ABSTRACT

This study developed and characterized a γ-aminobutyric acid (GABA)-enriched yogurt fermented by Levlactobacillus brevis CGMCC1.5954. The GABA content in the yogurt was 147.36 mg/100 mL, which was 317.06% higher than that of the control group. Furthermore, there was a significant improvement in the aroma, hardness, adhesion, cohesiveness, and gelatinousness of yogurt. The chromatography and metabolomics analyses further confirmed the high GABA content in yogurt and its nutritional value, and the metabolic pathway for GABA production by L. brevis 54 was identified. A total of 58 volatile flavor compounds were identified using headspace solid-phase microextraction-gas chromatography-mass spectrometry, of which 2-nonanone and 2-heptanone may be responsible for the high odor score of GABA-enriched yogurt. This study developed a nutritious and unique GABA-enriched flavored yogurt, summarized the metabolic pathway of GABA, and provided a flavor fingerprint that could guide the production of specifically flavored yogurts.

Key words: lactic acid bacteria, yogurt, HS-SPME-GC-MS, γ-aminobutyric acid

INTRODUCTION

Yogurt is made by fermenting milk with Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus as starters and is popular with consumers for its unique flavor and high nutritional value (Aryana and Olson, 2017). The Food and Agriculture Organization of the United Nations and the World Health Organization define probiotics as live microorganisms that are capable of producing beneficial functions for the host when consumed in sufficient quantities (Morelli and Capurso, 2012). As probiotic yogurt can enhance the immune function of the body, promote digestion, and improve the intestinal environment. The probiotic industry has ushered a windfall for the global probiotic market in the postepidemic era, which is expected to experience a compound annual growth rate of 6.9% (Fan et al., 2021; Macnaughtan et al., 2020). Therefore, developing and characterizing a yogurt with a unique flavor and potential probiotic function is very important.

Gamma-aminobutyric acid (GABA) is a nonprotein AA produced by the removal of a molecule of the carboxyl group from glutamate and its sodium salt, catalyzed by the enzyme glutamic acid decarboxylase (GAD; Möhler, 2012; Bonomi et al., 2020). The functions of GABA are different in different organisms, such as an inhibitory neurotransmitter in the animal central nervous system and a protective mechanism against stress in plants (Xu et al., 2017). Recent studies have found that GABA can regulate mood, and improve sleep disturbances and memory capacity by increasing monoamine transmitter levels or binding to its receptors (Bagheri et al., 2019). For example, behavioral tests in mice have shown that long-term administration of GABA-rich green tea can alleviate despair in mice under stress (Teng et al., 2017). In contrast, the levels of GABA in biological tissues are very low and difficult to extract from natural organisms, so it is essential to find an alternative method to obtain GABA. Microbial fermentation offers the advantages of higher yields, lower costs, and less environmental impact than the chemical synthesis of GABA using pyrrolidone with solid base ring-opening methods (Boonstra et al., 2015). Therefore, the addition of exogenous glutamate or monosodium glutamate and the adjustment of optimal catalytic conditions for GAD may be an effective method to increase the production of GABA synthesis by microorganisms.
Furthermore, GABA-enriched food supplements and fermented foods have emerged as a result of increased research into GABA. There are 3 main methods to study GABA-enriched yogurts. The first is to add pure GABA directly to cow milk, but this is costly, and a GABA concentration over 0.5% (wt/vol) can lead to large amounts of whey deposits, which has a detrimental effect on the texture of the yogurt (Liu et al., 2015). The next option is to add plant-based ingredients, such as sprouted brown rice and beans, to introduce GABA directly into the fermentation matrix. However, this is highly susceptible to undesirable flavors, and the stability of soya milk is average (Fan et al., 2022b). The last is to screen for GABA-producing lactic acid bacteria (LAB) as strains for yogurt fermentation, but the screening process is tedious and full of chance, whereas our laboratory prescreened a strain of L. brevis CGMCC 1.5954 (L. brevis 54) with high GABA production and showed the ability to alleviate anxiety and mild depression in animal studies (Wu et al., 2021). For example, Abd El-Fattah et al. (2018) used 4 strains of Lactocaseibacillus rhamnosus B-1445, O-114, YC-X11, and Lactobacillus helveticus Lh-B 02, which were screened for GABA conversion, to coculture and ferment for 5 h to obtain yogurt with a GABA content of 571.0 µg/mL. The highest GABA yield of 99.63 µg/mL was achieved in the yogurt produced using Lactocaseibacillus paracasei as the probiotic and fructose galactose and inulin as the prebiotic (Garavand et al., 2022). However, the GABA content of the resulting product was relatively low. Therefore, L. brevis 54 was expected to ferment yogurt with a distinctive flavor, GABA-enriched, and potential probiotic properties.

In this study, the factors affecting the production of GABA by L. brevis 54 and enzymatic activity of the optimum conditions were first explored. Second, a GABA-enriched yogurt was developed based on the GABA-producing conditions of L. brevis 54. Finally, HPLC-MS and, headspace solid-phase microextraction (HS-SPME)-GC-MS combined with metabolomics were used to characterize the metabolic pathways of flavor compounds and GABA in yogurt, which could guide the production of specifically flavored yogurts.

**Materials and Methods**

**Materials**

Streptococcus thermophilus ABT-T and Lactobacillus delbrueckii ssp. bulgaricus BNCC 336436 were purchased from American Type Culture Collection (Manassas, VT). Leivilactobacillus brevis CGMCC1.5954 was screened by our laboratory and stored in the China General Microbiological Culture Collection Center (Beijing, China). Methanol, acetonitrile, and anhydrous sodium acetate were purchased from Macklin’s MACKLIN, Tedia, and Aladdin Biochemical Ltd., respectively. The sucrose and milk powder were provided by Angie Yeast Co. and Apatamil. Glycine, tyrosine, phenyl isothionitrate, triethylamine, glacial acetic acid, sodium L-glutamate (L-MSG), and hexane were all manufactured by Sinopharm Chemical Reagent Co.

**Determination of GABA Production Capacity of LAB**

The activated S. thermophilus (M17), L. bulgaricus (MRS), and L. brevis 54 (MRS) were added individually and at a 1:1:1 ratio at 1% (vol/vol) total inoculum to liquid medium containing 1% (wt/vol) L-MSG, respectively, and then incubated at 37°C for 48 h. The fermentation broth was centrifuged at 4°C for 5 min at 7,104 × g and the supernatant was passed through a 0.22-µm filter membrane. Then, 500 µL of the filtered sample, 250 µL of 0.2 mol/L phenyl isothionithioate-acetonitrile, and 1.0 mol/L triethylamine-acetonitrile were added and left at room temperature for 1 h. The 50 µL of 20% acetic acid was added and mixed before adding 1.0 mL of hexane and mixed for 1 min. After standing, the lower layer was aspirated and filtered through a 0.22-µm membrane. Finally, the GABA content of the fermentation broth was determined by HPLC according to the method described by Wu et al. (2021). Calculate the glutamate conversion rate according to Equation [1]:

\[
\text{Glutamate conversion ratio} = \frac{(G_L - G_0)}{(F_L - F_0)} \times 100% ,
\]

where \(G_L\) is the GABA content in the fermentation broth after addition of monosodium L-glutamate; \(G_0\) is the GABA content in the fermentation broth without addition of monosodium L-glutamate; \(F_L\) is the amount of monosodium L-glutamate added; and \(F_0\) is the amount of L-glutamate remaining after fermentation.

**Factors Influencing the Production of GABA by L. brevis 54**

The activated L. brevis 54 was inoculated at 1% (vol/vol) into 4 groups of media. Group A was de Man, Rogosa, and Sharpe (MRS) medium containing 0, 1, 1.5, 2, 2.5, and 3% (wt/vol) L-MSG; group B was Ca\(^{2+}\) concentrations of 0, 0.5, 1, 1.5, and 2.0 mmol/L with 1% L-MSG MRS medium; group C was MRS medium containing 0, 0.5, 1, 1.5, 2, 2.5, and 3% (wt/vol) glycine; and group D was MRS medium containing 0, 0.5, 1, 1.5, 2, 2.5, and 3% (wt/vol) tyrosine. The cultures were
then incubated at 37°C for 48 h and the GABA content in the fermentation broth was determined by HPLC. This was used to determine the effect of L-glutamate, calcium ion concentration, glycine, and tyrosine addition on the GABA production of *L. brevis* 54.

**Enzymatic Properties of GAD**

**Preparation of GAD.** The fermentation broth of *L. brevis* 54 was centrifuged at 4°C for 10 min at 7,104 × g. After discarding the supernatant, the broth was washed twice with 50 mmol/L sodium acetate buffer solution (pH 4.2–4.8), again discarding the supernatant before adding 100 mL of acetone (−20°C), mixed for 5 min, and then filtered with a vacuum pump and a cloth funnel to obtain LAB powder. In total, 1.0 g of LAB powder was dissolved in 50 mmol/L sodium acetate buffer solution and crushed for 20 min using an ultrasonic cell crusher with 220 W, 5 s ultrasonic operation time, and 10 s intervals. The protein concentration standard curve and protein concentration of crude enzyme solution were determined using a BCA protein assay kit (Thermo Scientific) according to the manufacturer’s instructions.

**Factors Influencing GAD Activity.** In brief, 10 g/L of the reaction substrate L-MSG was added to 50 mmol/L of sodium acetate, and the pH was adjusted using acetic acid. An aliquot 1 mL of the above solution was mixed with 1 mL of the crude enzyme solution and kept at a constant temperature for 10 min before boiling to terminate the reaction and determine the GABA content by HPLC. The effect of pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), reaction temperature (35, 40, 45, 50, 55, and 60°C), and coenzyme ions [1 mmol/L of Na+, K+, Ca2+, Mn2+, Mg2+, Zn2+, and pyridoxal phosphate (PLP)] on the activity of GAD were investigated.

**Thermal Stability and Kinetic Parameters of Enzymes.** The centrifuge tube containing 6 mL of enzyme solution (50 mmol/L sodium acetate solution and 10 g/L L-MSG) was placed at 30, 40, 50, 60, and 70°C, from which 1 mL of enzyme solution was sampled at 30, 60, 90, 120, and 180 min. The reaction was stopped by boiling immediately after 10 min at the above optimum temperature and the GABA content was determined by HPLC.

Under optimum conditions, 500 µL of the reaction system was added to 5 µL of enzyme solution and different concentrations (6–100 mM) of a substrate (S), and the reaction was terminated by boiling for 10 min. The amount of product was measured, and the reaction rate at different concentrations was calculated as V.

The Lineweaver-Burk double inverse method was used to calculate *K*ₘ and *V*ₐₘₙₐₓ, with 1/S as the horizontal coordinate and 1/V as the vertical coordinate (Yap et al., 2003).

**Preparation of GABA-Enriched Flavored Yogurt**

The activated *S. thermophilus*, *L. bulgaricus*, and *L. brevis* 54 were adjusted to a bacterial concentration of 1 × 10⁹ cfu/mL using sterile saline solution. The milk was homogenized at 65°C with 11% (wt/vol) whole milk powder, 6% (wt/vol) sucrose, and L-MSG, then pasteurized in a water bath at 95°C for 10 min and cooled in a water bath at 42°C. *S. thermophilus*, *L. bulgaricus*, and *L. brevis* 54 were added and homogenized, placed in an incubator for fermentation, and stored at 4°C for 12 h. The amount of inoculum (3, 4, 5, 6, and 7%), strain mix (*L. brevis* 54: *S. thermophilus*: *L. bulgaricus* = 1:1:1, 2:1:1, 3:1:1, 1:2:1, and 1:1:2), fermentation time (4, 5, 6, 7, 8, and 9 h), temperature (36, 38, 40, 42, 44, and 46°C), and the amount of L-MSG added (0.1, 0.15, 0.20, 0.25, and 0.30% wt/vol) on the GABA content in fermented milk was determined.

**Quality of GABA-Enriched Fermented Milk**

Two types of yogurts were prepared, group A was the control containing only the yogurt starter and group B was the GABA-enriched yogurt developed in this study. First, the squeeze mode of texture property analyzer (Stable Micro System) was used to determine the texture of yogurt at a preset speed of 1.0 mm/s, test speed of 1.0 mm/s, posttest speed of 5.0 mm/s, trigger force of 5.0 g, and test distance of 10.0 mm. Second, the pH and acidity of yogurt were determined according to the method described by Fan et al. (2022a). The amount of each type of LAB in yogurt was determined using a selective plate colony counting method. Of these, the *S. thermophilus* in the yogurt was incubated using an M17 agar medium in an aerobic incubator at 42°C for 24 h, and *L. bulgaricus* was incubated using MRS agar (pH 5.2) in an anaerobic incubator at 43°C for 72 h, whereas *L. brevis* 54 was incubated using an MRS medium at pH 5.5 at 35°C for aerobic incubation for 36 h. The sensory evaluation applied in this study was approved by the Ningbo University Institutional Review Board. The sensory evaluation was conducted by the quantitative descriptive analysis method presented in Table 1. The sensory properties of yogurt were assessed by 20 professionally trained personnel (10 males and 10 females) based on 4 quality characteristics: color, taste, odor, and tissue state.
In brief, 100 mg of fermented yogurt sample was added to a 2-mL sterile centrifuge tube with 200 µL of methanol and methyl tert-butyl ether and mixed vigorously for 60 s. The mixed samples were centrifuged at 12,000 × g for 10 min at 4°C and then passed through a 0.22-µm filter membrane. The filtered samples were used for HPLC-MS analysis, which was performed according to the method proposed by Peng et al. (2022).

Nonvolatile Flavor Compounds

The nonvolatile flavor compounds in the yogurt were extracted using methanol and methyl tert-butyl ether and mixed vigorously for 60 s. The mixed samples were centrifuged at 12,000 × g for 10 min at 4°C and then passed through a 0.22-µm filter membrane. The filtered samples were used for HPLC-MS analysis, which was performed according to the method proposed by Peng et al. (2022).

Volatile Flavor Compounds

The volatile flavor compounds in the yogurt were extracted using HS-SPME, and its content was analyzed by relative quantification. A 5-g yogurt sample was added into an extraction flask, and the volatile flavor compounds were extracted at 55°C and 350 rpm for 1 h. The Agilent 8890 GC System with 5977B/MSD was used to analyze the volatile compounds in the extracts. The GC-MS analysis was performed using helium (He) as the carrier gas at a flow rate of 1.0 mL/min and an inlet temperature set at 250°C. The starting temperature was 35°C and held for 5 min, then increased at a rate of 5°C/min to 140°C, held for 2 min, and increased again at a rate of 10°C/min to 250°C and held for 3 min (Tian et al., 2017).

Statistical Analysis

All experiments were performed with 3 replicates. Duncan’s multiple range analysis was used to analyze the variability between samples. The principal component analysis (PCA) plots and heat maps were generated by Simca software (MKS Data Analytics Solutions), and the metabolic pathways were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG).

RESULTS AND DISCUSSION

GABA Production Capacity of LAB

As displayed in Figure 1A, the 3 strains of L. brevis 54, S. thermophilus ABT-T, and L. bulgaricus BNCC 336436 used in this experiment had GABA production capacity, but the GABA production capacity of L. brevis 54 was significantly higher than that of the other 2 strains (P < 0.01). Furthermore, the L-glutamate conversion rate of L. brevis 54 was 54.22%, approximately 4.5 times higher than that of the other 2 strains. It may be because L. brevis 54 was selected from 21 strains of GABA-producing LAB, which had the most capability of converting monosodium glutamate (Wu et al., 2021). When the 3 strains were mixed in a 1:1:1 ratio, the GABA content of the mixed ferment was higher than that of the single strain ferment (P < 0.05). This is consistent with previous studies where mixed cultures of S. thermophilus and L. bulgaricus, isolated from commercially available yogurt, had higher GABA than that of a single strain (Hayakawa, 1997). This may be due to the symbiotic effect of S. thermophilus and L. bulgaricus, where the polypeptides produced from the breakdown of casein by L. bulgaricus can be further hydrolyzed into short peptides and AA by S. thermophilus, whereas the formic acid, folate, and CO2 produced by S. thermophilus can in return promote the growth of L. bulgaricus (Sieuwerts et al., 2010).

Factors Influencing the Production of GABA by L. brevis 54

As a precursor to GABA production by LAB, glutamate significantly affected GABA production by L. brevis 54. As demonstrated in Table 2, the amount of GABA produced by L. brevis 54 increased with
The γ-aminobutyric acid (GABA) production capacity of lactic acid bacteria (LAB) and factors influencing the enzymatic properties of glutamic acid decarboxylase. (A) The GABA production capacity of Leuvalactobacillus brevis CGMCC1.9554 (L. brevis 54), Lactobacillus delbrueckii ssp. bulgaricus (L. bulgaricus), Streptococcus thermophilus ABT-T, and mixed strains. (B) Effect of pH on the activity of glutamic acid decarboxylase. (C) Effect of temperature on the activity of glutamic acid decarboxylase. (D) Thermal stability of glutamic acid decarboxylase. (E) Effect of metal ions on enzyme activity. (F) Kinetic curves for enzymatic reactions, where S denotes the substrate concentration and V is the reaction rate (i.e., the enzyme activity). The letters a, b, c indicate significant differences, where $P < 0.05$ indicates a significant difference and $P > 0.05$ indicates a nonsignificant difference. Data are expressed as mean ± SD.

The addition of L-MSG and then decreased, reaching a maximum value of $13.24 \pm 1.53$ g/L at 2.5% (wt/vol) L-MSG. The decrease in GABA production at 3% (wt/vol) was probably due to the accumulation of glutamic acid in the cells, which prevented the synthesis of GABA (Villegas et al., 2016).

Activation of glutamate decarboxylase by calcium-binding calmodulin (CaM) is required for the normal regulation of glutamate and GABA metabolism in plants. The interaction of CaM with the C-terminal structural domain of GADase induces dimerization of the enzyme, which is associated with Ca$^{2+}$-dependent enzymes (Yap et al., 2003). In Table 2, low concentrations (0.5–1.0 mmol/L) of Ca$^{2+}$ significantly ($P < 0.05$) increased the GABA content of the solution compared with the lack of Ca$^{2+}$ addition, however, when the Ca$^{2+}$
concentration was too high, the GABA content significantly decreased. It may be due to the high osmolarity directly inhibiting the biosynthesis of GABA by *L. brevis* 54 (Lyu et al., 2018). When glycine was added to the medium, the ability of *L. brevis* 54 to produce GABA was increased to some extent, but the highest value was only 1.75 ± 0.30 g/L, much lower than the GABA production with L-MSG as the substrate.

Tyrosine is a conditionally essential and ketogenic glycogenic AA for the human body, with a role in mood regulation and stimulation of the nervous system, and can also be used as a nutritional supplement (Paul and Mukhopadhyay, 2004). The experimental results of tyrosine addition to the medium demonstrated a similar trend as glycine, with the maximum yield of only 10% when glutamic acid was the substrate. Amino acids have been reported to promote the growth of LAB (Solvval et al., 2019). However, glycine and tyrosine revealed weak effects in promoting the growth of *L. brevis* 54 to produce GABA, whereas the addition of glutamate, a substrate for GABA synthesis, had the greatest effect.

### Enzymatic Properties of GAD

Glutamic acid decarboxylase can catalyze the α-decarboxylation of glutamic acid to produce GABA, a key enzyme in the synthesis of GABA in living organisms (Lyu et al., 2018). Therefore, it is crucial to explore its enzymatic properties for GABA production in *L. brevis* 54. The BCA kit determined the protein concentration of the extracted GAD crude enzyme solution. The standard curve equation was defined as $y = 0.0002x + 0.1194$ ($R^2 = 0.9995$) and the measured protein concentration was 3.282 g/L.

The pH is a key factor affecting enzyme activity, as it may change the dissociation state of the group in the active site. When the pH was gradually increased from 3.5, GAD activity gradually increased and then decreased. When the pH value was 4.5, GAD had the maximum enzymatic activity (Figure 1B). This result is consistent with the activity of LAB GAD as determined by Huang et al. (2018). This is mainly due to the ability of the Gad B structure in bacteria to regulate the effect of pH on catalytic activity through the 14 residues at the C-terminus (Tramonti et al., 2002).

Enzymes are mostly proteins by nature, and its activity is significantly influenced by temperature (Yap et al., 2003). As displayed in Figure 1C, the enzymatic activity of GAD depicted an overall increase and then decrease as the temperature of the reaction system increased to a maximum of 55°C. The optimum germination temperature of the foxtail millet (*Setaria italica* L.) GAD is 40°C, which is inconsistent with our results, mainly because of the differences in the structure and, therefore, the enzymatic properties of GAD from different sources (Bai et al., 2009). The thermal stability of enzymes is an important property and is mainly related to the stability of its spatial conformation. As displayed in Figure 1D, the enzymatic activity was above 80% at lower temperatures (30, 40, and 50°C). When the enzyme is exposed to 60°C, the activity decreases with time. At 70°C, GAD was completely inactivated after 240 min.

The cofactors of enzymes are generally metal ions and organic substances of small molecular weight, which perform functions such as the transfer of hydrogen ions, electrons, and some chemical groups, which can bind to the corresponding ligands and change the conformation of the active site, thus making it more favorable to bind to the substrate to form a more stable complex. The addition of PLP has a promotive effect on the activity of this GAD and the highest activity, whereas several other metal ions inhibit the enzyme to some extent (Figure 1E). The PLP acts as a cofactor for GAD and has a PLP-binding region in the GAD structure; hence it increases the activity of GAD (Zhang et al., 2007). We demonstrated that high concentrations of Ca²⁺ have an inhibitory effect on the activity of enzymes, which is consistent with the effect of adding of Ca²⁺ to the medium on GABA production by *L. brevis* 54.
Enzyme kinetics is the study of the ability of enzymes to bind substrates and catalyze reaction rates, with rate equations at the heart of any kinetic study. To provide a simple and clear reaction rate for glutamate decarboxylase, a double inverse graphing method (Lineweaver-Burk graphing) was used to obtain a straight line with a horizontal axis intercept of $-1/K_m$ and a vertical axis intercept of $1/V_{\text{max}}$, using the Equation $1/v = (K_m/V_{\text{max}}) (1/S) + 1/V_{\text{max}}$. The data measured at an optimum pH of 4.5 and temperature of 50°C are displayed in Figure 1F. The intercepts of horizontal and vertical axes were converted to give a $K_m$ of 0.98 mM, and a $V_{\text{max}}$ of 7.53 U/mg. The $K_m$ of the enzyme was 0.98 mM and the $V_{\text{max}}$ was 7.53 U/mg.

Preparation of GABA-Enriched Flavored Yogurt

The fermentation temperature affects not only the activity of GAD but also the growth performance of LAB and thus the quality of yogurt. As illustrated in Figure 2A, with the increase in fermentation temperature, the GABA content in yogurt increased and then decreased, reaching a maximum value of 51.22 ± 1.11 (mg/100 g) at 42°C. Meanwhile, the number of viable $L.\ brevis$ 54 reached a maximum value of 8.72 log (cfu/mL). The results may be due to the yogurt’s highest activity of the starter at 42°C, creating a more suitable environment for $L.\ brevis$ 54 to grow and thus allowing an increased GABA production and from section 3.3 that GAD in $L.\ brevis$ 54 has high activity at this temperature, whereas too high a fermentation temperature would inhibit the growth of LAB and thus reduce the GABA content of yogurt (Fan et al., 2022a).

The GABA content in yogurt reached a maximum of 71.71 ± 2.25 mg/100 mL when the inoculum was 6% (wt/vol; Figure 2B). From the GABA Production Capacity of LAB section, $L.\ brevis$ 54 was the main GABA-producing bacterium in yogurt, which gradually increased when its numbers increased; however, when the inoculum was too large, it led to competition between LAB species for limited nutrients; hence the low pH induced a decreased GABA production in yogurt (Villegas et al., 2016). Fermentation time not only influences the production of GABA by LAB but also significantly affects the texture and flavor formation of yogurt (Nsogning Dongmo et al., 2017). As the fermentation time increased, the pH of yogurt gradually decreased and the number of viable $L.\ brevis$ 54 increased, whereas GABA reached a maximum value of 114.07 ± 4.67 (mg/100 g) by 8 h (Figure 2C).

As discussed in the GABA Production Capacity of LAB section, a mixture of the 3 bacteria in an MRS medium increases GABA production; however, the effect of the different strain ratios on the GABA content of yogurt is not yet known. When $L.\ brevis$ 54: $S.\ thermophilus$: $L.\ bulgaricus$ was equal to 3:1:1, the GABA content in the yogurt was up to 147.36 mg/100 mL (Figure 2D), 52.54% higher than the previous optimized result (Chen et al., 2018). The ratio of $L.\ brevis$ 54 determined the GABA content of yogurt, which indicated that the flora in the yogurt made excellent use of the monosodium glutamate to convert it to GABA.

The addition of L-MSG had a significant effect on GABA in the fermentation broth (Factors Influencing the Production of GABA by $L.\ brevis$ 54 section), but as demonstrated in Figure 2E, there was no significant increase in GABA content in the fermented milk as the monosodium glutamate content increased. This may be due to the short fermentation time of yogurt, during which the amount of monosodium glutamate available to $L.\ brevis$ 54 reached saturation, and therefore the further increase in monosodium glutamate did not increase production (Villegas et al., 2016; Chen et al., 2018). In addition, the amount of L-MSG used as an additive in food is a key factor as residual L-MSG imparts a distinctly fresh taste to the yogurt; therefore, a minimum addition of 0.10% (wt/vol) was chosen (Solval et al., 2019).

Quality of GABA-Enriched Fermented Milk

The differences between the GABA-enriched flavored yogurt developed in this experiment and conventional fermented yogurt (containing only a starter as a control) were compared in terms of texture, viable bacteria count, sensory evaluation, and GABA content. The GABA-enriched yogurt had higher tissue status and odor scores than the control group (Table 3). Compared with the control group, the GABA-rich yogurt (147.36 mg/100 mL) developed in this experiment had 317.06% higher GABA content (Figure 2G). Furthermore, Husin et al. (2020) reported a GABA content of 59.00 mg/100 g in yogurt fermented with $L.\ plantarum$ Taj-Apis362 in combination with conventional fermenters. Moreover, $L.\ brevis$ KCTC 3320 and soybean sprouts (1 cm) were used to ferment soy flour yogurt with a GABA content of 120.38 mg/100 mL (Hwang et al., 2018). The GABA content of the yogurt developed in this study was significantly higher in comparison, probably due to the $L.\ brevis$ 54 being a high GABA-producing LAB, the abundant substrate, and suitable enzyme reaction conditions for GABA production.

In contrast, $L.\ brevis$ 54 had little effect on the growth of $S.\ thermophilus$ and $L.\ bulgaricus$. This was consistent with Gu et al. (2020), who used $L.\ paracasei$ IMC502 cocultured with a conventional starter to find that probiotics did not affect the number of viable bacteria in the starter. As displayed in Table...
Figure 2. Production of γ-aminobutyric acid (GABA)-enriched yogurt. (A) Effect of fermentation temperature on GABA content, viable bacterial count, and pH of yogurt. (B) Effect of the amount of inoculum. (C) Effect of the fermentation time. (D) Effect of strain combination. (E) Effect of sodium L-glutamate. *L. brevis* 54 = *Levilactobacillus brevis* CGMCC1.5954. The letters a, b, c indicate significant differences, where $P < 0.05$ indicates a significant difference and $P > 0.05$ indicates a nonsignificant difference. Data are expressed as mean ± SD.
3, the GABA-enriched yogurt fermented by \textit{L. brevis} 54 revealed significantly higher hardness, adhesiveness, cohesiveness, and gumminess than those of the control group but no significant effects on chewiness, resilience, and springiness. This is because LAB can produce extracellular polysaccharides when fermenting yogurt, which consists mainly of galactose, mannose, arabinose, and xylose, which typically increase the viscosity of yogurt. Therefore, the \textit{L. brevis} 54 may be a strain of LAB that is more prone to extracellular polysaccharides production (Pachekrepapol et al., 2017; Fan et al., 2022a).

### Nonvolatile Flavor Compounds

The HPLC-MS was combined with metabolomics to analyze nonvolatile flavor compounds and its metabolic pathways in GABA-enriched yogurt fermented by \textit{L. brevis} 54. In the PCA score plot (Figure 3A), samples in the same group were clustered together, indicating its repeatability and data reliability. In contrast, the larger distance between groups A and B indicated greater differences in nonvolatile metabolites in the 2 types of yogurts (Wang et al., 2021), which were strictly distinguished along PCA1 (30.7%) and PCA2 (22.6%).

A heat map was drawn to distinguish the different nonvolatile metabolites between the 2 groups of yogurts (Figure 3B). For the screening of differential metabolites, a \( P \)-value \( \leq 0.05 \), variable importance in the projection (VIP) \( \geq 1 \), and fold change \( \geq 1.5 \) or \( \leq 0.667 \) were generally considered to be statistically significant (Tian et al., 2017; Gu et al., 2020; Peng et al., 2022).

Comparing group B (GABA-enriched yogurt) with group A (control, containing only starter), a total of 84 differential metabolites were screened, of which 30 were significantly downregulated and 54 were significantly upregulated. The GABA, l-glutamic acid, and succinic acid semialdehyde, related to GABA biosynthesis, were upregulated by 3.35, 3.07, and 2.48 folds, respectively. The pyridoxal 5-phosphate was downregulated by 0.66 folds which acts as a multifunctional coenzyme involved in GABA biosynthesis (Lyu et al., 2018; Villegas et al., 2016).

In addition, l-tryptophan, l-cysteine, l-proline, l-threonine, l-arginine, l-leucine, l-isoleucine, l-allothreonine, and l-glutamic acid were significantly upregulated by 1.99, 1.58, 3.07, 1.90, 1.83, 2.04, 1.51, 1.71, and 1.52 folds, respectively. Of these, l-tryptophan, l-threonine, l-isoleucine, and l-leucine are EAA that cannot be synthesized by the body or are synthesized at a rate that is far from adequate for the needs of the body and must therefore be supplied by dietary protein intake (Hu and Guo, 2021). On the other hand, l-arginine is an EAA for premature babies (Solon-Biet et al., 2019). Many organisms prefer l-type AA, which are more easily digested and absorbed. Furthermore, AA such as leucine, tryptophan, and tyrosine positively affect the growth of LAB (Yonezawa et al., 2010; Newstead, 2019). This may be one of the reasons for the high GABA content in mixed fermented yogurt. The results indicated that the GABA-rich yogurt produced by the fermentation of \textit{L. brevis} 54 was of high nutritional value.
Figure 3. Principal component (PC) analysis (PCA) score plot and a heat map of nonvolatile metabolites. (A) PCA score plot; (B) heat map of nonvolatile metabolites. Group A: control group, containing starter only; group B: experimental group, γ-aminobutyric acid (GABA)-enriched yogurt.
By analyzing the metabolic pathways of the differential metabolites, 20 metabolic pathways with high impact were identified in *L. brevis* 54 (Figure 4A). The GABAergic synapse, d-glutamine and d-glutamate metabolism, and alanine, aspartate, and glutamate metabolism pathways associated with GABA metabolism indeed appeared significantly upregulated. The metabolic pathways associated with GABA synthesis and metabolism in *L. brevis* 54 are summarized in Figure 4B. In addition, the significant upregulation of 9 AA in yogurt may be due to changes in the valine, leucine, and isoleucine biosynthesis, β-alanine metabolism, and alanine, aspartate, and glutamate metabolism metabolic pathways.

**Volatile Flavor Compounds**

The addition of probiotic LAB may positively affect the volatile flavor compounds in yogurt (Tian et al., 2017). In addition, in the sensory evaluation of the 2 yogurts, the one with the addition of *L. brevis* 54 scored highest on the odor. Therefore, HS-SPME-GC-MS was used to analyze the differentially volatile flavor compounds in the 2 yogurts. As shown in the graphical PCA analysis (Figure 5A), the groups proximity and far distance indicated excellent sample reproducibility and large differences in volatile flavor compounds between groups (Nsogning Dongmo et al., 2017). The volatile flavor compounds in the 2 yogurts were strictly differentiated along the PCA1 (68.9%) and PCA2 (29.18%).

The relative values of the volatile flavor substance content in the 2 yogurts were subjected to metabolite hierarchical cluster analysis (Figure 6). A total of 58 volatile flavor compounds were identified in the yogurt, including alcohols, aldehydes, ketones, carbonyl compounds, organic acids, hydrocarbons, sulfur compounds, and other compounds, which were more than the volatile flavor compounds identified in other probiotic-containing yogurts using the same analytical method (Gu et al., 2020; Huang et al., 2020; Zhang et al., 2022). Most of the flavor compounds in yogurt were produced by the lipolysis of milk fat and microbial transformation of lactose and citric acid (Özcelik et al., 2016). Short-chain fatty acids such as butyric acid in yogurt have been produced from SFA and have contributed significantly to the flavoring of yogurt (Chen et al., 2017). Unsaturated fatty acids are oxidized into hydroperoxides in the presence of free radicals, which are rapidly decomposed into hexanal or unsaturated aldehydes. Biogenic amines and nitrogen-containing compounds were derived from proteins and AA, whereas sulfur-containing compounds were derived from organic compounds (Ott et al., 2000).

Of the 58 volatile flavor compounds, 2-heptanone, benzene, 1,2,4,5-tetramethyl-, benzene, 1-ethyl-2,4-dimethyl-, naphthalene, benzene, 2-ethyl-1,4-dimethyl-, cyclopentasiloxane, decamethyl-, and 2-nonanone were significantly upregulated, whereas benzene, 4-ethyl-1,2-dimethyl-, 2-heptanol, (S)-, indan, 1-methyl-, benzene, 1,2,3,5-tetramethyl-, pentanal, 2-methyl-, 4-methylpropionophenone, and undecane, 4,7-dimethyl- were significantly downregulated (Figure 5B). Among them, 2-nonanone had a fresh, sweet, green, cheesy, and fruity odor; pentanal, 2-methyl- had an ether-like, green and vegetal nature with a fruity flavor (Lubbers et al., 2004); cyclopentasiloxane, decamethyl- had a slight characteristic odor and deodorizing properties, as it is an excellent carrier of oils and active ingredients and is often used in cosmetics and personal care products; benzene, 2-ethyl-1,4-dimethyl- has an aromatic hydrocarbon odor; 2-heptanol, (S)- had a mushroom and cheese smell; 2-heptanone presented a cheese, fruity, coconut, waxy, green aroma (Cheng, 2010). The significant upregulation of 2-nonanone and 2-heptanone, which are typical aroma compounds of yogurt and contribute significantly to the flavor of yogurt, indicated their role in the high odor scores of GABA-enriched yogurt during sensory evaluation (Aryana and Olson, 2017).

**CONCLUSIONS**

This study developed and characterized a GABA-enriched yogurt fermented by *L. brevis* 54. The maximum GABA content in the yogurt was obtained when the fermentation temperature was 42°C; the inoculum was 6% (vol/vol), the fermentation time was 8 h. *L. brevis* 54: *S. thermophilus*: *L. bulgaricus* 54: *L. brevis* 54: 3:1:1, and L-MSG was added at 0.10% (wt/vol). At this point, the GABA content in the yogurt was 317.06% higher than that in the control group and 52.54% higher than in the previous studies. Further, there was a significant improvement in the aroma, hardness, adhesion, cohesive- ness, and gelatinousness of yogurt. HPLC-MS further confirmed the presence of high GABA in the yogurt and the nutrition of yogurt. The GABA in yogurt was mainly related to GABAergic synapse, d-glutamine and d-glutamate metabolism, and alanine, aspartate, and glutamate metabolism pathways of *L. brevis* 54. A total of 58 volatile flavor substances were identified using HS-SPME-GC-MS, of which 2-nonanone and 2-heptanone may be responsible for the high odor score of GABA-enriched yogurt in the sensory evaluation. This study developed a nutritious and unique GABA-enriched flavored yogurt, summarizes the metabolic pathway of GABA, provided a flavor compounds fin-
Figure 4. The enriched pathways were involved in metabolites and the γ-aminobutyric acid (GABA) synthesis and metabolism pathway in *Lactobacillus brevis* CGMCC1.9554 (*L. brevis* 54). (A) The enriched pathways involved metabolites in group B compared with the control A. (B) The GABA synthesis and metabolism pathway in *L. brevis* 54. Group A: control group, containing starter only; group B: experimental group, GABA-enriched yogurt. PC = principal component; PCA = principal component analysis.
Figure 5. Principal component analysis (PCA) and variable importance in the projection (VIP) value for volatile flavor substances in yogurt. (A) PCA analysis; (B) comparison of groups B and A yielded important variables for changes in yogurt volatile flavor substances. Group A: control group, containing starter only; group B: experimental group, γ-aminobutyric acid (GABA)-enriched yogurt. VIP, which is the importance of a variable to the model, describes the overall contribution of each variable to the model, where the threshold is set at VIP >1. Red for upward adjustment, blue for downward adjustment. Red indicates an increase in content compared with the control, whereas blue indicates a decrease.
gerprint, and provided theoretical guidance to produce specifically flavored yogurts.

ACKNOWLEDGMENTS

This work was supported by the Natural Science Funding of China (31972048, 32272339; Beijing, China), the outstanding youth fund of Zhejiang Province (LR22C200001; Hangzhou, China), and National Key R&D Program of China (2021YFD2100104; Beijing, China). Xiankang Fan, writing original draft, data curation, investigation, methodology, formal analysis; Luyun Yu, conceptualization, writing review and editing; Daodong Pan, conceptualization, project administration, funding acquisition; Zihang Shi, methodology; Chunwei Li, data curation; Zhen Wu, formal analysis,
Fan et al.: CHARACTERIZATION OF FLAVORED FERMENTED YOGURT


ORCIDs

Xiankang Fan https://orcid.org/0000-0002-3462-9443

Zhen Wu https://orcid.org/0000-0002-3607-6440

Daodong Pan https://orcid.org/0000-0002-5299-644X