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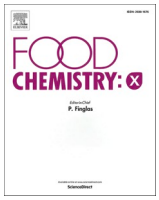

# Characterizations of lactic acid bacteria derived from pickles and the effects of fermentation on phenolic compounds in peony flowers

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ABSTRACT

This study aims to characterize lactic acid bacteria (LAB) in pickles and investigate the effect of lactic acid fermentation on phenolic compounds in peony flowers. Six strains of *Lactobacillus plantarum* and one strain of *Weissella* identified by 16S rRNA sequencing met the safety standards confirmed by metabolite safety assessment and antibiotic resistance analysis. NPLP12 exhibited excellent fermentation characteristics and its tolerance, adhesion, and antioxidant indicators all demonstrated its potential as probiotics and starter. After fermentation with NPLP12, the content of total phenols (15.2 %) and flavonoids (22.7 %) in the liquid extract of peony flowers was significantly increased, and the antioxidant activity was also enhanced. Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS) analysis confirmed that apigenin 7-O-glucoside and kaempferol-3-O-glucoside were key synergistic components. This study provides a reference for the screening of peony flower fermentation strains, the utilization of peony flower resources and the development of functional peony flower fermentation beverages.

## 1. Introduction

Plant-based foods are increasingly being valued in contemporary dietary plans due to their rich bioactive ingredients that promote health. However, these phytochemicals often exhibited anti-nutritional properties, digestive enzyme inhibition effects, and adverse sensory characteristics (Gaur & Gänzle, 2023). Fermentation, as a mature and economical biological processing technology, has shown great potential in improving the unique bitterness and astringency associated with plant-based food systems, thereby enhancing their overall acceptability (Ruiz Rodríguez et al., 2021). Recent studies have shown that the enzymatic conversion mediated by lactic acid bacteria (LAB), particularly through the catalytic action of esterases, decarboxylases, and reductases, promotes the release and structural modification of complex polyphenolic macromolecules. This biotransformation process has been proven to enhance the functional efficacy and bioavailability of these plant chemicals (Gaur & Gänzle, 2023; Leonard, Zhang, Ying, Adhikari, & Fang, 2021). It is worth noting that floral resources are an underutilized but highly nutritious food matrix, rich in various bioactive compounds that promote human health benefits.

Peony (*Paeonia suffruticosa* Andr.) is a common traditional flower in China, which is often used for ornamental and medicinal purposes. The earliest history of peony can be traced back to the Book of Songs over 3000 years ago and it was used for medicinal purposes during the Qin and Han Dynasties. (Yin et al., 2022). Peony flower (PF) has been used as a traditional food since the Song Dynasty due to its high nutritional content, including protein, trace elements and vitamins. The PF of the "Fengdan" variety was officially listed as a new food ingredient by the National Health Commission of China in 2013. As a potential functional food, “Fengdan” flower exhibits strong antioxidant properties due to its rich content of flavonoid glycosides and phenolic compounds, which can regulate the expression of antioxidant enzymes and enhance cell viability (Xiang et al., 2019). In the field of plant fermentation, various plant resources including fruits, vegetables, and grains are increasingly being used in the production of functional beverages to fully utilize their nutritional and bioactive ingredients (Zhao et al., 2024). However, there have been few reports on the research related to the lactic acid fermentation beverage of "Fengdan" flower.

LAB exhibit multiple physiological regulatory functions, including regulating gastrointestinal flora, improving digestion, reducing serum

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cholesterol and resisting tumors. Based on this, scholars have conducted extensive development and application research on the LAB resources in pickles (Behera et al., 2020). Huang et al. (2024) obtained 50 strains of LAB from pickled asparagus and screened for *L. plantarum* JGS49 through probiotic properties for asparagus juice fermentation. The results showed that L. *plantarum* JGS49 fermentation could increase the contents of total phenols and total flavonoids as well as antioxidant activity of asparagus juice. As a powerful carrier for LAB and bioactive compound supplements, LAB fermented foods can help live bacteria exert probiotics effects (Diez-Ozaeta & Astiazaran, 2022). However, the key to ensuring the probiotic effect of LAB was whether they can survive in the human body. Some requirement for identifying LAB as an effective probiotic include tolerance to gastric acid, bile and adhesion to intestinal epithelial cells (Rastogi et al., 2019). Although LAB were generally considered healthy and harmless, reliable identification and safety assessment of microorganisms used as fermentation agents or probiotics are still necessary.

Functional plant-fermented beverages rich in polyphenolic compounds have broad research prospects. In this study, the 16S rRNA identification and a series of *in vitro* characterizations were used to screen LAB from traditional Chinese pickles for the fermentation of peony flowers, ensuring their reliability as probiotics and beneficial starter. LAB with excellent fermentation characteristics were used to ferment peony flowers to elucidate the changes in phenolic compounds and antioxidant activity before and after fermentation. The root bark of peonies has been extensively studied as a traditional medicinal herb, but the health benefits of the abundant phenolic compounds in peony flowers are often overlooked, leading to the waste of a large amount of peony flower resources. This study not only provides ideas for the utilization of peony flower resources, but also provides theoretical basis for the development of advantageous peony flower fermentation strains and new functional peony flower fermentation beverages.

## 2. Materials and methods

### 2.1. Materials

*Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* (*S. aureus*) ATCC 25923 were preserved in the College of Food and Bioengineering, Henan University of Science and Technology. LAB were isolated from pickles in Inner Mongolia, China. “Fengdan” PF were collected from Luoyang, China. MRS broth, MRS agar, tryptone soy agar (TSA), tryptone soy broth (TSB), peptone and beef extract were purchased from Qingdao Haibo Biotechnology Co., Ltd. (Qingdao, China).

Ferric chloride ($FeCl_3$), calcium carbonate ($CaCO_3$), sodium nitrite ($NaNO_2$), ethanol, xylene, sodium hydroxide (NaOH), methanol, sodium carbonate ($Na_2CO_3$) and aluminum chloride ($AlCl_3$) were purchased from Tianjin DeEn Chemical Reagent Co., Ltd. (Tianjin, China). L-tyrosine, bromocresol purple, L-histidine, L-lysine and pepsin (10, 000 u/mg) were bought from Shanghai Lanji Technology Development Co., Ltd. Antimicrobial susceptibility test paper and sterile sheep blood were purchased from Changde Bickman Biotechnology Co., Ltd. (Changde, China). Bile salts was purchased from Beijing Solarbio Technology Co., Ltd. (Beijing, China). The Folin-Ciocalteu reagent, 2, 2′-diphenyl-1-picrylhydrazyl (DPPH), 2, 4, 6-tris (2-pyridyl)-*s*-triazine (TPTZ), 2, 2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2, 5, 7, 8-tetramethyl-chromane-2-carboxylic acid (Trolox) were sourced from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Mass spectrometry grade methanol and formic acid were sourced from Thermo Fisher Scientific Reagent Co., Ltd. (Waltham, Massachusetts, USA). The chemical standards (gallic acid, rutin and quercetin) were provided by Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China).

### 2.2. Identification and growth characteristics of LAB

The pickle juice was mixed into PBS to obtain a diluent. Subsequently, the diluent was evenly coated on MRS agar plate and incubated at 37 °C for 48 h. Colonies with similar characteristics to LAB were purified on MRS agar (0.1 % $CaCO_3$) plates. Gram staining method was used for further observation. Activated LAB was centrifuged (8, 000 ×*g*, 4 °C, 10 min) and the supernatant was discarded. The DNA extraction kit was used to extract DNA from LAB and universal primers 27F and 1492R were used for PCR amplification. The amplified product was subjected to agarose gel electrophoresis and sequencing was completed by Sangon Biotech Co., Ltd. (Shanghai, China). The 16S rRNA sequence was compared with nucleotide sequences in the NCBI database and MEGA 7 was used to construct a phylogenetic tree.

The $OD_{600}$ value of the activated LAB was adjusted to about 0.5 and inoculated in MRS broth at 0.01 % inoculum at 37 °C. The $OD_{600}$ values were measured every 2 h from 0 to 24 h and every 4 h from 24 to 48 h. A pH meter was used to measure the pH value.

### 2.3. The safety test of LAB

The indole test, gelatin liquefaction test and hemolytic activity of LAB were based on the previous methods with slight modifications (Kang et al., 2019). Indole test: LAB was inoculated into tryptophan broth and cultured at 37 °C for 48 h. The Kovac reagent was added dropwise to tryptophan fermentation broth and a red color indicates a positive result. *E. coli* was used as a positive control and tryptophan broth medium without LAB inoculation was used as a negative control. The gelatin liquefaction test: LAB was inoculated into gelatin culture medium at 37 °C for 48 h, and stored at 4 °C for 2 h to fully solidify the gelatin. *S. aureus* was used as the positive control, while sterile physiological saline was used as the negative control. The liquefaction phenomenon of gelatin at room temperature was determined to be positive. Hemolytic activity: LAB was streaked and inoculated into MRS agar containing sterile sheep blood at 37 °C for 48 h. *S. aureus* was used as positive control. β - Hemolysis: Red blood cells completely dissolve and a transparent hemolysis circle appears around the colony. α - hemolysis: A green semi-transparent hemolysis circle appears around the colony. γ - hemolysis: No hemolytic activity.

The determination of biogenic amine and antibiotic sensitivity was modified according to the method reported by Zhang et al. (2023). Biogenic amine: Bromocresol purple (0.06 g/L) was added to MRS agar containing L-tyrosine (10.00 g/L) and MRS broth containing L-histidine (10.00 mg/L) and L-lysine (10.00 g/L), respectively. LAB was inoculated into different MRS media at 37 °C for 48 h. Purple indicates a positive reaction and *E. coli* was used as positive control. Antibiotic sensitivity: Activated LAB were uniformly coated on MRS agar plates (approximately $10^8$ CFU/mL) and dried at room temperature. Aseptic forceps were used to place antibiotic trays at equal distances. The inhibition zone was measured after incubation at 37 °C for 24 h. The determination method referred to the “Performance Standards for Antimicrobial Susceptibility Testing” (CLSI M100) published by the Clinical and Laboratory Standards Institute (CLSI). The antibiotic sensitivity of LAB was marked as sensitive (S), moderate (I), or resistant (R).

### 2.4. Tolerance of LAB

The acid and bile salt tolerance of LAB was assessed according to the report by Nandha and Shukla (2023) with slight alterations. Acid tolerance: The activated LAB was resuspended in MRS broth (approximately $10^8$ CFU/mL) with pH adjusted to 3.0 by HCl (0.5 M) and subjected to colony counting at 37 °C for 0, 1 and 2 h. Bile salt tolerance: Activated LAB was inoculated into MRS broth containing bile salt (0.3 % *w/v*) and the concentration was adjusted to about $10^8$ CFU/mL. The number of living cells was counted after incubated at 37 °C for 0, 90 and 180 min. The simulated gastric fluid (SGF) tolerance of LAB was assessed

based on the method reported by Han et al. (2017) with slight alterations. One mL LAB suspension ($10^8$ CFU/mL) and 9 mL SGF were mixed and incubated at 37 °C. Then living cells were counted at 0, 90 and 180 min, respectively.

### 2.5. Aggregation potential of LAB

The method reported by Zuo et al. (2015) was used to assess the auto-aggregation and co-aggregation capacities of LAB, with slight alterations.

Activated LAB was centrifuged (8, 000 ×*g*, 10 min, 4 °C) to harvest cells. The cells were rinsed with sterile saline and resuspended in PBS (pH 7.4) and the $OD_{600}$ was adjusted to approximately 0.5 ($A_0$). The $OD_{600}$ ($A_t$) of the supernatant was measured at 37 °C for 1, 2 and 4 h.

$$\text{Auto}-\text{aggregation}\ (\%) = 100 \times (A_0 - A_t)/A_0 \quad (1)$$

An equal volume (1 mL) of cell suspension of LAB and pathogenic bacteria (*S. aureus* and *E. coli*) ($OD_{600} = 0.5 \pm 0.05$) were mixed in a colorimetric dish and $OD_{600}$ ($A_0$) was immediately measured. The $OD_{600}$ ($A_t$) of the supernatant was measured at 37 °C for 1, 2 and 4 h.

$$\text{Co}-\text{aggregation}\ (\%) = 100 \times (A_0 - A_t)/A_0 \quad (2)$$

The determination of LAB hydrophobicity was slightly modified based on the method reported by Nandha and Shukla (2023). Activated LAB was centrifuged (4 °C, 8, 000 ×g, 10 min) to obtain cells, which was rinsed twice with PBS. The cell concentration was adjusted to $OD_{600} = 0.5 \pm 0.05$ by PBS. Then, 1 mL of xylene was added to 3 mL of cell suspension and shaken for 3 min, followed by incubation at 37 °C for 1 h. The aqueous phase was collected to determine the absorbance at a wavelength of 600 nm ($OD_{final}$).

$$\text{Surface hydrophobicity}\ (\%) = 100 \times \left(OD_{initial} - OD_{final}\right)/OD_{initial} \quad (3)$$

### 2.6. Antioxidant activity of LAB

The preparation of cell-free supernatant, intact cells and cell-free extract followed the method reported by Han et al. (2017). The determination of DPPH radical scavenging capacity of LAB was modified based on the method proposed by Kim et al. (2022). DPPH (0.2 mM) and the sample were mixed at 1:2 and reacted for 30 min. Absorbance was measured at 517 nm ($OD_{sample}$). The control group ($OD_c$) was added with an equal amount of PBS.

$$\text{Radical scavenging activity}\ (\%) = 100 \times \left(OD_c - OD_{sample}\right)/OD_c \quad (4)$$

### 2.7. PF fermentation treatment and sample preparation

PF and deionized water were mixed in a ratio of 1:25 (m/m) for juicing and filtered through gauze. Sucrose was added to peony flowers liquid (PFL) at a content of 5 % (m/v). The $OD_{600}$ of LAB was adjusted to 0.5 and inoculated into pasteurized (60 °C and 30 min) PFL at a dose of 3 % (*v*/v) for incubation at 37 °C for 72 h. The bacterial count, pH and total acidity of peony flower fermentation liquid (PFFL) were tested every 12 h.

A previously reported method was used to extract polyphenols from PFL and PFFL (Xiang et al., 2019). Briefly, 5 mL of sample was added to 20 mL of pure ethanol and shaken at 25 °C for 1 h. The extracts were centrifuged and the supernatant was collected. Then, 75 % ethanol was added to the remaining precipitate to repeat the extraction process. The ethanol extract was evaporated by rotation to obtain the solid sample, which was redissolved in methanol solution (50 %).

### 2.8. Determination of the total phenolic content (TPC) and total flavonoid content (TFC)

The Folin-Ciocalteu assay and aluminum chloride colorimetric method were used to measure the TPC and TFC in extracts, as described by Li et al. (2023). The TPC was represented by gallic acid equivalent (μg GAE/mL) and TFC was represented by rutin equivalent (μg RE/mL).

### 2.9. Quantification of phenolic compounds

The Accucore C18 column (100 mm × 3 mm) was used as the analytical column. 10 μL of phenol extraction solution was injected for analysis and eluted using the C18 column. The gradient elution procedure and mass spectrometry conditions referred to the method previously reported (Yuan et al., 2022). Qualitative and quantitative analysis of phenolic compounds was completed by comparing the UV spectrum and mass spectrometry data with our previous reports (Xiang et al., 2019). The standard curve was drawn using standard samples of gallic acid (y = 1338.8x - 478.94, $R^2 = 0.9916$) and quercetin (y = 422.03x - 279.45, $R^2 = 0.9444$). Gallic acid and its derivatives were quantitatively analyzed by gallic acid, and flavonoid glycosides were quantitatively analyzed by quercetin. The content of each compound was calculated in microgram per milliliter (μg/mL).

### 2.10. Antioxidant capacity of PFFL

The DPPH radical scavenging activity, $ABTS^{\cdot+}$ radical scavenging capacity and ferric reducing antioxidant potential (FRAP) of the fermentation liquid were determined with the method described by Yuan et al. (2022). The standard curve was drawn with Trolox, and the result was expressed as micromolar Trolox equivalents per milliliter of sample (μmol TE/mL).

### 2.11. Statistics analysis

The experiments were repeated three times and the mean ± standard deviation represented the final result. The statistical analysis was completed using SPSS 20.0 software and the difference was considered statistically significant when $p < 0.05$.

## 3. Result and discussion

### 3.1. Screening and identification of LAB

LAB, as a key functional microbial community widely present in pickles, have a certain improvement effect on the sensory characteristics and shelf life of pickles. In addition, the bioactive metabolites of these microorganisms have the potential to promote health (Behera et al., 2020). In this study, seven target strains named NPLP11, NPLP12, NPLP13, NPLP15, NWP16, NPLP19 and NPLP26 with LAB characteristics were isolated and screened from traditional Chinese pickles. As shown in Fig. 1A, the colony colors of the 7 strains were creamy white and creamy yellow and their shapes were circular with varying sizes. The presence of a calcium solubilization zone around the colony indicated the production of extracellular organic acids. All the tested strains were rod-shaped under microscopic observation and Gram staining showed positive results. The 16S rRNA of 7 strains were sequenced and compared against known isolates based on NCBI BLAST searches. NPLP11, NPLP12, NPLP13, NPLP15, NPLP19 and NPLP26 were identified as *Lactobacillus plantarum* (*L. plantarum*) and NPW16 was identified as *Weissella*, as confirmed by the phylogenetic tree analysis (Fig. 1B). The 16S rRNA gene sequences of 7 bacterial strains have been stored in GenBank under accession numbers PQ443843 to PQ443849. In this study, *L. plantarum* was the key microorganism for fermenting vegetables. However, as studied by Song et al. (2021), *Weissella* dominated the fermentation of green onion and leaf mushroom in Korean kimchi due to the influence of food type on the distribution of LAB in fermented foods. Xiao et al. (2020) found that the main microbial community in three typical fermented vegetables from different regions of China was also dominated by *lactobacilli*, but *Lactobacillus sakei* and *Lactobacillus*

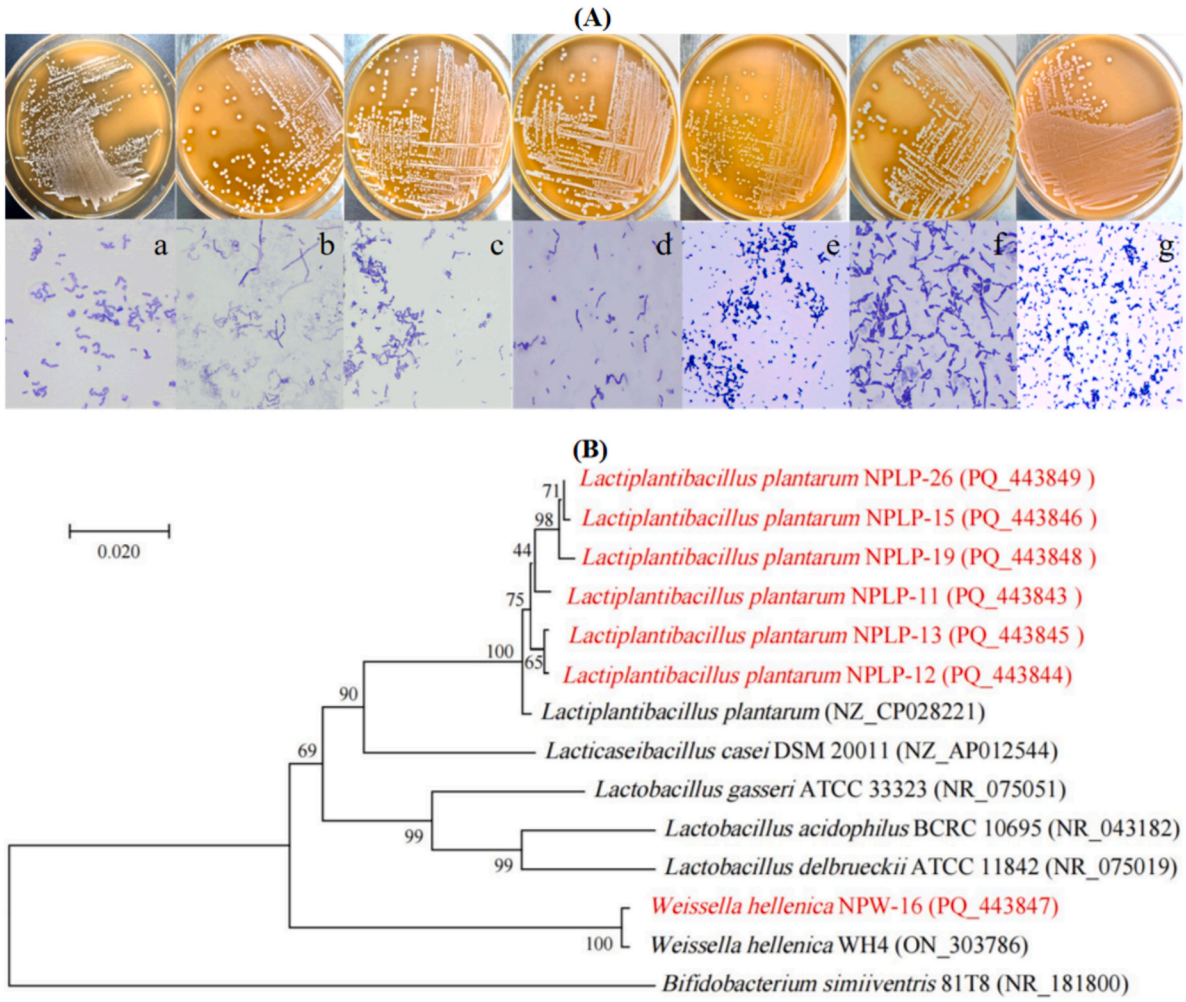


**Fig. 1.** Colony morphology (A) and phylogenetic tree (B) of lactic acid bacteria. The a-g were strains NPLP11, NPLP12, NPLP13, NPLP15, NPW16, NPLP19 and NPLP26, respectively. According to the 16S rRNA gene sequence of the strain, the phylogenetic tree was constructed using MEGA 7 software. *Bifidobacterium* 81 T8 was used an outgroup.

*acetotolerans* were considered the core functional microbial communities, which may be due to regional differences.

### 3.2. Growth and acid production characteristics of LAB

The growth characteristics of LAB were shown in Figure S1 A. Seven strains of LAB showed typical S-shaped growth curves in MRS broth, but there were differences in their growth characteristics. Between 0 and 4 h, all strains were in a lag growth phase, with NPLP11, NPLP12, NPW16 and NPLP19 completing metabolic accumulation and entering the logarithmic phase after 4 h, while NPLP13, NPLP15 and NPLP26 entered the logarithmic phase after 6 h. The $OD_{600}$ of NPW16 reached 1.75 at 14 h, indicating the earliest entry into the stationary phase. The reduction of nutrient substrates and the accumulation of metabolites limited the reproduction of LAB in the fermentation anaphase. The bacterial number of 7 strains of LAB continued to increase between 14 and 28 h, but the trend slowed down. Except for a slight increase in the bacterial count of NPLP12, NPLP13 and NPLP19, the other four LAB strains tended to stabilize after 28 h. The colony count of all LAB remained at its maximum at 48 h, and the bacterial concentrations of NPLP12 and NPLP19 were at their highest levels. However, the colony count of NPW16 was significantly ($p < 0.05$) lower than that of other LAB at this time, followed by NPLP26.

As shown in Figure S1B, pH of 7 LAB strains fermented in MRS broth for 48 h showed a brief increase at 0–4 h. This phenomenon may be related to *lactobacilli* converting pyruvate to acetate through aerobic respiration metabolic pathways, as reported in the study by Forestier et al. (2014). After 4 h, the pH of 7 strains of LAB began to decline. During the period of 6–12 h, the pH of tested strains began to significantly decrease and the downward trend slowed down after 12 h. The acid production rate of LAB slowed down in the later stage of fermentation and the pH tended to stabilize. NPLP11 showed the best acid production ability at 48 h, followed by NPLP12 and NPLP13. The limited growth of NPW16 was the main reason for its significantly ($p < 0.05$) lower acid production capacity compared to other tested strains.

During the fermentation process, LAB produce acid to inhibit the growth of other harmful bacteria and compete for nutrients in the fermentation substrate. There was a significant negative correlation between the number of LAB colonies and pH changes. LAB reproduce rapidly in logarithmic phase, and their acid production capacity was enhanced. Compared with *Weissella*, *L. plantarum* showed superior reproductive and acid production abilities in this report. The comprehensive growth and acid production capacity of NPLP12 indicate its superior fermentation potential.

### 3.3. Safety analysis of LAB

Indole was produced by the metabolism of tryptophan by tryptophan indole-lyase in gut microbiota. Although indoles were crucial in improving intestinal and even systemic diseases, it posed a security

threat to the kidney, gastrointestinal, nervous and cardiovascular systems (Ye et al., 2022). Indole was not detected in the 7 strains of LAB in this report (Fig. 2A). The proteolytic activity of gelatinase induced degradation of connective tissue was worth noting because gelatinase may trigger inflammation by hydrolyzing collagen (Ali et al., 2023). *S. aureus* was used as a positive control in the gelatin liquefaction test. As shown in Fig. 2B, 7 strains of LAB were unable to liquefy gelatin. Biogenic amines are formed by the removal of hydroxyl groups from amino acids by amino decarboxylase in bacteria. High concentrations of biogenic amines can cause neurotoxicity and blood pressure disorders (Kim et al., 2022). In this experiment, *E. coli* as a positive control caused the culture medium containing amino acids (L-tyrosine, L-lysine, L-histidine) to turn purple, while the culture medium for the growth of 7 strains of LAB turned yellow (Fig. 2C-E). The results showed that 7 strains of LAB did not produce putrescine, cadaverine and histamine. *Enterococcus* has been reported to be α-hemolytic in previous studies, therefore the evaluation of hemolytic activity was used to exclude the pathogenicity of 7 strains of LAB (Grujović et al., 2024). *S. aureus* showed obvious hemolysis circles on the blood agar plate and was detected as β-hemolysis (Fig. 2F). There were no hemolysis circles on the blood agar plates grown by 7 strains of LAB, which were detected as γ-hemolysis. The resistance of LAB to Erythromycin (E), Ciprofloxacin (CIP), Trimoproxim-sulfamethoxazole (SXT), Tetracycline (TET), Penicillin (PEN), Chloramphenicol (C), Ampicillin (AMP), Lincomycin (MY), Gentamicin (GEN) and Ceftriaxone (CTR) were investigated in this report (Table S1). The results showed that the tested strains were sensitive to E, PEN and C, resistant to CIP and MY and moderately sensitive to CTR.

### 3.4. Tolerance analysis of LAB

Whether probiotics can colonize in the intestine depends on their tolerance to acids, SGF and bile salts in the gastrointestinal environment. Tested strains showed varying degrees of tolerance to acid, SGF and bile salts (Table 1). The survival rates of NPLP11, NPLP12, NPLP13, NPLP15 and NPLP26 were greater than 60 % at pH 3 for 1 and 2 h ($p < 0.05$), with NPLP15 exhibiting the highest survival rates of 90.20 % and 77.54 %, respectively. However, NPW16 exhibited the lowest survival rate ($p < 0.05$), with survival rates 20.93 % and 36.25 % lower than NPLP15, respectively. *L. plantarum* exhibited better acid resistant ability compared to the *Weissella* and this phenotypic difference has been validated in the report by Wang et al. (2018). Lee et al. (2024) found that the accumulation of intracellular $NH_4^+$ and the enhancement of ATPase activity played important roles in the acid resistance of L. *plantarum*. The survival rate of the tested strains in SGF decreased in varying degrees with the extension of culture time. The survival rates of NPLP12 (82.56 %), NPLP13 (76.13 %) and NPLP15 (81.80 %) were higher than those of NPLP11 (59.38 %), NPLP19 (51.48 %) and NPLP26 (50.20 %) in SGF for 90 min ($p < 0.05$) and the NPW16 with the lowest survival rate was 17.78 % ($p < 0.05$). Under SGF for 180 min, NPW16 was not detected, while NPLP12 and NPLP15 showed higher survival rates than other strains, with survival rates of 57.97 % and 65.84 %, respectively. Bile salts can achieve antibacterial effects by disrupting microbial cell membranes, with concentrations typically ranging from 0.03 % to 0.3 % in the gastrointestinal tract of animals. The tolerance of LAB to 0.3 % bile salt was studied to enable LAB to enter the intestine and exert its probiotic effects. The results showed that the survival rates of NPLP12, NPLP13 and NPLP15 in 0.3 % bile salt MRS broth were higher than those of NPLP11, NPLP 19 and NPLP 26 ($p < 0.05$) and the survival rates were all above 50 %. The survival rate of NPW16 at 0.3 % bile salt for 180 min was only 34.79 %, which was higher than the 4 strains of LAB isolated by

**Table 1**
Acid, simulated gastric fluid and bile salt tolerance of lactic acid bacteria (%). Values are means of three replicates ± SD; [a-d]Within the same column, different letters indicate significant differences ($p < 0.05$). -: Undetected.

| Species | Acid (pH = 3) | | Simulated gastric fluid | | Bile salt (0.3 %) | |
|---|---|---|---|---|---|---|
| | 1 h | 2 h | 90 min | 180 min | 90 min | 180 min |
| NPLP11 | 81.64 ± 13.27$^{ab}$ | 62.94 ± 5.34$^{ab}$ | 59.38 ± 4.64$^{b}$ | 32.85 ± 4.15$^{c}$ | 72.59 ± 6.30$^{b}$ | 46.49 ± 5.26$^{bc}$ |
| NPLP12 | 86.20 ± 6.73$^{ab}$ | 66.56 ± 5.29$^{ab}$ | 82.56 ± 6.57$^{a}$ | 57.97 ± 1.26$^{ab}$ | 77.59 ± 0.94$^{ab}$ | 54.56 ± 4.19$^{ab}$ |
| NPLP13 | 84.44 ± 3.71$^{ab}$ | 62.25 ± 4.43$^{ab}$ | 76.13 ± 9.75$^{a}$ | 53.41 ± 2.62$^{b}$ | 84.89 ± 5.58$^{a}$ | 58.79 ± 4.87$^{a}$ |
| NPLP15 | 90.20 ± 2.81$^{a}$ | 77.54 ± 9.44$^{a}$ | 81.80 ± 5.28$^{a}$ | 65.84 ± 5.97$^{a}$ | 74.82 ± 1.01$^{ab}$ | 50.77 ± 5.01$^{abc}$ |
| NPW16 | 69.27 ± 3.73$^{b}$ | 41.29 ± 2.08$^{c}$ | 17.78 ± 2.52$^{c}$ | – | 55.63 ± 6.21$^{c}$ | 34.79 ± 1.26$^{d}$ |
| NPLP19 | 81.66 ± 4.05$^{ab}$ | 58.56 ± 9.02$^{b}$ | 51.48 ± 4.42$^{b}$ | 27.88 ± 3.79$^{c}$ | 66.58 ± 4.17$^{b}$ | 42.16 ± 2.53$^{cd}$ |
| NPLP26 | 79.05 ± 2.92$^{ab}$ | 60.56 ± 5.90$^{ab}$ | 50.20 ± 6.10$^{b}$ | 19.31 ± 1.45$^{d}$ | 68.45 ± 3.30$^{b}$ | 44.62 ± 1.94$^{bc}$ |

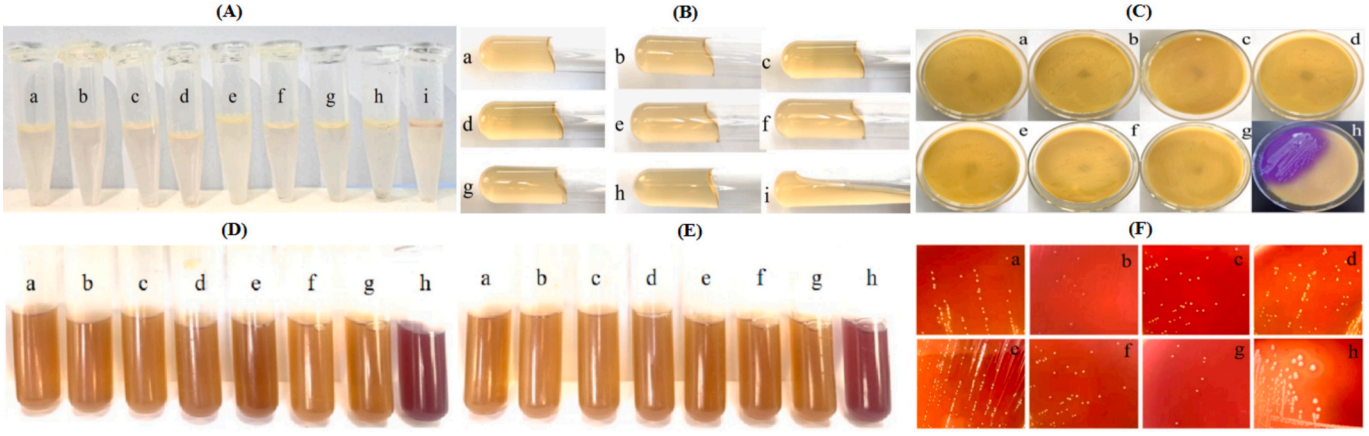


**Fig. 2.** Results of indole (A), gelatin liquefaction (B), Biogenic amine (C-E) and hemolytic activity (F) test of lactic acid bacteria. The a-i in Figs. A and B were negative control, NPLP11, NPLP12, NPLP13, NPLP15, NPW16, NPLP19, NPLP26 and positive control, respectively. The a-h in Figs. C—F were NPLP11, NPLP12, NPLP13, NPLP15, NPW16, NPLP19, NPLP26 and positive control, respectively. The indole test of the strain was negative in yellow and positive in red. The culture media in C, D, E, and F were supplemented with L-tyrosine, L-lysine, L-histidine and sterile sheep blood, respectively. Purple was positive and yellow was negative in the biogenic amine test. β-hemolysis: Red blood cells were completely dissolved and a transparent hemolysis circle appears around the colony. α-hemolysis: A green semi-transparent hemolysis circle appears around the colony. γ-hemolysis: No hemolytic activity. The positive control in indole test and biogenic amine test was *E. coli*, and the positive control in gelatin liquefaction and hemolytic activity test was *S. aureus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cai et al. (2022) from aquaculture systems.

### 3.5. Aggregative potential of LAB

The auto-aggregation ability of LAB was crucial for factors such as biofilm formation, colonization, and virulence of microorganisms (Shehata et al., 2024). The auto-aggregation, co-aggregation and hydrophobicity of LAB were studied to clarify their potential to colonize the intestine and maintain intestinal balance. With the extension of time, the auto-aggregation, co-aggregation and hydrophobicity of the tested strains were increased to varying degrees, which was time and strains dependent (Table 2). The auto-aggregation rate of the tested strains reached a minimum of 10.80 % at 2 h, while the lowest level of auto-aggregation rate of *Enterococcus faecalis* reported by Albayrak and Duran (2021) was below 10 %. The co-aggregation of LAB and pathogenic bacteria helps the host to avoid infection by pathogenic bacteria, a viewpoint that has been validated in previous reports (Sakoui et al., 2022). The copolymerization ability of the tested strains with *S. aureus* and *E. coli* was significant (1.37 % - 37.91 %), which depended on the time, the type of LAB and pathogenic strains. NPLP15 showed the best co-aggregation with *S. aureus* and *E. coli* at 4 h, with aggregation rates of 35.54 % and 37.91 %, respectively. The hydrophobicity of 7 strains of LAB were validated using xylene, ranging from 16.18 % to 53.35 %. The hydrophobicity was highest in NPLP13 with 53.35 % followed by NPLP12, NPLP15 and NPLP26 with 49.66 %, 50.73 % and 49.63 %, respectively. The results indicated that the *L. plantarum* has a greater advantage in host colonization capacity compared to *Weissella* in this report. The tolerance of the tested strains in this study was positively correlated with their aggregation potential and hydrophobicity, which was reflected in the lower tolerance, aggregation potential and hydrophobicity of NPW16 and NPLP19 compared to other strains.

### 3.6. Antioxidant activity of LAB

The DPPH radical scavenging ability was used to evaluate the *in vitro* antioxidant activity of LAB. All LAB's cell-free supernatant and cell-free extract showed antioxidant activity, but the antioxidant activity of intact cells was not detected (Fig. 3). The abundant organic acids in cell-free supernatant explain this phenomenon, but exopolysaccharides secreted by LAB may also be metabolites that provide antioxidant activity (Wang et al., 2017). NPLP15's cell-free supernatant and cell-free extract showed the best antioxidant capacity, while NPW16's cell-free supernatant and cell-free extract exhibited the lowest DPPH radical scavenging ability. The DPPH radical scavenging ability of the cell-free supernatant of NPLP15 was 11.25 %, significantly ($p < 0.05$) higher than

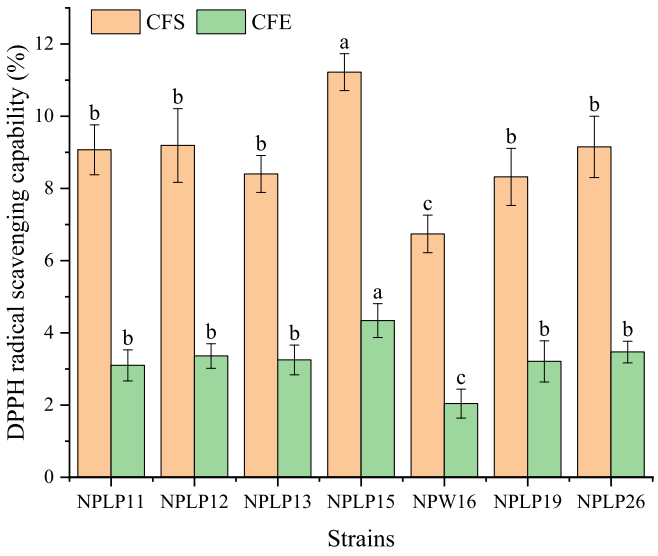


**Fig. 3.** DPPH radical scavenging capacity of cell-free supernatant (CFS) and cell-free extract (CFE) of lactic acid bacteria. Different letters of the same indicator represent significant differences ($p < 0.05$).

NPLP11 (9.07 %), NPLP12 (9.19 %), NPLP13 (8.40 %), NPW16 (6.74 %), NPLP19 (8.32 %) and NPLP26 (9.15 %) and there were no significant ($p > 0.05$) differences in the antioxidant activity among NPLP11, NPLP12, NPLP13, NPLP19 and NPLP26.

### 3.7. Growth characteristics of NPLP12 in PFL

After comprehensively compared the safety, growth characteristics and probiotic properties of 7 strains of LAB, NPLP12 was used to ferment PFL. The changes in acidity, pH and LAB colony count of PFL during the fermentation process from 0 to 72 h were investigated (Fig. 4). The number of live bacteria in probiotic beverages should be above $10^6$–$10^7$ CFU/mL to better utilize the probiotic effect and improve human health (Mojikon et al., 2022). The experimental results showed that NPLP12 grew normally and produced acid in PFL, maintaining above 8.0 lg CFU/mL at the end of fermentation. The decrease in pH value and the accumulation of total acidity depended on the growth of NPLP12. With the rapid increase in bacterial count and the accumulation of total acidity, the pH value also showed a significant downward trend between 0 and 36 h. The growth of NPLP12 reached a stationary phase after 36 h and the colony count peaked at 8.59 ± 0.05 lg CFU/mL before slowly

**Table 2**
Adhesion ability of lactic acid bacteria. $^{a\text{-}c}$Within the same indicators in the same row and $^{A\text{-}E}$Within the same column, values of different letters represent significant differences ($p < 0.05$).

| Species | Auto-aggregation (%) | | | Co-aggregation (%) | | | | | | Hydrophobic-ity (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *S. aureus* | | | *E. coli* | | | |
| | 1 h | 2 h | 4 h | 1 h | 2 h | 4 h | 1 h | 2 h | 4 h | |
| NPLP11 | $6.32 \pm 1.62^{ABb}$ | $10.80 \pm 1.35^{Cb}$ | $25.70 \pm 5.03^{ABa}$ | $6.09 \pm 1.84^{ABc}$ | $19.39 \pm 3.51^{ABb}$ | $34.83 \pm 2.94^{Aa}$ | $7.74 \pm 0.34^{Bc}$ | $17.14 \pm 2.48^{Bb}$ | $31.46 \pm 1.65^{Ba}$ | $45.41 \pm 2.29^{B}$ |
| NPLP12 | $8.63 \pm 2.72^{ABb}$ | $19.16 \pm 3.68^{ABa}$ | $25.01 \pm 4.43^{ABa}$ | $6.97 \pm 1.55^{ABc}$ | $15.32 \pm 2.07^{BCb}$ | $26.52 \pm 2.22^{CDa}$ | $4.91 \pm 1.38^{Bc}$ | $23.61 \pm 2.18^{Ab}$ | $36.46 \pm 2.42^{Aa}$ | $49.66 \pm 3.49^{AB}$ |
| NPLP13 | $11.00 \pm 2.76^{Ab}$ | $20.70 \pm 1.91^{Aa}$ | $26.02 \pm 4.30^{ABa}$ | $6.08 \pm 0.54^{ABc}$ | $16.70 \pm 2.10^{BCb}$ | $30.00 \pm 3.21^{BCa}$ | $5.03 \pm 1.69^{Bc}$ | $15.01 \pm 2.64^{Bb}$ | $28.52 \pm 1.32^{Ba}$ | $53.35 \pm 2.76^{A}$ |
| NPLP15 | $9.22 \pm 2.83^{Ac}$ | $22.89 \pm 3.17^{Ab}$ | $30.10 \pm 4.11^{Aa}$ | $4.78 \pm 1.71^{Bc}$ | $24.45 \pm 4.07^{Ab}$ | $35.54 \pm 4.07^{Aa}$ | $11.63 \pm 2.00^{Ac}$ | $25.03 \pm 1.28^{Ab}$ | $37.91 \pm 2.17^{Aa}$ | $50.73 \pm 6.67^{AB}$ |
| NPW16 | $4.52 \pm 1.35^{Bb}$ | $13.67 \pm 5.26^{BCa}$ | $19.86 \pm 1.18^{Ba}$ | $1.65 \pm 0.48^{Cc}$ | $7.08 \pm 0.83^{Db}$ | $14.17 \pm 1.07^{Ea}$ | $1.37 \pm 0.39^{Cc}$ | $4.81 \pm 0.57^{Db}$ | $12.06 \pm 0.81^{Da}$ | $16.18 \pm 1.90^{C}$ |
| NPLP19 | $9.43 \pm 3.08^{Ac}$ | $19.19 \pm 1.17^{ABb}$ | $25.99 \pm 1.19^{ABa}$ | $5.18 \pm 0.71^{ABc}$ | $11.73 \pm 3.60^{CDb}$ | $24.11 \pm 1.86^{Da}$ | $6.11 \pm 1.68^{Bc}$ | $10.98 \pm 2.93^{Cb}$ | $17.97 \pm 0.92^{Ca}$ | $20.92 \pm 3.14^{C}$ |
| NPLP26 | $7.48 \pm 1.96^{ABb}$ | $17.34 \pm 2.21^{ABa}$ | $21.56 \pm 3.54^{Ba}$ | $7.96 \pm 2.30^{Ac}$ | $19.70 \pm 1.81^{ABb}$ | $34.01 \pm 3.05^{ABa}$ | $7.80 \pm 2.52^{Bc}$ | $18.07 \pm 0.73^{Bb}$ | $35.91 \pm 4.93^{Aa}$ | $49.63 \pm 2.87^{AB}$ |

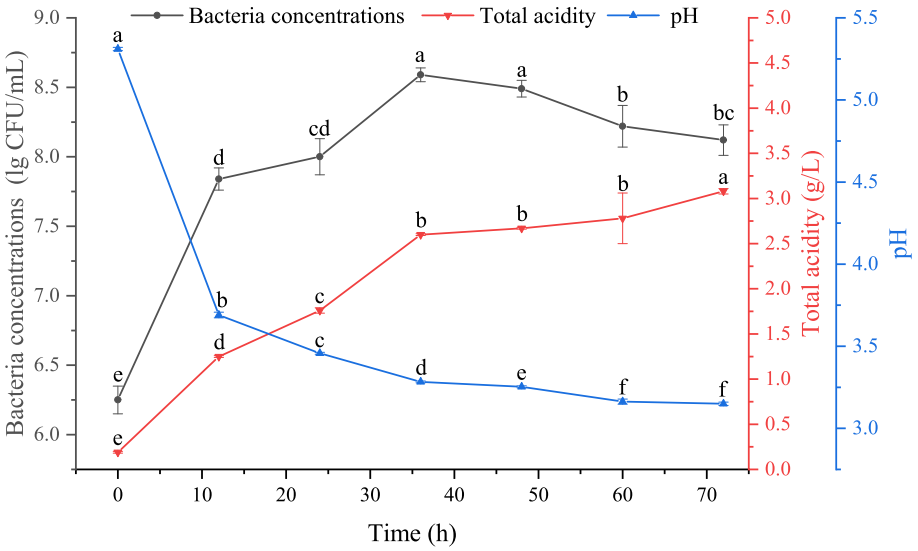


**Fig. 4.** Changes in bacterial concentrations, total acidity and pH value of NPLP12 in peony flower liquid during fermentation. Different letters of the same indicator represent significant differences ($p < 0.05$).

decreasing. At this point, although the pH decrease tended to be slow, organic acids continued to accumulate. Many studies suggested that fermentation improves the benefits of food to human health by altering the chemical structure of plant components. Park et al. (2021) fermented *Opuntia humifusa* with yuza peel and guava leaves by lactic acid fermentation to reduce glycemic index and enhance functional activity.

### 3.8. Effects of fermentation on TPC and TFC

Phenolic compounds have been found to have favorable physiological activities and significant improvement effects on human health. The changes of TPC and TFC in PFFL at 12, 36, and 60 h were shown in Table 3. The phenolic content of PFL fermented by *L. plantarum* NPLP12 showed an overall increasing trend. TPC continued to increase after fermentation, reaching a peak of 296.60 μg GAE/mL at 36 h, which was significantly ($p < 0.05$) higher than unfermented PFL by 29 μg GAE/mL. The TFC of PFFL slightly increased at 36 h and 60 h, but there was no statistical difference. Research has shown that changes in phenolic compounds are closely related to the strain and fermentation substrate: TPC and TFC were significantly increased in Xu Xiang/Hong Yang kiwi fruit juice fermented by *L. plantarum* Lp90, while *L. acidophilus* La85 showed the opposite trend (Wang et al., 2022). The increase in TPC and TFC was due to enzymatic hydrolysis of plant cell walls as well as fermentation promoting the release of phenolic substances and the conversion of conjugated phenols into free forms (Shang et al., 2022).

**Table 3**
Changes in TPC, TFC, phenolic compounds and antioxidant activities in peony flower liquid before and after fermentation. Results are expressed as mean ± SD. "*" Indicating significant differences in the same indicator ($p < 0.05$).

| | Fermentation time (h) | Before fermentation | After fermentation |
|---|---|---|---|
| TPC (μg GAE/mL) | 12 | 267.6 ± 5.86 | 296.23 ± 12.84* |
| | 36 | | 296.60 ± 5.76* |
| | 60 | | 276.48 ± 4.21 |
| TFC (μg RE/mL) | 12 | 44.11 ± 4.62 | 42.20 ± 3.44 |
| | 36 | | 49.45 ± 1.79 |
| | 60 | | 47.67 ± 2.47 |
| Phenolic acids (μg/mL) | | | |
| galloyl hexose | 36 | 5.12 ± 0.38* | 4.37 ± 0.06 |
| gallic acid | | 7.78 ± 0.85* | 1.82 ± 0.09 |
| methyl gallate | | 4.43 ± 0.05* | 0.25 ± 0.01 |
| methyl digallate | | 6.99 ± 0.12 | 7.58 ± 0.47 |
| Flavonoids (μg/mL) | | | |
| kaempferol-3,7-di-O-glucoside | 36 | 115.89 ± 1.41* | 72.12 ± 2.76 |
| isorhamnetin-3,7-di-O-glucoside | | 10.58 ± 0.22 | 12.66 ± 0.16* |
| eriodictyol-O-glucoside | | 29.42 ± 0.38* | 24.43 ± 0.37 |
| apigenin-7-O-glucoside | | 39.15 ± 2.49 | 51.12 ± 3.83* |
| kaempferol-3-O-glucoside | | 114.27 ± 3.80 | 139.48 ± 4.91* |
| Antioxidant activities (μmol TE/mL) | | | |
| DPPH | 36 | 1.45 ± 0.04 | 1.66 ± 0.09* |
| $ABTS^{+}$ | | 1.64 ± 0.05 | 1.79 ± 0.03* |
| FRAP | | 3.17 ± 0.06 | 3.40 ± 0.05* |

### 3.9. Effects of fermentation on phenolic compounds

In this study, 9 phenolic compounds were identified based on our previous reports (Xiang et al., 2019). Peaks 1–9 were identified as galloyl hexose, gallic acid, methyl gallate, eriodictyol-O-glucoside, kaempferol-3,7-di-O-glucoside, isorhamnetin-3,7-di-O-glucoside, methyl digallate, apigenin-7-O-glucoside and kaempferol-3-O-glucoside, respectively (Fig. 5). The PFL fermented for 36 h was used to quantify 9 phenolic compounds, with unfermented PFL serving as the control group (Table 3). The content of galloyl hexose, gallic acid, methyl gallate, kaempferol-3,7-di-O-glucoside and eriodictyol-O-glucoside showed a significant ($p < 0.05$) decrease trend after fermentation. But methyl digallate increased by 0.59 μg/mL, isorhamnoide-3,7-di-O-glucoside increased by 2.08 μg/mL, luteolin 7-O-glucoside increased by 25.21 μg/mL and apigenin 7-O-glucoside increased by 11.96 μg/mL. The decrease in galloyl hexose after fermentation may be due to the conversion of LAB into gallic acid during metabolism, a biotransformation process mentioned by Rabhi et al. (2015). Tannase and gallic acid decarboxylase are involved in the metabolism of gallic acid and methyl gallate, which may be the reason for the decrease in gallic acid and methyl gallate. Reverón et al. (2017) has also proposed that the presence of gallic acid and methyl gallate can induce the expression of genes encoding tannase (tanBLp) and gallic acid decarboxylase (lpdC). The increase in methyl digallate may be due to the dehydration condensation of methyl gallate and gallic acid, which was another reason for the decrease in methyl gallate and gallic acid. The increase in kaempferol-3-O-glucoside may be due to the hydrolysis of kaempferol-3,7-di-O-glucose during fermentation, a similar hydrolysis

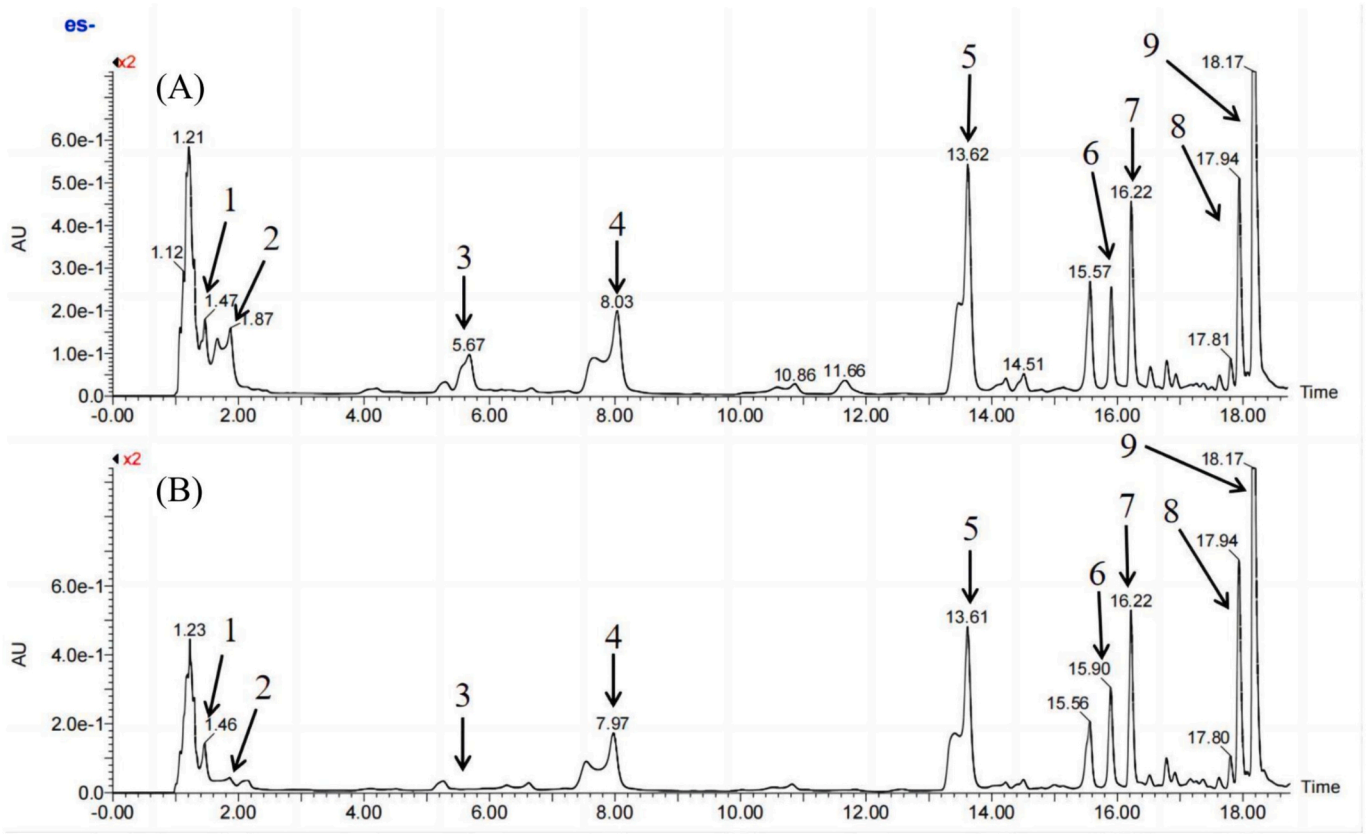


**Fig. 5.** UPLC chromatograms of phenolic compounds in peony flower liquid before (A) and after (B) fermentation at 280 nm detection wavelength. Compounds were confirmed by comparing with standards or putatively identified by UV spectra, MS and MS/MS data. The 1–9 were identified as galloyl hexose, gallic acid, methyl gallate, eriodictyol-O-glucoside, kaempferol-3,7-di-O-glucoside, isorhamnetin-3,7-di-O-glucoside, methyl digallate, apigenin-7-O-glucoside and kaempferol-3-O-glucoside, respectively.

process of glycosidic described in Schneider's report (Schneider & Blaut, 2000), which may also be the reason for the increase of apigenin-7-O-glucoside content.

### 3.10. Effect of fermentation on antioxidant activity

The flavonoids in PF have high antioxidant activity and may have potential roles in antioxidant activity in diet. The antioxidant activity of fermented PFL significantly ($p < 0.05$) increased and the DPPH radical scavenging ability, $ABTS^{\cdot+}$ scavenging ability and FRAP value increased by 0.21, 0.15 and 0.23 μmol TE/mL, respectively (Table 3). The main phenolic compounds in PF are flavonoids, among which kaempferol-3,7-O-diglucoside and kaempferol-3-O-glucoside contribute the main antioxidant activity, followed by kaempferol-O-glucoside and apigenin 7-O-glucoside. Galloyl hexose, gallic acid, methyl gallate, kaempferol-3,7-di-O-glucide and eriodictyol-O-glucoside are negatively correlated with the antioxidant activity of PFL, while methyl digallate, isorhamnetin-3,7-di-O-glucide, kaempferol-3-O-glucoside and apigenin-7-O-glucoside are negatively correlated with the antioxidant activity of PFL. In previous reports, the total flavonoid glycoside content and antioxidant activity in seabuckthorn apple juice inoculated with *L. plantarum* also showed a significant upward trend (Tkacz et al., 2020).

## 4. Conclusions

In this report, 6 strains of L. *plantarum* and 1 strain of *Weissell*a were screened from pickles through colony characteristics and 16S rRNA identification. NPLP12 and NPLP19 showed the best reproductive ability, while NPLP11 had the strongest acid production ability at 48 h, followed by NPLP12 and NPLP13. The general safety of 7 strains of LAB were verified through indoles and gelatin liquefaction, biogenic amine, hemolytic activity and antibiotic sensitivity tests. *L. plantarum* had better tolerance compared to *Weissella*, and NPW16 couldn't survive in SGF for 180 min. The comprehensive tolerance of NPLP12 was superior to other strains. The auto-aggregation and co-aggregation rate of NPLP15 reached their maximum values at 4 h, while NPLP13 had the best hydrophobicity. The DPPH radical scavenging ability of the cell-free supernatant was significantly greater than that of cell-free extract, but the antioxidant activity of intact cells was not detected. NPLP15's cell-free supernatant exhibited the highest antioxidant activity, with a DPPH radical scavenging ability of 11.25 %. NPLP12 can release phenolic compounds from PF and the TPC and TFC of PFL reach their maximum values after 36 h of fermentation. After fermentation, the contents of methyl digallate, isorhamnetin-3,7-di-O-glucoside, kaempferol-3-O-glucoside and apigenin-7-O-glucoside in PFL increased by 0.59, 2.08, 25.21 and 11.96 μg/mL, respectively. Flavonoids contribute to the main antioxidant activity of PFL. The DPPH radical scavenging ability, $ABTS^{\cdot+}$ radical scavenging ability and FRAP value of PFL was significantly increased after fermentation. The increase in the content of kaempferol-3-O-glucoside and apigenin-7-O-glucoside made the main contribution to the enhancement of antioxidant activity. In summary, all tested strains had the potential to be safely used as probiotics and fermentation agents, except for NPW16. The phenolic compounds and antioxidant activity of PFL significantly increased after fermentation by NPLP12. It can be further developed as fermented functional beverage in the future.

## CRediT authorship contribution statement

**Yangyang Yang:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Yunfeng Xu:** Writing – review &

editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Xiangxiang He:** Investigation, Formal analysis. **Mingyan Guo:** Methodology, Investigation. **Junliang Chen:** Conceptualization. **Lei Luo:** Resources, Formal analysis. **Jinle Xiang:** Writing – review & editing, Supervision, Resources, Investigation, Formal analysis.

## Declaration of competing interest

There are no known competitive financial interests or personal relationships that need to be declared.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102430.

## Data availability

Data will be made available on request.

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