

Microbial fermentation alters the phytochemical profile of Ginkgo biloba leaves

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ABSTRACT

Ginkgo biloba contains diverse natural products. This study employed microbial fermentation to alter phytochemical profiles in *Ginkgo biloba* leaves (GBL). Four microorganisms—*Lactobacillus plantarum* SCTM-1, *Saccharomyces cerevisiae* GLLB-3, *Aspergillus niger* ZYDC-1, and *Aspergillus cristasukae* LNAC-1—were used individually and in a consortium (YXSL: *L. plantarum* + *S. cerevisiae*). All the microbial fermentation changes the GBL metabolite profiles, including total polyphenols, flavonoids, and polysaccharides. Especially, compared to unfermented GBL, YXSL increased total polyphenols, flavonoids, and polysaccharides by 76.0 %, 170.6 %, and 20.9 %, respectively. Metabolomic analysis identified 116 differentially expressed secondary metabolites, mainly in plant secondary metabolite, phenylpropanoid, and flavonoid biosynthesis pathways. YXSL specifically enhanced flavonoid production via microbial biotransformation, with crocetin content increasing 104.5 % through hydrolysis of crocin. This work highlights microbial potential to boost GBL's bioactive components and provides a basis for improving phytochemical yields via fermentation.

1. Introduction

Ginkgo biloba L. (GBL), commonly known as ginkgo, contains abundant secondary metabolites, including flavonoids, terpenoids, polyphenols, polysaccharides, and organic phenolic acids (Liu et al., 2022). GBL has been widely used in oriental medicine and found to exhibit therapeutic effects such as improvement of blood circulation, neuro-protection, anti-cancer, antioxidation, and anti-inflammation (Biernacka et al., 2024; Hu et al., 2024; Tao et al., 2022). However, the absorption and metabolism of flavonoid glycosides from GBL highlight the challenges associated with their bioavailability. Mostly, ginkgo flavonoids are hydrolyzed by cytosolic β -glucosidase in the small intestine, converting them into aglycones before absorption. These aglycones then undergo phase II metabolism in the liver, involving glucuronidation,

sulfation, and methylation, which can influence their therapeutic efficacy (Tao et al., 2022). These limitations significantly constrain the broader application of GBL in medicinal and food products.

Biotransformation utilizes enzymatic and microbial processes to structurally modify specific compounds into related derivatives (M. Guo et al., 2023; Xin et al., 2023). Probiotic fermentation of Chinese herbal medicines offers multiple benefits, including the generation of novel bioactive components, enhancement of existing bioactive compounds, reduction of toxicity, and improvement of flavor (J. Zhang et al., 2024; X. Zhang, Miao, Pan, et al., 2023). This process involves probiotic-derived hydrolases that degrade plant cell wall, thereby increasing the bioavailability of bioactive constituents (Lin et al., 2024; L. Wang et al., 2016). For instance, *Aspergillus* (e.g., cellulases, proteases, and polyphenol oxidases) catalyzes the breakdown of cellulose and

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bioconversion of phytochemicals into bioactive compounds with enhanced nutritional values (Q. Li et al., 2018). Similarly, fermentation of goji juice by *Lactiplantibacillus plantarum* D and F significantly increases total polyphenol and flavonoid contents while producing beneficial secondary metabolite indole-3-lactic acid (Xie et al., 2023). Lactic acid bacteria secrete extracellular enzymes, such as proteases, lipases, and phosphatases (García-Cano et al., 2020), which collectively contribute to more efficient plant matrix degradation and nutrient release.

Probiotic solid-state fermentation has been applied to various herbs and plant-based foods. Certain lactic acid bacteria are applied in the fermentation of various foods, enhancing their nutritional and sensory attributes. This application significantly boosts antioxidant activity, particularly through phenolic compounds. When moringa leaves are utilized as a substrate and subjected to submerged fermentation using lactic acid bacterial strains, both their phenolic compound content and total flavonoids increased (Razola-Díaz et al., 2025). *S. cerevisiae* is one of the most widely used microorganisms in herbal fermentation, and its fermentation enhances the hypoglycemic activity of *Ge-Gen-Qin-Lian* decoction (Yan et al., 2018). Filamentous fungi secrete multiple enzymes during fermentation while simultaneously generating distinct flavor profiles and bioactive compounds. Fermentation of GBL with *Aspergillus niger* enhanced the contents of total flavonoids, reduced sugars and crude protein, while significantly improved the odor characteristics compared to the unfermented GBL (J. Wang et al., 2018).

Phenolic compounds, terpenoids, and alkaloids have been found to exhibit bioactivities, particularly antioxidant, antimicrobial, and anti-inflammatory properties (Hilal et al., 2024). Microbial fermentation modifies the secondary metabolite profile of plant materials through various biochemical transformations including hydrolysis, methylation, and carboxylation reactions (Gulsunoglu-Konuskan and Kilic-Akyilmaz, 2022). Efficient bioconversion of *Epimedium* flavonoids has been primarily achieved by probiotics and specific lignocellulases (X. Zhang et al., 2024). Metabolomic analysis of GBL has enabled comprehensive profiling of its chemical constituents, revealing the enriched metabolic pathways associated with flavonoids, terpenoids, carbohydrates, amino acids, and polyphenolic compounds (J. Guo et al., 2022; Han et al., 2021; He et al., 2021). The bioactive constituents of GBL present a complex chemical profile, with flavonoids and terpenoids representing the primary bioactive classes. A synergistic approach of integrating UPLC-MS/MS with transcriptome sequencing analysis has enabled the comprehensive characterization of pharmacologically dominant flavonoids in GBL (He et al., 2021). Elucidating the precise mechanisms underlying the biotransformation of GBL bioactive compounds would significantly advance the comprehensive utilization of this medicinal plant.

This study explored the impact and mechanistic underpinnings of probiotic-mediated fermentation on GBL. We firstly screened four probiotic strains by quantifying their lignocellulose activities. Subsequently, five fermentation groups were established: four single-strain fermentations using *Lactobacillus plantarum* SCTM-1, *S. cerevisiae* GLLB-3, *A. niger* ZYDC-1, and *A. cristatus* LNAC-1, respectively, and one co-culture group combining *L. plantarum* SCTM-1 and *S. cerevisiae* GLLB-3. Comprehensive biochemical assays were performed to quantify total phenolic content, total flavonoids, and polysaccharide levels. Additionally, untargeted metabolomic profiling was employed to characterize the metabolic shifts induced by probiotic fermentation.

2. Materials and methods

2.1. Materials, reagents, and microbial strains

The GBL was bought from Pizhou, Jiangsu province. The GBL were mechanically crushed and passed through a 40-mesh standard sieve. After being dried at 65 °C to a constant weight, the samples were collected and stored at 4 °C. Gallic acid (Purity ≥ 98 %), rutin (Purity ≥

98 %), and D-glucose (Purity ≥ 99.5 %) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China).

The potato dextrose broth (PDB) medium was prepared using 3.5 % potato dextrose dry powder. The MRS medium was composed of 1 % peptone, 0.4 % yeast extract, 0.5 % beef extract powder, 2 % glucose, 1 % Tween 80, 0.2 % KH₂PO₄, 0.5 % CH₃COONa, 0.2 % C₆H₅O₇ (NH₄)₃, 0.02 % MgSO₄, and 0.005 % MnSO₄. The yeast extract peptone dextrose (YPD) medium was prepared with 1 % yeast extract, 1 % peptone, and 2.0 % glucose. All chemicals and reagents were bought from Beijing Aoboxing Biotechnology Co., LTD.

L. plantarum SCTM-1 (Genbank accession number of 16S rRNA gene is PQ578293) was isolated from Chinese sauerkraut in Guiyang, Guizhou (China). *S. cerevisiae* GLLB-3 (Genbank accession number of ITS gene is PP466939) was isolated from Liubao tea in Guilin, Guangxi (China). *A. niger* ZYDC-1 (Genbank accession number of ITS gene is PP466956) was isolated from black bean sauce in Zunyi, Guizhou (China). *A. cristatus* LNAC-1 (Genbank accession number of ITS gene is PP425863) was isolated from fuzhuan tea in Anhua, Hunan (China).

2.2. The evaluation of microbial lignocellulose degradation

The modified medium, composed of 0.2 % KH₂PO₄, 0.1 % MgSO₄·7H₂O, 0.1 % (NH₄)₂SO₄, 3.5 % peptone, 3.0 % yeast extract, and 20 % agar, was used for microbial cultivation (Ezeilo et al., 2020). To test the lignocellulase activity of the microbes, 10 g/L of carboxymethyl cellulose (CMC), beechwood xylan, or pectin was added separately, serving as selective media (CMC-agar, xylan-agar, and pectin-agar, respectively) to detect cellulase, xylanase, and pectinase activities, respectively. PDB liquid medium was used to cultivate *A. niger* ZYDC-1, and *A. cristatus* LNAC-1. MRS liquid medium was used to cultivate *L. plantarum* SCTM-1. YPD liquid medium was used to cultivate *S. cerevisiae* GLLB-3. The cultivation temperature was 30 °C or 37 °C, and the culture time was 24 h. Culture broth (10 µL) was transferred onto culture plates and incubated at 30 °C or 37 °C for 3 days. Strains producing cellulase, xylanase, and pectinase were detected using the Congo red staining method, which manifested as light red and orange-red colored clear zones surrounding the colonies (Zhang, Miao, Tang, et al., 2023). All experiments were performed in triplicate to ensure higher reliability.

2.3. Fermentation of GBL with selected microbial strains

A. niger LNAC-1 and *A. cristatus* ZYDC-1 were inoculated on PDA medium and cultured at 30 °C for 5 days. Mycelia were then scraped off using a sterilized inoculating shovel and resuspended in sterile water. The resulting mycelial suspension was filtered through an eight-layer filter paper funnel under sterile conditions. The filtered spore suspension was collected into sterile tubes. After quantifying the spore concentration microscopically, the suspension was adjusted to a concentration of 10⁹ CFU/mL. For *L. plantarum* SCTM-1 and *S. cerevisiae* GLLB-3, single colonies were individually inoculated into MRS or YPD media (5 mL) and cultured for 6–8 h until reaching the logarithmic growth phase. Subsequently, 500 µL of the logarithmic-phase cultures (1 % inoculum volume) were transferred into 50 mL of fresh corresponding media and incubated for 24 h. The resulting cultures were spread onto appropriate agar plates for colony counting, then diluted with sterile water to achieve a final concentration of 10⁹ CFU/mL. 18 g of GBL was weighed and mixed with 12 mL of the respective bacterial suspension, resulting in a water content of 40 %. Fermentation was conducted at 30 °C for 7 days. The GBL fermented by *L. plantarum* SCTM-1, *S. cerevisiae* GLLB-3, *A. niger* ZYDC-1, and *A. cristatus* LNAC-1 were assigned as YXLp group, YXSc group, YXAn group, and YXAc group, respectively. The GBL fermented with *L. plantarum* SCTM-1 and *S. cerevisiae* GLLB-3 was named as YXSL group. *L. plantarum* SCTM-1 and *S. cerevisiae* GLLB-3 were mixed at a 1:1 ratio. Similarly, 18 g of GBL was weighed and mixed with 12 mL of mixed bacterial suspension (containing both

strains), achieving a water content of 40 %. The fermentation was carried out at 30 °C for 7 days. The control group (YXKC) was GBL added with an equal amount of sterile water suspension. The triplicate was implemented during GBL fermentation. After fermentation is complete, the material was placed in a 55 °C oven to dry until a constant weight is achieved, in order to effectively stop the fermentation process.

2.4. Determination of bioactive components in fermented GBL

The GBL dried after fermentation in Section 2.3 was used for the determination of total phenolic content, total flavonoid content, and total polysaccharide content. The total phenolic content of all samples was analyzed using the Folin–Ciocalteu colorimetric method (Lin et al., 2025). Specifically, 3.0 g of each sample was added to 30.0 mL of 75 % ethanol, followed by ultrasonic treatment (200 W, 60 °C, 120 min) and centrifugation (5000 × g, 10 min) to collect the supernatant for total polyphenol determination. For the assay, 1 mL of the prepared sample solution was mixed with 2 mL of 12 % Na₂CO₃ solution and 1 mL of Folin–Ciocalteu reagent. After thorough mixing and a 30-second standing period, the mixture was diluted to 25 mL with distilled water. The solution was then protected from light and allowed to react for 2 h. Absorbance was measured at 765 nm, and the total phenolic content was determined by fitting the measured value to a standard curve. Gallic acid ($\geq 98\%$ purity) was used as the standard for this assay.

The total flavonoid content of all samples was analyzed using the AlCl₃ colorimetric method (Lin et al., 2025). Similarly, the total flavonoid content was determined following the same procedure as described for total phenolics. Accurately 5 mL of the sample extract was added into a test tube, using 5 mL of distilled water as the blank control. To each tube, 0.3 mL of 5 % NaNO₂ solution was added and mixed thoroughly, and then stood for 6 min. Next, 0.3 mL of 10 % Al(NO₃)₃ solution was added and mixed well. The mixture was stood for another 6 min. Subsequently, 4 mL of 4 % NaOH solution was added, and diluted to 10 mL with distilled water. The mixture was stood for 15–20 min. Finally, the absorbance at 510 nm was measured, and the total flavonoid content was evaluated. Rutin ($\geq 98\%$ purity) was used as the standard for total flavonoid determination.

The total content of polysaccharides was determined by the phenol–sulfuric acid method (Yue et al., 2022). 3.0 g of each sample was added to 30.0 mL of distilled water, subjected to ultrasonic extraction (200 W, 80 °C, 30 min), and centrifuged (5000 × g, 10 min) to collect the supernatant. Three volumes of anhydrous ethanol were then added, and the mixture was stored at 4 °C for 12 h. The resulting precipitate was washed twice with 75 % ethanol, resuspended in 25 mL of distilled water, and used as the test solution. For the assay, 100 μL of phenol and 500 μL of concentrated sulfuric acid were added to 200 μL of the prepared test solution. After thorough mixing and a 5-minute standing period, the mixture was incubated in a boiling water bath (100 °C) for 15 min. Absorbance was measured at 490 nm, and the total polysaccharide content was determined by fitting the measured value to a standard curve. D-glucose ($\geq 99.5\%$ purity) was used as the standard for total polysaccharide determination.

2.5. Qualitative and quantitative analysis of secondary metabolites

The metabolomics analysis of the samples was performed as described previously (Lin et al., 2025). Each fermented samples (50 mg) were thoroughly mixed with 1000 μL of extraction solution containing an internal standard (methanol:acetonitrile:water volume ratio = 2:2:1, and internal standard concentration was 20 mg/L), and vortexed for 30 s. After adding a steel ball, the samples were subjected to grinding at 45 Hz for 10 min, followed by ultrasonication for 10 min in an ice water bath. The samples were then centrifuged to obtain the supernatant. All supernatants were analyzed using UPLC (Acquity I-Class PLUS, Waters) coupled with an MS/MS system (Xevo G2-XS QTOF, Waters). Chromatographic separation of the compounds was achieved using an

Acquity UPLC HSS T3 column (1.8 μm 2.1 × 100 mm, Waters). The mobile phase, consisting of 0.1 % formic acid aqueous solution (A), and 0.1 % formic acid dissolved in acetonitrile solution (B), was used in both positive ionization mode (ESI⁺) and negative ionization mode (ESI⁻). The injection volume was 2 μL.

The raw data, collected using MassLynx V4.2, were processed by Progenesis QI software for feature detection, chromatographic alignment, and other data processing operations. Subsequently, Progenesis QI was used for compound identification utilizing databases such as METLIN, KEGG, HMDB, LipidMaps, and Biomarker.

2.6. Statistical analysis

After normalizing the original peak area information with the total peak area, principal component analysis and spearman correlation analysis were used to judge the repeatability of the samples within a group and the quantity control samples. Based on the grouping information, fold changes were calculated and compared, and Student's *t*-test was used to calculate the difference of each compound. The R language package rolls were used to perform OPLS-DA modeling and 200 times permutation tests were used to verify the reliability of the model. The variable importance in projection (VIP) of the model was calculated using multiple cross-validation. Differential metabolites were screened by integrating fold changes, *p*-values, and VIP values of the OPLS-DA model, with the criteria set as FC > 2, *p*-value < 0.05, and VIP > 1. The hypergeometric distribution test was used for the enrichment analysis of the KEGG pathway to identify statistically significant differential metabolites. Data are expressed as mean ± standard deviation ($m \pm S.D.$) ($n = 3$). Comparisons between groups were made using the *t*-test, with **P* < 0.05 indicating statistical significance.

3. Results and discussion

3.1. Determination of enzyme activity of fermentation strain

A. niger ZYDC-1, and *A. cristasukae* LNAC-1 demonstrated cellulase activity (Fig. S1c and d), suggesting their potential for cellulose degradation. Both *L. plantarum* SSTM-1 and *A. niger* ZYDC-1 exhibited xylanase activity (Fig. S2a and c), indicating these two bacteria might have the ability to degrade hemicellulose. *S. cerevisiae* GLLB-3 and *A. cristasukae* LNAC-1 showed pectinase activity (Fig. S3b and d), indicating their ability to degrade pectin. These findings suggest that microorganisms produce various glycoside hydrolases that could enhance the enzymatic degradation of cell walls, and facilitate the release of bioactive natural compounds from plant biomass (Yu et al., 2025).

3.2. Variations of total phenolic, total flavonoid, and total polysaccharides after fermentation

Comparative analysis revealed a 44.0–90.5 % significant increase in total phenolic content across all experimental groups compared to the YXKC control group (Fig. 1A). The YXAn group demonstrated the highest phenolic increase among the experimental groups, while the YXAn group showed the lowest phenolic increase effect. This increase aligns with other findings that microbial fermentation enhances plant-derived phenolic availability (Lin et al., 2025). Generally, plants contain two forms of natural phenolic compounds: soluble-free and insoluble-bound phenolics. Some phenolic compounds, such as caffeic acid, *p*-coumaric acid, and ferulic acid, exist in a bound state within plants (Roasa et al., 2021). During fermentation, microorganisms may produce esterases, proteases, pectinases, cellulases, α-amylases, xylanases, and β-glucosidases, and these enzymes can catalyze the breakdown of structural polysaccharides and proteins in the substrate cell wall, thereby releasing available phenolic compounds (Bei et al., 2018). The enzymatic release of bound phenolics from the cell wall matrix and

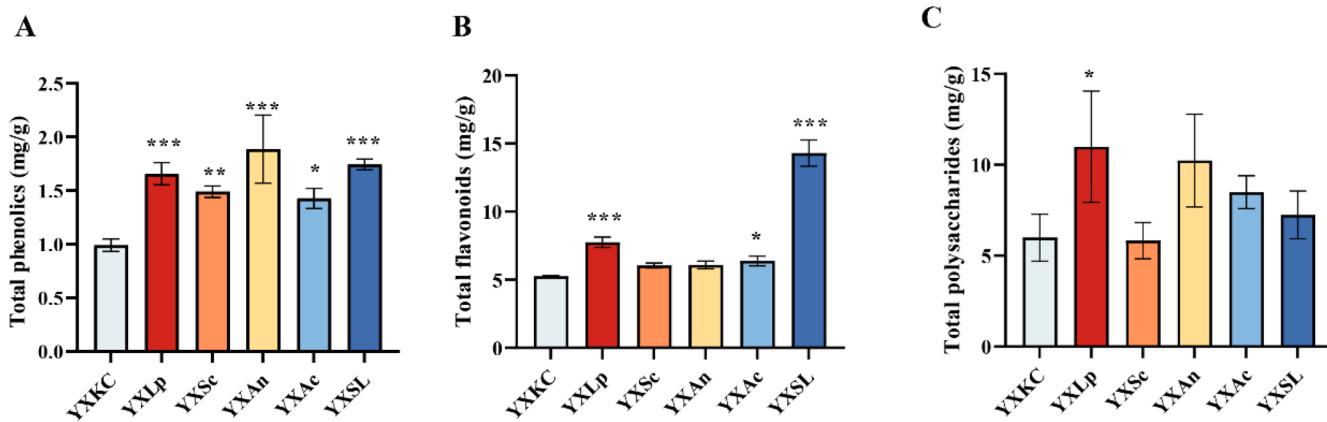


Fig. 1. Changes in natural product profiles of GBL after fermentation. (A) Variation in total phenolic content; (B) Variation in total flavonoid content; (C) Variation in total polysaccharide content. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the possible degradation of polymeric polyphenols into extractable subunits may have collectively contributed to the observed increase in total phenolic content (Acin-Albiac et al., 2021).

The groups of YXSL, YXLp, and YXAc facilitated significantly higher flavonoid content compared to the control (YXKC), with YXSL displaying the highest concentration (Fig. 1B). In particular, *L. plantarum* SSTM-1 monoculture (YXLp) and *S. cerevisiae* GLLB-3 monoculture (YXSc) exhibited a flavonoid content increase by 47.2 % and 15.1 %, respectively. The co-culture of *L. plantarum* SSTM-1 and *S. cerevisiae* GLLB-3 groups (YXSL) showed a 170.6 % increase in total flavonoid content (Fig. 1B). These results align with a previous study that microbial fermentation generally enhances flavonoid bioavailability. The enzymatic basis for this enhancement may involve glycosidase activity in microorganisms, which facilitates the release of flavonoid aglycones from the glycosidic complexes (Wang et al., 2018). These findings confirm that flavonoid accumulation is positively correlated with both fermentation strategies and microbial specificity.

Polysaccharides constitute crucial bioactive components in plant biomass. Comparative analysis revealed elevated total polysaccharide content in all experimental groups compared to the YXKC control group, except for the *S. cerevisiae* GLLB-3 monoculture (YXSc) (Fig. 1C). Specifically, the *L. plantarum* SSTM-1 monoculture (YXLp) demonstrated a 83.6 % increase in polysaccharide content. This enhancement might be attributed to the probiotic-mediated release of oligosaccharide prebiotics through cell wall lysis. *S. boulardii* fermentation of *Dioscorea*

opposita Thunb yields novel low-molecular-weight polysaccharides with enhanced digestion, antioxidant activity, and radioprotective properties (Shao et al., 2022). The co-culture group (YXSL) showed minimal polysaccharide accumulation compared to the YXLp group, potentially due to metabolic substrate competition wherein *S. cerevisiae* GLLB-3 utilized polysaccharides synthesized by *L. plantarum* SSTM-1 as a preferential carbon source.

3.3. Fermentation alters the metabolite profiles of GBL

Both unfermented and fermented GBL contain abundant metabolites, including phenols, flavonoids, and terpenes. Metabolite profiling identified 4549 compounds in fermented GBL samples, including carboxylic acids and derivatives ($n = 283$), prenol lipids ($n = 268$), flavonoids ($n = 107$), phenols ($n = 71$), and isoflavonoids ($n = 29$) (Fig. 2A). Principal component analysis (PCA) revealed distinct metabolic clustering patterns, where PC1 and PC2 explained 40.6 % and 13.8 % of the total variance, respectively. This ordination differentiated all experimental groups (YXLp, YXSc, YXAn, YXAc, and YXSL) from the control group (YXKC) (Fig. 2B) demonstrating microbial biotransformation effects. Cluster analysis further indicated metabolic similarity among YXLp, YXAn, and YXAc groups. In contrast, YXSc and co-culture YXSL groups formed independent clusters, suggesting strain-specific modulation of GBL composition through mono-fermentation and co-fermentation strategies.

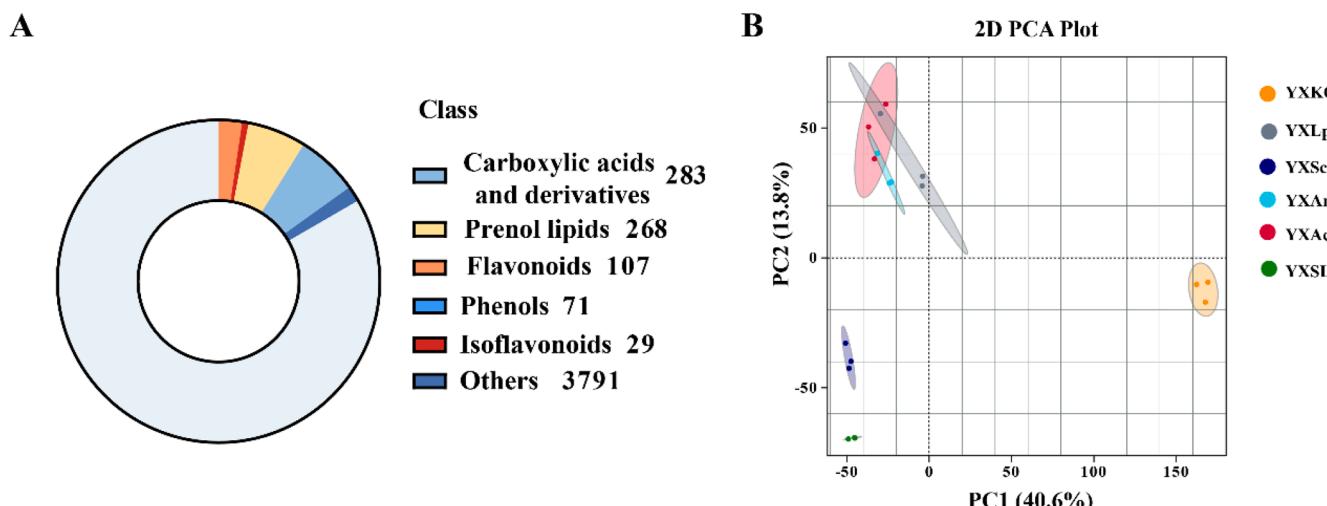


Fig. 2. Fermentation alters the metabolite profiles of GBL. (A) Overview of metabolites in the unfermented and fermented GBL; (B) PCA analysis of secondary metabolites in different groups.

Differential metabolites between groups were identified through OPLS-DA. The OPLS-DA score plot exhibited significant intergroup separation (Fig. S4A–E), confirming distinct metabolomic signatures between fermented and unfermented GBL. The metabolic profiles of fermented GBL were distinct from the unfermented sample. Comparative analysis identified a total of 595 shared metabolites across all five fermentation groups (Fig. 3A), with unique metabolic fingerprints characterizing each treatment. Especially, the co-culture group YXSL contained 124 exclusive metabolites, demonstrating superior biosynthetic diversity.

There were 832 different metabolites between YXKC and YXLp groups (Fig. 3B), including carboxylic acids and derivatives ($n = 48$), prenol lipids ($n = 44$), flavonoids ($n = 33$), phenols ($n = 12$), iso-flavonoids ($n = 8$), and others ($n = 687$). There were 1176 different metabolites between YXKC and YXSc groups (Fig. 3C), such as carboxylic acids and derivatives ($n = 69$), prenol lipids ($n = 54$), flavonoids ($n = 44$), phenols ($n = 20$), isoflavonoids ($n = 10$), and others ($n = 979$). There were 1001 differential metabolites between YXKC and YXAn (Fig. 3D), while 1018 differential metabolites existed between YXKC and YXA (Fig. 3E). A total of 1218 differential metabolites were identified between YXKC and YXSL (Fig. 3F), including carboxylic acids and derivatives ($n = 70$), prenol lipids ($n = 59$), flavonoids ($n = 42$), phenols ($n = 20$), isoflavonoids ($n = 12$), and various other compounds ($n = 1015$). A similar observation was reported in a previous study, which found that the composition of unfermented summer-autumn green tea differs from that of lab-fermented summer-autumn green tea (Zheng et al., 2024). The significant changes in metabolite further confirm that microbial fermentation can be used as a robust strategy for phytochemical modification.

3.4. Secondary metabolites changes after microbial fermentation

KEGG pathway analysis revealed the presence of differential metabolites in GBL. Following fermentation, the metabolites were predominantly enriched in pathways associated with plant secondary metabolism ($n = 54$), including phenylpropanoid biosynthesis ($n = 44$), flavone and flavonol biosynthesis ($n = 39$), and flavonoid biosynthesis ($n = 35$) (Fig. 4A). The metabolic of flavone and flavonol was the most significantly impacted pathway, followed closely by the flavonoid

metabolic pathway (Fig. 4B–F).

Differential metabolite pathway analysis using the KEGG database has been demonstrated to be a powerful approach for elucidating the changes in the abundance of phenolic compounds, flavonoids, and terpenes influence biological pathways (S. Li et al., 2023). According to KEGG pathway analysis, 116 differential secondary metabolites were screened (Table S1), including those involved in metabolism of secondary metabolites (37), phenylpropanoid metabolism ($n = 29$), flavone and flavonol metabolism ($n = 28$), and flavonoid metabolism ($n = 22$). YXKC, YXLp, YXSc, YXAn, YXA, and YXSL were divided into six distinct clusters. Certain secondary metabolites, including phenylpropanoic acids, phenols, cinnamic acids, sugars and glycosides, flavonoids, and terpenoids in GBL, were significantly upregulated or downregulated after fermentation (Fig. 5). Compared with unfermented GBL, the levels of phenylpropionic acid and phenols (coniferyl acetate, chavicol, and ferulic acid) were upregulated after fermentation. The levels of flavonoids or terpenoids, including beta-D-Gentibiosyl crocetin, beta-D-Glucosyl crocetin, ginsenoside Rb1, kaempferol 3-O-glucoside, apigenin 7-O-neohesperidoside, and quercetin 3-(2G-xylosylrutinoside), were downregulated. The glycosyl groups of GBL bioactive compounds were hydrolyzed and removed, thereby increasing their lipophilicity and improving human absorption rate (X. Zhang, Miao, Pan, et al., 2023). β -Glucosidase can hydrolyze phenolic glycosides, liberating their aglycones and enabling direct absorption across the epithelium (Day et al., 2000). Luteolin and quercetin aglycones demonstrated significantly greater efficacy than their 3-, 4-, and 7-O-glucoside derivatives in inhibiting hydroperoxide accumulation in membrane bilayers. Notably, glycosylation at the 4-position exhibited a stronger suppressive effect compared to substitution at the 3- or 7-positions (Heim et al., 2002). Quercetin glycosides could be hydrolyzed to the quercetin aglycone during absorption by lactase phlorizin hydrolase or cytosolic β -glucosidase, after which the aglycone enters the intestinal epithelial cells via passive diffusion (Gee et al., 2000). Metabolomic profiling reveals that probiotic fermentation alters the abundance of flavonoid and phenolic profiles, accompanied by the release of bioactive metabolites. This metabolic reprogramming highlights the transformative role of microbial fermentation in enhancing the bioavailability and functional properties of plant-derived phytochemicals.

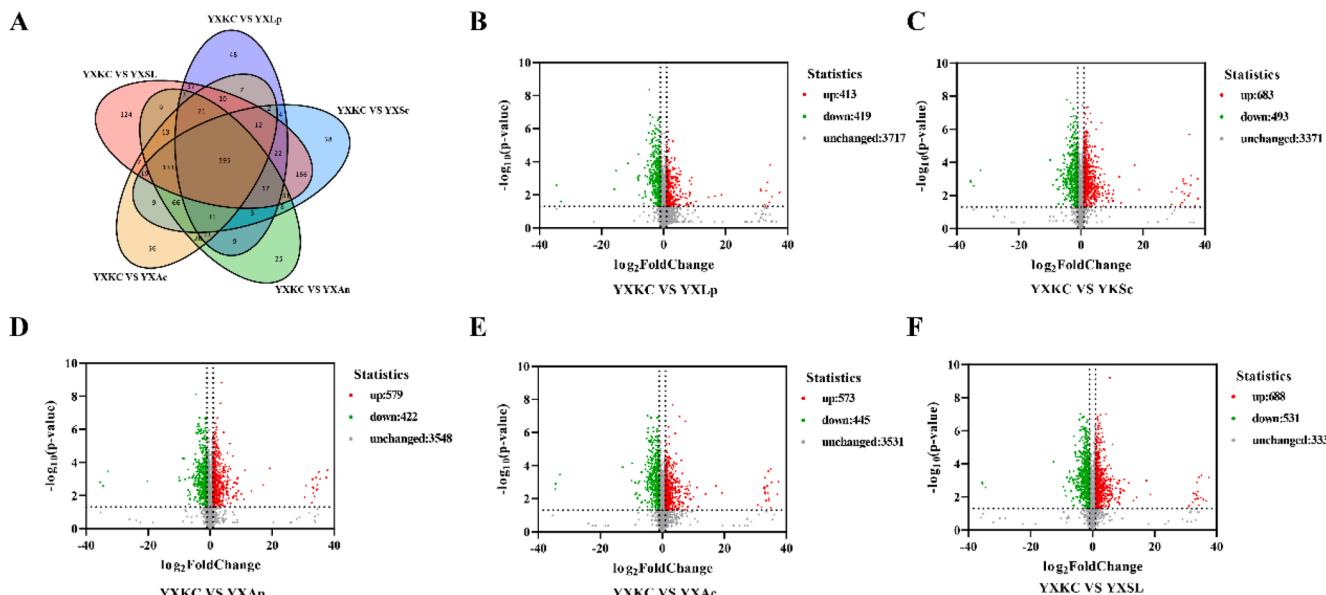


Fig. 3. Differential metabolites between groups. (A) Venn diagram of metabolite profiles of unfermented and fermented GBL, and the volcano plots illustrate differential secondary metabolites in pairwise comparisons: (B) YXKC vs. YXLp, (C) YXKC vs. YXSc, (D) YXKC vs. YXAn, (E) YXKC vs. YXA, and (F) YXKC vs. YXSL. The x-axis represents the \log_2 fold change, y-axis shows the $-\log_{10}(p\text{-value})$, representing the statistical significance of the changes. Red dots indicate significantly upregulated metabolites; Green dots represent significantly downregulated metabolites; Gray dots denote unchanged metabolites.

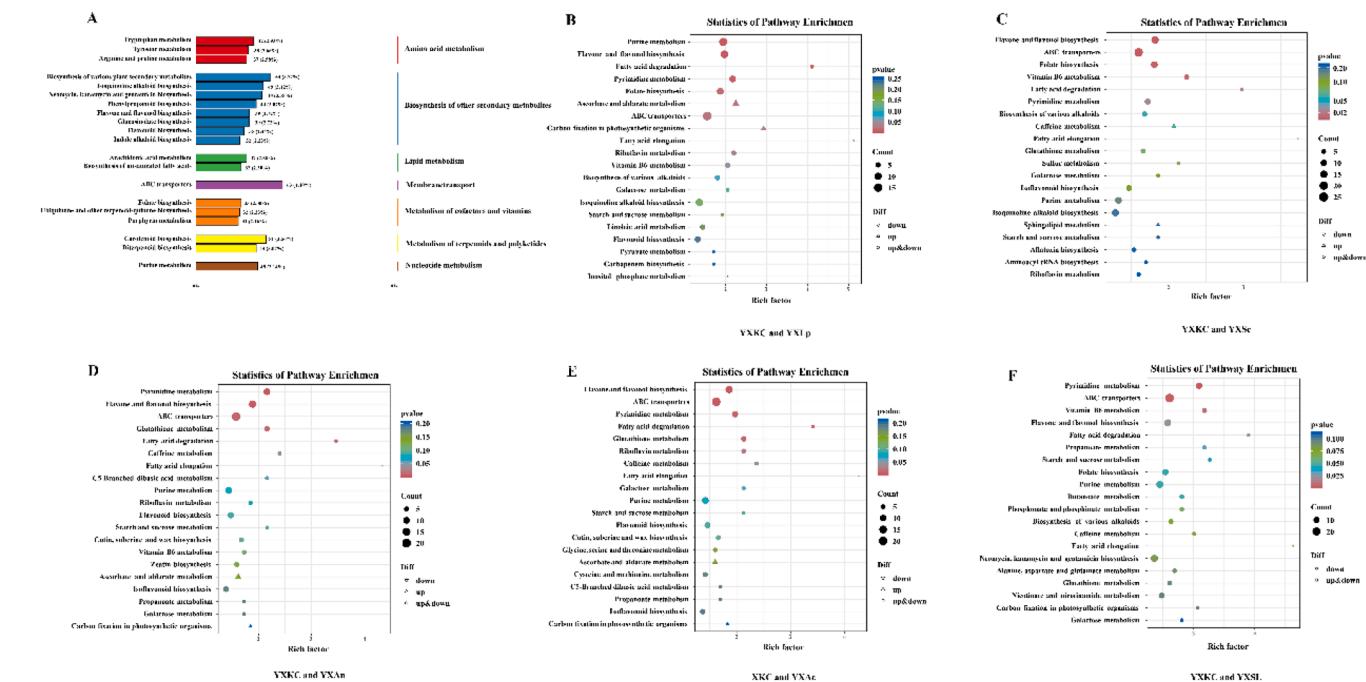


Fig. 4. The changes of detailed metabolites after microbial fermentation. (A) KEGG pathway classification maps after fermentation of GBL; Enriched bubble maps illustrating the impact factors of metabolic pathways in different comparisons: (B) YXKC vs. YXLp, (C) YXKC vs. YXSc, (D) YXKC vs. YXAn, (E) YXKC vs. YXAc, and (F) YXKC vs. YXSL. The x-axis represents the rich factor, and y-axis lists the metabolic pathways. The size of the bubbles corresponds to the number of metabolites involved, while the color gradient represents the *p*-value, with red indicating higher significance.

3.5. Changes in flavonoid profile after microbial fermentation

GBL flavonoids have multiple health-promoting functions in humans, such as anti-inflammatory, anti-cancer, and antioxidant activities (Boateng, 2022). The total flavonoids in the GBL were significantly increased by YXSL group (Fig. 1B), with the KEGG metabolic pathway to screen the secondary metabolites of flavonoids. A total of 68 metabolites were screened, including 13 flavonoid glycosides, 5 flavones, 2 flavans, 2 O-methylated flavonoids, 1 hydrolyzable tannin, 1 hydroxycinnamic acid, 1 hydroxycinnamic acid derivatives, 1 hydroxyflavonoids, and 1 pyranocoumarins (Fig. 6A).

After fermentation, quercetin 3-(2G-xylosylrutinoside), isoquercitrin, apigenin 7-O-neohesperidoside, apigenin 7-O-beta-D-glucoside, quercitrin, luteolin 7-O-beta-D-glucoside, and kaempferol 3-O-glucoside decreased significantly. The glycosyl hydrolases derived from the microorganisms remove the glycosyl groups of flavonoids. Therefore, the removal of glucosides from the natural products through fermentation represents a key mechanism for enhancing the antioxidant activity of plant-based foods (Hur et al., 2014). The health benefits of fermented soy foods are attributed to the antioxidant activity of specific compounds that undergo structural modification or release after bacterial hydrolysis. Glycosylated isoflavones are converted to aglycones by fermentation. Compared to unfermented soybeans, fermented soy foods contain more glycoside ligands as the main isoflavone structure (Hubert et al., 2008). Fermentation of red sorghum with *Lactobacillus* spp. led to the release of eriodictyol and naringenin from eriodictyol-7-O-glucoside and naringenin-7-O-glucoside, respectively (Svensson et al., 2010). Yeasts, such as *Saccharomycetaceae* var. *Pichia kluveri*, partially hydrolyzes rutinose moiety in flavanones. This suggests that microorganisms have diverse effects on flavonoids after GBL fermentation (Escudero-López et al., 2013).

Certain microorganisms can hydrolyze the covalent bonds between cell walls and insoluble bound phenolics, subsequently dissociate the phenolic constituents, break the glycosidic bonds of the flavonoids, and release flavonoid aglycones (Dulf et al., 2016). Flavonoids, such as

kaempferol, quercetin, naringenin, luteolin, and rutin and their derivatives are the main secondary differential metabolites. The content of luteolin 7-O-beta-D-glucoside in flavonoids decreased significantly (Fig. S5), while the content of luteolin after fermentation was higher than that in unfermented GBL. Luteolin protects the nervous system, reduces the occurrence of neuropathy, and improves memory and cognitive function through its antioxidant and anti-inflammatory effects (Deng et al., 2025). The relative luteolin content in the YXLp, YXSc, YXAn, YXAc, and YXSL groups increased by 5.97 times, 7.21 times, 7.85 times, 7.64 times, and 5.04 times, respectively (Fig. S5).

Naringenin significantly increased after fermentation (**p* < 0.001), possibly because naringenin-7-glucoside was hydrolyzed to naringenin. Meanwhile, the naringenin content in the YXLp, YXSc, YXAn, YXAc, and YXSL groups increased by 46.05 times, 33.14 times, 42.06 times, 54.86 times, and 41.61 times, respectively (Fig. S5). Prunin, also known as naringenin 7-O-beta-D-glucoside, is a glycosyl derivative of naringenin. Fermentation of red sorghum with *Lactobacillus* sp. generates naringenin from naringenin 7-O-glucoside (Svensson et al., 2010). Kaempferol 3-O-beta-D-glucosyl-(1→2)-beta-D-glucoside (Sophoraflavonoloside) was generated from the reaction of kaempferol 3-O-glucoside in the YXLp and YXSc groups. Sophoraflavonoloside increased by 3.15 times and 4.32 times in the YXLp and the YXSc groups, respectively. In the YXSL group, using co-culture of *L. plantarum* SSTM-1 and *S. cerevisiae* GLLB-3 strains, it increased by 5.87 times (Fig. S5).

3.6. Changes in terpene profile after fermentation

Terpenoids play an important role in lowering blood sugar and protecting the liver and have anti-inflammatory, antioxidant, anti-fatigue, antiviral, and anticancer activities (Y. Li et al., 2023). Ginsenosides are believed to be the primary beneficial components of ginseng, but their oral bioavailability is low. The reason lies in multiple sugar groups enveloping the ginsenosides to form large-molecular-weight polysaccharide-bound saponins, which impede absorption and metabolism. Microbial-based methods or enzymatic hydrolysis can be

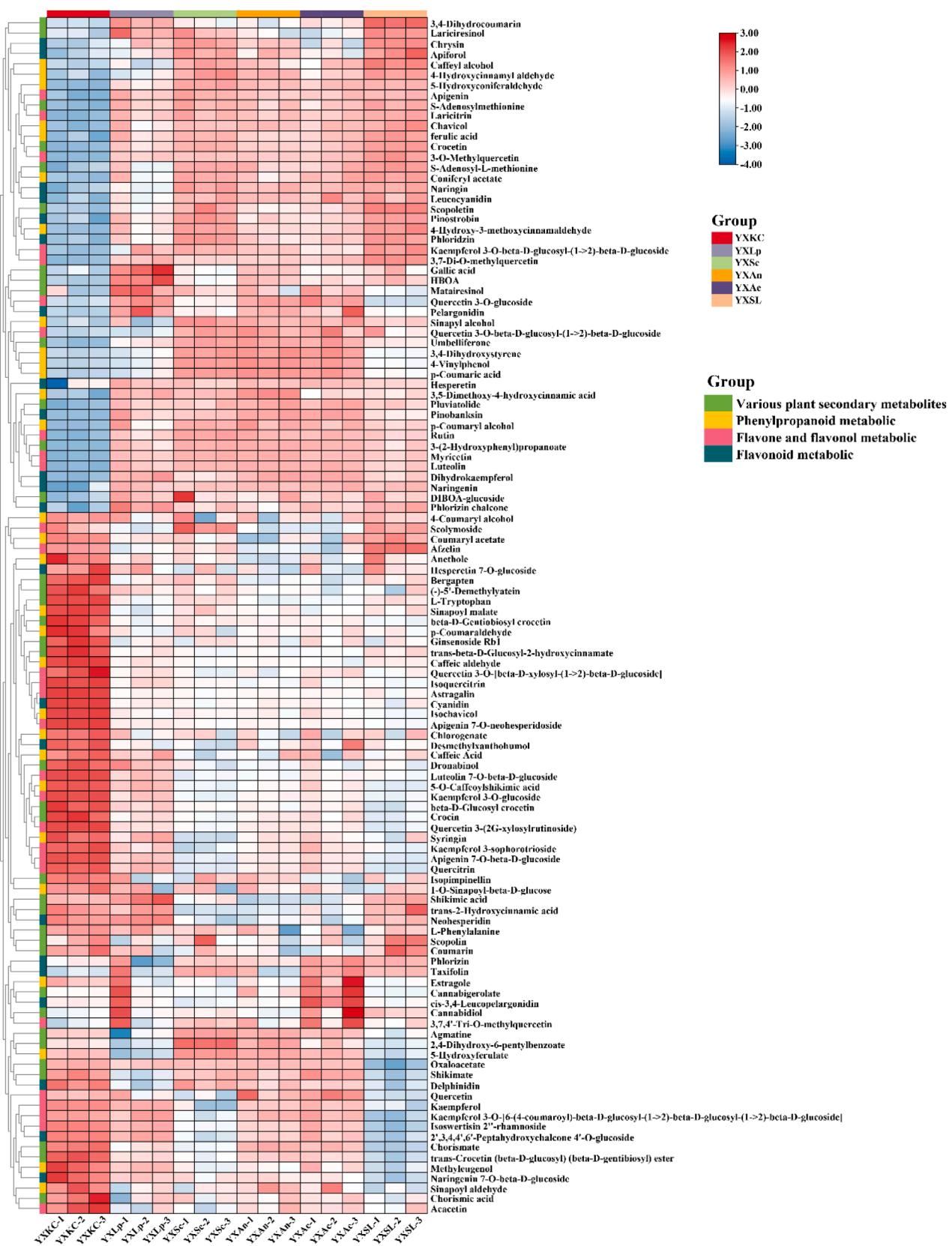


Fig. 5. Heatmap visualization of differentially expressed secondary metabolites in the groups of YXKC, YXLp, YXSc, YXAn, YXAc, and YXSL. The color gradient represents the standardized abundance of metabolites, with red indicating higher expression levels and blue indicating lower expression levels. Functional classifications of metabolites are indicated on the left, representing pathways involved in plant secondary metabolites, including flavonoid metabolites, phenylpropanoid metabolites, and flavone and flavonol metabolites.

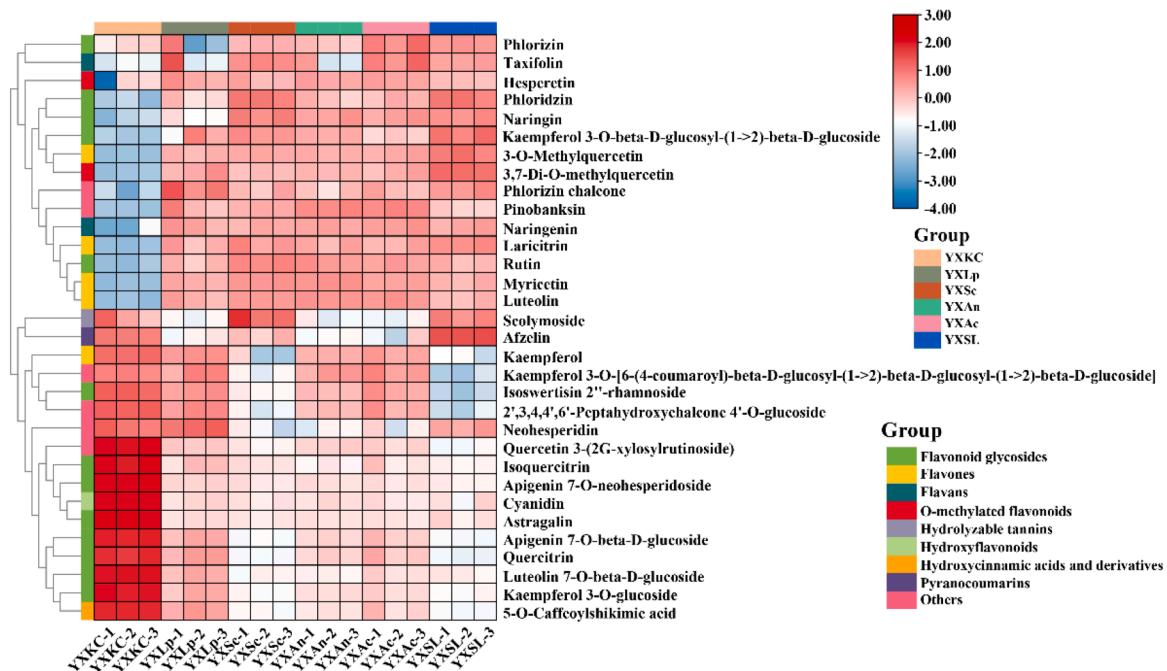
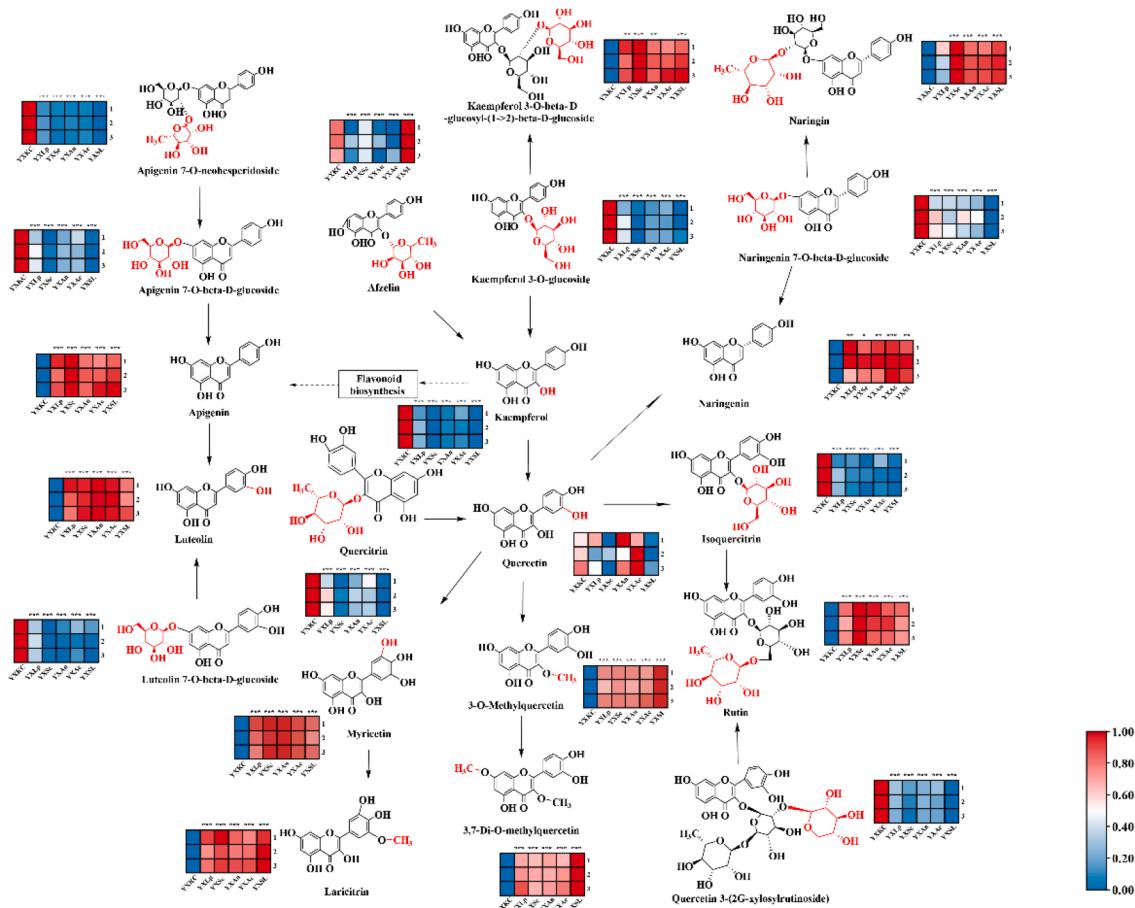
A**B**

Fig. 6. The flavonoid changes after microbial fermentation. (A) Heatmap of different flavonoids after GBL fermentation; (B) Proposed metabolic pathways of flavonoids during GBL fermentation. The color gradient represents the standardized abundance of metabolites, with red indicating higher expression levels and blue indicating lower expression levels. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

employed to selectively hydrolyze the sugar moieties on the saponins, thereby significantly enhancing their bioavailability. *A. niger* XD101 could transform Rb1 into the more easily absorbed ginsenoside CK via its extracellular β -glucosidase (Jiang et al., 2021). After fermentation, ginsenoside Rb1, ginsenoside Rc, and ginsenoside Rh3 were significantly reduced ($*p < 0.001$). Among them, the relative content of ginsenoside Rh3 was the highest (Fig. 7A). *L. plantarum* KCCM 11613P can secrete β -glucosidase, which converts ginsenosides Rb2 and Rb3 in red ginseng into the more biologically active ginsenoside Rd. Ginsenoside Rd is known to possess significant lipid peroxidation inhibitory activity and can modulate the expression of antioxidant enzymes, thereby exhibiting immunomodulatory and neuroprotective functions (Y. Zhang et al., 2024). Various microorganisms have been confirmed to influence the bioactivity of fermentation products through diverse biotransformation pathways (such as glycosylation, deglycosylation, methylation, glucuronidation, and sulfate incorporation), ultimately generating numerous novel metabolites with high biological activity (L. Li et al., 2020; Y. Zhang et al., 2024).

Prolonged administration of high doses of *Radix Bupleuri* has been associated with toxicity in the liver, kidneys, and hematopoietic system. Saikosaponins and essential oils are considered as the primary compounds responsible for these adverse effects (F. Yang et al., 2017). Rat or human gut microbiota with hydrolyzing activity, such as *Eubacterium* sp. A-44, can induce the deglycosylation of saikosaponin, from saikosaponin a, b1, b2, c, d, and g into prosaikogenin F, A, D, E, G, and H, and the corresponding saikogenin F, A, D, E, G, and H, respectively (X. Li et al., 2018). After fermentation, saikosaponin B3, saikosaponin E, and saikosaponin F were significantly reduced ($*p < 0.001$). Saikosaponin E levels were reduced by 99 % in the YXLp, YXSc, YXAn, YXAc, and YXSL groups (Fig. 7A), indicating that the microbial fermentation in this study has dehydrolyzation effects.

Crocin, a diester of two disaccharide gentiobiose compounds, is

found in *Crocus sativus* and *Gardenia jasminoides*. Crocetin, the aglycone of crocin, is a brick-red crystal containing an apocarotenoid dicarboxylic acid, which forms the central core of crocin (Asai et al., 2005; Umigai et al., 2011). Crocetin has been reported as a potential bioactive compound, and the hydrolysis of crocin to crocetin by intestinal microbiota is a key step for its pharmaceutical effects (Shakya et al., 2020). Crocin might be hydrolyzed into low-glycosylated saponin molecules through the pathway of crocin \rightarrow beta-D-Gentiobiosyl crocetin \rightarrow beta-D-Glucosyl crocetin \rightarrow crocetin during the fermentation (Fig. 7B).

After fermentation by the YXSL group, the relative contents of crocin, beta-D-Gentiobiosyl crocetin, beta-D-Gentibiosyl crocetin, and beta-D-Glucosyl crocetin decreased by 56.27 %, 65.64 %, 45.78 %, and 36.8 %, respectively ($*p < 0.001$). The glycosyl groups in the side chains of these four compounds were removed, suggesting that *L. plantarum* SCTM-1 and *S. cerevisiae* GLB-3 might secrete certain glycosidase during fermentation. Additionally, crocetin content was increased by 104.5 % in the YXSL group, exceeding the levels observed in the other groups. This suggests that the presence of two distinct microbial strains in the YXSL group may enhance the bioconversion process. Crocins are poorly absorbed following oral administration, whereas crocetin exhibits improved bioavailability (Xi et al., 2007). Crocetin functions as an antioxidant with anti-inflammatory and anti-apoptotic properties, contributing to its cardioprotective effects (José Bagur et al., 2017). Thus, the microbial transformation from crocin to crocetin would benefit GBL pharmaceutical activity.

4. Conclusions

This study confirms the beneficial effects of fermenting dried GBL with four microbial strains, demonstrating that fermentation significantly modulates the profile of secondary metabolites. Through

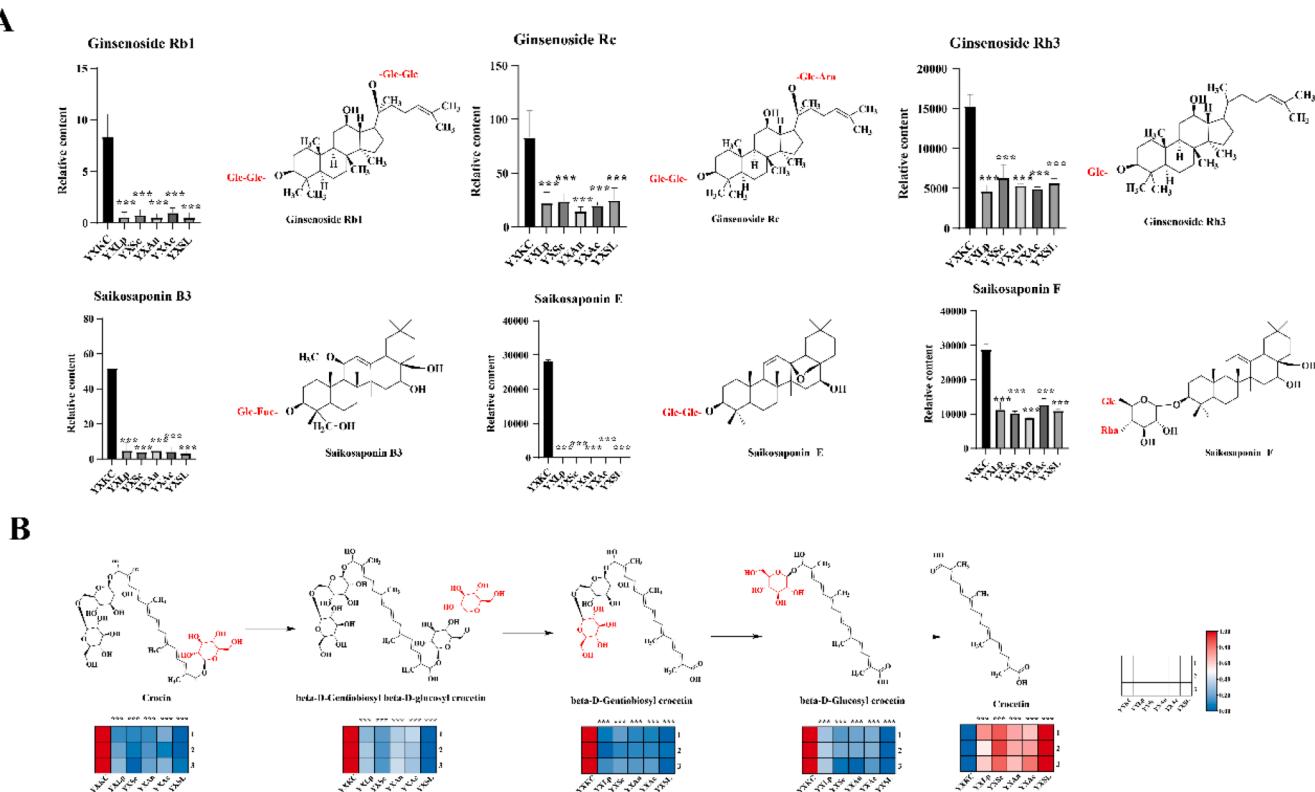


Fig. 7. Changes in terpenoids and their relative content after fermentation. (A) Changes of different types of ginsenoside and saikosaponin after fermentation; (B) Proposed biotransformation pathway of crocin during GBL fermentation. The color gradient represents the standardized abundance of metabolites, with red indicating higher expression levels and blue indicating lower expression levels. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

screening and verification, four functional strains harboring detected lignocellulase activities were selected and implemented in the solid-state fermentation protocol. Following the fermentation of GBL, levels of key active components—including total polyphenols and total flavonoids—increased significantly, accompanied by a rise in total polysaccharide content. These changes might collectively enhance the pharmaceutical properties of GBL. KEGG enrichment pathway analysis of secondary metabolites revealed proposed biotransformation pathways of phenylpropanoids, flavonoids, flavone, and flavonol, which may assist in identifying effective microorganisms for GBL fermentation. The flavone/flavonol biosynthetic pathway emerged as the most significantly impacted pathway. In the future, the elucidation of strain-specific biotransformation mechanisms and the development of optimized fermentation strategies will improve the quality and bioactivity of GBL.

Ethical statement - studies in humans and animals

This study did not involve experiments with human subjects or animals.

CRediT authorship contribution statement

Zhanqiang Yan: Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Wenhui Niu:** Software, Methodology, Investigation, Data curation. **Yingkui Liu:** Writing – review & editing. **Lingbo Qu:** Supervision, Resources, Project administration, Methodology. **Santosh Pandit:** Writing – review & editing. **Ivan Mijakovic:** Writing – review & editing. **Yongjun Wei:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

There are no conflicts of interest to declare in this study.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fufo.2025.100764](https://doi.org/10.1016/j.fufo.2025.100764).

Data availability

Data will be made available on request.

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