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Production of Functional Vinegar Enriched with γ -Aminobutyric Acid through Serial Co-Fermentation of Lactic Acid and Acetic Acid Bacteria Using Rice Wine Lees

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Abstract: Functional vinegar with high γ -aminobutyric acid (GABA) content was manufactured through a two-stage serial co-fermentation of rice wine lees, a by-product of Korean rice wine, using lactic acid bacteria (LAB) and acetic acid bacteria (AAB). The first LAB fermentation elevated GABA content by utilizing monosodium glutamate (MSG) as a precursor. *Lactiplantibacillus plantarum* KS2020 converted up to 10% of MSG into GABA and indicated a GABA content of 65.49 mg/g. The concentration of LAB-fermented rice wine lees was then optimized for the second co-fermentation, and *Acetobacter aceti* was used to produce vinegar. Co-fermentation using 40% first LAB-fermented rice wine lees yielded vinegar with 55.34 mg/g acetic acid and 22.61 mg/g GABA. The temperature-dependent reduction in GABA in GABA-enriched vinegar followed the Arrhenius relationship during storage, with an activation energy of 9.94 kcal/mol (20–35 °C, $R^2 = 0.99$). The GABA present in the vinegar showed evidence of a temperature-/time-dependent decrease, decreasing by 40% over five months. This study first proved the higher GABA-enriched vinegar production from rice wine lees using *Lb. plantarum* KS2020 and *A. aceti*.

Keywords: rice wine lees; GABA-enriched vinegar; *Acetobacter aceti*; *Lactiplantibacillus plantarum*



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1. Introduction

Rice wine lees, a by-product of Korean rice wine, comprises yeast and rice koji. It is rich in diverse metabolites and essential nutrients, including carbohydrates, proteins, dietary fiber, vitamins, minerals, and organic acids [1]. Despite its high nutritional value, rice wine lees is merely utilized as a fertilizer, feed for livestock, or is relegated as industrial waste. The recent advancements in hygienic practices within traditional liquor production, particularly in the context of rice koji, have prompted efforts to recognize rice wine lees as a viable post-filtration nutritional source. Researchers have been exploring the potential use of rice wine lees as a food additive and food fermentation substrate [2,3]. The abundance of essential elements, such as carbon and nitrogen, in rice wine lees facilitates microbial proliferation, thus underscoring its significant potential in the fermentation industry [4]. Given the escalating costs associated with agricultural raw materials, a growing emphasis is being placed on optimizing the utilization of food resources [5–7]. This highlights the importance of recognizing and capitalizing on the valuable attributes of rice wine lees, positioning it as a valuable resource in the broader context of sustainable food production and economic efficiency.

Vinegar, a widely utilized acidic condiment and traditional medicine [8], is produced via a fermentation process that transforms alcohol derived from grains or fruits into acetic acid [9]. The domestic vinegar market is experiencing a surge in demand for premium and diverse products, leading to the utilization of vinegar variants for various applications beyond traditional seasoning. With the expansion of the functional food market, research

on high-quality fermented vinegar enriched with functional additives serves to address consumer preferences for health-oriented food products [10,11].

γ -Aminobutyric acid (GABA) is a non-protein amino acid acting as an inhibitory neurotransmitter in the mammal central nervous system. It is known to enhance brain blood flow, support brain cell metabolic functions [12], alleviate stress, regulate blood pressure, and improve sleep. GABA has found its way into various Korean products such as GABA rice. Additionally, fermented kelp enriched with GABA is acknowledged as a functional food material with the ability to regulate blood pressure [13,14].

While natural sources like green tea, rice germ, and germinated brown rice contain GABA, the levels are often insufficient to achieve desired effects. However, lactic acid bacteria (LAB) involved in the fermentation of kimchi, a representative fermented food, demonstrate excellent GABA production [15]. GABA production was also performed by the yeast in okara (soybean residue) [16]. Hence, several studies have focused on optimizing fermentation conditions using *Lactiplantibacillus plantarum*, a strain used in kimchi fermentation, to achieve high GABA concentrations in natural food sources [17–19].

Overall, it is thought that sake lees will promote LAB growth and increase GABA production through yeast metabolites [20,21]. It is hypothesized that the production of GABA and the optimization of vinegar using rice wine lees will not only enhance the added value of rice wine lees but also utilize the residual alcohol while maintaining the functional properties of rice wine lees. In this study, we aimed to achieve a high concentration of GABA via the serial co-fermentation of rice wine lees using LAB and acetic acid bacteria (AAB), and evaluate the storage stability of functional GABA in vinegar. Our findings highlight a novel co-fermentation technique for the production of GABA-enriched functional vinegar from rice wine lees.

2. Materials and Methods

2.1. Materials

The rice wine lees utilized in this study were sourced from Daemyung traditional liquor (Daegu, Republic of Korea), and yeast extract (Y.E.) was procured from Choheung (Gyeonggi, Republic of Korea). Glucose and L-monosodium glutamate (MSG) were supplied by Daehan Jedang (KS H-2003, Incheon, Republic of Korea) and CJ Jeil Jedang (Seoul, Republic of Korea), respectively. Potassium phosphate dibasic was obtained from Serim Foods (Gyeonggi, Republic of Korea) and utilized in the experiment. The rice wine was acquired from Daegu Bullo Takju (Daegu, Republic of Korea), and Prethanol A, an alcohol, was sourced from Deoksan Pharmaceutical (Daejeon, Republic of Korea). Furthermore, apple vinegar (12% acetic acid) was purchased from Ottogi, situated in Gyeonggi, Republic of Korea. Most of raw materials were sterilized, while unsterilized rice wine was used.

2.2. Preparation of Starter Cultures

Lactiplantibacillus plantarum KS2020 (KCCM 12782P) was selected as the strain due to its isolation from kimchi and its high GABA production capability [22]. The strain underwent cultivation on an MRS agar plate at 30 °C for 2 days. Subsequently, it was inoculated into 100 mL of MRS broth (Difco, New Jersey, USA) and placed in a constant temperature incubator at 30 °C for 24 h. *Acetobacter aceti* (KCCM 40229P) was cultivated on an MRS agar plate at 30 °C for 48 h. Following this, a single colony was introduced into 100 mL of a rice wine mixture with 5% apple vinegar. The resulting mixture underwent cultivation with shaking in a 30 °C shaking incubator for 72 h, ultimately yielding the AAB fermentation starter. This process was designed to harness the unique qualities of these strains for GABA production and vinegar optimization in the context of this study's objectives.

2.3. GABA and Vinegar Production

To optimize conditions for 1st LAB fermentation, 300 g of rice wine lees were combined with 300 mL of distilled water and homogenized for 15 min at 3600 rpm using a hand mixer (HR1673, Philips, Budapest, Hungary). The homogenized rice wine lees underwent

sterilization at 80 °C for 20 min. The culture broth comprised 2% (*w/v*) glucose, 8–12% (*w/v*) MSG, 1% (*w/v*) yeast extract, and 0.2% (*w/v*) potassium diphosphate in 50% sterilized rice wine lees. Subsequently, a 1% (*w/v*) starter of *Lb. plantarum* KS2020 was inoculated and cultivated at 30 °C for 7 days.

For the AAB fermentation, the 1st fermented rice wine lees (40%, 45%, 50%) were mixed with 15% rice wine, 4% prethanol A, and 5% apple vinegar. The mixture underwent inoculation with 15% (*v/v*) *A. aceti* starter and then was cultured at 30 °C and 160 rpm for 5 days using a shaking incubator (SI-900R, JeioTech. Co., Ltd., Daejeon, Republic of Korea).

2.4. Moisture Content Assay

The moisture content of the rice wine lees was measured utilizing an infrared moisture analyzer (FD-660 Infrared Moisture Analyzer, Seoul, Republic of Korea). The sample quantity was set at 5 g and analyzed in an aluminum dish. The moisture content was expressed as a percentage (%) [23].

2.5. Alcohol Quantification

To quantify the alcohol content in the sample, 100 mL of the sample was placed in a flask and heated to 80 °C using a heating mantle (GLOBAL LAB, Gyeonggi, Republic of Korea). Approximately 100 mL of the condensed water was collected, cooled to 15 °C, and measured using a hydrometer (So Scientific, Seoul, Republic of Korea). The obtained values were then converted to percentages (%).

2.6. Reducing Sugars Assay

The content of reducing sugar was determined through a colorimetric method using DNS reagent [24]. The absorbance was measured with a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Watford, UK) at a wavelength of 550 nm. A glucose solution served as the standard, and the concentration was expressed in mg/mL.

2.7. Measurement of Viable Bacterial Counts

Viable bacterial counts for *Lb. plantarum* and *A. aceti* were determined using the standard plate count method [25]. The procedure involved a serial dilution method and selective medium. MRS agar plates were used for lactic acid bacteria. Acetic acid bacteria were grown on a modified acetic acid-ethanol agar plate consisting of 1.5% glucose, 0.2% Y.E., 0.3% peptone, 5% acetic acid, 3% ethanol, and 1% agar [26]. Fermentation samples were serially diluted, and were spread on selective medium. Incubation took place at 30 °C for 2 days, and the viable cell count was expressed as log CFU/mL.

2.8. pH and Titratable Acidity

The pH was measured with a pH meter (SevenEasy pH, Mettler-Toledo AG, Schwerzenbach, Switzerland) [27]. Titratable acidity was determined by diluting 1 mL of the sample with 9 mL of distilled water and titrating with 0.1 N NaOH until a pH of 8.3 was reached. The equivalent weights of lactic acid and acetic acid were then used to calculate the total titratable acidity (% *v/v*).

2.9. Qualitative Analysis of GABA

MSG consumption and GABA production were assessed through the thin layer chromatography (TLC) method using a silica gel 60 F254 plate (Merck KGaA, Darmstadt, Germany). The sample was centrifuged at 15.7 rcf for 15 min to obtain a supernatant. A total of 2 µL of each supernatant was spotted on the plate and subsequently eluted using a developing solvent (an organic layer composed of *n*-butyl alcohol: glacial acetic acid: distilled water in a 3:1:1 ratio). After drying, the silica gel plate was treated with a coloring reagent (0.2% ninhydrin) and then dried at 100 °C. This methodology allowed for the visualization and quantification of MSG consumption and GABA production in the samples.

2.10. Quantitative Analysis of the Free Amino Acid Content

GABA and the free amino acids in the serial co-fermented broth were analyzed using high-performance liquid chromatography (HPLC) with a Waters 2475 instrument (Waters, Milford, CT, USA). The dried sample underwent derivatization at room temperature for 30 min and was then mixed with solvent A (140 mM NaHAc, 0.15% TEA, 0.03% EDTA, 6% CH₃CN, pH 6.1). Subsequently, the mixture was filtered through a 0.45 µm syringe filter (Genie, New York, NY, USA). The sample was injected using an HPLC auto-sampler (1100 series; Hewlett Packard, Palo Alto, CA, USA) connected to a C18 column (Nova-Pak 4 µm; Waters, Milford, MA, USA) [28]. The mobile phase (A, Waters AccQ Taq Eluent; B, 60% Acetonitrile; C, Tertiary distilled water) was applied for 25 min at a flow rate of 1 mL/min. The amino acid content was determined by measuring the absorbance at 250 nm using a UV detector (Waters, Milford, CT, USA).

2.11. Measurement of Organic Acids

A quantitative analysis of organic acids was conducted using HPLC with a Waters 1695 instrument (Waters Co., Milford, MA, USA) equipped with a CAPCELL PAK C18 column (UG120, 150 × 4.6, 5 µm). For each serial co-fermented broth, 1 g was stirred with 100 mL of distilled water and then filtered using a 0.2 µm syringe filter (Genie, New York, NY, USA). The injected sample was monitored with a UV detector (Waters series 2487) at 210 nm. The mobile phase consisted of solvent A (50 mM sodium hexasulfonate, 20 mM H₃PO₄) and solvent B (distilled H₂O). The flow rate was 1.0 mL/min, with a sample injection volume of 10 µL. This methodology enabled the precise measurement of organic acid concentrations in the samples.

2.12. Quantitative Analysis of Sodium

The sodium content of serial co-fermented vinegar was determined using the dry ashing method with inductively coupled plasma optical emission spectroscopy (ICP-OES) on an Optima 7000DV instrument by Perkin Elmer (Waltham, MA, USA) [29]. The samples underwent carbonization and incineration within the temperature range of 550–600 °C. Subsequently, 8–10 mL of HCl solution was added to the dried material and heated until dissolved, followed by filtration. The resulting solution was then diluted to a volume of 100 mL using distilled water, and this solution was used as the test solution for analysis.

$$\text{Sodium content (mg/100 g)} = C \times V \times D/S \times 0.1$$

C: concentration measured by ICP-OES (mg/kg); V: total volume of solution (mL); D: dilution factor measured by ICP-OES; S: sample (g).

2.13. Determination of Storage Stability of GABA

To evaluate the stability of GABA in serial co-fermented vinegar, a modified shelf life experiment was employed. The vinegar product including GABA was sterilized at 80 °C for 25 min to deactivate bacteria and then placed at temperatures of 35, 30, and 25 °C during 5 months. Sampling was conducted every month, and GABA content was measured through HPLC. Measured values were expressed in %, and each condition was expressed as a linear equation using time (Month, *x*-axis) and GABA content (% , *y*-axis).

$$KT = A_0 - A_e$$

where K: reaction rate constant value; T: storage time; A₀: initial GABA content; and A_e: measured GABA content after time *t*.

The slope of a linear equation for each temperature was set as the K value, and the lnK value was derived through a natural logarithm. The activation energy value (E_a) was

calculated using the Arrhenius equation. This method allowed for the evaluation of GABA stability under various storage conditions.

$$\ln K = -(E_a/R)(1/T) + \ln A$$

where A: Arrhenius constant; E_a : activation energy (cal/mol); R: gas constant (1.986 cal/mol); T: absolute temperature ($^{\circ}\text{C} + 273$); and K: reaction rate constant value.

2.14. Statistical Analysis

Viable bacterial count, pH, and acidity measurements were conducted in triplicate, and the resulting data were presented as the mean value $\pm$ standard deviation. The statistical analysis was performed using two-way analysis of variance (ANOVA) with SPSS Ver. 27.0 (SPSS INC., Chicago, IL, USA). Duncan's multiple range test ($p < 0.05$) was applied to identify significant differences among the samples.

3. Results and Discussion

3.1. Physicochemical Composition of Rice Wine Lees

The solid and alcohol content of the rice wine lees were 30.74%, and 7.26%, respectively. Additionally, its pH and acidity were 3.96 and 0.78%, respectively. The reducing sugar and sodium content were 51.22 mg/mL and 0.06 ± 0.00 mg/g, respectively (Table 1).

Table 1. Physicochemical analysis of rice wine lees.

Parameter	Content
Soluble solid content (%)	$30.74 \pm 0.23^{(1)}$
Alcohol content (%)	7.26 ± 0.12
pH	3.96 ± 0.02
Acidity (%)	0.78 ± 0.10
Reducing sugar content (mg/mL)	51.22 ± 0.18

⁽¹⁾ All values are mean $\pm$ standard deviation of three replicates ($n = 3$).

Rice wine lees of previous studies had a moisture content of 65.5% [30]. Another study showed that the sugar content of lees was 3.2% [1]. These compositional differences were likely dependent upon the yeast fermentation in alcohol production and the difference in raw materials applied. In this study, it was crucial to enhance the reproducibility of raw materials by developing products using rice wine lees, a by-product. To optimize fermentation, water was diluted before entering lactic acid bacteria (LAB) fermentation to adjust the alcohol content and sugar content.

3.2. Physicochemical Properties of the First LAB Fermented Rice Wine Lees

The initial viable *Lb. plantarum* KS2020 counts in fermented rice wine lees were 7.06 log CFU/mL. The counts increased to 9.57, 9.54, and 9.40 log CFU/mL after 1 day in the presence of 8%, 10%, and 12% monosodium glutamate (MSG), respectively. Subsequently, LAB growth remained relatively high with counts of 9.23, 9.25, and 9.18 log CFU/mL after 7 days (Figure 1A). Additionally, an increase in the salt content (up to 5%) in the culture broth had no significant impact on LAB growth [31].

The pH values on day 0 of LAB fermentation were 5.97, 5.53, and 6.01 for the three MSG concentrations, which gradually decreased to 4.88 and 4.97 after 1 day, increasing, however, to 6.42 and 6.55 after 7 days, in the presence of 8% and 10% MSG, respectively. In the presence of 12% MSG, the pH decreased to 5.04 over 3 days, and then rose to 5.80 after 7 days (Figure 1B). Similar trends were observed for acidity, which was measured to be 0.96%, 0.83%, and 1.03%, for the three conditions on day 0 (Figure 1C). The higher acidity of initial culture broth was due to the organic acids present in yeast-fermented rice wine lee. In the presence of 8% and 10% MSG, acidity increased to 2.09% and 1.97% after 1 day, followed by a decrease to 0.68% and 0.41% after 7 days, respectively. Notably, 12%

MSG caused acidity to increase to 2.38% over 3 days, followed by a decrease to 0.92% after 7 days. Microbial GABA production in the presence of MSG involves the decarboxylation of glutamate by glutamate decarboxylase, with the concomitant consumption of protons [32], which results in an increased pH. The MSG content in the culture broth may influence the glutamate conversion rate, as shown by a 12% MSG concentration significantly slowing down the GABA production rate compared to the other two conditions.

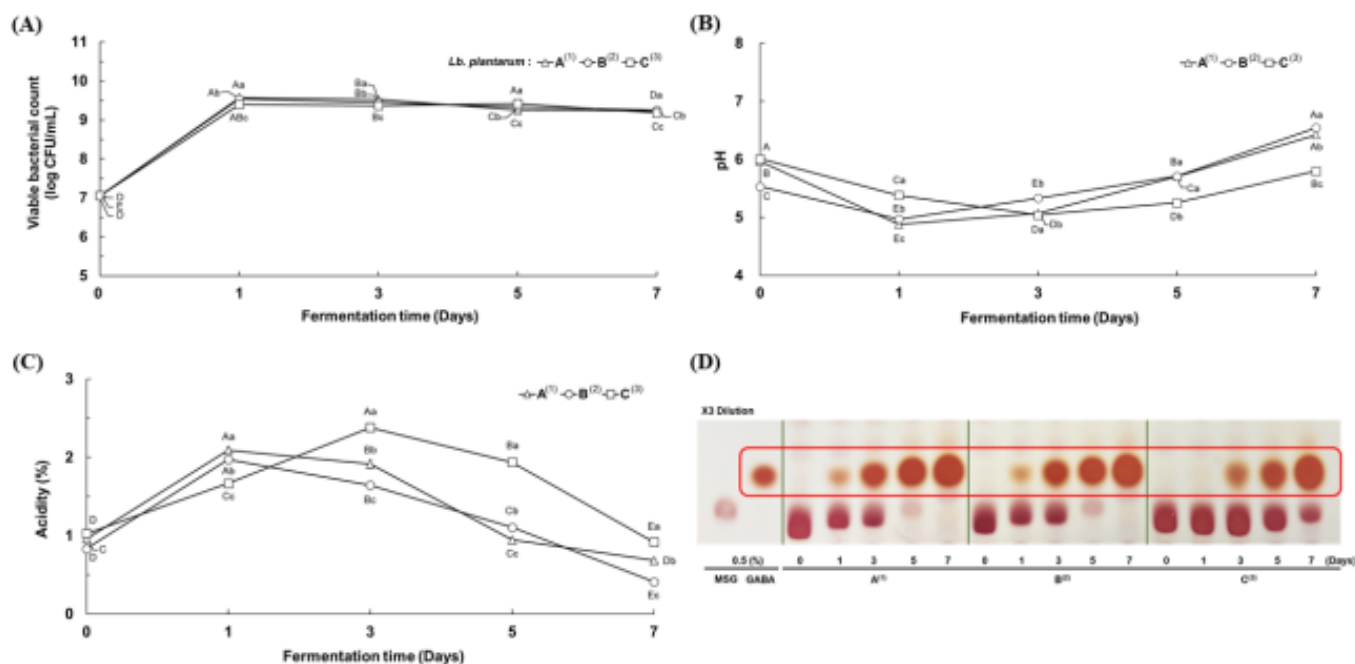


Figure 1. Viable bacterial count, pH, acidity, and TLC of rice wine lees fermented by *Lb. plantarum* KS2020. (A) Viable bacterial count of *Lb. plantarum* in first LAB fermentation. (B) pH of first LAB fermentation. (C) Acidity of first LAB fermentation. (D) TLC of first LAB fermentation. ⁽¹⁾ A: 8% MSG; ⁽²⁾ B: 10% MSG; ⁽³⁾ C: 12% MSG. MSG: Monosodium L-glutamate. The error bars indicate standard deviations. Different letters denote statistical difference ($p < 0.05$). $n = 3$ for each group.

When GABA was produced in the first fermentation using *Lb. plantarum* KS2020, the GABA production was greatly influenced by the period of fermentation according to the MSG content (Figure 1D). The conversion of 8% and 10% MSG to GABA started from the first day of LAB fermentation, and was completed in 7 days. The addition of 12% MSG resulted in some MSG remaining in the culture broth, which implied that 10% MSG is the optimal condition for GABA production via the LAB fermentation of rice wine lees.

HPLC analysis confirmed the complete conversion of 8% and 10% MSG to GABA within 7 days. While the addition of 8% MSG produced 48.31 mg/g GABA with 3.32 mg/g glutamic acid, the addition of 10% MSG led to the production of 65.49 mg/g GABA with 5.08 mg/g glutamic acid. The GABA content after the enzyme and LAB-mediated co-fermentation of *Laminariales* extract was 0.93 mg/g [33]. Recombinant *Bacillus subtilis* produced 2.26 mg/g GABA [34]. The highest production of GABA was obtained with 4.31 g/L by the yeast *Kluyveromyces marxianus* C21 in okara (soybean residue) [16]. Compared to other fermentation studies, the higher production of GABA was optimized by *Lb. plantarum* KS2020 in rice wine lees.

3.3. Physicochemical Properties of the Second Serial Co-Fermented Rice Wine Lees

The initial viable bacterial count of *Lb. plantarum* KS2020 in the second AAB fermentation containing 40%, 45%, and 50% of the first LAB-fermented product was 7.249 log CFU/mL, suggesting a substantial decrease (Figure 2A). The viable bacterial

count of *Lb. plantarum* KS2020 reduced to 4.83–5.30 log CFU/mL during the 5 days of the second AAB fermentation. Studies have reported a significant growth inhibition of *Lb. plantarum* KS2020 in the presence of 5.0% ethanol [35]. Thus, the initial alcohol content may have caused a decrease in *Lb. plantarum* KS2020 counts. Since the viable bacterial count of *Lb. plantarum* KS2020 was 9.54 log CFU/mL in the first LAB-fermented product, it can be inferred that the majority of the viable bacteria acted as parabiotics. Studies have reported immune-enhancing effects of the parabiotic LAB, which induce concentration-dependent immunomodulatory effects in an excessively lipopolysaccharide-stimulated macrophage model [36]. In contrast, the initial count of viable *A. aceti* was 5.07 log CFU/mL in the serial co-fermented product containing 40%, 45%, and 50% of the first LAB-fermented product, which increased over 1 day to 5.79, 6.09, and 6.11 log CFU/mL, respectively. After that, the viable bacterial count of AAB was greatly decreased, showing 4.71–4.83 log CFU/mL (Figure 2B).

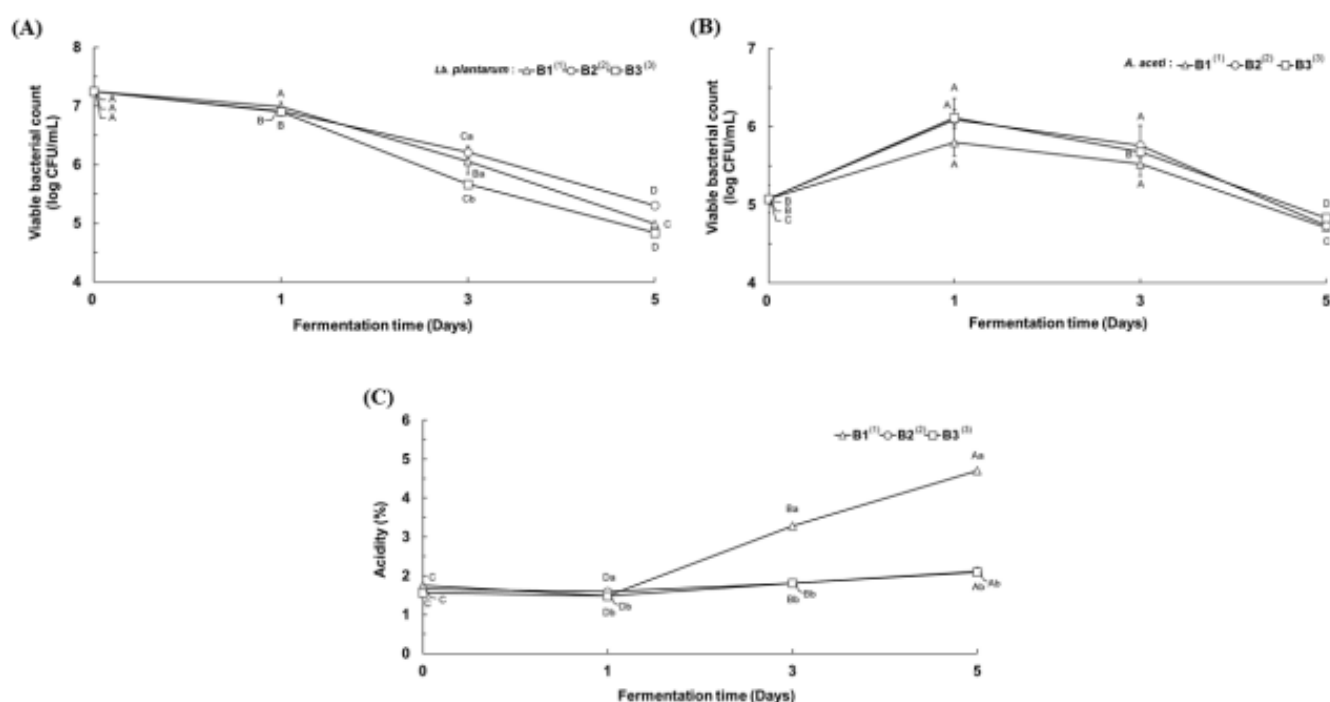


Figure 2. Viable bacterial count, pH, and acidity of *Lb. plantarum* and *A. aceti* in co-fermented rice wine lees vinegar by *Lb. plantarum* KS 2020 and *A. aceti*. **(A)** Viable bacterial count of *Lb. plantarum* in second fermentation. **(B)** Viable bacterial count of *A. aceti* in second fermentation. **(C)** Acidity in second fermentation. ⁽¹⁾ B1: First fermented rice wine lees (40%); ⁽²⁾ B2: first fermented rice wine lees (45%); ⁽³⁾ B3: first fermented rice wine lees (50%). LAB fermentation was performed with rice wine lees containing 10% MSG. The error bars indicate standard deviations. Different letters denote statistical difference ($p < 0.05$). $n = 3$ for each group.

Acidity was consistently maintained between 1.56 and 1.76% on day 1 of the second AAB fermentation containing 40%, 45%, and 50% of the first LAB fermentation product (Figure 2C). Subsequently, the serial co-fermented product containing 40% of the first LAB-fermented product exhibited a rapid increase in acidity for up to 5 days of AAB fermentation. In contrast, the addition of 45% and 50% of the first LAB-fermented product led to lower acetic acid production, with final acidity reaching 2.12% and 2.09%, respectively. In conclusion, serial co-fermentation using 40% of the first LAB-fermented rice wine lees was suitable for vinegar production during the second AAB fermentation. The addition of 10% MSG during the first LAB fermentation increased the sodium content of the culture broth, and the second AAB co-fermentation could be affected by the first LAB-fermented product. For substrate oxidation, *A. aceti* utilizes enzymes involved in acetic acid produc-

tion, such as alcohol dehydrogenase and aldehyde dehydrogenase. Overall, the higher sodium content of the LAB-fermented product may have resulted in reduced bacterial activity [37,38]. Furthermore, acetic acid production in vinegar fermentation was inhibited by the first LAB-fermented product.

The alcohol content in the culture broth containing 40%, 45%, and 50% of the first LAB-fermented product before the second AAB fermentation were estimated as 5.1%, 5.4%, and 5.6%, respectively (Table 2). Over 5 days of AAB co-fermentation, the alcohol content decreased to 0.0% in the presence of 40% of the first LAB-fermented product, which was probably oxidized and converted into acetic acid by the *A. aceti*. In contrast, in the presence of 45% and 50% of the first LAB-fermented product, 1.8% and 2.5% residual alcohol was observed, respectively. In the vinegar industry, up to 1% of ethanol is sometimes left behind to prevent the oxidation of acetic acid [39]. However, for both conditions, the residual alcohol content exceeded the acceptable level for commercial vinegar products, indicating that fermentation by *A. aceti* was not effective.

Table 2. The alcohol and sodium content of rice wine lees vinegar co-fermented by *Lb. plantarum* KS2020 and *A. aceti*.

First Fermented Rice Wine Lees Content	Alcohol Content (%)		Sodium (Na) Content (mg/g)
	0 Day	5 Days	5 Days
40%	5.1 ± 0.0 ⁽¹⁾	0.0 ± 0.0	5.83 ± 0.04
45%	5.4 ± 0.0	1.8 ± 0.2	7.68 ± 0.15
50%	5.6 ± 0.0	2.5 ± 0.1	8.03 ± 0.06

LAB fermentation was performed with rice wine lees containing 10% MSG. ⁽¹⁾ All values are mean ± standard deviation of three replicates (n = 3).

3.4. Quantitative Properties of the Serial Co-Fermented Vinegar Product

The sodium contents of rice wine lees vinegar produced by *Lb. plantarum* KS2020 and *A. aceti* are shown in Table 1. When 40%, 45%, and 50% of the first LAB-fermented products were used, the sodium content was 5.83 mg/g, 7.68 mg/g, and 8.03 mg/g, respectively. Given that the only vinegar produced by serial co-fermentation that met commercial specifications was obtained upon the addition of 40% of the first LAB-fermented product, it is plausible that a sodium content of 7.68 mg/g or higher in the LAB-fermented product may inhibit the second AAB fermentation by *A. aceti*.

The final co-fermented product obtained on inclusion of 40% of the first LAB-fermented product contained 3.61 mg/g and 22.61 mg/g of glutamic acid and GABA, respectively (Table 3A). However, the GABA content of the final rice wine lees vinegar was slightly lower due to dilution following the addition of the first LAB-fermented product.

The organic acid levels were assessed pre- and post fermentation of vinegar optimization conditions to ascertain alterations in organic acids during AAB fermentation (Table 3B). Pre-fermentation, the initial acetic acid levels were 10.42 mg/g, with lactic acid concentrations at 14.11 mg/g. Oxalic acid and citric acid were slightly detected, while malic acid was present in trace quantities at 1.04 mg/g. Acetic acid notably surged to 52.21 mg/g post AAB fermentation, meeting the domestic vinegar standard of 4.0–20.0% acetic acid. Conversely, the other organic acids displayed a diminishing trend, with lactic acid decreasing by over two-fold. This decline is attributed to lactic acid peroxidation by *A. aceti* in the absence of alcohol [40]. In this experiment, the alcohol content was 0% from day 3 of fermentation, with AAB growing in the absence of alcohol for an additional 2 days of fermentation.

Table 3. (A) Quantitative analysis on the GABA and glutamic acid content for rice wine lees first fermented by *Lb. plantarum* KS2020 and rice wine lees vinegar co-fermented by *Lb. plantarum* and *A. aceti*. (B) Compositional analysis of rice wine lees and rice wine lees vinegar co-fermented by *Lb. plantarum* KS2020 and *A. aceti*.

(A)		
Condition	Glutamic Acid (mg/g)	GABA (mg/g)
First LAB fermentation (10% MSG)	5.08 ± 0.21 ⁽¹⁾	65.49 ± 3.41
Second AAB fermentation (first fermented product: 40%)	3.61 ± 0.22	22.61 ± 0.61
(B)		
Organic Acid (mg/g)	First Fermented Rice Wine Lees (Addition 10% MSG)	
	Before AAB Fermentation	After AAB Fermentation
Acetic acid	10.42 ± 0.18 ⁽¹⁾	52.21 ± 5.53
Lactic acid	14.11 ± 0.10	4.81 ± 0.16
Malic acid	1.04 ± 0.06	0.00 ± 0.00
Oxalic acid	0.02 ± 0.00	0.00 ± 0.01
Citric acid	0.00 ± 0.00	0.00 ± 0.00

⁽¹⁾ All values are mean ± standard deviation of three replicates (n = 3).

3.5. Evaluation of GABA Stability in Vinegar

The loss rate of GABA was confirmed using the Arrhenius equation to determine GABA stability in rice wine lees vinegar co-fermented by LAB and AAB for a storage period of 5 months. The reaction rate constant values (K) at 25, 30, and 35 °C in the Arrhenius equation were 0.1189, 0.1614, and 0.2051, respectively. Subsequently, lnK was −2.1295, −1.8239, and −1.5843, and the Arrhenius equation based on the lnK and the reciprocal of absolute temperature was generated. The activation energy (Ea) for GABA degradation was calculated from the slope (Ea/R: gas constant; Table 4), which was −5006.3 (Figure 3). The activation energy of 9.94 kcal/mol was obtained by multiplying the slope by the gas constant, i.e., 1.986 kcal/mol.

Table 4. GABA stability test modeling in rice wine lees vinegar co-fermented with *Lb. plantarum* KS2020 and *A. aceti* using the Arrhenius equation.

°C (K)		R ²	1/T (K ^{−1})	K	lnK
25 (298)	y = −0.1189x + 2.4238	0.9078	0.003356	0.1189	−2.129472475
30 (303)	y = −0.1614x + 2.6486	0.7409	0.003300	0.1614	−1.823869523
35 (308)	y = −0.2051x + 2.6629	0.7585	0.003247	0.2051	−1.584257614

°C (K): absolute temperature; K: reaction rate constant value.

An accelerated test to evaluate the stability of multivitamin liquid products showed the decomposition of vitamin A according to the zero-order kinetics and vitamins B₁ and C according to the first-order kinetics. The activation energies of vitamins A, B₁, and C obtained using the Arrhenius equation were 26.27, 21.67, and 23.50 kcal/mol, respectively [41]. Reactions with a higher Ea have a steeper slope and higher reaction rate (K), resulting in greater sensitivity to temperature change. The loss of GABA in rice wine lees vinegar products shows lower activation energy values than vitamins, indicating that GABA in vinegar is less stable compared to the liquid vitamins when stored at room temperature.

The thermal degradation of GABA in *Monascus*-fermented rice follows the first-order reaction kinetics and Arrhenius relationship with an activation energy value of 24.2 kcal/mol (pH 3.4, 80–121 °C, r² = 0.96) [42]. A reduction in pH and continuous heating

resulted in a decrease in E_a . The vinegar optimized in this experiment has a low pH and is believed to have reduced GABA levels due to long-term storage at a constant temperature.

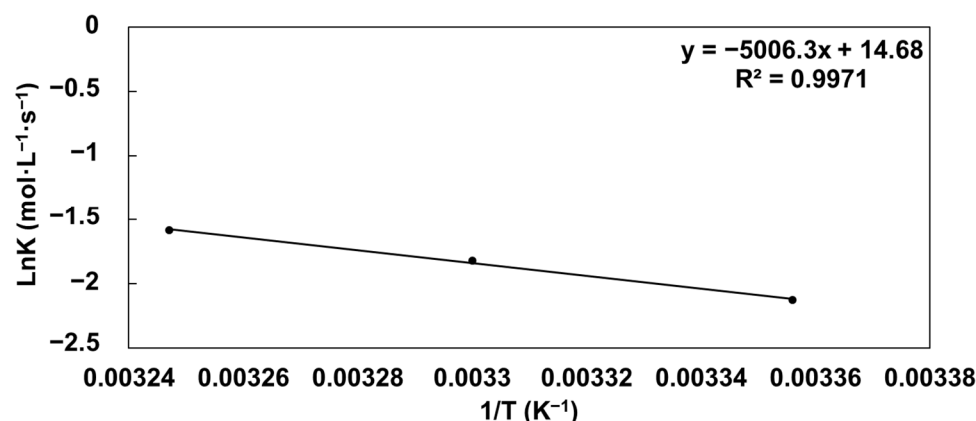


Figure 3. GABA stability test modeling in rice wine lees vinegar co-fermented with *Lb. plantarum* KS2020 and *A. aceti* using the Arrhenius equation.

However, *Monascus*-fermented rice and rice wine lees vinegar show a significant difference in activation energy. GABA alone exhibits stability under heat treatment and varying pH levels; however, its stability may be compromised through interactions with other molecular components within the food matrix [43]. Recently, the reduction in GABA content in the GABA-enriched dark chocolates was attributed to the Maillard reaction between GABA and sugar. This suggested that GABA enrichment was the most efficient way to compensate for the reduction in GABA [44]. To date, there has been little research on the stability of GABA in various foods, and additional research is needed to study the stability of GABA in food.

In summary, rice wine lees as a by-product was utilized to develop the valuable food ingredient by fermentation. The higher GABA-enriched rice wine lees vinegar could be produced through a two-stage serial co-fermentation using *Lb. plantarum* KS2020 and *A. aceti*. As a bioactive compound, GABA in vinegar was gradually decreased depending upon temperature and storage time.

4. Conclusions

This study investigated GABA production via LAB fermentation using rice wine lees, a by-product of rice wine. Subsequently, functional vinegar enriched with GABA was developed by optimizing serial co-fermentation with *A. aceti*. The first LAB fermentation had a viable *Lb. plantarum* KS2020 count of 9.25 log CFU/mL regardless of MSG concentration. However, noticeable variations in physicochemical properties (i.e., pH and acidity) were observed. The addition of 10% MSG was suitable for GABA production through the lactic acid fermentation of rice wine lees, with a yield of 6.55%.

The first LAB-fermented product containing 5% spirit and 5% apple vinegar was used for the second AAB fermentation. The concentration of the first LAB fermentation product influenced acid production during AAB fermentation; 40% concentration of the first LAB fermentation product optimally enhanced GABA yield (2.26%) along with efficient acetic acid production (4.7%), and no evidence of alcohol remnants. However, during 5 months of storage of the final product at 25–35 °C, the GABA content gradually decreased to 1.84–1.64% in rice wine lees vinegar. Additionally, it demonstrated a lower activation energy (9.94 kcal/mol) compared to other similar studies.

Taken together, our data indicate that the nutritional content of rice wine lees has positive consequences in the production of high concentrations of GABA by LAB. The serial AAB fermentation of rice wine lees enriched with GABA met the vinegar standards specified in the Food Code. Additionally, the present study demonstrates the weak stability of GABA in vinegar. However, it also indicates a novel method for enhancing GABA

production through the utilization of rice wine lees, a by-product, and producing functional vinegar through two-stage co-fermentation.

5. Patents

Development of functional vinegar materials containing higher GABA using traditional liquor by-product rice wine lees/21 November 2022.

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