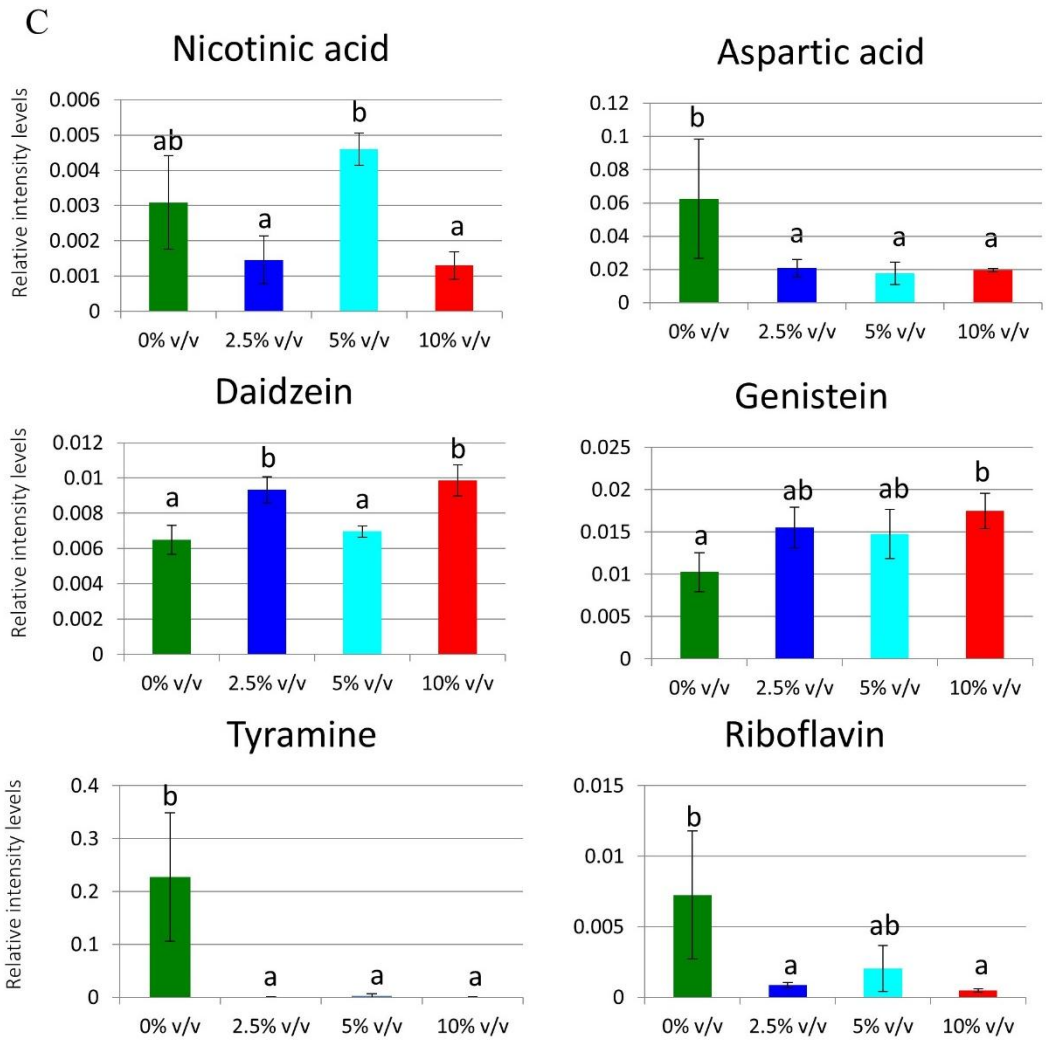


Figure 3.2. Principal component analysis (PCA) results of different inoculum size in tempe soaking using GC-MS analysis. (A) Score plot of different tempes. Green squares indicate water-soaked (0% v/v) tempe, blue squares indicate *L. plantarum*-soaked (2.5% v/v) tempe, turquoise squares indicate *L. plantarum*-soaked (5% v/v) tempe, and red squares indicate *L. plantarum*-soaked (10% v/v) tempe. (B) Loading plot of tempe in GC-MS analysis. Yellow color indicate amino acids, purple color indicate fatty acids, orange color indicate organic acids, blue color indicate others, and green color indicate sugars. (C) Bar graph of important metabolites. The vertical axis indicates relative intensity. The horizontal axis indicates samples. The error bar shows the standard deviation from three biological

replicates. Groups labeled with different letters are significantly different using Tukey adjustment ($p < 0.05$).

Research has indicated that variations in inoculum size in soy fermented foods, such as soy sauce and natto, can significantly impact the quality of the final product (Pramanda et al., 2023; Yang et al., 2021). In the context of fermentation processes, a lower initial concentration of bacteria results in an extended reproduction time and a reduced fermentation rate. This reduction can impede the effective accumulation of fermentation products (Sood et al., 2011). In the case of soybean soaking, the results shown in Figure S2 indicate that different inoculum sizes affect the pH of the soak water, even within the first 3 hours. This variation in pH could impact the overall microbial community present and ultimately influence the quality of the soybean itself. Higher inoculation size results in a robust and rapid bacterial metabolism, necessitating the consumption of a significant portion of available nutrients to sustain this accelerated growth and mitigate the accumulation of fermentation byproducts. However, the substantial production of metabolic waste may lead to an expedited process of bacterial senescence, consequently diminishing the functionality of the bacterial cells (Steiner, 2021).

The use of different inoculum sizes of *L. plantarum* during the soaking step of tempe affected the resulting metabolome of the tempe. However, it did not have any impact on several bioactive compounds, such as daidzein and genistein, nor the reduction of biogenic amine tyramine.

3.4 Conclusion

In conclusion, using different species of lactic acid bacteria during the soaking step of tempe production significantly affects the metabolite profile observed during the final

fermentation, including antinutrient raffinose and bioactive compounds like GABA. Additionally, variations in inoculum size do not demonstrate a notable impact on bioactive compounds and biogenic amine tyramine. This study offers insights that can assist tempe manufacturers in advancing product development within the food industry. For example, the selection of lactic acid bacteria strains utilized for tempe soaking for different purposes and consideration of inoculum size used. It is important to note that the research has certain limitations, as it primarily addresses the compounds detectable by GC-MS. Therefore, we recommend conducting further investigations to expand the range of metabolite classes using alternative analytical platforms. This approach will enhance our understanding of the functional effects of microbial interventions in soaking on tempe.

Chapter 4

Conclusion and future perspectives

4.1 Conclusion

This research dissertation employs metabolomics approach to investigate the impact of microbial interventions in soaking step of tempe production. The first strategy is to evaluate the effect of lactic acid bacteria and yeast as microbial intervention in soaking step and lactic acid as chemical addition in soaking step to tempe metabolome. LAB-soaked soybean tempe demonstrated a significantly lower concentration of the biogenic amine tyramine compared to both water-soaked and yeast-soaked soybean tempe. Unlike tempe produced from soybeans treated with chemical additive of lactic acid during the soaking step, tempe made through microbial intervention exhibited a decreased accumulation of sugars, such as raffinose. Additionally, microbial intervention during the soaking step was found to significantly enhance the relative levels of several bioactive metabolites, including vitamin B_3. LAB-soaked soybean tempe is preferred due to its lower content of biogenic amines, less flatulence-inducing antinutrients, and increased relative levels of bioactive metabolites.

Furthermore, the second strategy is by using fermentation engineering in the form of varying bacterial species and inoculum sizes applied in the tempe soaking step. Application of different bacterial species and inoculum size in the microbial intervention for soaking tempe resulted in diverse metabolite profiles in the final product. The differentiation of lactic acid bacteria species significantly influences the metabolome of tempe, specifically

affecting the biogenic amine and bioactive compounds. In contrast, variations in inoculum size do not demonstrate a notable impact. This emphasizes the significance of the soaking step in the production of tempe, which is an often-overlooked step, and how alterations to this process can affect the metabolite profile of the final product. Much of the microbiological research on tempe has predominantly concentrated on the tempe fermentation starter, rather than the acid fermentation starter.

The metabolomics approach provides a thorough analysis of the metabolite profile of tempe that has been altered through various microbial interventions during the soaking process. This comprehensive approach not only highlights the beneficial effects of these treatments, but also identifies potential drawbacks, allowing for a balanced understanding of the overall impact on tempe's quality.

4.2 Future perspectives

The results of this research emphasize the promising role of microbial interventions during the soaking process of soybeans. The influence of microbial intervention on the composition of the microbial community in tempe can be elucidated through a metagenomic approach. This innovative approach not only enhances the quality of the soybeans but also holds significant potential for improving various soy-based fermented foods such as soy sauce, natto, and miso. By leveraging the benefits of microbial activity, we can find unique flavors or nutritional enhancements in these products.

In order to achieve more applicable results, it is essential to undertake larger-scale optimization efforts. Furthermore, a thorough analysis of the complex interactions between microflora and various microbial interventions through metagenomics approach is

necessary to better understand their effects and ensure food safety. Sensory analysis is also warranted to determine the acceptability of the final product to consumers.

References

Aguirre, L., Garro, M.S., Savoy de Giori, G., 2008. Enzymatic hydrolysis of soybean protein using lactic acid bacteria. Food Chem. 111, 976–982. https://doi.org/10.1016/j.foodchem.2008.05.018

Aguirre, L., Hebert, E.M., Garro, M.S., Savoy de Giori, G., 2014. Proteolytic activity of Lactobacillus strains on soybean proteins. Lwt 59, 780–785. https://doi.org/10.1016/j.lwt.2014.06.061

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. Compr. Rev. Food Sci. Food Saf. 20, 1717–1767. https://doi.org/10.1111/1541-4337.12710

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. https://doi.org/10.1016/J.LWT.2020.109322

Arroyo-López, F.N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., García-García, P., Querol, A., Garrido-Fernández, A., 2012. Yeasts in table olive processing: Desirable or spoilage microorganisms? Int. J. Food Microbiol. 160, 42–49. https://doi.org/10.1016/j.ijfoodmicro.2012.08.003

Ashenafi, M., Busse, M., 1991. The microflora of soak water during tempeh production from various beans. J. Appl. Bacteriol. 70, 334–338. https://doi.org/10.1111/j.1365-2672.1991.tb02945.x

Astuti, M., Meliala, A., Dalais, F.S., Wahlqvist, M.L., 2000. Tempe, a nutritious and healthy food from Indonesia. Asia Pac. J. Clin. Nutr. 9, 322–325. https://doi.org/10.1046/j.1440-6047.2000.00176.x

Badan Pusat Statistik, 2022. Statistik Indonesia 2022.

Bodor, E.T., Offermanns, S., 2008. Nicotinic acid: An old drug with a promising future. Br. J. Pharmacol. 153, 68–75. https://doi.org/10.1038/sj.bjp.0707528

Cao, C.C., Feng, M.Q., Sun, J., Xu, X.L., Zhou, G.H., 2019. Screening of lactic acid bacteria with high protease activity from fermented sausages and antioxidant activity assessment of its fermented sausages. CYTA - J. Food 17, 347–354. https://doi.org/10.1080/19476337.2019.1583687

Carrau, F., Medina, K., Fariña, L., Boido, E., Dellacassa, E., 2010. Effect of Saccharomyces cerevisiae inoculum size on wine fermentation aroma compounds and its relation with assimilable nitrogen content. Int. J. Food Microbiol. 143, 81–85. https://doi.org/10.1016/j.ijfoodmicro.2010.07.024

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 J. Microbiol. Biotechnol. 18, 159–163. https://doi.org/10.1023/A:1014451728868

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 J. Microbiol. 47, 941–948. https://doi.org/10.1016/j.bjm.2016.06.003

Dahlan, H.A., Nambu, Y., Putri, S.P., Fukusaki, E., 2022. Effects of Soaking Tempe in Vinegar on Metabolome and Sensory Profiles. Metabolites 12. https://doi.org/10.3390/metabo12010030

de Reu, J.C., Rombouts, F.M., Nout, M.J.R., 1995. Influence of acidity and initial substrate temperature on germination of Rhizopus oligosporus sporangiospores during tempe manufacture. J. Appl. Bacteriol. 78, 200–208. https://doi.org/10.1111/j.1365-2672.1995.tb02843.x

Deepika Priyadarshani, W.M., Rakshit, S.K., 2011. Screening selected strains of probiotic lactic acid bacteria for their ability to produce biogenic amines (histamine and tyramine). Int. J. Food Sci. Technol. 46, 2062–2069. https://doi.org/10.1111/j.1365-2621.2011.02717.x

Denter, J., Bisping, B., 1994. Formation of B-vitamins by bacteria during the soaking process of soybeans for tempe fermentation. Int. J. Food Microbiol. 22, 23–31. https://doi.org/10.1016/0168-1605(94)90004-3

Drulyte, D., Orlien, V., 2019. The Effect of Processing on Digestion of Legume Protein. Foods 8, 1–9.

EFRIWATI, SUWANTO, A., RAHAYU, G., NURAIDA, L., 2013. Population Dynamics of Yeasts and Lactic Acid Bacteria (LAB) During Tempeh Production. HAYATI J. Biosci. 20, 57–64. https://doi.org/10.4308/hjb.20.2.57

Elhalis, H., Chin, X.H., Chow, Y., 2023. Soybean fermentation: Microbial ecology and starter culture technology. Crit. Rev. Food Sci. Nutr. 0, 1–23. https://doi.org/10.1080/10408398.2023.2188951

Fan, J., Lu, Y., Li, X., Huang, J., Dong, L., Luo, J., Tian, F., Ni, Y., 2025. Omics analysis of key pathway in flavour formation and B vitamins synthesis during chickpea milk fermentation by Lactiplantibacillus plantarum. Food Chem. 463. https://doi.org/10.1016/j.foodchem.2024.141083

Fathima, A.M., Chuang, D., Laviña, W.A., Liao, J., Putri, S.P., Fukusaki, E., 2018. Iterative cycle of widely targeted metabolic profiling for the improvement of 1-butanol titer and productivity in Synechococcus elongatus. Biotechnol. Biofuels 11, 1–12. https://doi.org/10.1186/s13068-018-1187-8

Feng, X.M., Eriksson, A.R.B., Schnürer, J., 2005. Growth of lactic acid bacteria and Rhizopus

oligosporus during barley tempeh fermentation. Int. J. Food Microbiol. 104, 249–256. https://doi.org/10.1016/j.ijfoodmicro.2005.03.005

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. Sci. Rep. 12, 1–8. https://doi.org/10.1038/s41598-022-22943-1

Gaya, P., Peirotén, Á., Landete, J.M., 2017. Transformation of plant isoflavones into bioactive isoflavones by lactic acid bacteria and bifidobacteria. J. Funct. Foods 39, 198–205. https://doi.org/10.1016/J.JFF.2017.10.029

Genet, B.M.L., Xiao, H., Christensen, L.F., Laforce, I.N., Mohammadifar, M.A., Bang-Berthelsen, C.H., Hansen, E.B., 2023. Selection of proteolytic LAB starter cultures for acidification of soy based dairy alternatives. Lwt 184, 115082. https://doi.org/10.1016/j.lwt.2023.115082

Gillman, P.K., 2018. A reassessment of the safety profile of monoamine oxidase inhibitors: elucidating tired old tyramine myths. J. Neural Transm. 125, 1707–1717. https://doi.org/10.1007/s00702-018-1932-y

Hata, Y., Yamamoto, M., Nakajima, K., 1991. Effects of Soybean Oligosaccharides on Human Digestive Organs: Estimatio of Fifty Percent Effective Dose and Maximum Non-Effective Dose Based on Diarrhea. J. Clin. Biochem. Nutr. 10, 135–144.

Hossain, T.J., 2022. Functional genomics of the lactic acid bacterium Limosilactobacillus fermentum LAB-1: metabolic, probiotic and biotechnological perspectives. Heliyon 8, e11412. https://doi.org/10.1016/j.heliyon.2022.e11412

Huo, C., Yang, X., Li, L., 2023. Non-beany flavor soymilk fermented by lactic acid bacteria: Characterization, stability, antioxidant capacity and in vitro digestion. Food Chem. X 17, 100578. https://doi.org/10.1016/j.fochx.2023.100578

Iman, M.N., Haslam, D.E., Liang, L., Guo, K., Joshipura, K., Pérez, C.M., Clish, C., Tucker, K.L., Manson, J.A.E., Bhupathiraju, S.N., Fukusaki, E., Lasky-Su, J., Putri, S.P., 2024. Multidisciplinary approach combining food metabolomics and epidemiology identifies meglutol as an important bioactive metabolite in tempe, an Indonesian fermented food. Food Chem. 446. https://doi.org/10.1016/j.foodchem.2024.138744

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. J. Biosci. Bioeng. 136. https://doi.org/10.1016/j.jbiosc.2023.05.012

Jung, S., Hwang, H., Lee, J.H., 2019. Effect of lactic acid bacteria on phenyllactic acid production in kimchi. Food Control 106, 106701. https://doi.org/10.1016/j.foodcont.2019.06.027

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. J. Biosci. Bioeng. 126, 411–416. https://doi.org/10.1016/j.jbiosc.2018.03.020

Keuth, S., Bisping, B., 1993. Formation of vitamins by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation. J. Appl. Bacteriol. 75, 427–434. https://doi.org/10.1111/j.1365-2672.1993.tb02798.x

Kumari, S., Krishnan, V., Sachdev, A., 2015. Impact of soaking and germination durations on antioxidants and anti-nutrients of black and yellow soybean (Glycine max. L) varieties. J. Plant Biochem. Biotechnol. 24, 355–358. https://doi.org/10.1007/s13562-014-0282-6

Kustyawati, M.E., 2009. Kajian Peran Yeast dalam Pembuatan Tempe. Agritech J. Fak. Teknol. Pertan. UGM 29, 64–70.

Lacy, B.E., Gabbard, S.L., Crowell, M.D., 2011. Pathophysiology, evaluation, and treatment of bloating: Hope, hype, or hot air? Gastroenterol. Hepatol. 7, 729–739.

Lai, Z., Tsugawa, H., Wohlgemuth, G., Mehta, S., Mueller, M., Zheng, Y., Ogiwara, A., Meissen, J., Showalter, M., Takeuchi, K., Kind, T., Beal, P., Arita, M., Fiehn, O., 2018. Identifying metabolites by integrating metabolome databases with mass spectrometry cheminformatics. Nat. Methods 15, 53–56. https://doi.org/10.1038/nmeth.4512

Lambo, M.T., Ma, H., Zhang, H., Song, P., Mao, H., Cui, G., Dai, B., Li, Y., Zhang, Y., 2024. Mechanism of action, benefits, and research gap in fermented soybean meal utilization as a high-quality protein source for livestock and poultry. Anim. Nutr. 16, 130–146. https://doi.org/10.1016/j.aninu.2023.10.003

Lehri, B., Seddon, A.M., Karlyshev, A. V., 2017. Potential probiotic-associated traits revealed from completed high quality genome sequence of Lactobacillus fermentum 3872. Stand. Genomic Sci. 12, 1–9. https://doi.org/10.1186/s40793-017-0228-4

Li, C., Rui, X., Zhang, Y., Cai, F., Chen, X., Jiang, M., 2017. Production of tofu by lactic acid bacteria isolated from naturally fermented soy whey and evaluation of its quality. LWT - Food Sci. Technol. 82, 227–234. https://doi.org/10.1016/J.LWT.2017.04.054

Li, P., Zhou, Q., Gu, Q., 2016. Complete genome sequence of Lactobacillus plantarum LZ227, a potential probiotic strain producing B-group vitamins. J. Biotechnol. 234, 66–70. https://doi.org/10.1016/j.jbiotec.2016.07.020

Magdalena, S., Hogaputri, J.E., Yulandi, A., Yogiara, Y., 2022. The addition of lactic acid bacteria in the soybean soaking process of tempeh. Food Res. 6, 27–33. https://doi.org/10.26656/fr.2017.6(3).304

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. J. Funct.

Foods 4, 594–601. https://doi.org/10.1016/J.JFF.2012.03.005

Meybodi, N.M., Mortazavian, A.M., Mirmoghtadaie, L., Hosseini, S.M., Yasini, S.A., Azizi, M.H., Nodoushan, S.M., 2019. Effects of microbial transglutaminase and fermentation type on improvement of lysine availability in wheat bread: A response surface methodology. Appl. Food Biotechnol. 6, 151–164. https://doi.org/10.22037/afb.v%vi%i.24359

Moa, H., Kariluoto, S., Piironen, V., Zhu, Y., Sanders, M.G., Vincken, J.P., Wolkers-Rooijackers, J., Nout, M.J.R., 2013. Effect of soybean processing on content and bioaccessibility of folate, vitamin B12 and isoflavones in tofu and tempe. Food Chem. 141, 2418–2425. https://doi.org/10.1016/j.foodchem.2013.05.017

Mulyowidarso, R.K., Fleet, G.H., Buckle, K.A., 1990. Association of bacteria with the fungal fermentation of soybean tempe. J. Appl. Bacteriol. 68, 43–47. https://doi.org/10.1111/j.1365-2672.1990.tb02546.x

Mulyowidarso, R.K., Fleet, G.H., Buckle, K.A., 1989. The microbial ecology of soybean soaking for tempe production. Int. J. Food Microbiol. 8, 35–46. https://doi.org/10.1016/0168-1605(89)90078-0

MULYOWIDARSO, R.K., FLEET, G.H., BUCKLE, K.A., 1991a. Changes in the concentration of carbohydrates during the soaking of soybeans for tempe production. Int. J. Food Sci. Technol. 26, 595–606. https://doi.org/10.1111/j.1365-2621.1991.tb02005.x

MULYOWIDARSO, R.K., FLEET, G.H., BUCKLE, K.A., 1991b. Changes in the concentration of organic acids during the soaking of soybeans for tempe production. Int. J. Food Sci. Technol. 26, 607–614. https://doi.org/10.1111/j.1365-2621.1991.tb02006.x

Naila, A., Flint, S., Fletcher, G., Bremer, P., Meerdink, G., 2010. Control of biogenic amines in food - existing and emerging approaches. J. Food Sci. 75.

https://doi.org/10.1111/j.1750-3841.2010.01774.x

Nelson, D., Thomas -Alberta, J., McIntyre, L., Sik Jang, S., Adamkowicz -Manitoba Health Leslie Hudson -Manitoba Agriculture Douglas Walker, B., LeBlanc -New Brunswick, J., Locke, S., Trefry -Nova Scotia Environment, D., Change Rick Kane -Nova, C., Collins, D., Stewart, E., Walzak -Prince Edward, S., Rose -Saskatchewan Health Authority, L., 2023. Safety of fermented foods. Assessing risks in fermented food processing practices and advice on how to mitigate them. Section 3.9 Tempeh. Environ. Heal. Serv. BC Cent. Dis. Control.

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 Sci. Nutr. 2, 768–785. https://doi.org/10.1002/fsn3.171

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. https://doi.org/10.3390/metabo12100991

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 Microbiol. 4, 165–172. https://doi.org/10.1016/0740-0020(87)90032-3

Nout, M.J.R., Kiers, J.L., 2005. Tempe fermentation , innovation and functionality : Update into the third millenium Tempe fermentation , innovation and functionality : update into the third millenium. J. Appl. Microbiol. 98, 789–805. https://doi.org/10.1111/j.1365-2672.2004.02471.x

Pannerchelvan, S., Rios-Solis, L., Faizal Wong, F.W., Zaidan, U.H., Wasoh, H., Mohamed, M.S., Tan, J.S., Mohamad, R., Halim, M., 2023. Strategies for improvement of gamma-

aminobutyric acid (GABA) biosynthesis via lactic acid bacteria (LAB) fermentation. Food Funct. 14, 3929–3948. https://doi.org/10.1039/d2fo03936b

Park, S.J., Kim, D.H., Kang, H.J., Shin, M., Yang, S.Y., Yang, J., Jung, Y.H., 2021. Enhanced production of γ-aminobutyric acid (GABA) using Lactobacillus plantarum EJ2014 with simple medium composition. Lwt 137, 110443. https://doi.org/10.1016/j.lwt.2020.110443

Pramanda, I.T., Saputro, M.N.B., Naidu, N.C., Devanthi, P.V.P., 2023. Starter cultures inoculation procedure changes microbial community structure during low-salt moromi fermentation. Food Res. 7, 96–102. https://doi.org/10.26656/fr.2017.7(S1).18

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. https://doi.org/10.3390/metabo13020300

Putri, S.P., Ikram, M.M.M., Sato, A., Dahlan, H.A., Rahmawati, D., Ohto, Y., Fukusaki, E., 2022. Application of gas chromatography-mass spectrometry-based metabolomics in food science and technology. J. Biosci. Bioeng. 133, 425–435. https://doi.org/10.1016/j.jbiosc.2022.01.011

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. https://doi.org/10.1007/s11306-019-1591-5

Qi, W., Hou, L.H., Guo, H.L., Wang, C.L., Fan, Z.C., Liu, J.F., Cao, X.H., 2014. Effect of salt-tolerant yeast of Candida versatilis and Zygosaccharomyces rouxii on the production of biogenic amines during soy sauce fermentation. J. Sci. Food Agric. 94, 1537–1542. https://doi.org/10.1002/jsfa.6454

Radita, R., Suwanto, A., Kurosawa, N., Wahyudi, A.T., Rusmana, I., 2021. Dynamics Of

Microbial Community During Tempeh Fermentation. Biotropia (Bogor). 28, 11–20. https://doi.org/10.11598/btb.2021.28.1.820

Radita, R., Suwanto, A., Kurosawa, N., Wahyudi, A.T., Rusmana, I., 2017. Metagenome analysis of tempeh production: Where did the bacterial community in tempeh come from? Malays. J. Microbiol. https://doi.org/10.21161/mjm.101417

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

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. J. Biosci. Bioeng. 132, 487–495. https://doi.org/10.1016/j.jbiosc.2021.07.001

Romulo, A., Surya, R., 2021. Tempe: A traditional fermented food of Indonesia and its health benefits. Int. J. Gastron. Food Sci. 26, 100413. https://doi.org/10.1016/j.ijgfs.2021.100413

Roopashri, A.N., Varadaraj, M.C., 2014. Hydrolysis of fatulence causing oligosaccharides by α-D-galactosidase of a probiotic lactobacillus plantarum MTCC 5422 in selected legume fours and elaboration of probiotic attributes in soy-based fermented product. Eur. Food Res. Technol. 239, 99–115. https://doi.org/10.1007/s00217-014-2207-y

Samson, R.A., Van Kooij, J.A., De Boer, E., 1987. Microbiological quality of commercial tempeh in The Netherlands. J. Food Prot. 50, 92–94. https://doi.org/10.4315/0362-028X-50.2.92

Satılmış, M.K., Öztürk, H.İ., Demirci, T., Denktaş, B., Akın, N., 2023. Revealing the proteolytic characteristics of lactobacillus, lacticaseibacillus, and lactiplantibacillus

isolates by in vitro and in situ perspectives. Food Biosci. 55. https://doi.org/10.1016/j.fbio.2023.103086

Shah, A.M., Tarfeen, N., Mohamed, H., Song, Y., 2023. Fermented Foods: Their Health-Promoting Components and Potential Effects on Gut Microbiota. Fermentation 9, 1–23. https://doi.org/10.3390/fermentation9020118

Shalaby, A.R., 1996. Significance of biogenic amines to food safety and human health. Food Res. Int. 29, 675–690. https://doi.org/10.1016/S0963-9969(96)00066-X

Sirilun, S., Sivamaruthi, B.S., Kesika, P., Peerajan, S., Chaiyasut, C., 2017. Lactic acid bacteria mediated fermented soybean as a potent nutraceutical candidate. Asian Pac. J. Trop. Biomed. 7, 930–936. https://doi.org/10.1016/j.apjtb.2017.09.007

Smith, T.K., 1990. Effect of Dietary Putrescine on Whole Body Growth and Polyamine Metabolism. Proc. Soc. Exp. Biol. Med. 194, 332–336. https://doi.org/10.3181/00379727-194-43100

Sood, S., Singhal, R., Bhat, S., Kumar, A., 2011. Inoculum Preparation, Second Edi. ed, Comprehensive Biotechnology, Second Edition. Elsevier B.V. https://doi.org/10.1016/B978-0-08-088504-9.00090-8

Steiner, U.K., 2021. Senescence in Bacteria and Its Underlying Mechanisms. Front. Cell Dev. Biol. 9, 1–15. https://doi.org/10.3389/fcell.2021.668915

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. Am. J. Clin. Nutr. 69, 135–139. https://doi.org/10.1093/ajcn/69.1.135

Tamam, B., Syah, D., Suhartono, M.T., Kusuma, W.A., Tachibana, S., Lioe, H.N., 2019. Proteomic study of bioactive peptides from tempe. J. Biosci. Bioeng. 128, 241–248. https://doi.org/10.1016/j.jbiosc.2019.01.019

Tamang, J.P., Anupma, A., Nakibapher Jones Shangpliang, H., 2022. Ethno-microbiology of Tempe, an Indonesian fungal-fermented soybean food and Koji, a Japanese fungal starter culture. Curr. Opin. Food Sci. 48, 100912. https://doi.org/10.1016/j.cofs.2022.100912

ten Brink, B., Damink, C., Joosten, H.M.L.J., Huis in 't Veld, J.H.J., 1990. Occurrence and formation of biologically active amines in foods. Int. J. Food Microbiol. 11, 73–84. https://doi.org/10.1016/0168-1605(90)90040-C

Thierry, A., Valence, F., Deutsch, S.M., Even, S., Falentin, H., Le Loir, Y., Jan, G., Gagnaire, V., 2015. Strain-to-strain differences within lactic and propionic acid bacteria species strongly impact the properties of cheese–A review. Dairy Sci. Technol. 95, 895–918. https://doi.org/10.1007/s13594-015-0267-9

Tian, X., Chen, H., Liu, H., Chen, J., 2021. Recent Advances in Lactic Acid Production by Lactic Acid Bacteria. Appl. Biochem. Biotechnol. 193, 4151–4171. https://doi.org/10.1007/s12010-021-03672-z

Ting Wong, C.G., Bottiglieri, T., Snead, O.C., 2003. GABA, gamma-hydroxybutyric acid, and neurological disease. Ann. Neurol. 54, S3–S12. https://doi.org/10.1002/ana.10696

Tofalo, R., Fusco, V., Böhnlein, C., Kabisch, J., Logrieco, A.F., Habermann, D., Cho, G.S., Benomar, N., Abriouel, H., Schmidt-Heydt, M., Neve, H., Bockelmann, W., Franz, C.M.A.P., 2020. The life and times of yeasts in traditional food fermentations. Crit. Rev. Food Sci. Nutr. 60, 3103–3132. https://doi.org/10.1080/10408398.2019.1677553

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). Int. J. Adv. Sci. Eng. Inf. Technol. 5, 103–106. https://doi.org/10.18517/ijaseit.5.2.494

Yang, Y., Lan, G., Tian, X., He, L., Li, C., Zeng, X., Wang, X., 2021. Effect of fermentation

parameters on natto and its thrombolytic property. Foods 10. https://doi.org/10.3390/foods10112547

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). Processes 11. https://doi.org/10.3390/pr11041161

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 Funct. 9, 1638–1646. https://doi.org/10.1039/c7fo01887h

Zhang, L., Hu, Y., Wang, X., Abiola Fakayode, O., Ma, H., Zhou, C., Xia, A., Li, Q., 2021. Improving soaking efficiency of soybeans through sweeping frequency ultrasound assisted by parameters optimization. Ultrason. Sonochem. 79, 105794. https://doi.org/10.1016/j.ultsonch.2021.105794

Zhang, Y.T., Yu, B., Lu, Y.H., Wang, J., Liang, J.B., Tufarelli, V., Laudadio, V., Liao, X.D., 2017. Optimization of the Fermentation Conditions to Reduce Anti-Nutritive Factors in Soybean Meal. J. Food Process. Preserv. 41, 1–7. https://doi.org/10.1111/jfpp.13114

List of publications

Original Paper

Riyanto, R.A., Fukusaki, E., Putri, S.P., 2024. Metabolite profiling highlights the effect of microbial intervention in the soaking step of tempe. *Int. J. Food Sci. Technol*. 59, 7414–7425. https://doi.org/10.1111/ijfs.17481

Conferences

1. Rifqi Ahmad Riyanto, Eiichiro Fukusaki, Sastia Prama Putri. Comparative GCMS-based Metabolomics of *Tempe* Under Chemical and Microbial Intervention During Soaking. Metabolomics Society Annual Conference, Japan (2024). Poster presentation
2. Rifqi Ahmad Riyanto, Eiichiro Fukusaki, Sastia Prama Putri. Metabolome of soybean subjected to microbial intervention-soaking. The Society of Biotechnology Japan (SBJ) 76th Annual Meeting (2024). Oral presentation
3. Rifqi Ahmad Riyanto, Eiichiro Fukusaki, Sastia Prama Putri. The impact of microbial interventions in *Tempe*-soaking step in several bioactive metabolites. Japan Society for Bioscience, Biotechnology, and Agrochemistry (JSBBA) Annual Meeting (2025). Poster presentation

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Supplementary Materials

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

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
1	Lysine	0.134679	Tyramine	0.160738
2	Unknown8	0.133572	Unknown39	0.149477
3	Leucine	0.132833	Xylonic acid	0.142033
4	Genistein	0.131915	Urocanic acid	0.139526
5	Phenylalanine	0.131342	Anthranilic acid	0.136662
6	Proline	0.130932	Cysteine	0.12897
7	Histidine	0.129464	2,6-Diaminopimelic acid	0.127284
8	Unknown43	0.129312	Nicotinic acid	0.12718
9	Cystathionine	0.128417	Aspartic acid	0.127063
10	beta-Lactose	0.126914	Glutamic acid	0.124505
11	Alanine	0.12614	Unknown51	0.124428
12	Unknown30	0.12578	2-Aminoadipic acid	0.119994
13	Valine	0.124967	Unknown20	0.118156
14	Isoleucine	0.124461	Unknown5	0.117509
15	Glycolic acid	0.12374	3-Phosphoglyceric acid	0.108487
16	Daidzein	0.122446	Unknown27	0.106791
17	Tyrosine	0.121276	Unknown31	0.0993099
18	Ribose	0.12041	Oxalic acid	0.0992345
19	Unknown37	0.12003	Mannitol	0.0982321
20	allantoic acid	0.119751	beta-Alanine	0.0860208
21	Glyoxylic acid	0.119244	3-Phenyllactic acid	0.0780329
22	Unknown41	0.118636	Uracil	0.0756983
23	Glyceric acid	0.117199	Melezitose	0.0752395
24	Unknown45	0.116716	Thymine	0.0729226
25	3-Hydroxy-3-Methylglutaric acid	0.116214	Unknown30	0.0708098
26	Unknown15	0.115977	Unknown12	0.0676225
27	Unknown9	0.115958	TDP-glucose	0.0674483
28	Tryptophan	0.114823	2-Aminoethanol	0.0660906
29	Unknown47	0.114001	Threitol	0.0649448
30	Threonine	0.112093	Unknown13	0.06285
31	Unknown17	0.108947	Meso erythritol	0.0614576
32	Linoleic acid	0.108898	Fumaric acid	0.0611907

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
33	Unknown40	0.107618	3-Hydroxy-3-methylbutanoic acid	0.0583775
34	Asparagine	0.107109	Unknown24	0.0571923
35	Uric acid	0.106471	3-α-Mannobiose	0.0536906
36	3-Hydroxy-3-methylbutanoic acid	0.106263	Unknown22	0.0533261
37	Unknown28	0.106063	Linoleic acid	0.047644
38	Unknown42	0.105757	Unknown34	0.0445721
39	2-Hydroxypyridine	0.104235	palmitic acid	0.0433434
40	Unknown1	0.103899	Genistein	0.0342252
41	Xanthine	0.103829	Malonic acid	0.0298603
42	Unknown46	0.103621	Unknown21	0.0296478
43	Threitol	0.102815	Xanthine	0.0287221
44	Unknown19	0.102504	Unknown52	0.0279638
45	Unknown10	0.101584	Melibiose	0.0241973
46	Ornithine	0.101486	Ornithine	0.0214323
47	Unknown2	0.100073	Unknown32	0.0204294
48	Glycine	0.0978005	Proline	0.01927
49	2,6-Pyridinedicarboxylic acid	0.0971515	3-Hydroxy butyrate	0.0182508
50	Melibiose	0.0971137	Glycerol	0.0164752
51	Unknown4	0.0962338	Unknown26	0.0153979
52	Unknown26	0.0944447	2-Aminobutyric acid	0.0149775
53	3-Hydroxy butyrate	0.0935526	Unknown8	0.0130578
54	2-Aminobutyric acid	0.0931187	Histidine	0.0124186
55	Glucono-1,5-lactone	0.0927482	Malic acid	0.0122538
56	2-Aminoadipic acid	0.0915551	Panose	0.0121692
57	Aspartic acid	0.0911807	Unknown44	0.0111249
58	Unknown20	0.0907765	Unknown36	0.0111234
59	Glutamic acid	0.0889521	Trehalose	0.00904727
60	2-hydroxyglutaric acid	0.0888532	Unknown18	0.00888382
61	Unknown5	0.0885188	Glycine	0.00680186
62	Unknown25	0.0884603	Glyceric acid	0.00646517
63	Succinic acid	0.0881738	Unknown46	0.00423347
64	Glycerol	0.0855702	Daidzein	0.00386855
65	Oxalic acid	0.0827677	Unknown47	0.00375851
66	Methionine	0.0825251	Pentasiloxane	0.00317067
67	beta-Alanine	0.079495	Unknown17	0.00224701
68	Unknown23	0.0793136	Unknown41	8.11E-05
69	Unknown34	0.0786397	Unknown38	-0.0054527
70	Lyxose	0.0773875	Glucose	-0.00732686

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
71	Adenosine	0.0759124	Phosphate	-0.00817891
72	Urocanic acid	0.0739165	Succinic acid	-0.0113735
73	Unknown50	0.0727298	Leucine	-0.012662
74	Unknown24	0.0720451	Methionine	-0.0140928
75	Allothreonine	0.0701855	Lysine	-0.014539
76	3-Phosphoglyceric acid	0.0697616	Ethyl-α-D-glucopyranoside	-0.0152694
77	4-Hydroxyphenylacetic acid	0.0694883	Adenosine	-0.0186535
78	Unknown18	0.0657475	Saccharic acid	-0.0208177
79	3-Phenyllactic acid	0.0649737	Asparagine	-0.0213877
80	Putrescine	0.0634808	3-Hydroxy-3-Methylglutaric acid	-0.0218919
81	Thymine	0.0630532	Phenylalanine	-0.0230621
82	Unknown6	0.0628284	2-hydroxyglutaric acid	-0.023283
83	Uracil	0.0617339	Unknown16	-0.0323439
84	Unknown29	0.0573501	Unknown43	-0.0324209
85	Mannitol	0.0567286	Maltitol	-0.0327822
86	Cysteine	0.0564244	Cystathionine	-0.0334623
87	3-Hydroxyanthranilic acid	0.0560909	Ribose	-0.0340253
88	Stearic acid	0.0553315	Sucrose	-0.0347845
89	Unknown39	0.0518313	Unknown4	-0.0354789
90	Meso erythritol	0.0505929	Allothreonine	-0.0396674
91	Phosphate	0.0466029	Lyxose	-0.0399642
92	Tyramine	0.0454588	beta-Lactose	-0.0409344
93	Malonic acid	0.045286	Glucono-1,5-lactone	-0.0468681
94	Unknown12	0.0452469	Alanine	-0.0474624
95	2,3-Butanediol	0.0429474	Unknown19	-0.0475136
96	2-Aminoethanol	0.0414244	Unknown29	-0.0487971
97	Fumaric acid	0.0380432	Putrescine	-0.0493228
98	Glucose	0.0379128	Serine	-0.0502999
99	Unknown21	0.036743	Unknown37	-0.0520557
100	Nicotinic acid	0.0353863	Stearic acid	-0.0524914
101	Xylonic acid	0.0350728	Valine	-0.0532667
102	Anthranilic acid	0.0346156	Isoleucine	-0.0547115
103	Unknown51	0.0343039	Unknown45	-0.0561003
104	2,6-Diaminopimelic acid	0.0331667	Isocitric acid	-0.0607368
105	Unknown7	0.0321626	2,6-Pyridinedicarboxylic acid	-0.0613726
106	Unknown35	0.0305634	Sorbitol	-0.0627489
107	Unknown11	0.0266333	3-Hydroxyanthranilic acid	-0.0646104
108	Unknown27	0.0234427	Glycolic acid	-0.0649231

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
109	Trehalose	0.020384	Unknown3	-0.0663007
110	Unknown38	0.0191605	Unknown15	-0.0697249
111	Unknown31	0.018219	allantoic acid	-0.0699315
112	Unknown33	0.0174915	Tyrosine	-0.0710381
113	Adenine	0.0145429	Glyoxylic acid	-0.0717674
114	4-Aminobutyric acid	0.0130689	Tryptophan	-0.0725533
115	Melezitose	0.012954	Unknown1	-0.0739752
116	TDP-glucose	0.0113863	Unknown28	-0.0753839
117	Unknown52	0.00876429	Threonine	-0.0778892
118	palmitic acid	0.00812056	Pinitol	-0.0797289
119	Inositol	0.00753706	Unknown40	-0.0804676
120	Unknown3	0.00751432	Unknown2	-0.0805445
121	Unknown13	0.00456166	Unknown9	-0.0815508
122	Unknown32	0.00344388	2-Hydroxypyridine	-0.0843696
123	Unknown22	0.00216103	Adenine	-0.0879047
124	3-α-Mannobiose	0.00104667	Raffinose	-0.0921496
125	Malic acid	-3.25E-05	Unknown48	-0.0942818
126	Turanose	-0.00149025	Unknown42	-0.0982019
127	Unknown44	-0.0018988	Uric acid	-0.10223
128	Serine	-0.00643984	Unknown25	-0.103178
129	N-acetyl- α-D-glucosamine 1-phosphate	-0.00819539	Unknown49	-0.10499
130	Maltitol	-0.010812	Inositol	-0.105317
131	Sorbitol	-0.0109239	4-Hydroxyphenylacetic acid	-0.106682
132	Guanine	-0.0146718	Unknown10	-0.106841
133	Lactic acid	-0.0171835	Unknown11	-0.107628
134	Unknown16	-0.0177351	Unknown7	-0.110647
135	Unknown14	-0.018546	Unknown50	-0.112292
136	Isocitric acid	-0.0195307	Turanose	-0.117735
137	Galactose	-0.0200794	Unknown53	-0.119754
138	Pentasiloxane	-0.0236032	2,3-Butanediol	-0.120465
139	Fructose	-0.0238396	Myo-Inositol	-0.120531
140	Panose	-0.0254302	Unknown14	-0.122981
141	Unknown48	-0.0277445	4-Aminobutyric acid	-0.129476
142	Citric acid	-0.0327531	Galactinol	-0.129913
143	Saccharic acid	-0.0377748	Galactose	-0.131977
144	Unknown53	-0.0386776	Fructose	-0.13334
145	D-Glucopyranoside	-0.0419459	Guanine	-0.134447
146	Galactinol	-0.0488156	Unknown6	-0.137158

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
147	Myo-Inositol	-0.0563169	Lactic acid	-0.141595
148	Ethyl- α-D-glucopyranoside	-0.0579331	Citric acid	-0.144695
149	Sucrose	-0.0665042	D-Glucopyranoside	-0.146575
150	Unknown49	-0.08085	N-acetyl- α-D-glucosamine 1-phosphate	-0.147648
151	Unknown36	-0.0874735	Unknown23	-0.148278
152	Raffinose	-0.107858	Unknown33	-0.148286
153	Pinitol	-0.118466	Unknown35	-0.15078

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

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
1	Pinitol	0.105462	Unknown39	0.187104
2	Raffinose	0.0962738	Sorbitol	0.173888
3	3-α-Mannobiose	0.0319558	Mannitol	0.171217
4	Unknown13	0.0268505	Xylonic acid	0.169516
5	Sucrose	0.0245538	Cysteine	0.166006
6	Unknown44	0.0236983	Glutamic acid	0.164647
7	Unknown22	0.0225126	Aspartic acid	0.164277
8	Maltitol	0.0218302	2-Aminoadipic acid	0.160257
9	Unknown36	0.0216763	Isocitric acid	0.159312
10	Unknown27	0.0214856	2,6-Diaminopimelic acid	0.159286
11	Malic acid	0.0207972	Unknown20	0.156865
12	Melezitose	0.0195414	Nicotinic acid	0.156474
13	Unknown32	0.0169973	Unknown51	0.156348
14	palmitic acid	0.0153496	Unknown5	0.156017
15	Unknown52	0.0146392	Unknown31	0.155964
16	Unknown49	0.00868004	Urocanic acid	0.15473
17	Unknown48	0.00740396	Tyramine	0.152072
18	D-Glucopyranoside	0.00582999	Unknown18	0.151797
19	Unknown38	0.00574075	3-Phenyllactic acid	0.146096
20	Anthranilic acid	0.00488062	Unknown24	0.142019
21	Trehalose	0.00453416	Pentasiloxane	0.1394
22	TDP-glucose	0.00419326	3-Phosphoglyceric acid	0.129268
23	Panose	0.00295885	Anthranilic acid	0.128568

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
24	Tyramine	-0.00108441	beta-Alanine	0.128353
25	Xylonic acid	-0.00579537	Threitol	0.126024
26	Unknown31	-0.00994408	TDP-glucose	0.116534
27	Adenine	-0.0111844	Citric acid	0.112572
28	Galactinol	-0.0113054	3-Hydroxy-3-methylbutanoic acid	0.112447
29	Malonic acid	-0.0129651	Oxalic acid	0.110849
30	2,6-Diaminopimelic acid	-0.0166245	Panose	0.109215
31	Inositol	-0.0171474	Unknown30	0.0967092
32	Nicotinic acid	-0.0172053	Unknown14	0.0954353
33	Unknown51	-0.0179325	2-Aminoethanol	0.0945562
34	Phosphate	-0.0198415	Fumaric acid	0.0749208
35	3-Phosphoglyceric acid	-0.0199435	Meso erythritol	0.0713691
36	Meso erythritol	-0.0210781	Guanine	0.0685817
37	Unknown21	-0.0211404	Daidzein	0.0669938
38	Fumaric acid	-0.0215997	Unknown12	0.0658918
39	Myo-Inositol	-0.022753	Uracil	0.0646473
40	Unknown12	-0.0252587	Thymine	0.0606469
41	Fructose	-0.0268233	Unknown27	0.0592069
42	Sorbitol	-0.0272385	Melezitose	0.0591896
43	Unknown39	-0.027925	Unknown17	0.0578132
44	Uracil	-0.0280248	Genistein	0.0567925
45	Thymine	-0.0308429	Galactose	0.0538605
46	Cysteine	-0.0356711	Unknown36	0.0519526
47	Urocanic acid	-0.0373689	Unknown34	0.0484503
48	3-Phenyllactic acid	-0.0406865	Glucose	0.0430333
49	Glycerol	-0.0415358	Ribose	0.0426534
50	3-Hydroxyanthranilic acid	-0.0424023	Turanose	0.0401802
51	Unknown29	-0.0431805	Myo-Inositol	0.0401009
52	Glucose	-0.0440893	Proline	0.0355364
53	Oxalic acid	-0.0460022	Unknown48	0.0338745
54	Stearic acid	-0.0481675	2-Aminobutyric acid	0.033409
55	2,3-Butanediol	-0.0532611	N-acetyl- α-D-glucosamine 1-phosphate	0.0314877
56	Mannitol	-0.0534065	Unknown13	0.0311817
57	Unknown34	-0.0537354	3-Hydroxy butyrate	0.0302747
58	Allothreonine	-0.0575567	Glucono-1,5-lactone	0.0299606

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
59	beta-Alanine	-0.0581989	Unknown21	0.028113
60	Melibiose	-0.0587861	Putrescine	0.026985
61	Lyxose	-0.0600158	Raffinose	0.0264256
62	Isocitric acid	-0.0600382	Xanthine	0.0260891
63	4-Aminobutyric acid	-0.060356	Unknown49	0.0255622
64	Putrescine	-0.0609575	palmitic acid	0.0240206
65	Pentasiloxane	-0.0616969	Ornithine	0.0228542
66	Succinic acid	-0.0625851	Histidine	0.0218757
67	Unknown5	-0.0630923	Unknown22	0.0208302
68	Glutamic acid	-0.0636943	Linoleic acid	0.0207541
69	Aspartic acid	-0.0640399	Glyceric acid	0.0194438
70	Adenosine	-0.0644789	Unknown41	0.0162645
71	Unknown20	-0.0667751	Asparagine	0.0147248
72	2-Aminoadipic acid	-0.0670533	Unknown8	0.0108571
73	Methionine	-0.0671614	2-hydroxyglutaric acid	0.00872402
74	Unknown26	-0.0697828	Unknown50	0.00654391
75	Lactic acid	-0.070242	Stearic acid	0.00653944
76	Unknown53	-0.070697	Unknown35	0.00585237
77	Glycine	-0.0728537	Unknown47	0.00128037
78	4-Hydroxyphenylacetic acid	-0.0741045	4-Aminobutyric acid	-0.000578219
79	Unknown18	-0.0741067	Unknown11	-0.00077947
80	Unknown24	-0.0741123	Fructose	-0.00119659
81	2-Aminobutyric acid	-0.0765591	Unknown26	-0.00221289
82	3-Hydroxy butyrate	-0.0778824	Unknown3	-0.00337648
83	Unknown16	-0.0779243	Glycolic acid	-0.00623819
84	2-Aminoethanol	-0.0802623	Leucine	-0.00736109
85	Linoleic acid	-0.0808669	Unknown4	-0.00998097
86	Saccharic acid	-0.0828886	3-α-Mannobiose	-0.0130356
87	Ornithine	-0.0830624	Maltitol	-0.014852
88	Xanthine	-0.0831657	Adenosine	-0.0148621
89	Unknown46	-0.0834606	Lysine	-0.016654
90	2,6-Pyridinedicarboxylic acid	-0.0847567	Methionine	-0.0175516
91	Threitol	-0.0891537	Unknown52	-0.0191783
92	Citric acid	-0.0915994	Phosphate	-0.0195367
93	Glucono-1,5-lactone	-0.0919504	Malonic acid	-0.0206606
94	Unknown25	-0.0937235	Glycerol	-0.0219219
95	Unknown14	-0.0941755	Sucrose	-0.0237603
96	3-Hydroxy-3-methylbutanoic acid	-0.0960945	Unknown44	-0.0240113

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
97	3-Hydroxy-3-Methylglutaric acid	-0.0968249	Unknown37	-0.0254118
98	Unknown7	-0.0972352	Unknown29	-0.0255383
99	Ethyl- α-D-glucopyranoside	-0.0973831	Phenylalanine	-0.0267962
100	Unknown47	-0.0978522	Malic acid	-0.0268508
101	2-Hydroxypyridine	-0.0983935	Serine	-0.0287038
102	Unknown17	-0.0994303	D-Glucopyranoside	-0.0294004
103	Unknown1	-0.10082	Unknown32	-0.0300801
104	Unknown40	-0.101143	Cystathionine	-0.0322351
105	Threonine	-0.101194	Lyxose	-0.0340042
106	Guanine	-0.10206	Unknown43	-0.0357785
107	2-hydroxyglutaric acid	-0.102192	Trehalose	-0.0370219
108	Asparagine	-0.102874	Pinitol	-0.0370768
109	Unknown10	-0.103891	Unknown19	-0.0406183
110	Glyceric acid	-0.104284	Melibiose	-0.0406505
111	Unknown2	-0.104706	Unknown38	-0.0417535
112	Unknown4	-0.105214	Adenine	-0.0421589
113	Unknown19	-0.105546	Glyoxylic acid	-0.0422157
114	Tryptophan	-0.105739	Unknown46	-0.0426036
115	Unknown28	-0.105888	Glycine	-0.0432753
116	Unknown23	-0.107506	Inositol	-0.0433623
117	Unknown30	-0.1081	Uric acid	-0.0449355
118	Unknown41	-0.109143	Allothreonine	-0.0489791
119	Unknown33	-0.110551	Alanine	-0.0507883
120	Unknown9	-0.111028	beta-Lactose	-0.0561878
121	Unknown6	-0.113033	Unknown25	-0.056441
122	Serine	-0.113368	Saccharic acid	-0.0577118
123	Unknown15	-0.114161	Galactinol	-0.0582733
124	Unknown42	-0.114273	Isoleucine	-0.0597571
125	allantoic acid	-0.114646	Unknown15	-0.0602517
126	Daidzein	-0.114725	Unknown2	-0.0623549
127	Unknown3	-0.114804	Unknown53	-0.0652512
128	Tyrosine	-0.115896	2,3-Butanediol	-0.0653152
129	Uric acid	-0.116775	4-Hydroxyphenylacetic acid	-0.0656441
130	Unknown50	-0.116952	Unknown6	-0.065984
131	beta-Lactose	-0.117164	Unknown1	-0.0667274
132	Valine	-0.117578	Unknown45	-0.0670431
133	Unknown37	-0.117651	Ethyl- α-D-glucopyranoside	-0.0679513

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
134	Turanose	-0.118143	Unknown42	-0.0683455
135	Isoleucine	-0.118149	Valine	-0.0690063
136	Unknown11	-0.118458	Succinic acid	-0.0718512
137	Genistein	-0.118535	Lactic acid	-0.0732177
138	Histidine	-0.118785	allantoic acid	-0.0748548
139	Galactose	-0.119111	Tyrosine	-0.0759934
140	Unknown45	-0.119138	3-Hydroxy-3-Methylglutaric acid	-0.0774019
141	Alanine	-0.119723	Unknown10	-0.0801571
142	Glyoxylic acid	-0.120516	3-Hydroxyanthranilic acid	-0.0814886
143	Cystathionine	-0.120694	Unknown23	-0.0815529
144	Proline	-0.120797	Unknown33	-0.0815773
145	Unknown43	-0.120849	Unknown28	-0.082413
146	Ribose	-0.121367	Tryptophan	-0.0881163
147	N-acetyl- α-D-glucosamine 1-phosphate	-0.122027	Unknown40	-0.0881845
148	Glycolic acid	-0.122596	Unknown9	-0.0884373
149	Unknown35	-0.123024	2,6-Pyridinedicarboxylic acid	-0.0914713
150	Unknown8	-0.123242	Unknown7	-0.0923016
151	Phenylalanine	-0.123482	Threonine	-0.0967774
152	Leucine	-0.125361	2-Hydroxypyridine	-0.103692
153	Lysine	-0.126956	Unknown16	-0.10755

Table S3. List of metabolites in volcano plot comparing raw soybean and lactic acid bacteria-soaked soybean tempe, which were significantly different based on *t*-test (*p*-value less than 0.05)

Fold Change 2 and more				
Number	Metabolite	Fold Change (FC)	log2FC	Minus log10 *p* value
1	Trehalose	1.07E+08	26.68	1.62
2	Maltitol	1.11E+05	16.76	1.39
3	Glycerol	4.12E+01	5.37	1.45
4	Glycine	3.14E+07	24.90	2.20

5	Malic acid	1.41E+06	20.43	2.05
6	beta-Lactose	2.09E+02	7.70	1.90
7	palmitic acid	7.48E+05	19.51	2.16
8	Phosphate	2.70E+06	21.37	2.83
9	Malonic acid	7.21E+05	19.46	2.42
10	Melibiose	4.59E+01	5.52	1.80
11	N-acetyl-alpha-D-glucosamine 1-phosphate	3.35E+00	1.75	3.19
12	Citric acid	8.66E+04	16.40	2.21
13	4-Aminobutyric acid	2.44E+06	21.22	2.53
14	Succinic acid	4.33E+07	25.37	2.47
15	beta-Alanine	1.21E+01	3.60	2.54
16	Fructose	1.43E+05	17.13	1.86
17	Xanthine	2.88E+03	11.49	2.30
18	2-hydroxyglutaric acid	2.30E+01	4.52	1.66
19	Sucrose	6.04E+02	9.24	1.51
20	Uracil	4.54E+07	25.44	1.97
21	Sorbitol	6.07E+04	15.89	4.34
22	Glyoxylic acid	9.35E+06	23.16	2.77
23	Lyxose	8.91E+06	23.09	2.07
24	Stearic acid	5.86E+05	19.16	2.37
25	2-Hydroxypyridine	4.70E+08	28.81	2.31
26	Galactinol	2.35E+00	1.23	1.40
27	allantoic acid	1.99E+08	27.57	2.46
28	Melezitose	4.76E+05	18.86	2.56
29	Glucono-1,5-lactone	5.35E+05	19.03	2.32
30	2-Aminoadipic acid	8.71E+02	9.77	2.79
31	Putrescine	5.14E+06	22.29	1.57
32	Panose	1.32E+05	17.01	1.79
33	3-Hydroxy butyrate	1.82E+02	7.51	2.28
34	3-Phenyllactic acid	1.23E+07	23.55	1.71
35	Thymine	7.35E+07	26.13	1.84
36	Saccharic acid	9.91E+05	19.92	3.04
37	Genistein	9.12E+01	6.51	2.93
38	Linoleic acid	7.37E+01	6.20	3.06
39	Glycolic acid	4.28E+01	5.42	2.15
40	Turanose	1.60E+07	23.93	1.94
41	Tyrosine	2.69E+03	11.40	2.42
42	Threitol	9.71E+01	6.60	1.99
43	Tryptophan	6.40E+01	6.00	1.97
44	Galactose	7.29E+05	19.48	2.26
45	TDP-glucose	1.40E+05	17.10	2.12
46	Daidzein	1.71E+01	4.10	2.26
47	Fumaric acid	3.05E+06	21.54	2.34
48	Xylonic acid	3.65E+05	18.48	2.83
49	Isocitric acid	1.63E+05	17.32	2.42
50	Threonine	2.09E+02	7.71	1.87
51	Cystathionine	1.58E+02	7.30	2.56

52	Lysine	2.25E+03	11.14	2.34
53	Oxalic acid	8.80E+00	3.14	2.50
54	Nicotinic acid	3.51E+06	21.74	2.03
55	Ribose	7.95E+01	6.31	2.13
56	Valine	4.31E+02	8.75	2.04
57	Uric acid	1.95E+07	24.22	2.25
58	Ornithine	2.64E+03	11.37	2.66
59	3-Hydroxy-3-Methylglutaric acid	1.34E+01	3.75	2.85
60	Glyceric acid	2.30E+02	7.84	1.43
61	Phenylalanine	4.57E+03	12.16	2.00
62	Isoleucine	2.40E+03	11.23	2.02
63	2,6-Diaminopimelic acid	5.36E+02	9.07	2.11
64	Mannitol	1.17E+05	16.84	1.36
65	Meso erythritol	3.05E+06	21.54	2.42
66	Adenine	2.02E+06	20.95	2.55
67	Cysteine	8.77E+02	9.78	2.69
68	Methionine	3.15E+03	11.62	2.17
69	Guanine	9.02E+00	3.17	1.53
70	Leucine	5.63E+03	12.46	2.16
71	Serine	8.13E+06	22.95	1.58
72	Aspartic acid	7.61E+01	6.25	2.58
73	Histidine	5.68E+03	12.47	3.26
74	Alanine	6.26E+02	9.29	2.88
75	3-Hydroxy-3-methylbutanoic acid	2.75E+06	21.39	1.88
76	Glutamic acid	5.53E+02	9.11	2.88
77	Proline	3.16E+03	11.63	2.08
78	Lactose	5.72E+05	19.12	1.50
79	Pentasiloxane	5.38E+05	19.04	2.91
80	Ethyl .alpha.-D-glucopyranoside	1.43E+06	20.44	2.00

Fold Change less than 0.5

Number	Metabolite	Fold Change (FC)	log2FC	Minus log10 *p* value
1	Raffinose	1.17E-02	-6.41	2.17
2	Pinitol	4.72E-02	-4.40	3.96

Not significant

Number	Metabolite	Fold Change (FC)	log2FC	Minus log10 *p* value
1	Tyramine	4.55E+00	4.55	0.74
2	Inositol	1.88E+01	18.80	0.99
3	Lactic acid	4.68E+00	4.68	1.06

4	Allothreonine	8.25E+00	8.25	0.70
5	Glucose	2.15E+01	21.54	0.74
6	2-Aminoethanol	1.70E+00	1.70	0.97
7	Urocanic acid	6.41E+00	6.41	1.18
8	Asparagine	2.28E+01	22.82	1.13
9	Anthranilic acid	8.66E-01	0.87	1.03
10	2-Aminobutyric acid	6.44E+00	6.44	0.77
11	3-Phosphoglyceric acid	5.46E+00	5.46	0.76
12	2,6-Pyridinedicarboxylic acid	9.32E+00	9.32	1.29
13	Adenosine	1.87E+01	18.74	1.28
14	4-Hydroxyphenylacetic acid	4.09E+00	4.09	1.00
15	3-Hydroxyanthranilic acid	8.16E+00	8.16	0.87
16	3-.alpha.-Mannobiose	2.01E+01	20.10	1.21
17	2,3-Butanediol	9.38E+00	9.38	0.82
18	Myo-Inositol	4.80E-01	0.48	0.65

Table S4. List of metabolites in volcano plot comparing raw soybean and yeast-soaked soybean tempe, which were significantly different based on *t*-test (*p*-value less than 0.05)

Fold Change 2 and more				
Number	Metabolite	Fold Change (FC)	log2FC	Minus log10 *p* value
1	Trehalose	3.84E+07	25.20	1.42
2	Tyramine	2.80E+03	11.45	1.36
3	Maltitol	1.08E+05	16.72	1.32
4	Glycerol	3.08E+01	4.94	1.83
5	Inositol	1.19E+05	16.86	1.45
6	Glycine	1.38E+07	23.72	2.32
7	Malic acid	9.99E+05	19.93	1.68
8	beta-Lactose	7.31E+01	6.19	2.00
9	palmitic acid	6.53E+05	19.32	2.45
10	Phosphate	1.58E+06	20.60	3.82
11	Malonic acid	5.70E+05	19.12	3.00
12	Melibiose	2.92E+01	4.87	2.17
13	Citric acid	1.23E+05	16.90	1.53
14	4-Aminobutyric acid	1.07E+06	20.03	2.01
15	Succinic acid	1.17E+07	23.47	1.80
16	Fructose	4.40E+04	15.42	1.91
17	Allothreonine	5.16E+01	5.69	1.62
18	2-hydroxyglutaric acid	1.92E+01	4.26	1.87
19	Uracil	4.77E+07	25.51	1.54
20	Glucose	4.11E+06	21.97	1.63
21	Glyoxylic acid	4.15E+06	21.99	1.64

22	Lyxose	1.59E+06	20.60	1.55
23	Stearic acid	4.56E+05	18.80	2.75
24	2-Aminoethanol	4.31E+00	2.11	1.79
25	allantoic acid	3.52E+07	25.07	1.36
26	2-Aminoadipic acid	1.92E+03	10.91	1.31
27	3-Phenyllactic acid	2.61E+07	24.64	1.35
28	Saccharic acid	4.40E+05	18.75	2.60
29	Genistein	8.54E+01	6.42	3.00
30	Linoleic acid	5.85E+01	5.87	2.24
31	Glycolic acid	2.47E+01	4.63	2.45
32	Turanose	1.19E+07	23.51	1.93
33	Urocanic acid	4.65E+02	8.86	2.07
34	Threitol	1.44E+02	7.17	1.44
35	Galactose	6.30E+05	19.26	2.39
36	Daidzein	1.70E+01	4.09	3.02
37	Isocitric acid	4.63E+05	18.82	1.37
38	Threonine	2.54E+01	4.67	1.57
39	Cystathionine	8.39E+01	6.39	1.49
40	Lysine	1.18E+03	10.20	2.62
41	Oxalic acid	1.79E+01	4.16	2.14
42	Asparagine	4.94E+06	22.24	2.10
43	Anthranilic acid	5.11E+01	5.68	1.66
44	Ribose	5.98E+01	5.90	1.91
45	2-Aminobutyric acid	8.66E+01	6.44	2.44
46	Valine	1.22E+02	6.93	1.68
47	3-Phosphoglyceric acid	1.22E+02	6.93	2.22
48	Uric acid	6.19E+06	22.56	2.22
49	3-Hydroxy-3-Methylglutaric acid	5.98E+00	2.58	2.48
50	Phenylalanine	2.18E+03	11.09	2.34
51	Isoleucine	6.71E+02	9.39	2.06
52	Adenine	7.10E+05	19.44	2.25
53	Guanine	8.75E+00	3.13	2.21
54	Leucine	3.05E+03	11.58	3.07
55	Serine	2.68E+06	21.35	1.51
56	Aspartic acid	1.96E+02	7.62	1.52
57	Histidine	4.60E+03	12.17	1.66
58	Alanine	2.13E+02	7.73	1.64
59	3-Hydroxy-3-methylbutanoic acid	3.54E+06	21.76	1.43
60	Glutamic acid	1.52E+03	10.57	1.60
61	Proline	2.55E+03	11.32	2.23
62	Lactose	2.84E+06	21.44	1.87
63	3-.alpha.-Mannobiose	2.66E+06	21.34	2.24
64	2,3-Butanediol	1.52E+01	3.93	1.52
65	Pentasiloxane	9.96E+05	19.93	1.43
66	Ethyl .alpha.-D-glucopyranoside	5.79E+05	19.14	1.58

Fold Change less than 0.5		
Number	Metabolite	log2FC

		Fold Change (FC)		Minus log10 *p* value
1	Raffinose	1.72E-01	-2.54	2.01
2	Pinitol	4.72E-02	-4.40	3.25
3	2,6-Pyridinedicarboxylic acid	6.32E-04	-10.63	1.86
4	3-Hydroxyanthranilic acid	1.76E-03	-9.15	1.69
Not significant				
Number	Metabolite	Fold Change (FC)	log2FC	Minus log10 *p* value
1	Lactic acid	3.08E+00	1.62	1.16
2	beta-Alanine	1.78E+01	4.16	0.92
3	Xanthine	3.54E+03	11.79	0.99
4	Sucrose	9.68E+02	9.92	1.08
5	Sorbitol	2.52E+05	17.94	1.01
6	2-Hydroxypyridine	8.02E+06	22.94	1.19
7	Galactinol	9.45E-01	-0.08	0.32
8	Melezitose	2.01E+06	20.94	1.09
9	Glucono-1,5-lactone	6.65E+05	19.34	1.15
10	Putrescine	4.86E+06	22.21	0.88
11	Panose	3.97E+05	18.60	1.03
12	3-Hydroxy butyrate	2.35E+02	7.88	0.93
13	Thymine	1.48E+08	27.14	0.84
14	Tyrosine	5.63E+02	9.14	1.10
15	Tryptophan	1.27E+01	3.67	1.20
16	TDP-glucose	9.46E+05	19.85	0.96
17	Fumaric acid	3.04E+07	24.86	0.70
18	Xylonic acid	4.56E+06	22.12	0.92
19	Nicotinic acid	4.07E+07	25.28	0.71
20	Ornithine	2.88E+03	11.49	0.89
21	Glyceric acid	1.21E+02	6.92	1.26
22	2,6-Diaminopimelic acid	1.30E+04	13.67	0.71
23	Mannitol	4.01E+05	18.62	1.30
24	Meso erythritol	3.54E+06	21.76	1.30
25	Cysteine	3.78E+03	11.89	0.90
26	Methionine	3.29E+03	11.69	0.67
27	Adenosine	4.84E+05	18.89	0.68
28	4-Hydroxyphenylacetic acid	4.01E+00	2.00	0.57
29	Myo-Inositol	1.22E+00	0.28	0.65
30	N-acetyl-alpha-D-glucosamine 1-phosphate	2.77E+00	1.47	2.67

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

ID	Metabolite name	t_R (min)[a]	RI[b]	Quant mass (*m/z*)	Similarity (%)	Library[c]
1	2,6-Diaminopimelic acid	15.807	2014.53	200.063	90.5	in-house
2	2,6-Pyridinedicarboxylic acid	13.305	1768.25	296.025	94.6	in-house
3	2-Aminoadipic acid	12.928	1733.5	260.08	90.9	in-house
4	2-Aminobutyric acid	5.969	1181.43	130.085	89.8	in-house
5	2-Aminoethanol	7.295	1276.5	174.09	99.5	in-house
6	2-hydroxyglutaric acid	11.31	1590.5	129.086	93.7	in-house
7	2-Hydroxypyridine	4.021	1038.75	117.1	83.8	in-house
8	2,3-Butanediol	4.137	1047.59	117.1	98.1	NIST11
9	3-alpha-Mannobiose	30.086	3965.46	205.05	81.2	in-house
10	3-Hydroxy butyrate	5.780	1167.95	147.131	93.6	in-house
11	3-Hydroxy-3-methylbutanoic acid	6.449	1215.73	131.140	89.4	in-house
12	3-Hydroxy-3-Methylglutaric acid	11.635	1618.52	147.112	94.4	in-house
13	3-Hydroxyanthranilic acid	14.563	1888.47	354.069	91.6	in-house
14	3-Phenyllactic acid	11.381	1596.39	193.073	91.6	in-house
15	3-Phosphoglyceric acid	14.009	1834.89	299.050	88.8	in-house
16	4-Aminobutyric acid	10.729	1542.11	174.100	98.5	in-house
17	4-Hydroxyphenylacetic acid	11.896	1641.36	179.100	85.9	in-house
18	Adenine	14.457	1878.20	264.050	82.3	in-house
19	Adenosine	21.295	2667.09	230.038	85.5	in-house
20	Alanine	4.962	1109.76	116.102	96.7	in-house
21	Allantoic acid	6.808	1241.55	171.068	98.7	in-house
22	Allothreonine	8.945	1399.10	117.083	82.1	in-house
23	Anthranilic acid	11.700	1624.16	266.063	92.2	in-house
24	Asparagine	14.563	1888.51	204.075	95.8	in-house
25	Aspartic acid	10.664	1536.71	232.072	99.2	in-house
26	beta-Alanine	9.439	1438.11	174.104	96.7	in-house
27	beta-Lactose	22.159	2785.57	204.038	97	in-house
28	Citric acid	14.134	1846.98	273.055	98.1	in-house
29	Cystathionine	17.869	2240.62	128.090	88.1	in-house
30	Cysteine	11.071	1570.58	220.038	90	in-house
31	Daidzein	23.286	2948.05	398.061	84.7	in-house
32	D-Glucopyranoside	14.225	1855.81	204.043	95	NIST11
33	Ethyl-alpha-D-glucopyranoside	15.399	1972.55	204.028	90	NIST11
34	Fructose	14.796	1911.50	103.088	99.5	in-house
35	Fumaric acid	8.331	1353.08	245.015	97.7	in-house
36	Galactinol	24.128	3075.01	204.034	91.8	in-house
37	Galactose	15.205	1952.97	147.112	99.4	in-house
38	Genistein	23.487	2977.89	471.075	85.4	in-house
39	Glucono-1,5-lactone	16.094	2045.03	147.098	91.2	in-house
40	Glucose	15.246	1957.07	147.100	98.9	in-house
41	Glutamic acid	11.831	1635.65	246.069	99.6	in-house

42	Glyceric acid	8.250	1347.04	147.136	88.7	in-house
43	Glycerol	7.459	1288.32	147.107	99.4	in-house
44	Glycine	7.845	1316.71	174.100	99.4	in-house
45	Glycolic acid	4.578	1081.20	147.129	88.9	in-house
46	Glyoxylic acid	15.256	1958.06	147.117	86.5	in-house
47	Guanine	17.029	2146.10	352.075	84.3	in-house
48	Histidine	15.078	1940.11	154.107	93.4	in-house
49	Inositol	16.898	2131.62	217.051	99	in-house
50	Isocitric acid	14.146	1848.07	245.027	81.8	in-house
51	Isoleucine	7.680	1304.31	158.153	84.2	in-house
52	Lactic acid	4.385	1066.44	147.103	99.9	in-house
53	Leucine	7.378	1282.48	158.153	93.2	in-house
54	Linoleic acid	17.640	2214.29	95.081	93.1	in-house
55	Lysine	15.098	1942.08	174.089	99.2	in-house
56	Lyxose	12.503	1694.56	103.058	93	in-house
57	Malic acid	10.281	1504.80	147.102	99.4	in-house
58	Malonic acid	6.394	1211.79	147.110	96.3	in-house
59	Maltitol	23.098	2920.19	204.034	86.3	in-house
60	Mannitol	15.379	1970.56	319.125	88	in-house
61	Melezitose	27.282	3597.53	361.100	94.2	in-house
62	Melibiose	23.308	2951.36	204.044	98.3	in-house
63	Meso erythritol	10.621	1533.11	217.068	89.3	in-house
64	Methionine	10.588	1530.37	176.071	96.9	in-house
65	Myo-Inositol	15.625	1995.42	318.113	90	NIST11
66	N-acetyl-alpha-D-glucosamine 1-phosphate	14.236	1856.80	117.110	90.1	in-house
67	Nicotinic acid	7.547	1294.62	180.034	90.9	in-house
68	Ornithine	14.050	1838.80	142.129	96	in-house
69	Oxalic acid	5.369	1138.69	147.136	84	in-house
70	palmitic acid	16.131	2048.99	117.071	95.8	in-house
71	Panose	28.638	3798.53	204.039	82.6	in-house
72	Pentasiloxane	6.058	1187.77	147.117	94	NIST11
73	Phenylalanine	11.873	1639.32	218.050	91.2	in-house
74	Phosphate	7.419	1285.43	299.034	99.4	in-house
75	Pinitol	14.364	1869.22	147.101	94	NIST11
76	Proline	7.694	1305.38	142.144	93.8	in-house
77	Putrescine	13.145	1753.48	174.074	87.7	in-house
78	Raffinose	26.756	3506.09	361.104	97.9	in-house
79	Ribose	12.675	1710.11	103.062	93.4	in-house
80	Saccharic acid	16.221	2058.53	333.092	95.8	in-house
81	Serine	7.135	1265.01	116.076	97.7	in-house
82	Sorbitol	15.474	1980.15	147.107	96.3	in-house
83	Stearic acid	17.922	2246.73	117.068	92	in-house
84	Succinic acid	7.892	1320.22	147.110	96.8	in-house
85	Sucrose	21.630	2712.28	361.100	97.9	in-house
86	TDP-glucose	13.510	1787.15	217.048	81.2	in-house
87	Threitol	10.511	1523.97	147.115	85.3	in-house
88	Threonine	8.995	1402.97	117.094	96.8	in-house

89	Thymine	9.110	1412.06	255.052	95.4	in-house
90	Trehalose	22.390	2818.23	361.104	98.3	in-house
91	Tryptophan	17.573	2206.54	218.043	97.8	in-house
92	Turanose	22.477	2830.57	204.034	84.7	in-house
93	Tyramine	14.946	1926.76	174.095	97.3	in-house
94	Tyrosine	15.259	1958.41	218.042	96.5	in-house
95	Uracil	8.262	1347.96	241.028	98.7	in-house
96	Uric acid	16.914	2133.42	441.106	90.2	in-house
97	Urocanic acid	15.728	2006.20	267.050	86.7	in-house
98	Valine	6.585	1225.51	144.156	97.8	in-house
99	Xanthine	16.068	2042.23	353.072	93.7	in-house
100	Xylonic acid	13.656	1800.66	292.114	94	in-house

[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

Table S6. Loading Score of PCA results of tempe with bacterial species variation in soaking step based on PC1 and PC2.

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
1	Phenylalanine	0.130008	Alanine	0.237322
2	3-Hydroxy butyrate	0.129664	2-Aminobutyric acid	0.211818
3	Tyrosine	0.129601	Tyramine	0.198057
4	2-Aminoethanol	0.129598	Threitol	0.185484
5	Lyxose	0.129492	Linoleic acid	0.177785
6	Xanthine	0.129368	Oleic acid	0.177152
7	Uracil	0.129108	Palmitic acid	0.164322
8	2-hydroxyglutaric acid	0.12875	Galactitol	0.163977
9	Lysine	0.128546	Glucose	0.153703
10	Cysteine	0.12844	Serine	0.153679
11	Glycine	0.12828	3-Phosphoglyceric acid	0.147844
12	Tryptophan	0.128071	Stearic acid	0.147374
13	Malonic acid	0.128015	Genistein	0.142054
14	Ornithine	0.127932	Phosphate	0.137885
15	Urocanic acid	0.126484	Glucono-1,5-lactone	0.136446
16	Oxalic acid	0.126326	Urea	0.131998
17	Threonine	0.125972	2-Aminoadipic acid	0.131299

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
18	Glycolic acid	0.125847	3-Hydroxy-3-Methylglutaric acid	0.126187
19	Galactose	0.125744	Meso-erythritol	0.125817
20	Valine	0.124793	Putrescine	0.123409
21	Leucine	0.124244	2,6-Pyridinedicarboxylic acid	0.120899
22	3-Phenyllactic acid	0.123761	Mannitol	0.119522
23	Isoleucine	0.123652	Thymine	0.112254
24	Hypoxanthine	0.122965	Glutamic acid	0.111578
25	Succinic acid	0.122852	Malic acid	0.111258
26	Methionine	0.122297	Trehalose	0.110248
27	beta-Lactose	0.121929	N-acetyl-alpha-D-glucosamine 1-phosphate	0.106417
28	2-Hydroxypyridine	0.121633	Fumaric acid	0.105402
29	Aspartic acid	0.118965	beta-Alanine	0.101159
30	Xylonic acid	0.118851	3-Hydroxy-3-methylbutanoic acid	0.0987987
31	Glutamic acid	0.11866	3-Hydroxyanthranilic acid	0.09018
32	Thymine	0.118618	Citric acid	0.0851917
33	3-Hydroxy-3-methylbutanoic acid	0.11856	Isocitric acid	0.0840338
34	Glyceric acid	0.114922	Hypoxanthine	0.0812365
35	Panose	0.114366	2-Hydroxypyridine	0.074703
36	Melibiose	0.11433	Glycerol	0.0696147
37	2,6-Pyridinedicarboxylic acid	0.113167	Aspartic acid	0.0692421
38	4-Hydroxyphenylacetic acid	0.112989	4-Aminobutyric acid	0.0650292
39	2-Aminoadipic acid	0.110817	Glycolic acid	0.0522619
40	beta-Alanine	0.109759	Xylonic acid	0.0502828
41	Pyruvic acid	0.109698	Galactose	0.0494839
42	Phosphate	0.107667	Tryptophan	0.0468032
43	Saccharic acid	0.106126	Malonic acid	0.0447785
44	Glucono-1,5-lactone	0.104038	Valine	0.0432767
45	Stearic acid	0.100713	Glycine	0.0411156
46	Nicotinic acid	0.0931736	Melezitose	0.0407086
47	palmitic acid	0.0911264	Urocanic acid	0.0400603
48	Linoleic acid	0.0822565	Lysine	0.0393182
49	Lactic acid	0.0799699	2-hydroxyglutaric acid	0.0340056
50	Oleic acid	0.07982	4-Hydroxyphenylacetic acid	0.0293066
51	Riboflavin	0.0763684	Daidzein	0.0272879

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
52	Anthranilic acid	0.0739512	Saccharic acid	0.0268358
53	Tyramine	0.0722095	Xanthine	0.0264546
54	2-Aminobutyric acid	0.0657167	Uracil	0.0256694
55	Inositol	0.0539049	Raffinose	0.0207218
56	Alanine	0.0486391	2-Aminoethanol	0.0192783
57	Uric acid	0.04628	Ornithine	0.0180753
58	cis-Aconitic acid	0.0309797	Lyxose	0.0168606
59	Maltitol	0.0246784	Tyrosine	0.0153348
60	3-Phosphoglyceric acid	0.0237299	3-Hydroxy butyrate	0.0113764
61	3-Hydroxy-3-Methylglutaric acid	-0.0137491	Phenylalanine	0.0098276
62	Isobutylamine	-0.0151658	Threonine	0.0087158
63	Threitol	-0.0306481	Cysteine	0.006959
64	Turanose	-0.050273	Sucrose	-2.11E-06
65	Serine	-0.0668482	Oxalic acid	-0.0032209
66	Galactitol	-0.0698851	Leucine	-0.0119538
67	Genistein	-0.0815718	Nicotinic acid	-0.0134486
68	4-Aminobutyric acid	-0.0856724	Isoleucine	-0.0173744
69	Glucose	-0.101283	Methionine	-0.0290477
70	Putrescine	-0.103034	Galactinol	-0.032714
71	Mannitol	-0.105443	Succinic acid	-0.0330092
72	Fumaric acid	-0.107552	Glyceric acid	-0.0418048
73	Trehalose	-0.10776	3-Phenyllactic acid	-0.0422999
74	Meso erythritol	-0.10794	beta-Lactose	-0.0452844
75	Malic acid	-0.108535	Riboflavin	-0.0668062
76	Urea	-0.114891	Turanose	-0.0670381
77	N-acetyl-alpha-D-glucosamine 1-phosphate	-0.118209	Uric acid	-0.072941
78	Isocitric acid	-0.120437	Pyruvic acid	-0.0997905
79	Galactinol	-0.1205	Panose	-0.106522
80	3-Hydroxyanthranilic acid	-0.121201	Melibiose	-0.109457
81	Sucrose	-0.121893	Lactic acid	-0.135175
82	Citric acid	-0.122098	Anthranilic acid	-0.147963
83	Glycerol	-0.122582	Inositol	-0.169061
84	Daidzein	-0.124987	cis-Aconitic acid	-0.176662
85	Raffinose	-0.125948	Maltitol	-0.228346
86	Melezitose	-0.126061	Isobutylamine	-0.269464

Table S7. Loading Score of PCA results of tempe with different inoculum size based on PC1 and PC2.

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
1	Xanthine	0.150008	Alanine	0.204499
2	Aspartic acid	0.149998	Glyceric acid	0.182803
3	2-Aminoethanol	0.148293	beta-Alanine	0.168863
4	Uracil	0.146605	4-Aminobutyric acid	0.168036
5	Thymine	0.146395	Urea	0.16508
6	Phenylalanine	0.145319	Serine	0.155993
7	Lyxose	0.145223	Fumaric acid	0.149806
8	Cysteine	0.141735	Pyruvic acid	0.148916
9	2-hydroxyglutaric acid	0.141404	Saccharic acid	0.146095
10	Glycine	0.140642	Threonine	0.135894
11	Lysine	0.140056	3-Hydroxy-3-Methylglutaric acid	0.131799
12	3-Hydroxy-3-methylbutanoic acid	0.139845	Malic acid	0.129518
13	Ornithine	0.136445	N-acetyl-alpha-D-glucosamine 1-phosphate	0.113769
14	Tryptophan	0.136417	Trehalose	0.113702
15	Glutamic acid	0.135033	2-Aminobutyric acid	0.113051
16	Methionine	0.134958	Phosphate	0.112644
17	Glycolic acid	0.134676	Tyrosine	0.112035
18	Malonic acid	0.134101	Turanose	0.110834
19	Stearic acid	0.133698	Meso erythritol	0.107351
20	Hypoxanthine	0.133609	Valine	0.106153
21	3-Hydroxy butyrate	0.132377	3-Hydroxy butyrate	0.101243
22	3-Phenyllactic acid	0.131128	beta-Lactose	0.100917
23	Leucine	0.131022	3-Phosphoglyceric acid	0.099129
24	Nicotinic acid	0.130208	Threitol	0.09856
25	2-Aminoadipic acid	0.129678	Isoleucine	0.09478
26	Succinic acid	0.129615	Tryptophan	0.094362
27	palmitic acid	0.127015	Genistein	0.0928854
28	Isoleucine	0.126753	Lactic acid	0.0891816
29	2,6-Pyridinedicarboxylic acid	0.126712	Putrescine	0.0884303
30	Valine	0.126524	Ornithine	0.0850754
31	Tyrosine	0.126226	Leucine	0.0847843
32	Galactose	0.124548	2,6-Pyridinedicarboxylic acid	0.084307

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
33	Phosphate	0.119747	Glycine	0.0821198
34	beta-Lactose	0.119289	Succinic acid	0.0809814
35	Glucono-1,5-lactone	0.117939	Glucose	0.0753361
36	Meso erythritol	0.11485	2-Aminoadipic acid	0.0753311
37	4-Hydroxyphenylacetic acid	0.11	Lysine	0.0715501
38	Threonine	0.109188	Glutamic acid	0.0710392
39	Linoleic acid	0.10636	Cysteine	0.0687094
40	2-Hydroxypyridine	0.100246	Malonic acid	0.0628392
41	Oleic acid	0.0979701	Nicotinic acid	0.0625492
42	3-Hydroxy-3-Methylglutaric acid	0.0925189	Glycerol	0.0592277
43	Oxalic acid	0.0870436	Methionine	0.0559368
44	Melibiose	0.0836186	3-Hydroxyanthranilic acid	0.0558439
45	2-Aminobutyric acid	0.0801375	Oleic acid	0.0524273
46	beta-Alanine	0.0789914	Lyxose	0.0504998
47	Riboflavin	0.0750274	Phenylalanine	0.0459541
48	Saccharic acid	0.0737503	Daidzein	0.0435577
49	Mannitol	0.0718783	Citric acid	0.0399175
50	Galactitol	0.0717226	2-Hydroxypyridine	0.0394311
51	Panose	0.0689061	Melibiose	0.0375471
52	Urocanic acid	0.0664641	Inositol	0.0367699
53	Tyramine	0.0529845	Sucrose	0.0363958
54	Uric acid	0.0511128	Stearic acid	0.0362273
55	Xylonic acid	0.0496425	Linoleic acid	0.0273229
56	Threitol	0.0439364	2-hydroxyglutaric acid	0.0191613
57	Isobutylamine	0.0414195	Isocitric acid	0.0113927
58	Anthranilic acid	0.0381129	Xanthine	0.0107611
59	Melezitose	0.0268548	Aspartic acid	0.0054297
60	Maltitol	0.0079604	Thymine	-0.0015759
61	Turanose	-0.0059727	palmitic acid	-0.0027885
62	cis-Aconitic acid	-0.013544	2-Aminoethanol	-0.0087969
63	Pyruvic acid	-0.0226605	Panose	-0.0146579
64	4-Aminobutyric acid	-0.0254684	3-Phenyllactic acid	-0.0320888
65	Inositol	-0.0328375	Uracil	-0.0476115
66	Alanine	-0.036196	Galactose	-0.0523964
67	Fumaric acid	-0.0467116	3-Hydroxy-3-methylbutanoic acid	-0.0629925
68	Isocitric acid	-0.0507687	Hypoxanthine	-0.0671769
69	Genistein	-0.0516841	cis-Aconitic acid	-0.0725647
70	Glyceric acid	-0.0558635	Isobutylamine	-0.077624

Number	Metabolite and Loading Score			
	Metabolite	PC1	Metabolite	PC2
71	Serine	-0.073801	Glycolic acid	-0.0812504
72	Malic acid	-0.0774647	Galactinol	-0.0858672
73	Urea	-0.082883	Galactitol	-0.0914452
74	N-acetyl-alpha-D-glucosamine 1-phosphate	-0.101742	4-Hydroxyphenylacetic acid	-0.107879
75	Putrescine	-0.10228	Raffinose	-0.112016
76	Citric acid	-0.10341	Glucono-1,5-lactone	-0.131314
77	Glucose	-0.114893	Urocanic acid	-0.143474
78	Lactic acid	-0.115001	Maltitol	-0.150524
79	Trehalose	-0.116333	Oxalic acid	-0.169734
80	Daidzein	-0.117271	Riboflavin	-0.17826
81	Raffinose	-0.117635	Uric acid	-0.18147
82	Galactinol	-0.12175	Melezitose	-0.189309
83	3-Phosphoglyceric acid	-0.123942	Mannitol	-0.190715
84	Sucrose	-0.124006	Xylonic acid	-0.1946
85	Glycerol	-0.132552	Tyramine	-0.199905
86	3-Hydroxyanthranilic acid	-0.139908	Anthranilic acid	-0.20495

Table S8. Annotation of metabolites in tempe extracts by GC-MS metabolomics, followed by the t_R, RI, quant mass, similarity score, and library.

ID	Metabolite name	t_R (min)[a]	RI[b]	Quant mass (*m/z*)	Similarity (%)	Annotation[c]
1	2,6-Pyridinedicarboxylic acid	13.538	1767.47	296.0875	87.1	in-house
2	2-Aminoadipic acid	13.165	1733.39	260.0945	81	in-house
3	2-Aminobutyric acid	6.151	1180.82	130.1125	80.5	in-house
4	2-Aminoethanol	7.491	1276.21	174.1265	98.9	in-house
5	2-Hydroxyglutaric acid	11.54	1590.75	129.1046	89	in-house
6	2-Hydroxypyridine	4.181	1038.02	152.0625	86.4	in-house
7	3-Hydroxy butyrate	5.961	1167.43	147.1308	79.2	in-house
8	3-Hydroxy-3-methylbutanoic acid	6.66	1216.9	147.1071	77.3	in-house
9	3-Hydroxy-3-Methylglutaric acid	11.862	1618.26	147.0933	87	in-house
10	3-Hydroxyanthranilic acid	14.812	1888.35	354.125	85.8	in-house
11	3-Phenyllactic acid	11.612	1596.68	193.1	80	in-house
12	3-Phosphoglyceric acid	14.267	1835.99	299.05	76.7	in-house

13	4-Aminobutyric acid	10.95	1541.98	174.1208	97.7	in-house
14	4-Hydroxyphenylacetic acid	12.195	1647.26	179.105	82.6	in-house
15	Alanine	5.137	1109.29	116.1028	93.6	in-house
16	Anthranilic acid	11.93	1624.22	266.05	76	in-house
17	Aspartic acid	10.883	1536.36	232.1115	97	in-house
18	beta-Alanine	9.653	1438.09	248.1588	91.4	in-house
19	beta-Lactose	22.454	2783.44	204.0813	94	in-house
20	cis-Aconitic acid	13.496	1763.63	147.1063	83.4	in-house
21	Citric acid	14.375	1846.32	273.1	94.8	in-house
22	Cysteine	11.295	1570.47	218.0917	76.2	in-house
23	Daidzein	23.611	2948.89	398.1133	75.6	in-house
24	Fumaric acid	8.531	1352.62	245.0647	95.7	in-house
25	Galactinol	24.453	3074.68	204.05	72.5	in-house
26	Galactitol	15.758	1982.99	217.07	73.2	in-house
27	Galactose	15.25	1931.87	147.0857	87.4	in-house
28	Genistein	23.811	2978.08	471.1395	77	in-house
29	Glucono-1,5-lactone	16.313	2040.55	147.0857	90.1	in-house
30	Glucose	15.31	1937.91	147.1	95.9	in-house
31	Glutamic acid	12.056	1635.22	246.1395	97.2	in-house
32	Glyceric acid	8.433	1345.28	189.1	80.3	in-house
33	Glycerol	7.655	1287.9	147.1	97.4	in-house
34	Glycine	8.045	1316.41	174.129	99.1	in-house
35	Glycolic acid	4.743	1080.34	147.1039	80.7	in-house
36	Hypoxanthine	14.064	1816.4	265.0889	77.7	in-house
37	Inositol	17.163	2131.19	305.1471	96.6	in-house
38	Isobutylamine	4.99	1098.87	174.225	81.1	in-house
39	Isocitric acid	14.389	1847.69	245.1083	74.9	in-house
40	Isoleucine	7.878	1303.91	158.15	93.1	in-house
41	Lactic acid	4.552	1065.92	147.0969	99.2	in-house
42	Leucine	7.573	1282.04	158.15	87.9	in-house
43	Linoleic acid	17.925	2215.46	95.11	92.5	in-house
44	Lysine	15.349	1941.82	156.1393	96	in-house
45	Lyxose	12.733	1694.12	103.07	86.9	in-house
46	Malic acid	10.495	1504.27	147.1	97.1	in-house
47	Malonic acid	6.581	1211.27	147.0921	91.4	in-house
48	Maltitol	23.413	2919.8	204.1028	80.4	in-house
49	Mannitol	15.642	1971.28	319.15	75.9	in-house
50	Melezitose	27.715	3603.47	361.1	86.7	in-house
51	Melibiose	23.627	2951.12	204.095	96.2	in-house
52	Meso erythritol	10.833	1532.28	147.1	96.6	in-house
53	Methionine	10.807	1530.15	176.0889	93.9	in-house
54	N-acetyl-alpha-D-glucosamine 1-phosphate	14.474	1855.87	147.1111	83.2	in-house
55	Nicotinic acid	7.754	1294.92	180.0611	74.9	in-house, STD
56	Oleic acid	17.97	2220.57	117.075	87.4	in-house
57	Ornithine	14.294	1838.55	142.1269	93.4	in-house

58	Oxalic acid	5.548	1138.29	147.0857	73.7	in-house
59	palmitic acid	16.393	2048.92	117.0671	92.4	in-house
60	Panose	29.418	3826.03	204.09	72	in-house
61	Phenylalanine	11.065	1551.46	120.1	86.9	in-house
62	Phosphate	7.616	1285.13	299.0658	96	in-house
63	Putrescine	13.383	1753.37	174.14	71	in-house
64	Pyruvic acid	4.376	1052.73	174.0962	80.7	in-house
65	Raffinose	27.103	3505.58	361.1531	95.3	in-house
66	Riboflavin	23.214	2890.87	204.14	null	STD
67	Saccharic acid	16.482	2058.31	333.1342	89.7	in-house
68	Serine	7.33	1264.67	116.0833	90.9	in-house
69	Stearic acid	18.197	2246.48	117.0677	86.5	in-house
70	Succinic acid	8.09	1319.75	147.0917	94	in-house
71	Sucrose	21.926	2710.62	361.15	93.9	in-house
72	Threitol	10.736	1524.27	147.05	85.9	in-house
73	Threonine	7.863	1302.8	117.1077	95.1	in-house
74	Thymine	9.319	1411.95	255.0938	89.8	in-house
75	Trehalose	22.699	2817.69	361.15	95	in-house
76	Tryptophan	18.168	2243.22	202.1	86.3	in-house
77	Turanose	22.777	2828.83	204.0571	77.3	in-house
78	Tyramine	15.202	1927.08	174.1286	96.4	in-house
79	Tyrosine	15.513	1958.37	218.1	91.4	in-house
80	Uracil	8.466	1347.73	241.0833	95.4	in-house
81	Urea	7.003	1241.35	147.1	99.1	in-house
82	Uric acid	17.178	2132.84	441.1464	78.1	in-house
83	Urocanic acid	15.992	2006.79	267.15	75.2	in-house
84	Valine	4.875	1090.23	156.05	70.5	in-house
85	Xanthine	16.325	2041.85	353.1273	89.1	in-house
86	Xylonic acid	13.889	1799.62	292.1464	86.7	in-house

[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

STD in annotation column means confirmed metabolites with authentic standard

Figure S1. The changes in pH during soybean soaking. WSB: water-soaked soybean; LASB: acid-soaked soybean; LBSB: lactic acid bacteria-soaked soybean; and YSB: yeast-soaked soybean

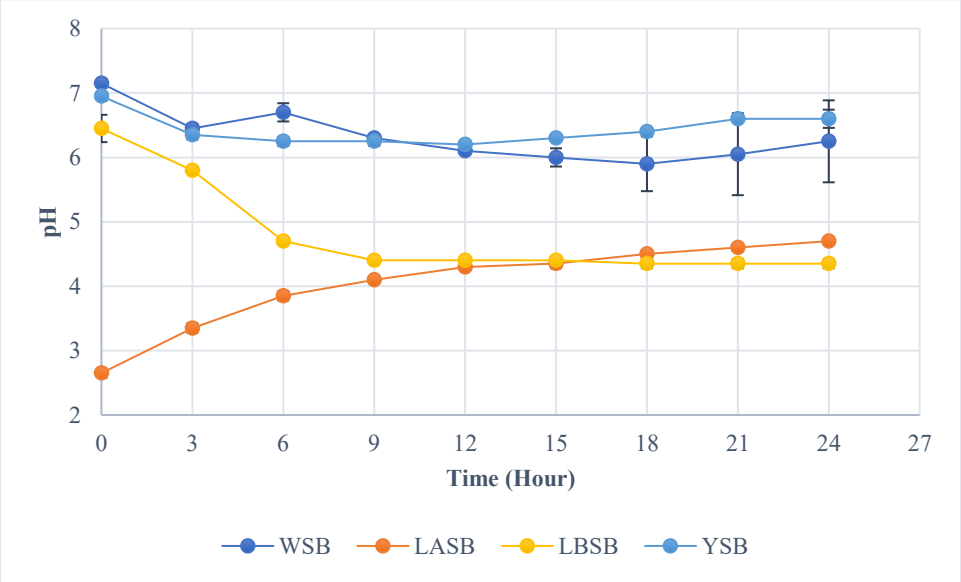

Figure S2. The changes in pH during soybean soaking using different inoculum size of *L. plantarum*