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Bioprocessing of Mulberry Leaf Juice With High-GABA Producing *Lactobacillus plantarum*: A Strategy for Flavor, GABA Enrichment, and Antioxidant Enhancement

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ABSTRACT

The purpose of this work is to isolate γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) from traditional Chinese pickles and to evaluate their fermentation characteristics and functional effects in a mulberry leaf juice system. Six GABA-producing strains were isolated, comprising five *Lactobacillus plantarum* strains and one *Enterococcus faecium* strain. These strains demonstrated significant probiotic properties, exhibiting traits such as acid tolerance, bile salt resistance, in vitro gastrointestinal resistance, cell surface hydrophobicity, auto-aggregation, γ-hemolysis, and antibiotic sensitivity. The GABA content in fermented mulberry leaf juice ranged from 0.663 ± 0.007 to 0.879 ± 0.023 mg/mL, which was 9.61 to 12.74 times that of the initial content. Mulberry leaf juice's flavor, α-glucosidase inhibitory activity, DPPH and hydroxyl radical scavenging capacity, and ferric-reducing antioxidant power (FRAP) were all greatly improved by the LAB fermentation. A quality evaluation model for fermented mulberry leaf juice was developed using principal component analysis (PCA), identifying *L. plantarum* L10 as the optimal probiotic strain for fermentation.

1 | Introduction

Mulberry (*Morus alba* L.) leaves are significant source of medicine and food. This perennial species belongs to the Moraceae family. Rich in nutrients and bioactive compounds like polyphenols, polysaccharides, and alkaloids, mulberry leaves exhibit antioxidant, lipid-lowering, and anti-inflammatory properties, earning them recognition as a medicinal and edible plant from China's National Health Commission (Han et al. 2025a; Ma et al. 2022; Meng et al. 2025; Yu and Shi 2021). Consequently, mulberry leaf-based products such as beverages and teas have gained market popularity (Xue et al. 2025; Han et al. 2025b). Fresh mulberry leaves contain substantial moisture, fiber, carbohydrates, protein, and provide essential minerals and vitamins, alongside functional components including GABA (Tian et al. 2025).

Glutamic acid decarboxylase, commonly abbreviated as GAD, mediates the decarboxylation reaction of glutamate, thereby yielding GABA—a type of nonprotein amino acid. It has several health advantages, including as neuroprotective, anxiolytic, and antihypertensive properties (Kwon et al. 2025). However, its natural concentration in mulberry leaves is relatively low (2.45–122.5 mg/100 g DW), despite the presence of its precursor, glutamic acid (L. Zhang et al. 2014). As endogenous GABA production declines with age, dietary supplementation through GABA-enriched products presents a promising strategy to counteract age-related deficiencies (Cuypers et al. 2020; Iorizzo et al. 2024).

Conventional strategies to augment GABA in plant materials, such as abiotic stress or solid-state fermentation, often face limita-

tions in scalability and process control. Liquid-state fermentation offers a promising alternative for the production of uniform, beverage-type products. LAB are promising candidates for GABA production and a food additive that is generally recognized as safe (GRAS) (Langa et al. 2025). Strains like *Lacticaseibacillus paracasei* and *Lactobacillus plantarum* have demonstrated high GAD activity, isolated from traditional fermented foods (Cui et al. 2020; Montagano et al. 2025; Yin et al. 2026). Their inherent acid tolerance and plant substrate utilization capabilities are hypothesized to not only drive efficient GABA production but also to synergistically improve the flavor profile and enhance other functional properties of mulberry leaf juice.

Therefore, this work was designed to isolate GABA-producing LAB from Chinese pickles and systematically evaluate their performance in fermented mulberry leaf juice. We hypothesize that the fermented mulberry leaf juice with LAB strains will lead to a multifunctional beverage characterized by synergistic enhancements in GABA content, antioxidant and antidiabetic activities, and overall sensory acceptability. This research provides a novel strategy for the valorization of mulberry leaves by creating a health-promoting product that integrates probiotics, bio-enriched GABA, and inherent leaf bioactives.

2 | Materials and Methods

2.1 | Materials and Reagents

Fresh mulberry leaves were obtained from a sericulture farm in Jinhua City, Zhejiang Province, China. The sampling time was from April to May each year, at 9 a.m. The leaves were all collected at the same maturity stage, specifically selecting the fourth to eighth leaves from the top of the branch to ensure consistency in physiological development. Traditional Chinese Pickles originated from the agricultural market in Wuxi, China. MRS broth was purchased from Haibo Biotechnology Company (Qingdao, China). Fengtai Biotechnology Co. Ltd. (Shanghai, China) supplied the GABA standard and phosphate-buffered saline (PBS).

2.2 | Isolation of LAB Strains

The pickles were diluted consecutively using 0.85% NaCl (w/v) and inoculated onto MRS agar, followed by incubation for 48 h at 37°C. White colonies were isolated and streaked three times for purification to obtain pure cultures. Colony morphology was observed and recorded. Preliminary identification of the isolated strains was conducted using Gram staining and the catalase test. The strains were preserved in tubes containing 25% glycerol at −80°C.

2.3 | Screening of GABA-Producing LAB

2.3.1 | Screening

MRS medium, containing 10 g/L MSG, was inoculated with LAB strains at a 2% level and then incubated statically for 72 h at 37°C. The method of Bai et al. with some modifications, thin-layer chromatography (TLC) was used to conduct a preliminary screening of the bacteria that produce GABA (Bai et al. 2025). The developing solvent consisted of *n*-butanol:water:glacial acetic acid in a 4:1:1 (v/v/v) ratio, supplemented with 0.4% (w/v) indole-3-acetic acid. The reaction was stopped when the developing solvent reached 1 cm from the top edge of the silica gel plate. For color development, the plate was subsequently incubated at 90°C for 10 min in an oven. GABA was visualized on the TLC plate, and MSG and GABA standard samples were used as controls for comparison.

2.3.2 | Quantitative Determination of GABA Content by UPLC

A microporous filter (aqueous phase, 0.22 μm) was used to filter the fermentation broth supernatant for further examination. GABA content was quantitatively determined by using the HPLC with an automatic pre-column *o*-phthalaldehyde (OPA) derivatization method, with detailed methods provided in the Supporting Information (Chen et al. 2024).

2.4 | 16S rRNA Gene Analysis

Ezup column-based kit for extracting bacterial genomic DNA was used to extract DNA from the target strain. PCR amplification was conducted with universal bacterial primers: forward 27F (AGAGTTTGATCMTGGCTCAG) and reverse 1492R (GGTTACCTTGTTACGACTT). The reaction mixture (25 μL total volume) consisted of 1 μL forward primer, 1 μL reverse primer, 1 μL template DNA, 12.5 μL PCR mix, and 9.5 μL double-distilled water. The cycling conditions followed the method described by W. Zhang et al. (2022). The amplification products were confirmed by gel electrophoresis and subsequently sent for sequencing at Shanghai Sangong Biotechnology Company. The resulting sequences were subjected to BLAST analysis for comparison.

2.5 | Probiotic Potential Characterization

2.5.1 | Acid and Bile Salts Tolerances

Referring to the method proposed by Xia et al. (2021), incorporating some adjustments, the tolerance of LAB strains to low pH and bile salts was evaluated. Specifically, after centrifugation and washing with PBS buffer, the bacterial cells were inoculated at 5% (v/v) into MRS broth adjusted to pH 3.0 or containing 0.3% (w/v) bile salts, followed by incubation for 3 h. Viable cell counts were then determined by plating appropriate dilutions onto MRS agar, expressed as log colony-forming units per milliliter (log CFU/mL). The survival rate was reported as:

$$\text{Survival rate } (\%) = (\log N / \log N_0) \times 100 \quad (1)$$

where log$N$ and logN_0 correspond to the logarithmic values of the terminal and initial viable cell populations, respectively.

2.5.2 | In Vitro Gastrointestinal Fluid Tolerances

The tolerances of in vitro gastrointestinal fluids were assessed based on the method proposed by W. Zhang et al. (2022), with slightly modifications. Specifically, after washing with PBS buffer, the bacterial cells were inoculated at 5% (v/v) into artificial gastric

fluid (PBS containing 3 g/L pepsin, pH 3.0) and incubated for 3 h. The bacteria were then collected, washed again with PBS, and transferred into artificial intestinal fluid (PBS containing 1 g/L trypsin, pH 8.0) for a further 3 h of incubation. Finally, viable cell counts were determined by formula (1).

2.5.3 | Cell Surface Hydrophobicity and Auto-Aggregation Activity

The hydrophobicity and auto-aggregation ability of LAB were determined with reference to literature methods (X. Li, Zhu, et al. 2022; Fan et al. 2022), with minor adjustment. Specifically, hydrophobicity was evaluated using the microbial adhesion to solvents method. The cells were resuspended in 0.1 M KNO_3 (pH 6.2) and adjusted to an OD_{600} of 0.8, recorded as A_0. Then 3 mL of the bacterial suspension was mixed with 1 mL of xylene, left to stand for 3 h, and the OD_{600} of the aqueous phase was measured as A_t. Auto-aggregation ability was assessed by adjusting the bacterial suspension to an OD_{600} of 0.8 with PBS, recorded as A_0, followed by static incubation at 37°C for different time intervals (2, 4, 6, and 8 h), and measuring the OD_{600} of the supernatant at each time point as A_t. Both indicators were reported as:

$$\text{Indicator } (\%) = [1 - (A_t/A_0)] \times 100 \quad (2)$$

where A_0 denotes the initial OD_{600} value, A_t represents the OD_{600} value at each incubation time point.

2.5.4 | Antibiotic Susceptibility

The strains were evenly disseminated on MRS agar plates after being grown to an amount of 8.0 log CFU/mL. Place antibiotic susceptibility sheets on the surface of the agar, including gentamicin (10 μg), ampicillin (10 μg), ciprofloxacin (5 μg), vancomycin (30 μg), kanamycin (30 μg), tetracycline (30 μg), erythromycin (15 μg), penicillin (10 units). Measurements of the inhibitory zone diameters were made, and the results were interpreted using the criteria put forward by Charteris et al. (1998).

2.5.5 | Hemolysis Test

Blood agar plates were streaked with a suitable volume of the activated bacterial suspension and incubated for 24 h at 37°C (Rwubuzizi et al. 2025). Hemolysis around the bacterial colonies was assessed for its presence or absence. Nonhemolytic strains showed no effect (γ-hemolysis) or exhibited a greenish area (α-hemolysis), while hemolytic strains formed a clear zone of lysis (β-hemolysis).

2.6 | Preparation of Mulberry Leaf Juice for Fermentation

Fresh tender mulberry leaves are washed with clean water, drained of excess moisture. The mulberry leaves are juiced in a blender with a water-to-juice ratio of 1:2 (w/v). The resulting juice is filtered using 100-mesh filter cloth and subsequently pasteurized at 80°C for 10 min. A 2% LAB inoculum is added to the mulberry leaf juice containing 0.6% (w/v) MSG for fermentation (approximately 7.0 log CFU/mL), with a control group that does not receive LAB inoculation. Because the GABA precursor substances in mulberry leaves are limited, which the control group without added MSG only a 1.51-fold increase, with GABA content reaching 0.173 mg/mL. Adding 0.6% MSG is based on the results of preexperiments, which took into account factors such as fermentation efficiency, product accumulation, and flavor coordination. The mixture has been incubated at 37°C for 72 h, in a temperature-controlled incubator.

2.7 | Viable Cell Counts

The usual plate counting method was used to perform the viable cell counts test. A continuous dilution of fermented mulberry leaf juice as conducted with sterile saline, plated onto MRS agar plates to incubate.

2.8 | Total Phenol Content and Total Flavonoid Content

Total phenol content (TPC) of fermented mulberry leaf juice was determined using the Folin–Ciocalteu method (Murai et al. 2025). The determination of total flavonoid content (TFC) is based on the method proposed by Jia et al. (2025), with slightly adjustment. Specifically, the first step is to mix 1 mL of appropriately diluted fermented mulberry leaf juice with 1 mL of water, then add 0.4 mL of 50 g/L $NaNO_2$ solution. The second step is to add 0.4 mL of 100 g/L $Al(NO_3)_3$ solution. The first and second step are each mixed and incubated for 6 min. The final step is to add 4 mL of 40 g/L NaOH and 3.2 mL of water, and the mixture was allowed to react for 15 min before measuring the absorbance at 510 nm. The outcomes quantified as rutin equivalents (RE) /mL.

2.9 | Flavor Profile Analysis Using Electronic Nose

The ultra-fast GC electronic nose equipped with both a DB-5 nonpolar column and a DB-1701 low-to-medium polarity column, was used for simultaneous dual-column analysis. Each sample was injected and the retention indices were determined using a reference C6–C16 alkane standard solution. Compounds detected by the system were analyzed using Alphasoft V 12.44 data processing software and the Aroma Chem Base database. A 5 mL sample of fermented mulberry leaf juice was transferred to a 20 mL headspace vial. The electronic nose's parameters were set up based on the method proposed by Krishnan et al. (2026). With some modifications, the details can be found in Supporting Information.

2.10 | Analysis of Biological Activity

2.10.1 | α-Glucosidase Inhibition Activity

The blood glucose-lowering activity of fermented mulberry leaf juice was evaluated based on an α-glucosidase inhibition assay (Yue et al. 2022), with slightly adjustment. Briefly, a mixture

containing 50 μL of diluted juice and 50 μL of 0.2 U/mL α-glucosidase was pre-incubated for 5 min in a 96-well plate prior to the reaction at 37°C. Subsequently, 50 μL of 4 mM p-NPG solution was added to each well to start the reaction. The amount of p-nitrophenol (p-NP) generated by p-NPG hydrolysis was measured by measuring the absorbance at 405 nm after 100 μL of 0.2 M Na_2CO_3 was added to stop the reaction. The following formula was used to determine the α-glucosidase inhibitory activity:

$$\text{Inhibition } (\%) = [1 - (A_s - A_b)/A_c] \times 100 \quad (3)$$

where A_b refers to the value of without α-glucosidase group, while A_c and A_s correspond to the values of the group without the sample and the test sample group, respectively.

2.10.2 | Determination of Antioxidant Capacity

To comprehensively evaluate the in vitro antioxidant activity of the mulberry leaf juice, four methods were employed. DPPH radical scavenging activity (Teixeira et al. 2024), ABTS radical scavenging activity (S. Wang et al. 2024), and hydroxyl radical scavenging activity (K. Li et al. 2023). All scavenging activity assays used 0.1 mg/mL ascorbic acid (VC) solution as the positive control. A commercial FRAP assay kit (M78793; Merck) was used and the procedure was carried out according to the instructions provided. FRAP capacity was expressed as Trolox equivalents (TE/mL).

2.11 | Quality Evaluation Model for Fermented Mulberry Leaf Juice

PCA is a multivariate statistical method based on dimensionality reduction. Its purpose is to transform multiple original variables into a few comprehensive components (principal components [PCs]). This transformation reduces data dimensions and complexity while retaining key information in the original dataset. PCA was employed to evaluate the fermentation characteristics and bioactivity of mulberry leaf juice, considering seven key indicators: GABA yield, strain growth before and after fermentation, α-glucosidase inhibition activity, FRAP and DPPH, ABTS and hydroxyl radical scavenging activity. PCs with eigenvalues greater than 1 were selected for further analysis. By analyzing the score functions between each PC and various indicators, a comprehensive quality evaluation model of fermented mulberry leaf juice was established. The variance contribution rate of each PC was used as the weight in this model, allowing for comprehensive scoring and ranking of different fermentation strains (Lan et al. 2023).

2.12 | Statistical Analysis

All experiments were performed in three independent biological replicates. The mean ± standard is used to present the results. PCA, multiple comparisons, and analysis of variance were performed using SPSS 26.0. PCA and data visualization were done using Origin 2024b. Data normality and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. One-way ANOVA was used for datasets that satisfied these criteria, and Duncan's post-hoc test was used to further analyze significant differences ($p < 0.05$).

3 | Results and Discussion

3.1 | LAB Strains Screening and Identification

A total of 97 putative LAB strains were isolated and purified from traditional Chinese pickles. As illustrated in Figure 1A, the colonies exhibited characteristic morphology: circular, convex, off-white, and moist surfaces with entire margins. Microscopic examination revealed rod-shaped to coccobacillary cellular morphology. Gram-positive, catalase-negative isolates were subjected to preliminary screening via TLC. As demonstrated in Figure 1B, 54 LAB strains showed migratory spots co-eluting with the GABA standard, indicating potential GABA production.

GABA content in MSG-supplemented MRS broth was quantified via HPLC as shown Figure 1C,D. Strain-dependent production variability was observed: 5 strains exceeded 0.8 mg/mL, 6 strains produced 0.5~0.8 mg/mL, 23 strains yielded 0.3–0.5 mg/mL, and 20 strains showed ≤0.3 mg/mL. Six high-yield GABA-producing strains phenotypically similar but statistically distinct strains were selected for further analysis, with GABA concentrations ranging 0.68 ± 0.01–1.36 ± 0.02 mg/mL. Notably, *L. plantarum* L10 demonstrated the highest production (1.36 ± 0.02 mg/mL). LAB strains exhibit significant capacity to convert MSG into GABA, and extensive screening has identified several LAB strains with notable GABA production from various fermented foods. For instance, according to Yogeswara et al. (2020). *L. plantarum* FNCC 260 produced 809.2 mg/L of GABA content after incubating 60 h, which was isolated from fermented foods. In a separate study, *Enterococcus faecium* BS5 was isolated from dairy products by Bs et al. (2021), which displayed effective GABA production capabilities.

Figure 1E displays the agarose gel electrophoresis used in amplifying and detecting the bacterial 16S rRNA gene. The amplification product had an approximate length of 1500 bp. The NCBI database's BLAST program was used to assess the sequencing findings' sequence similarity. As detailed in Table 1, five strains were identified as *L. plantarum*, and one strain was identified as *E. faecium*.

3.2 | Probiotic Properties

3.2.1 | Cell Surface Hydrophobicity and Auto-Aggregation Ability of LAB

Reflecting the capability of strains to adhere to hydrocarbon surfaces, cell surface hydrophobicity plays a crucial role in the adhesion of LAB to gastrointestinal epithelial cells (Meena et al. 2022). This hydrophobicity is influenced by surface proteins and is a key factor in the specific adhesion of cells to both biological and non-biological surfaces (Kustos et al. 2002). LAB strains of cell surface hydrophobicity, as shown in Figure 2(A), were observed in xylene, with values ranging from 4.43 ± 0.13% to 49.86 ± 2.09%. This is similar to the properties of the potential probiotic LAB isolated by Abushelaibi et al. from raw camel milk (Abushelaibi

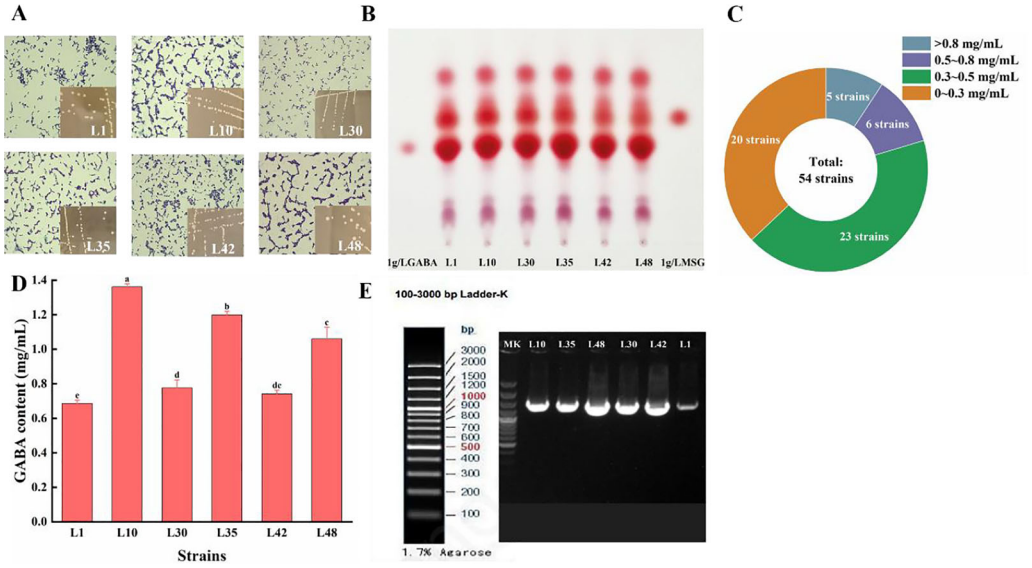

FIGURE 1 | Isolation and identification of LAB: (A) Colony characteristics and Gram-stained microscopic observation of the selected strains; (B) screening of GABA-producing LAB by thin-layer chromatography (TLC); (C) the distribution of GABA-producing strains, with different colors representing the range of GABA production; (D) screening of high-yield GABA-producing strains by HPLC; (E) gel electrophoresis of PCR products from isolated strains. Different letters in the figure indicate significant differences ($p < 0.05$). The value is presented as mean ± SD of three replicates. L1, *Lactobacillus plantarum* L1; L10, *L. plantarum* L10; L30, *L. plantarum* L30; L35, *L. plantarum* L35; L42, *L. plantarum* L42; L48, *Enterococcus faecium* L48.

TABLE 1 | Molecular identification of LAB strains by 16S rRNA gene sequence.

Strains	Homologous strains	Nucleotide similarity	Identification results	Accession number
L1	*Lactobacillus plantarum* (LRCC5195)	99.80%	*L. plantarum*	PV635988
L10	*L. plantarum* (CUDS1902)	100%	*L. plantarum*	PV635989
L30	*L. plantarum* (SK156)	99.87%	*L. plantarum*	PV635990
L35	*L. plantarum* (Ni1325)	99.74%	*L. plantarum*	PV635991
L42	*L. plantarum* (AD73)	99.87%	*L. plantarum*	PV635992
L48	*Enterococcus faecium* (LMEM34)	97.03%	*E. faecium*	PV635993

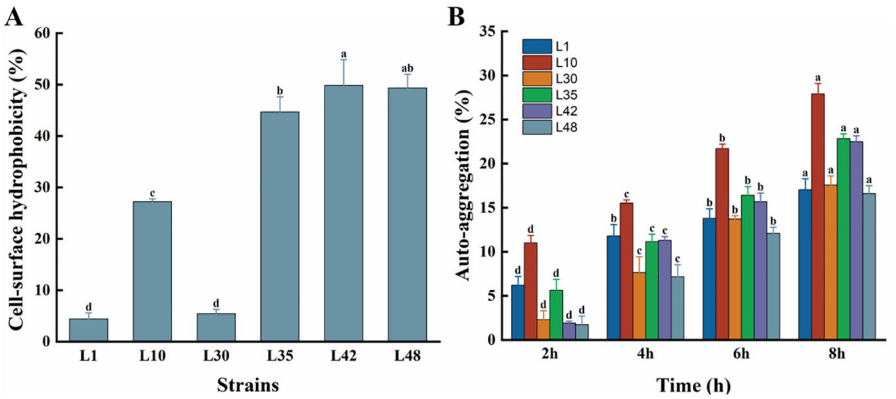

FIGURE 2 | Probiotic properties of LAB: (A) Cell-surface hydrophobicity; (B) auto-aggregation. Different letters in the figure indicate significant differences ($p < 0.05$). The value is presented as mean ± SD of three replicates.

TABLE 2 | Tolerance test of LAB strains: (A) acid and bile salt tolerance, (B) in vitro gastrointestinal tolerance.

(A)

Strains	Initial concentration (log CFU/mL)	Acid tolerance (pH3.0)		Bile salt tolerance (0.3%)	
		3 h (log CFU/mL)	Survival rate (%)	3 h (log CFU/mL)	Survival rate (%)
L1	8.04 ± 0.69^{a}	6.33 ± 0.55^{b}	78.67	6.07 ± 0.46^{b}	75.42
L10	9.62 ± 0.88^{a}	8.28 ± 0.75^{b}	85.99	8.11 ± 0.77^{b}	84.26
L30	9.02 ± 0.76^{a}	8.05 ± 0.70^{b}	89.23	8.17 ± 0.77^{bc}	90.57
L35	8.78 ± 0.78^{a}	8.34 ± 0.75^{bc}	94.89	8.21 ± 0.75^{c}	93.48
L42	9.73 ± 0.85^{a}	8.01 ± 0.71^{b}	82.30	8.08 ± 0.71^{b}	83.03
L48	8.72 ± 0.75^{a}	8.30 ± 0.73^{b}	95.20	8.16 ± 0.65^{bc}	93.52

(B)

Strains	Initial concentration (log CFU/mL)	Artificial gastric fluid (pH 3.0)		Artificial intestinal fluid (pH 8.0)	
		3 h (log CFU/mL)	Survival rate (%)	6 h (log CFU/mL)	Survival rate (%)
L1	8.04 ± 0.69^{a}	6.16 ± 0.47^{b}	76.60	6.02 ± 0.45^{b}	74.83
L10	9.62 ± 0.88^{a}	8.43 ± 0.72^{b}	87.57	8.34 ± 0.70^{b}	86.66
L30	9.02 ± 0.76^{a}	8.20 ± 0.69^{b}	90.85	8.13 ± 0.70^{b}	90.13
L35	8.78 ± 0.78^{a}	8.43 ± 0.73^{b}	95.95	8.32 ± 0.74^{bc}	94.69
L42	9.73 ± 0.85^{a}	8.37 ± 0.73^{b}	86.01	8.19 ± 0.68^{b}	84.17
L48	8.72 ± 0.75^{a}	8.39 ± 0.74^{b}	96.16	8.33 ± 0.77^{b}	95.53

Note: Different superscript letters within a row indicate significant differences ($p < 0.05$). Data are expressed as mean ± SD of three replicates.

et al. 2017). *L. plantarum* L42 and *E. faecium* L48 exhibited higher hydrophobicity at 49.86 ± 2.09% and 49.32 ± 2.67%, respectively, while *L. plantarum* L1 and L30 demonstrated much lower hydrophobicity, below 5%.

Auto-aggregation is another key indicator of the ability to adhere to intestinal epithelial cells (Beldarrain-Iznaga et al. 2021). As shown in Figure 2B, over time, the isolated LAB's auto-aggregation rise, a trend consistent with that reported by Ruiz-Moyano et al. (2019). *L. plantarum* L10 exhibited the strongest auto-aggregation ability, reaching 27.91 ± 1.19% after standing for 8 h. This auto-aggregation capacity is higher than 17.62% of the probiotic strain *P. pentosaceus* K41 reported by Topçu et al. (2020).

3.2.2 | The Acid and Bile Salt Tolerance of LAB

As indicated in Table 2A, when cultured under conditions of pH 3.0 and 0.3% bovine bile salts for 3 h, the survival rates ranged from 78.67% to 95.20% and from 75.42% to 93.52%, respectively. LAB maintain their intracellular pH homeostasis primarily through the action of proton-pumping ATPases (H^+-ATPases), which expel excess protons from the cytoplasm. In addition, the production of alkali compounds, the presence of amino acid decarboxylase systems (e.g., the glutamate decarboxylase system), and changes in cell membrane composition contribute to survival under low pH conditions. The finding on acid resistance align with those reported by Ahire et al. and Chen et al. for selected probiotic LAB (Ahire et al. 2021; Chen et al. 2024). Similarly, key mechanism of bile salt tolerance is the expression of bile salt hydrolase (BSH) enzymes, which deconjugate bile acids, reducing their toxicity. The results on bile salt resistance are consistent with what Topçu et al. (2020) found for probiotic LAB isolated from dry-cured meat products.

3.2.3 | In Vitro Gastrointestinal Tolerance of LAB

Pepsin and trypsin are included to allow for a more physiologically accurate simulation of human gastric and intestinal fluid environments. As shown in Table 2B, In artificial gastric and intestinal fluids, the selected LAB's survival rates varied from 76.60% to 96.16% and from 74.83% to 95.53%, respectively. The high survival rates in simulated gastric fluid can be attributed to the strains' inherent acid tolerance mechanisms, which allow them to maintain viability despite the combined challenge of low pH and proteolytic enzyme activity. Similarly, survival in simulated intestinal fluid indicates robust resistance not only to bile salts but also to pancreatic enzymes. This suggests the presence of protective cell envelope structures, such as a robust peptidoglycan layer and surface proteins, that resist enzymatic degradation. These results are comparable to the survival rates observed for *Lactobacillus rhamnosus* ZYN-

TABLE 3 | The antibiotic susceptibility of LAB strains.

Antibiotics	Strains					
	L1	L10	L30	L35	L42	L48
Gentamicin (10 μg)	R	R	R	R	R	R
Ampicillin (10 μg)	S	S	S	S	S	S
Ciprofloxacin (5 μg)	R	R	R	R	R	R
Vancomycin (30 μg)	R	R	R	R	R	R
Kanamycin (30 μg)	R	R	R	R	R	R
Tetracycline (30 μg)	I	S	I	S	S	S
Erythromycin (15 μg)	S	S	S	S	S	S
Penicillin (10 U)	S	S	S	S	I	S

Abbreviations: I, intermediate; R, resistant; S, sensitive.

0417 and *L. plantarum* ZYN-0221 isolated from blueberries by Cong et al. (2024) from blueberries, which exhibit good probiotic characteristics. These findings suggest that six LAB strains have good potential for survival in the gastrointestinal tract.

3.2.4 | Hemolysis Ability and Antibiotic Susceptibility

Six LAB strains displayed γ-hemolysis, indicating that they were nonhemolytic and suggesting a high level of safety. Microbial resistance, which is a serious threat to both medicine and food production, has emerged as a finding of the extensive use of antibiotics. Table 3 presents the antibiotic susceptibility profiles of the isolates. In this study, all strains showed resistance to gentamicin, ciprofloxacin, kanamycin, and vancomycin, compatible with earlier research showing that the majority of LAB exhibit such resistance (Abriouel et al. 2015; X. Li, Zhu, et al. 2022). Conversely, the isolates were sensitive or moderately sensitive to ampicillin, tetracycline, erythromycin, and penicillin. The resistance of the isolated LAB to ciprofloxacin, vancomycin, and aminoglycoside antibiotics (such as gentamicin and kanamycin) is generally considered intrinsic, encoded by chromosomal genes, and the risk of resistance gene transfer is very low (Jose et al. 2015; T. Li et al. 2020). Evidence suggests that vancomycin resistance is caused by the absence of the normal D-Ala–D-Ala dipeptide targets in the peptidoglycan of LAB, and aminoglycoside resistance is caused by the shortage of cytochrome-mediated electron transport in LAB, which prevents drug absorption (Argyri et al. 2013).

It is noteworthy that among the isolates obtained in this study was one strain of *E. faecium* L48. As opportunistic pathogens, enterococci warrant careful evaluation within a food safety framework due to their potential pathogenicity and transferable antibiotic resistance. In preliminary phenotypic assessments, *E. faecium* L48 from traditional fermented food exhibited γ-hemolysis and sensitivity to ampicillin (Table 3), characteristics that align with initial safety screening criteria for microorganisms intended for certain food applications (Merzoug et al. 2025; Hanchi et al. 2018). Since the primary aim of this study was to screen for optimal starter cultures for mulberry leaf juice fermentation, and *L. plantarum* L10 demonstrated superior fermentation performance and was therefore selected as the candidate for further investigation and application, more in-depth genomic safety profiling of *E. faecium* L48 was not conducted. We recognize that this is a shortcoming of the current work, and systematic molecular safety evaluation of food-derived enterococci remains an important direction for future research. Based on the context of the intrinsic resistance mechanisms described earlier, the resistance profile observed for strains in this study is considered to pose a low risk of horizontal gene transfer.

3.3 | Properties of Fermented Mulberry Leaf Juice

3.3.1 | Viable Cell Counts in Fermented Mulberry Leaf Juice

The adaptability and growth conditions of LAB for fruit and vegetable juices vary depending on the strain. Y. Liu et al. (2022) compared the fermentation effects of four LAB strains on sea buckthorn juice and found that the *Lactococcus lactis* Lc-630 group had the highest viable cell count. Sun et al. (2022) utilized five LAB strains for the fermentation of pumpkin juice, and after 12 h of fermentation, *L. paracasei* CICC20245 exhibited the largest viable cell count of 9.04 log CFU/mL. As shown in Figure 3A, over extended fermentation time, the population of viable LAB cells showed a pattern of initial growth followed by a decline. This overall trend is consistent with the variation in viable cell count observed by Wu et al. (2020) when fermenting apple juice with six LAB strains. After 24 h of fermentation, all six LAB strains displayed maximum viable cell counts, with *L. plantarum* L10 strain exhibiting the highest count at 10.01 ± 0.01 log CFU/mL. Subsequently, due to a lack of energy substances during the fermentation process, leading to an observed reduction in viable cell count. By the end of the 72-h fermentation, all LAB counts exceeded 8 log CFU/mL reaching the standard for viable cell counts in probiotic fermentation products (Aragon-Alegro et al. 2007). This indicates that all six LAB strains possess good growth capabilities in mulberry leaf juice.

3.3.2 | GABA Content in Fermented Mulberry Leaf Juice

The initial GABA content in mulberry leaf juice was 0.069 ± 0.003 mg/mL, and this could be enriched through

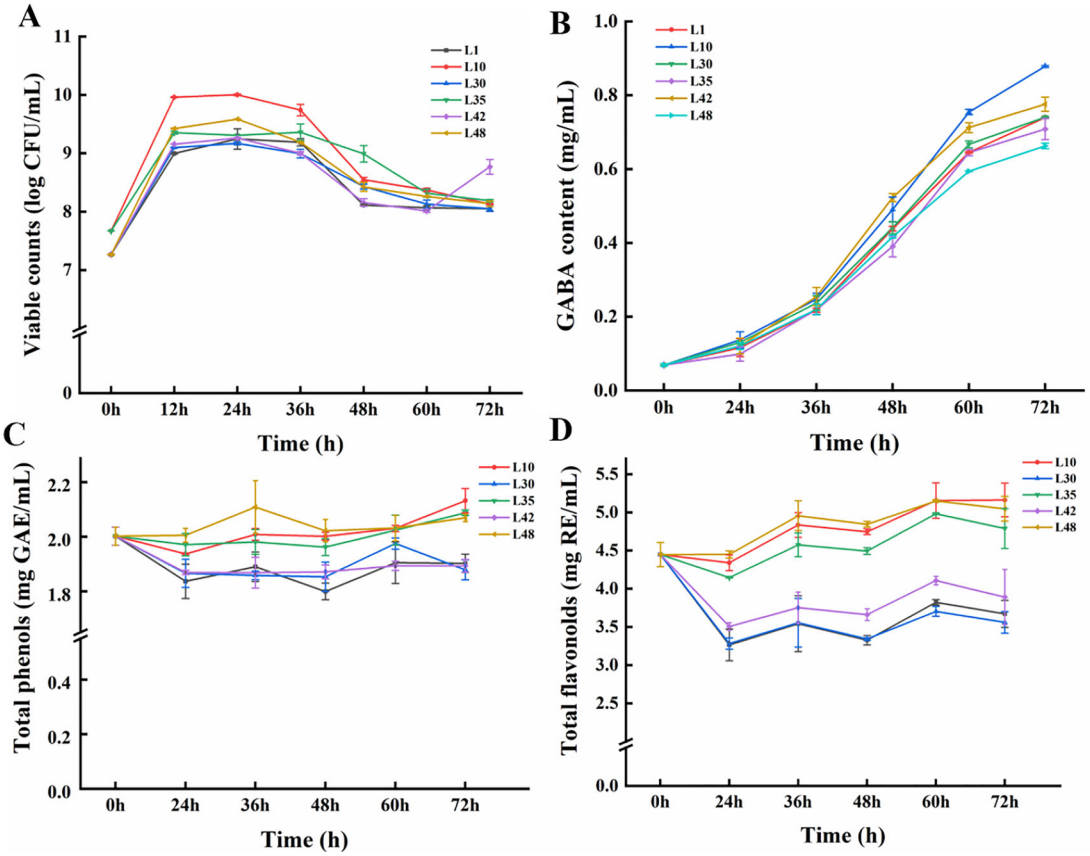

FIGURE 3 | LAB enriches GABA and determination of phytochemicals in mulberry leaf juice: (A) The viable count of the six LAB strains; (B) GABA content; (C) total phenols content; (D) total flavonoids content.

fermentation with six strains of LAB. The GABA content gradually increased over time, with a noticeable increase after 24 h of fermentation shown in Figure 3B. As the pH and energy substances in the system decrease, while the activity of GAD increases; after 72 h of fermentation, the GABA content in fermented mulberry leaf juice ranged from 0.663 ± 0.007 to 0.879 ± 0.023 mg/mL. Notably, the mulberry leaf juice fermented with *L. plantarum* L10 exhibited the highest GABA content of 0.879 ± 0.023 mg/mL, which was 12.74 times higher than the initial level. Similarly, an approximate increase of 40 mg GABA per 100 mL was reported in lychee juice following 40-h fermentation with *L. plantarum* HU-C2W (D. Wang et al. 2021).

3.3.3 | TPC and TFC in Fermented Mulberry Leaf Juice

The fermentation of mulberry leaf juice with different LAB strains resulted in divergent alterations in TPC and TFC, underscoring a critical strain-specificity, which is consistent with previous studies (Isas et al. 2020; Lee et al. 2021). According to Figure 3C,D, TPC and TFC in fermented mulberry leaf juice ranges from 1.88 ± 0.04 to 2.17 ± 0.04 mg GAE/mL and from 3.67 ± 0.17 to 5.16 ± 0.22 mg RE/mL, respectively. Strains *L. plantarum* L10, L35, and *E. faecium* L48 significantly enhanced both TPC and TFC, with L10 yielding the most prominent increases. Among the tested samples, the fermented mulberry leaf juice with *L. plantarum* L10 resulted in the highest total phenolic and flavonoid content, measuring 2.17 ± 0.04 mg GAE/mL and 5.16 ± 0.22 mg RE/mL, representing increases of 6.50% and 16.05% compared to the unfermented mulberry leaves juice. In contrast, fermentation with *L. plantarum* L1, L30, and L42 led to a reduction in these compounds. We propose that this divergence stems from the equilibrium between two opposing metabolic processes: the liberation versus the bioconversion of phenolic compounds. The observed increases are likely driven by the robust enzymatic activities (e.g., *β*-glucosidases, esterases, tannases) of strains like *L. plantarum* L10, which hydrolyze complex phenolic glycosides and polymers, thereby releasing soluble, measurable phenolics and flavonoid aglycones. Conversely, the decreases induced by strains like *L. plantarum* L1 suggest a metabolic shift where the rate of liberated phenolics biotransformation into other undetected compounds (e.g., through decarboxylation or polymerization), surpasses the rate of their release.

3.3.4 | The Electronic Nose Sensory Evaluation of Fermented Mulberry Leaf Juice

Electronic nose technology utilizes electronic sensors to simulate human sensory perceptions, allowing for the characterization of the taste and aroma properties of samples (Jiang et al. 2025). We utilized electronic nose technology in combination with PCA and radar chart analysis to distinguish and characterize the volatile flavor compounds in mulberry leaf juice fermented by various LAB. The PCA results (Figure 4A) show that PC1 and PC2 account for 40.2% and 15.5% respectively, with a cumulative contribution

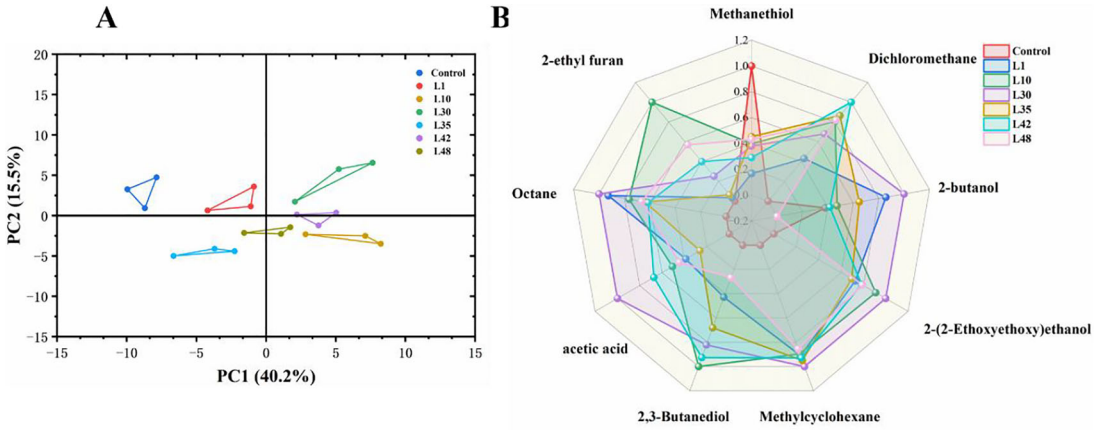

FIGURE 4 | The electronic nose sensory evaluation of fermented mulberry leaf juice. (A) PCA and (B) radar chart.

of 55.7%. This effectively captures the overall flavor disparities among the samples. Further findings from the radar chart analysis (Figure 4B) emphasized the variations in the relative concentrations of specific flavor compounds. All fermented mulberry leaf juice samples demonstrated increased levels of acetic acid and 2,3-butanediol, mainly stemming from the glycolytic and lactic fermentation pathways of LAB. Moreover, compounds such as 2-ethyl furan and methylcyclohexane exhibited significant differences among the samples, significantly influencing the flavor profile of the product (e.g., contributing fruity and sweet notes). The acidification induced by lactic acid fermentation modifies the sugar-to-acid ratio in mulberry leaf juice, mitigating undesirable grassy and bitter flavors, and facilitating the development of a more harmonious and pleasing taste profile (Z. Liu et al. 2025).

3.3.5 | α-Glucosidase Inhibitory Activity

α-Glucosidase is a key enzyme responsible for carbohydrate metabolism and the subsequent absorption of glucose in the intestine, directly affecting energy metabolism and the maintenance of blood glucose homeostasis (Cheng et al. 2025). Therefore, we evaluated the α-glucosidase inhibitory activity to investigate the hypoglycemic potential conferred by LAB-mediated fermentation. As illustrated in Figure 5A, the inhibitory activity was 50.61 ± 1.02%–58.02 ± 1.92%, which was 0.94 ± 0.14%–8.34 ± 0.44% higher than that of control. Among them, the mulberry leaf juice fermented with *L. plantarum* L42 had the most significant inhibitory effect on α-glucosidase, increasing by 8.34 ± 0.44%.

3.3.6 | Antioxidant Activity

To comprehensively assess the antioxidant activity of fermented mulberry leaf juice, four methods were employed: DPPH, ABTS, hydroxyl radical scavenging activity (using 0.1 mg/mL VC as a positive control, with values of 26.55 ± 0.57%, 46.83 ± 0.21%, and 27.11 ± 0.31%, respectively), and FRAP assays. As shown in Figure 5B–E, during the total antioxidant activity test, LAB fermentation did not result in any significant increase in ABTS radical scavenging activity. Among them, the mulberry leaf juice fermented with *E. faecium* L48 reduced the ABTS radical scavenging activity by 6.76 ± 0.13%. In contrast, the DPPH radical scavenging activity was significantly enhanced, in line with the results published by J. Li, Zhao, et al. (2022). The difference arises from distinct antioxidant mechanisms: ABTS radical scavenging mainly involves electron transfer, whereas DPPH radical scavenging relies on hydrogen atom transfer (Qi et al. 2021). The DPPH radical scavenging activity of fermented mulberry leaf juice was 56.64 ± 1.52% to 67.80 ± 0.32%, which was 1.08 ± 0.06%–12.24 ± 0.53% higher than that of control. Among them, the fermented mulberry leaf juice groups of *E. faecium* L48 and *L. plantarum* L10, respectively, significantly increased the DPPH radical scavenging activity by 12.24 ± 0.53% and 9.21 ± 0.60%. LAB fermentation can significantly enhance FRAP and hydroxyl radical scavenging ability, aligning with the reports by Muhialdin et al. (2020) and Wei et al. (2023). FRAP of fermented mulberry leaf juice was 11.49 ± 0.36 to 14.27 ± 0.12 μmol TE/mL, which was 1.46 ± 0.06–4.24 ± 0.31 μmol TE/mL higher than that of control. Among them, the fermentation of *E. faecium* L48 and *L. plantarum* L10, respectively, significantly increased FRAP by 4.24 ± 0.31 and 3.56 ± 0.28 μmol TE/mL. The hydroxyl radical scavenging activity of fermented mulberry leaf juice was 36.82 ± 0.27%–45.09 ± 1.22%, which was 3.18 ± 0.18%–11.44 ± 0.79% higher than that of control. Among them, the fermented mulberry leaf juice groups of *L. plantarum* L42 and *L. plantarum* L10, respectively, significantly increased the hydroxyl radical scavenging activity by 11.44 ± 0.79% and 11.21 ± 0.27%. It is crucial that the enhanced effects of these antioxidants are in line with the increase in TPC and TFC in high-yield strains. We posit that beyond elevating the quantifiable levels of phenolic compounds, LAB fermentation likely transforms their chemical composition, which may be of greater functional significance. The hydrolysis of complex glycosides into smaller, more easily bioavailable aglycones usually enhances hydrogen supply capacity and metal reduction ability, thereby directly improving antioxidant properties.

3.4 | PCA-Based Preferential Selection of Strains for Fermented Mulberry Leaf Juice

To identify the most suitable strain for mulberry leaf juice fermentation among six potential probiotic LAB strains, a PCA-based quality evaluation was performed. As illustrated in Figure 6A, three PCs were extracted, which collectively accounted for 96.49%

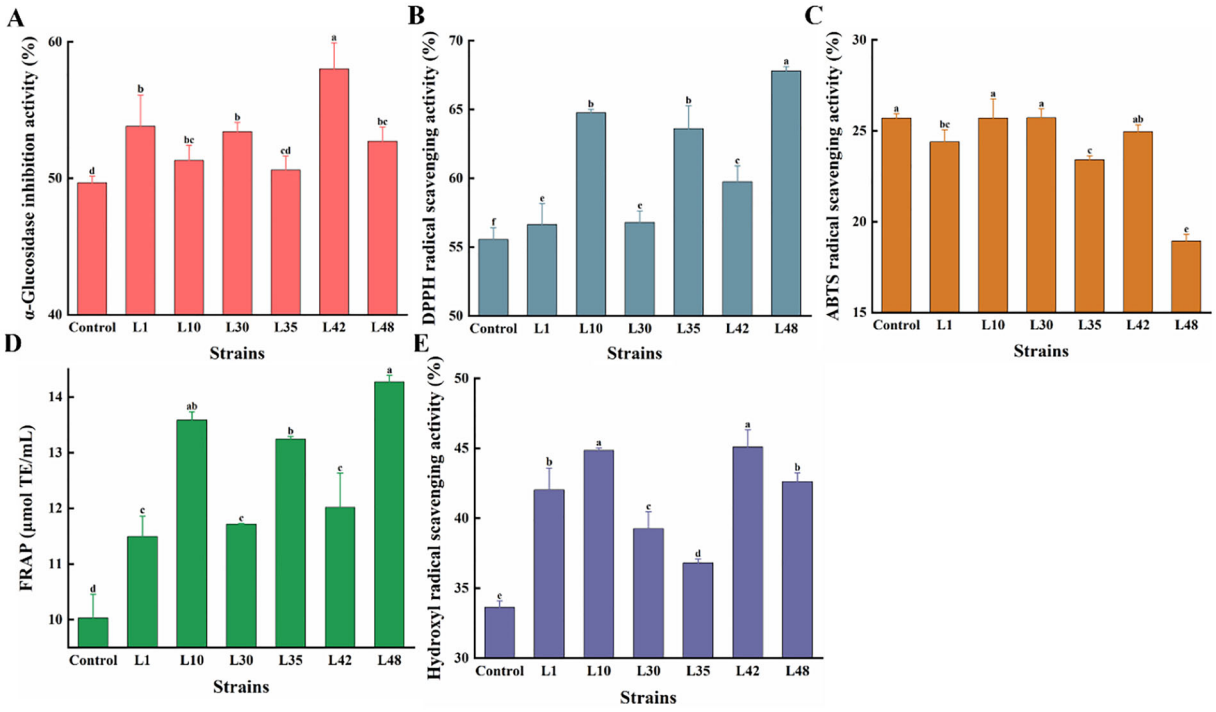

FIGURE 5 | α-Glucosidase inhibition and antioxidant activity of fermented mulberry leaf juice: (A) α-Glucosidase inhibition activity; (B) DPPH radical scavenging activity; (C) ABTS radical scavenging activity; (D) ferric-reducing antioxidant power (FRAP); (E) hydroxyl radical scavenging activity. Different letters in the figure indicate significant differences ($p < 0.05$). The value is presented as mean ± SD of three replicates.

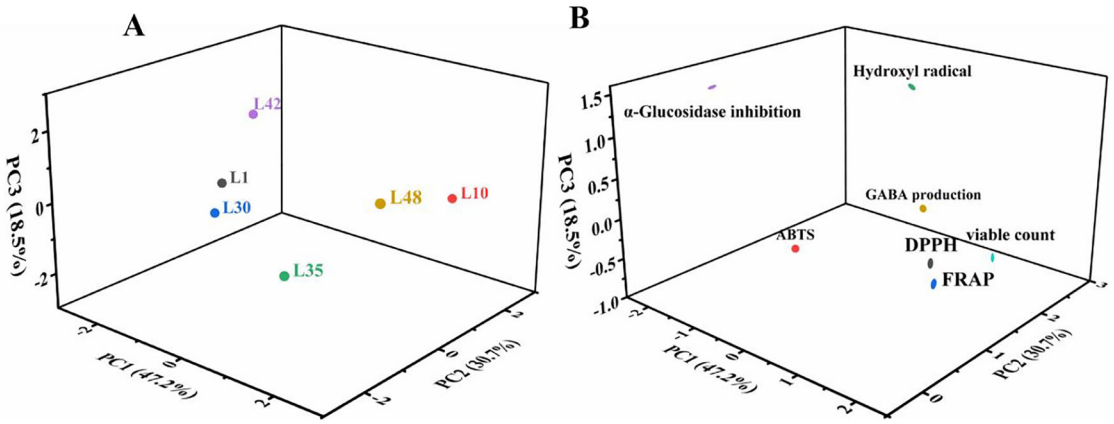

FIGURE 6 | PCA for optimal strain selection used for mulberry leaf juice fermentation: (A) PCA score plot; (B) PCA loading plot.

of the total variance. This indicates that these PCs effectively capture the essential information from the original variables and provide a comprehensive representation of the fermentation performance and bioactive properties of the fermented mulberry leaf juice. According to Figure 6B, PC1 primarily reflects variables associated with viable cell counts, DPPH radical scavenging activity, and FRAP values, while PC2 is mainly related to GABA yield and ABTS radical scavenging activity. PC3 predominantly represents hydroxyl radical scavenging activity and α-glucosidase inhibition rate.

Using the matrix coefficients and standardized data as shown in Table 4A, the scoring functions for the three PCs were derived:

$$Z_1 = 0.067X_1 + 0.287X_2 + 0.289X_3 - 0.151X_4 + 0.296X_5 + 0.123X_6 - 0.140X_7 \quad (4)$$

$$Z_2 = 0.477X_1 + 0.252X_2 - 0.095X_3 + 0.427X_4 - 0.076X_5 + 0.057X_6 - 0.188X_7 \quad (5)$$

$$Z_3 = 1l0.041X_1 - 0.014X_2 + 0.074X_3 - 0.165X_4 + 0.012X_5 + 0.594X_6 + 0.556X_7 \quad (6)$$

TABLE 4 | Comprehensive quality evaluation: (A) Component matrix and component coefficient matrix; (B) comprehensive quality evaluation of different LAB to ferment mulberry leaf juice.

(A)

Quality indices	Component matrix			Component coefficient matrix		
	1	2	3	1	2	3
GABA production	0.109	0.960	0.236	0.067	0.477	0.041
Viable count	0.874	0.442	0.010	0.287	0.252	−0.014
DPPH	0.931	−0.220	0.006	0.289	−0.095	0.074
ABTS	−0.532	0.823	−0.046	−0.151	0.427	−0.165
FRAP	0.966	−0.209	−0.084	0.296	−0.076	0.012
Hydroxyl radical	0.242	0.325	0.900	0.123	0.057	0.594
α-Glucosidase inhibition	−0.547	−0.129	0.808	−0.140	−0.188	0.556

(B)

Strains	Principal component scores (Z)			Comprehensive score (F)	Sort
	1	2	3		
L1	−0.251	0.104	0.482	0.003	5
L10	1.031	1.613	0.578	1.129	1
L30	−0.304	0.107	0.239	−0.069	6
L35	0.157	−0.188	−0.070	0.003	4
L42	−0.198	0.145	1.060	0.153	3
L48	0.709	−0.622	0.604	0.265	2

In the formula, Z_1–Z_3 denote the scores for the three PCs, while X_1–X_7 represent the standardized values for GABA yield, the change in viable cell count before and after fermentation, DPPH radical scavenging rate, ABTS radical scavenging rate, FRAP, hydroxyl radical scavenging rate, and α-glucosidase inhibition rate.

By using the variance contribution rates of each PC as weights, a comprehensive quality evaluation function (F) was derived Table 4B.

$$F = 0.489Z_1 + 0.318Z_2 + 0.192Z_3 \quad (7)$$

To compare the performance of different LAB strains, comprehensive scores and rankings of the fermented mulberry leaf juices were generated based on the evaluation model, leading to the subsequent order: L10, L48, L42, L35, L1, and L30. Among these, *L. plantarum* L10 demonstrated the highest comprehensive score, indicating its superior overall performance and identifying it as the most suitable potential probiotic to ferment mulberry leaf juice.

4 | Conclusion

Six GABA-producing LAB strains were successfully isolated from traditional Chinese pickles in this study, with *L. plantarum* L10 emerging as the most promising starter culture for mulberry leaf juice fermentation. Fermentation with *L. plantarum* L10 significantly enhanced the functional quality of mulberry leaf juice, markedly increasing its GABA content, TPC, TFC, and improving its flavor profile. Furthermore, the fermented mulberry leaf juice exhibited strengthened antioxidant and hypoglycemic activities.

A key contribution of this research is to advance both the theory and practice behind harnessing mulberry leaves for high-value applications. The establishment of a PCA-based quality evaluation model offers a reliable tool for rapid screening of efficient fermentation strains. Moreover, fermented mulberry leaf juice rich in GABA not only enhances blood sugar-lowering and antioxidant activities but also significantly improves flavor by reducing the grassy taste and increasing a sweet floral note. This makes it suitable as a base ingredient for various baked goods, functional beverages or health foods. To further optimize fermentation and scale up industrial applications, subsequent studies need to decipher the molecular mechanisms governing strain-substrate interactions and associated metabolic regulation.

Author Contributions

Jingjing Xie: conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing, data curation. **Siqi Yang**: formal analysis, investigation, writing – review and editing. **Lara Matia-Merino**: resources, writing – review and editing, project administration. **Jingjing Chen**: writing – review and

editing. **Hong (Sabrina) Tian**: conceptualization, writing – review and editing.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available on request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Materials: jfds70949-sup-0001-SuppMat.docx