Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Widely targeted metabolomics analysis of the effects of probiotic fermentation on the phytochemicals of kiwifruit juice and prediction of key metabolic pathways

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ARTICLE INFO

Article history:
Received 10 October 2023
Received in revised form 2 February 2024
Accepted 11 April 2024

Keywords:
Kiwifruit juice
Lactic acid bacteria
Mono- and mixed-fermentation
Metabolomics
Bioactive metabolites
Metabolic pathway

ABSTRACT

Metabolomics can be used to identify the changes and metabolic pathways of nutrients and flavor substances in foods at specific processing or fermentation stages. This study revealed the accumulation and degradation of phytochemicals during the fermentation of kiwifruit juice (KJ) by lactic acid bacteria (LAB) through widely targeted metabolomics and explored the impact of different fermentation strategies on the nutritional quality and functional characteristics of KJ. LAB utilized the original sugars, acids, amino acids, and nucleotides of the matrix to produce numerous bioactive compounds, including phenols, organic acids, free fatty acids, and vitamins, while achieving bacterial proliferation and reduced anti-nutritional factors such as alkaloids, thus improving the nutritional and functional properties of KJ. Moreover, compared with monoculture fermentation, mixed fermentation promoted the production of more biologically active phenols and alkaloids, among which vanillic acid and luteolin-8-*C*-arabinoside were the characteristic metabolites of mixed and mono-fermentation. Subsequently, the metabolic pathways of key metabolites were predicted, including the glycolytic Embden-Meyerhof-Parnas pathway, pentose phosphoketolase pathway, organic acid metabolic and arginine deiminase pathways for improving the acid resistance of LAB, and the phenolic-acid-based phenylalanine and tyrosine metabolic pathway.

1. Introduction

Actinidia chinensis Planch., commonly known as kiwifruit, is among the most commercially valuable fruits currently, with enormous popularity among consumers due to its rich nutrition and unique flavor[1]. With its high nutrient density, this fruit is abundant in bioactive phytochemicals, offering numerous potential health benefits to humans. Previous studies have shown that consuming kiwifruit provides antioxidant, antiobesity, and anti-inflammatory benefits and promotes intestinal function[2-3]. However, as a climacteric fruit, kiwifruit is difficult to store for a long time after reaching edible maturity and is highly susceptible to decay. Thus, the vigorous development of kiwifruit deep-processing products, especially those that are nutritious, healthy, and of high quality, is a viable solution to prevent the decay and waste of fresh fruit and also increase its value[4]. Current deep-processing products of kiwifruit mainly include fruit slices, juice, wine, jam, and vinegar. However, the types of kiwifruit processing products are relatively limited as a whole. Additionally, most current processing methods lead to a loss of nutrients and functional properties[2]. Thus, investigations into innovative processing technologies to enhance the

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Peer review under responsibility of Beijing Academy of Food Sciences.

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http://doi.org/10.26599/FSHW.2024.9250257

range of kiwifruit products and improve their nutritional qualities and health functions are essential.

Recently, the interest in developing new products and producing functional ingredients through fermentation has been growing[5]. Various studies have demonstrated that fermentation prolongs the shelf life of food and positively impacts its nutritional, sensory, and functional qualities[6]. Meanwhile, in some juice matrices, the final product obtained through mixed fermentation exhibits superior viable bacterial count, sensory and nutritional quality, and functional activity compared to mono-culture fermentation[7-9]. Fermentation by lactic acid bacteria (LAB), a "generally recognized as safe" bacterial strain, in various juice matrices has been widely studied[10-11]. Recent studies have shown kiwifruit to be a favorable substrate for LAB fermentation. This fermentation enhances the flavor, nutritional quality, and functional features of the kiwifruit matrix[12-13]. However, current studies on the impact of fermentation in the kiwifruit matrix mainly focus on the compositions and biotransformations of some important phenolic compounds[13-15]. Comprehensive investigations into the nutritional changes during kiwifruit matrix fermentation are insufficient, there is still a lack of research on the differential effects of fermentation by mono and mixed strains on the nutritional profile of the kiwifruit matrix.

Metabolomics can be used to qualitatively and quantitatively analyze all primary and secondary metabolites in foods so as to clearly identify their evolutions in foods at a specific processing or fermentation stage and explore the possible metabolic pathways involved[16]. In this study, widely targeted metabolomics based on ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) was used to investigate the dynamic changes of the different types of metabolites in kiwifruit juice (KJ) under mono- and mixed-culture fermentation strategies, identify the differentially accumulated metabolites (DAMs) and subsequently predict the metabolic pathways of the key metabolites. The mono- and mixed-culture fermentation strategies were pure *Lactobacillus brevis* fermentation and mixed-culture fermentation of *L. plantarum* and *L. brevis*, respectively. Both of them exhibited excellent sensory qualities and functional characteristics in our previous study[12]. The results provide valuable insights for developing and producing high-quality fermented KJ and may help further understand the metabolic rules and pathways of typical phytochemical components in fermented juice matrices.

2. Materials and methods

2.1 Plant material and juice preparation

Kiwifruit of the variety 'Ruiyu' (*A. deliciosa* cv. Ruiyu) was obtained from a commercial orchard in the Zhouzhi County of the Shaanxi Province and stored at (22 ± 2) ℃ until reaching the edible maturity stage required for juice preparation. At the edible maturity stage, the fruits had a firmness of (5 ± 1) N/cm^2 and soluble solids content (SSC) of (19.7 ± 0.1) °Brix. The kiwifruit was peeled, pressed into a pulp, and homogenized using a high-pressure homogenizer (ATS Engineering Ltd., Suzhou, China). Following centrifugation at 6 000 × *g* for 25 min, the pulp was separated and the supernatant was collected. The supernatant was divided into 250 mL Erlenmeyer flasks, with 100 mL in each flask. The flasks were sterilized in a boiling water bath for 1 min and cooled to below 45 ℃ prior to fermentation. The SSC of the KJ was (17.2 ± 0.1) °Brix and pH was 4.0 ± 0.1. The sterilized KJ met the microbiological safety criteria of the Chinese National Standard (CNS) GB 7101–2015.

2.2 Bacterial strains and juice fermentation

Lyophilized *L. plantarum* CICC 20265 (LP) and *L. brevis* CICC 20269 (LB) were purchased from the China Center of Industrial Culture Collection (CICC, Beijing, China). Both bacterial strains were grown in Man Rogosa Sharpe (MRS) broth and incubated at 37 ℃ for 12 h. The cell cultures were collected after cryogenic centrifugation at 2 000 × *g* for 7 min and resuspended in sterile saline to obtain active cell group cultures, which were then used to initiate the fermentation of KJ.

The pure LB and mixed culture (LB: LP = 2:1) suspensions were separately inoculated in sterilized KJ with initial cell concentrations of approximately 7.0 (lg (CFU/mL)). Cell concentrations were estimated by measuring the optical density at 600 nm ($OD_{600\,nm}$). The inoculated KJ was fermented at 37 ℃ for 36 h to obtain KJ fermented by LB (LBKJ) and a mixed culture of LP and LB (PBKJ). KJ cultured under the same conditions without adding LAB was used as control check (CK) (Fig. 1A).

2.3 Colony count analysis

Using the measurements performed by Lan et al.[12] as a reference, an $OD_{600\,nm}$ linear regression model was used to monitor variations in the colony counts in KJ during fermentation. Samples were collected every 6 h to estimate the colony counts.

2.4 pH, SSC, total sugar content (TSC), and titratable acidity (TA) measurements

The pH values and SSC of the KJ samples were measured using a pH meter (PHS-3E; Shanghai Leici Co. Ltd., China) and an Abbe refractometer (PAL-1; Atago Co., Tokyo, Japan), respectively. The TA was determined by sodium hydroxide titration. The TSC was determined *via* anthrone colorimetry, with the results expressed as mg glucose equivalents (GE)/mL.

2.5 Ascorbic acid content (AAC), total polyphenols content (TPC), total flavonoid content (TFC) and antioxidant activity analysis

The AAC of the KJ samples were determined by 2,6-dichloroindophenol titration, as per the CNS GB 5009.86–2016. The TPC and TFC were determined by Folin-Ciocalteu colorimetry and aluminum chloride colorimetry, respectively, and expressed as mg gallic acid equivalents (GAE)/L and mg catechol equivalents (CE)/L, respectively[12,17]. Antioxidant activities were characterized in terms of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP), with the results expressed as μmol trolox equivalents (TE)/mL[2].

2.6 Metabolite analysis

Widely targeted metabolomics was used to analyze the metabolites in the KJ samples and performed in collaboration with the Wuhan MetWare Biotechnology Co., Ltd. (http://www.metware.cn).

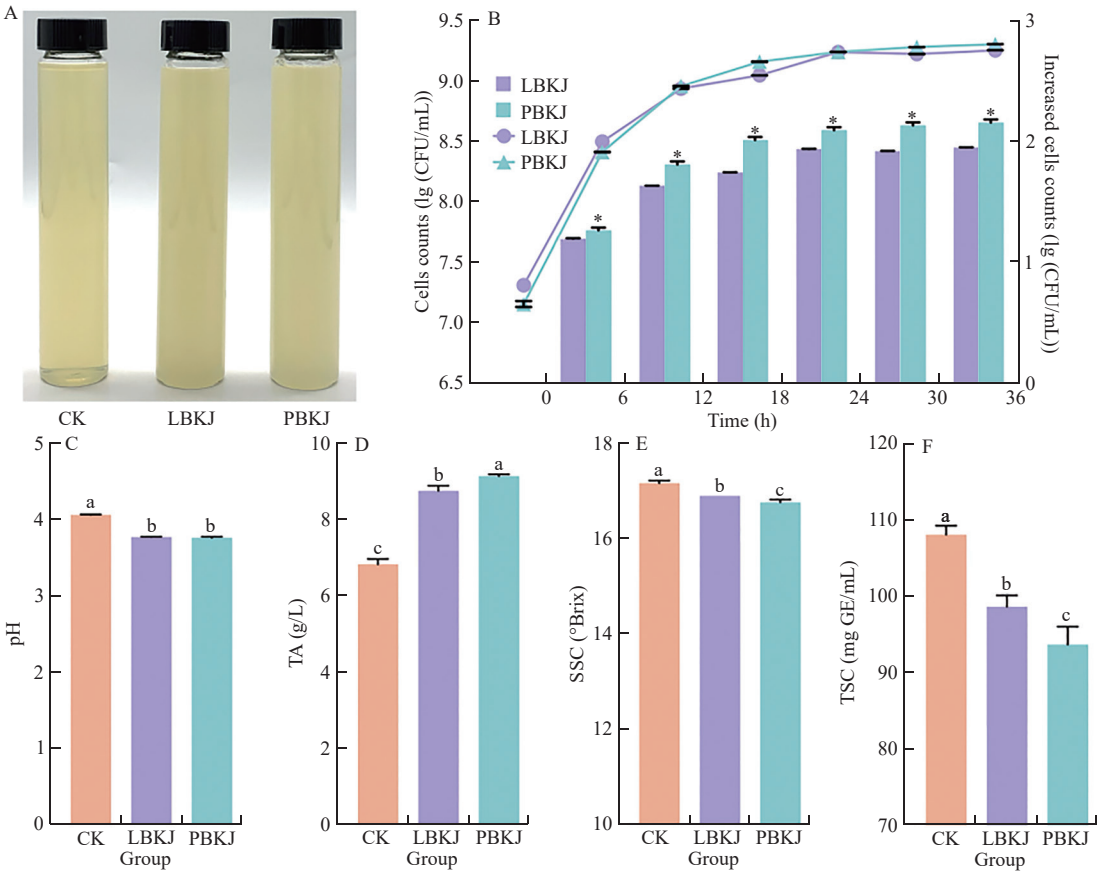

Fig. 1 (A) The appearance of CK, LBKJ and PBKJ. (B) The change of cell counts and increased cell counts of LBKJ and PBKJ during the fermentation. The line chart represents the cell counts; the bar chart represents increased cell counts; * indicates a significant difference ($P < 0.05$) between LBKJ and PBKJ at the same time point. (C) pH, (D) TA, (E)SSC and (F) TSC of KJ samples. Different lowercase letters indicate significant differences ($P < 0.05$) among CK, LBKJ and PBKJ.

2.6.1 *Sample extraction and profiling*

The lyophilized samples were ground into fine powders using MM 400 hybrid ball mill (Retsch, Germany) with a frequency of 30 Hz for 1.5 min. The powdered lyophilized samples (100 mg) were dissolved in 1.2 mL of 70% methanol. The mixtures were vortexed for 30 s every 30 min, which was repeated 6 times, and left overnight at 4 °C. Following centrifugation at 16 100 × *g* for 10 min, the supernatants were filtered through 0.22-μm pore filter membranes and analyzed by UPLC-ESI-MS/MS (UPLC, Nexera X2, Shimadzu, Co., Kyoto, Japan; MS, Q-trap 4500, Applied Biosystems, Toronto, Canada) with reference to the analytical conditions described by Su et al.[18].

2.6.2 *Tentative identification and quantification of metabolites*

The metabolites were identified based on the retention time, fragmentation pattern, and mass-to-charge ratio (*m/z*) using the Metware metabolomics database. The metabolites were quantified by scheduled multiple reaction monitoring, as described by Chen et al.[19]. The relative metabolite contents were determined by integrating the characteristic peak areas of the mass spectra.

2.6.3 *Screening of DAMs*

Orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed using MetaboAnalystR and used to extract the variable importance in projection (VIP) scores. The DAMs were screened based on the following criteria: fold change (FC) ≥ 2 or FC ≤ 0.5, VIP ≥ 1, and *P*-value < 0.05[18].

2.7 *Statistical analysis*

The data was sorted and analyzed using Microsoft Excel (version 16.4). SPSS (version 26.0) was used to perform analysis of variance and multiple comparisons. Correlation analysis, principal component analysis (PCA), and visualizations were performed on GraphPad Prism (version 9.3.1). Metabolomics data was analyzed and visualized using Adobe Illustrator (version 2020) and Metware Cloud (https://cloud.metware.cn/). All experiments were performed in triplicate.

3. Results and discussion

3.1 *Bacterial growth in KJ*

Probiotic viability levels of 6–7 (lg (CFU/mL or g)) of the carrier food product can positively affect human health when passing through the digestive tract[20]. As shown in Fig. 1B, LB exhibited satisfactory growth in KJ, where the cell counts increased exponentially in the first 18 h of fermentation and gradually stabilized, increasing to 9.20 (lg (CFU/mL)) after 36 h, which was a 1.94 (lg (CFU/mL)) increase compared with the initial level during fermentation. For PBKJ, the cell count increased by 2.14 (lg (CFU/mL)) after 36 h, which

was significantly higher than that of LBKJ ($P < 0.05$). This indicated the better fermentation characteristics of PBKJ. In a comparative study of mono and mixed grape juice fermentations, the fermentation characteristics of the mixed fermentation were also superior to those of the monoculture[9].

3.2 SSC, pH, TSC, and TA values of KJ

Figs. 1C-F show the changes in pH, TA, SSC, and TSC before and after KJ fermentation. LAB fermentation significantly decreased the pH, SSC, and TSC of KJ and significantly increased the TA ($P < 0.05$), indicating the utilization of the juice matrix carbohydrates by LAB to produce various acids such as organic and phenolic acids (PPs) [10]. Additionally, the TA of PBKJ was significantly higher and the TSC and SSC were significantly lower ($P < 0.05$) than those of LBKJ, indicating a higher LAB activity and more metabolite accumulation during mixed fermentation. This was consistent with the superior fermentation characteristics (Fig. 1B).

3.3 Overall determination of metabolites

UPLC-ESI-MS/MS data was used for widely targeted metabolomics analysis of CK, LBKJ, and PBKJ. The total ion chromatogram and intragroup correlation analysis (Fig. S1) demonstrated a high data reproducibility (Pearson correlation coefficient > 0.98) and reliability for each group. A total of 955 metabolites including 10 categories were tentatively identified in all KJ samples (Table S1), which included 380 polyphenols (Ps), 133 lipids (Ls), 88 amino acids and amino acid derivatives (AAs), 78 organic acids (Os), 75 saccharides and alcohols (SAs), 75 alkaloids (As), 50 nucleotides and nucleotide derivatives (Ns), 33 terpenoids (Ts), 18 vitamins (Vs), and 25 classified as "others" (OTs).

PCA was performed on the metabolomics data (Fig. 2A). The clear distinction between the fermented and non-fermented KJ on PC1 and inability to distinguish between both fermentation strategies indicated that PC1 could represent the effect of fermentation. The metabolomics data therefore demonstrated that fermentation significantly impacted the metabolites in KJ, while different fermentation strategies had little impact. This phenomenon could also be explained using a Venn diagram (Fig. 2B). In addition to the 843 metabolites found in all 3 KJ samples, CK had the most unique metabolites at 36; LBKJ and PBKJ shared 51 metabolites between themselves and 9 and 15 metabolites with CK, respectively.

3.4 Overall profiles of DAMs

OPLS-DA was used to obtain the DAMs among the different KJ samples (Fig. 2C). Predictive ability (Q^2) scores > 0.8 indicated the suitability and efficiency of the model. The DAM counts resulting from pairwise comparisons of CK *vs*. LBKJ, CK *vs*. PBKJ, and LBKJ *vs*. PBKJ were as follows: 258 (106 up-regulated and 152 down-regulated), 254 (109 up-regulated and 145 down-regulated), and 28 (19 up-regulated and 9 down-regulated), respectively (Figs. 2D-F). In total, 276 DAMs were identified among all the KJ samples (Table S2), including 77 Ls, 48 Ps, 35 AAs, 30 Ns, 28 Os, 25 As, 14 SAs, 9 Vs, 3 Ts, and 7 OTs. Among these, 236 DAMs showed differential changes and consistent trends on comparing CK with both LBKJ and PBKJ, with 140 and 96 DAMs significantly down-regulated and up-regulated, respectively. These DAMs may significantly impact the sensory, nutritional, and functional properties of KJ. Therefore, further grouping analysis was performed on these significantly up-regulated and down-regulated DAMs.

3.5 Differentially accumulated Os and SAs

LAB converts carbohydrates into acids and produces various metabolites. A significant up-regulation of most differentially accumulated Os (23 of 28) were observed after LAB fermentation (Fig. 3A), which was consistent with the previously observed decrease in pH and increase in TA (Figs. 1C-D). During the fermentation, a total of 8 Os DAMs were produced and significantly up-regulated, including oxalic acid (O1), *L*-lactic acid (O2), 2-hydroxybutyric acid (O10), 2-hydroxyisobutyric acid (O11), pyrrole-2-carboxylic acid (O14), 2-hydroxy-2-methylbutyric acid (O20), 2-picolinic acid (O22), and mevalonic acid (O52) (Fig. 3A and Table S2). Among these, O2 was the main end-product of carbohydrate metabolism by LAB, mainly produced *via* the Embden-Meyerhof-Parnas (EMP) and pentose phosphoketolase (PK) pathways (Fig. 3C). Additionally, the O10, O11, O22, 2-hydroxyisocaproic acid (O36), 2-hydroxy-4-methylpentanoic acid (O37), *DL*-3-phenyllactic acid (O57), and shikimic acid (O63) contents were significantly higher in PBKJ than those in LBKJ ($P < 0.05$), with O22 only significantly up-regulated in CK *vs*. PBKJ (Fig. 3A and Table S2). Some butyric, valeric, and caproic acids may be produced as a result of branched-chain amino acid (BCAA) metabolism[21]. This study also found that BCAAs, including *L*-leucine (AA12), *L*-isoleucine (AA13), and *L*-valine (AA5), were significantly down-regulated after fermentation (Fig. 4A). Moreover, α-ketoglutaric acid (O44), succinic anhydride (O3), and 3-methylmaleic acid (O51) were only significantly up-regulated in CK *vs*. LBKJ (Fig. 3A). O44, which is an intermediate product within the tricarboxylic acid cycle and nitrogen and amino acid metabolism as well as a synthetic precursor for some amino acids, has various physiological functions, including anti-inflammatory and anti-aging roles[22].

Fermentation significantly down-regulated five differentially accumulated Os, namely *L*-malic acid (O39), fumaric acid (O15), 6-aminocaproic acid (O30), *L*-citramalic acid (O50), and decanoic acid (O61). O15 was completely metabolized under both fermentation strategies and could be degraded *via* two pathways: 1) succinic acid (O17) generation by succinate dehydrogenase (SDH); and 2) O39 production by fumarate hydroxylase (FH) and its subsequent metabolism into O2 by malolactic enzyme (ME) (Fig. 3C)[21,23]. These pathways were confirmed by the significant up-regulation in O17 and O2 and the significant down-regulation of O39 after fermentation. Furthermore, O50 and O61 were only significantly down-regulated in CK compared with LBKJ (Fig. 3A and Table S2), indicating that both may be the characteristic metabolites of monoculture fermentation. Overall, LAB fermentation promoted extensive Os production, thereby increasing the acidity and changing the sensory properties of the juice, while also helping to prolong the shelf life of products. Different fermentation strategies caused varying changes in organic acid monomers.

In total, 14 SAs were identified as DAMs, with 4 and 10 significantly down-regulated and up-regulated, respectively (Fig. 3B). These DAMs were mainly involved in carbohydrate metabolism by LAB through the EMP and PK pathways. The 4 significantly down-regulated DAMs, 3-dehydro-*L*-threonic acid (SA5), *N*-acetyl-*D*-mannosamine (SA40), 1-(*sn*-glycero-3-phospho)-1*D*-myo-inositol (SA55), and lactobiose (SA63), mainly functioned as carbon sources of

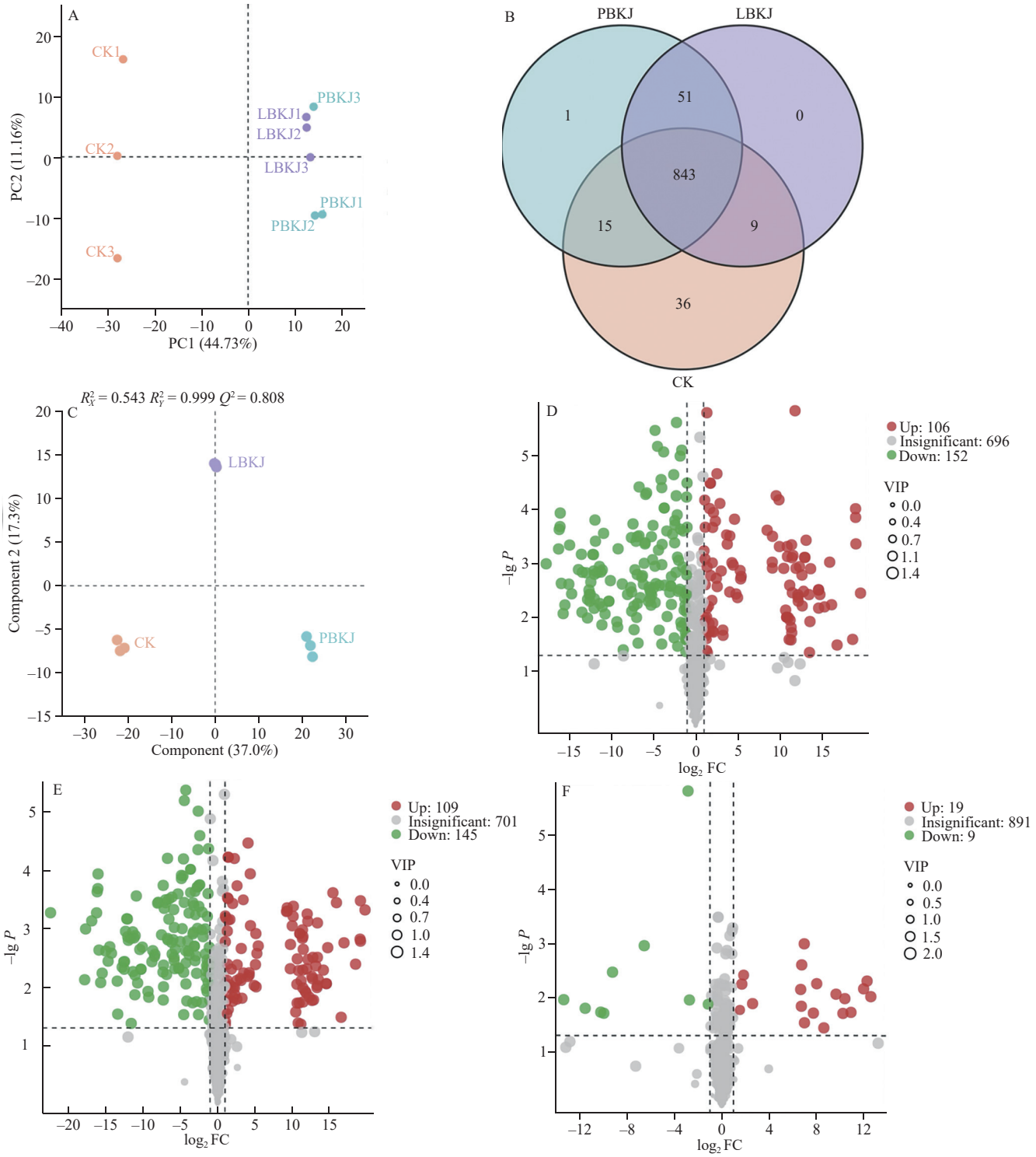

Fig. 2 (A) PCA of the 955 identified metabolites; (B) Venn diagram of metabolites of CK, LBKJ and PBKJ; (C) the score plots of OPLS-DA and volcano plots of DAMs on (D) CK *vs*. LBKJ, (E) CK *vs*. PBKJ and (F) LBKJ *vs*. PBKJ.

energy for LAB during fermentation. Meanwhile, the down-regulation of SA40 might also be due to phosphorylation and epimerization during fermentation to form *N*-acetylglucosamine-6-phosphate[24]. Furthermore, the main intermediates during carbohydrate metabolism, dihydroxyacetone phosphate (SA17), 3-phospho-*D*-glyceric acid (SA28), and *D*-fructose-1,6-biphosphate (SA57), were significantly up-regulated in CK compared with LBKJ and PBKJ (Fig. 3C). However, the main hexose carbohydrates used by LAB during fermentation, including *D*-glucose (SA20), *D*-galactose (SA22), *D*-fructose (SA23), and *D*-mannose (SA24), showed no significant changes as a result of fermentation (Table S1). This was consistent with the results of elderberry juice fermentation[11]. On the one hand, this was because acid metabolism was more active in low-pH environment; on the other hand, KJ was rich in glycosides, such as flavonol glycosides, which might be decomposed by hydrolytic enzymes produced by LAB during fermentation to produce free sugars, thereby balancing the sugar

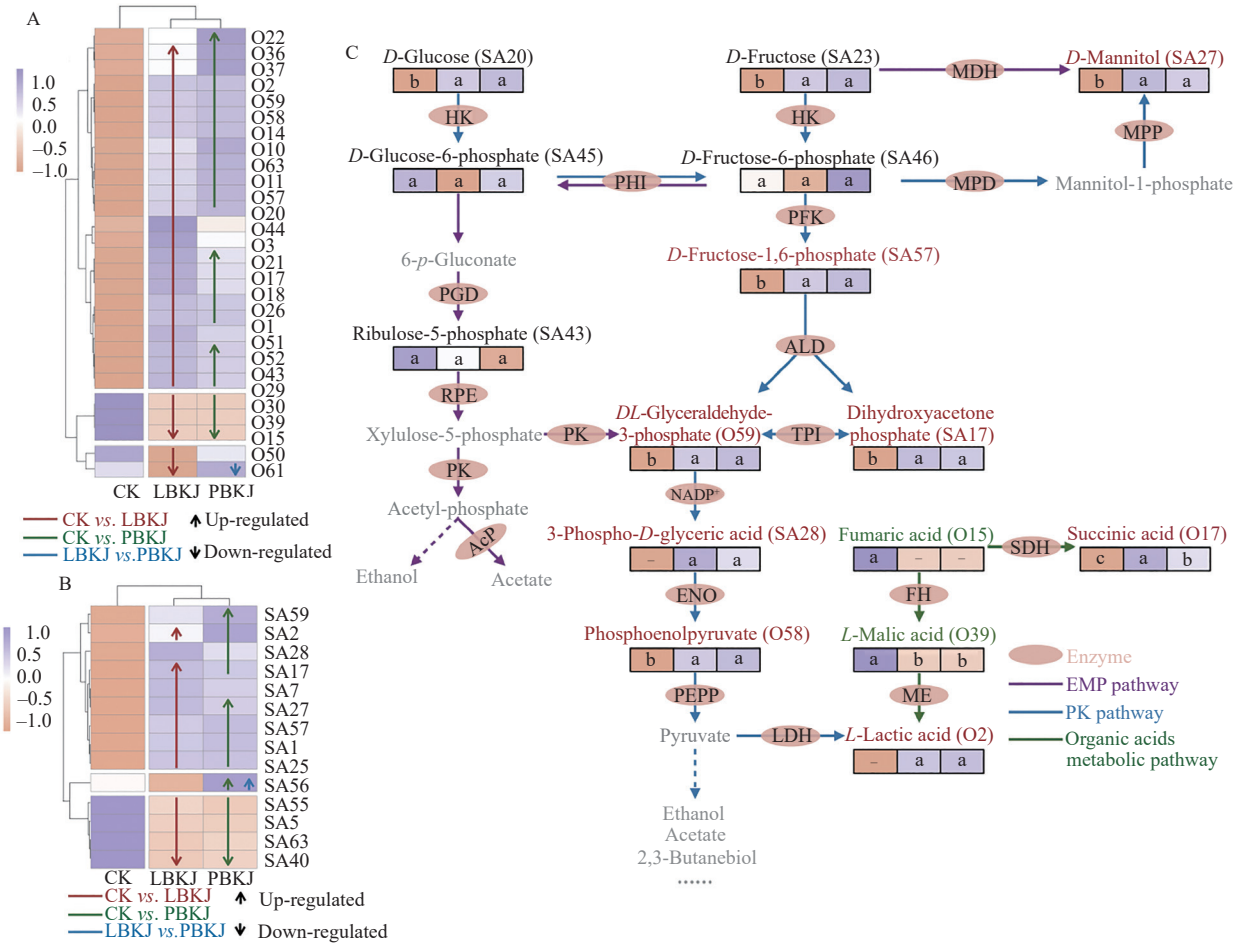

Fig. 3 Heatmap of DAMs of (A) Os, (B) SAs and (C) their possible main metabolic pathways. The red words: significantly up-regulated DAMs; green words: significantly down-regulated DAMs; black words: non-DAMs and grey words: undetected substances. The shifting shades from orange to purple represent peak areas changing from small to large, and different lowercase letters indicate significant differences ($P < 0.05$) among samples. ALD, fructose-bisphosphate aldolase; ENO, enolase; HK, hexokinase; LDH, lactate dehydrogenase; MPD, mannitol 1-phosphate dehydrogenase; MPP, mannitol-1-phosphate phosphatase; $NADP^+$, glyceraldehyde-3-phosphate dehydrogenase; PEPP, phosphoenolpyruvate phosphatase; PFK, 6-phosphofructokinase; PGD, phosphogluconate dehydrogenase; PHI, phosphohexose isomerase; RPE, ribulose-phosphate-3-epimerase; TPI, triose-phosphate isomerase.

consumption during fermentation[25]. Moreover, two polyols of KJ, dulcitol (SA25) and *D*-mannitol (SA27), were significantly up-regulated after fermentation (Fig. 3B). As nutritive sweeteners, these could enhance the flavor of KJ and offer various health benefits such as antioxidant and anti-inflammatory effects. In particular, SA27 is a low-glycemic index non-metabolizable sweetener that is widely used to treat individuals with controlled caloric intake requirements, such as those with diabetes. Most of SA27 was synthesized from SA23 by mannitol 2-dehydrogenase (MDH) during the LAB fermentation (Fig. 3C) [26]. Lactic acid is widely known to be the final product of LAB fermentation and is usually the product of metabolizing sugars, especially hexoses such as glucose, galactose, and fructose. However, their content showed no significant change after fermentation, with only 4 and 10 SAs that were significantly down-regulated and up-regulated, respectively. Additionally, some OAs were significantly down-regulated, including O39 and O15. Studies have shown that these OAs also serve as a carbon sources for fermentation, resulting in lactic acid production[11]. Therefore, within the low pH environment of the KJ matrix, LAB may prefer metabolizing acids instead of conventional sugars to adapt to low pH stress. This phenomenon has also been observed in other highly acidic food matrices[11,23].

3.6 *Differentially accumulated AAs*

As precursors of phenols, volatile aroma substances, and flavor compounds, amino acids can be metabolized by LAB during fermentation and help improve the functional characteristics and sensory qualities of food[27]. As shown in Fig. 4A, most amino acids were significantly down-regulated during KJ fermentation, including BCAAs such as *L*-valine (AA5), *L*-leucine (AA12) and *L*-isoleucine (AA13), which are precursors of volatile compounds such as acids, aldehydes, alcohols, and esters[28]. Our recent research also confirmed that LAB fermentation helps produce esters, alcohols, ketones, and Ts in KJ, thereby improving its aroma profile[12]. Additionally, the aromatic amino acids, including *L*-phenylalanine (AA35), *L*-tyrosine (AA45), and *L*-tryptophan (AA60) were degraded during LAB metabolism through transamination to produce phenols and indole substances. Indole substances are a type of As that positively affect the human body. Aromatic amino acids can also be degraded through decarboxylation to produce biogenic amines such as tryptamine, tyramine, and phenylethylamine, which can be harmful to the human body[21].

LAB hydrolyzes proteins and oligopeptides in food matrices through endopeptidases and exopeptidases to produce peptides and release

terminal amino acids[29]. Therefore, 10 DAMs in the form of amino acids and their derivatives were significantly up-regulated after fermentation (Fig. 4A), among which the contents of *L*-ornithine (AA16), *S*-allyl-*L*-cysteine (AA30), *N*-acetyl-*L*-leucine (AA38), and *γ*-glutamyl-*L*-valine (AA71) in PBKJ were significantly higher than those of LBKJ ($P < 0.05$). AA16 also showed significant up-regulation in the pairwise comparison of CK *vs*. PBKJ, indicating that AA16 generation was more active in PBKJ. Notably, LAB mainly metabolized *L*-arginine (AA41) to AA16 and carbamoyl phosphate *via* the arginine deiminase (ADI) pathway (Fig. 4B), wherein carbamoyl phosphate further forms ATP and NH_3 through carbamate kinase (CKase), thus providing energy for LAB metabolism and regulating the pH of the fermentation substrate[21,29]. This may be another metabolic pathway to help LAB adapt to KJ matrices under low pH conditions. In addition, amino acid acetylation was also common during KJ fermentation, which was reflected in the significant up-regulation of *N*-acetyl-*L*-threonine (AA31), *N*-acetyl-*L*-leucine (AA38), N^6-acetyl-*L*-lysine (AA47), and *N*-acetyl-*L*-methionine (AA54) (Fig. 4A). During fermentation, *γ*-glutamyl-*L*-valine (AA71) and *γ*-glutamylmethionine (AA78) were also significantly up-regulated (Fig. 4A); *γ*-glutamyl dipeptides have been shown to impart a kokumi taste, which positively affects the flavor characteristics of food[30]. In general, LAB utilized the rich protein and amino acid resources of KJ as a nitrogen source during fermentation, while provided energy for metabolism and produced numerous flavor substances, phenols, and other functional components, which effectively improved the flavor and functional properties of KJ.

3.7 Differentially accumulated Ps

Ps are the most abundant secondary metabolites in KJ. As shown in Fig. 5A, compared with that of CK, the TPC of KJ increased significantly after fermentation ($P < 0.05$). This could be due to the hydrolysis of complex phenolics or phenolic polymers into simpler forms during fermentation, thereby increasing the phenolic content of the juice[31]. The DPPH and FRAP of fermented KJ were also significantly higher, mainly due to the increased TPC during fermentation. Meanwhile, the antioxidant capacity of LBKJ was significantly higher than that of PBKJ due to the significantly higher TPC in LBKJ (Fig. 5A). In total, 380 Ps were detected in all KJ samples, including 167 flavonoids (PFs), 161 PPs, 25 lignans (PLs), 19 coumarins (PCs), and 8 tannins (PTs) (Table S1).

3.7.1 PFs

PFs are the most abundant phenolic compounds in kiwifruit. As shown in Fig. 5A, the TFC of KJ increased significantly after fermentation ($P < 0.05$), but showed no significant difference between the two fermentation strategies ($P > 0.05$). Metabolomics identified only 12 PFs as DAMs, including 2 flavonols (PF(II)s), 6 flavanones (PF(V)s), one flavonoid carbonoside (PF(VI)), and 3 chalcones (PF(VII)s) (Fig. 5C). Most (8 of 12) were significantly up-regulated under both fermentation strategies, which was consistent with the observed change in TFC.

3.7.1.1 PF(II)s

Among the detected PF(II)s, the contents of 12 increased significantly after fermentation (Table S1). Among these, kaempferol-3-*O*-(2″-*O*-acetyl) glucuronide (PF(II)32) and isorhamnetin-3-*O*-sophoroside (PF(II)54) were identified as DAMs and showed a significant up-regulation in fermented KJ compared with CK (Fig. 5C). Both differentially accumulated PF(II)s may be synthesized by monoglycosylated PF(II)s during glycosyltransferase metabolism[25].

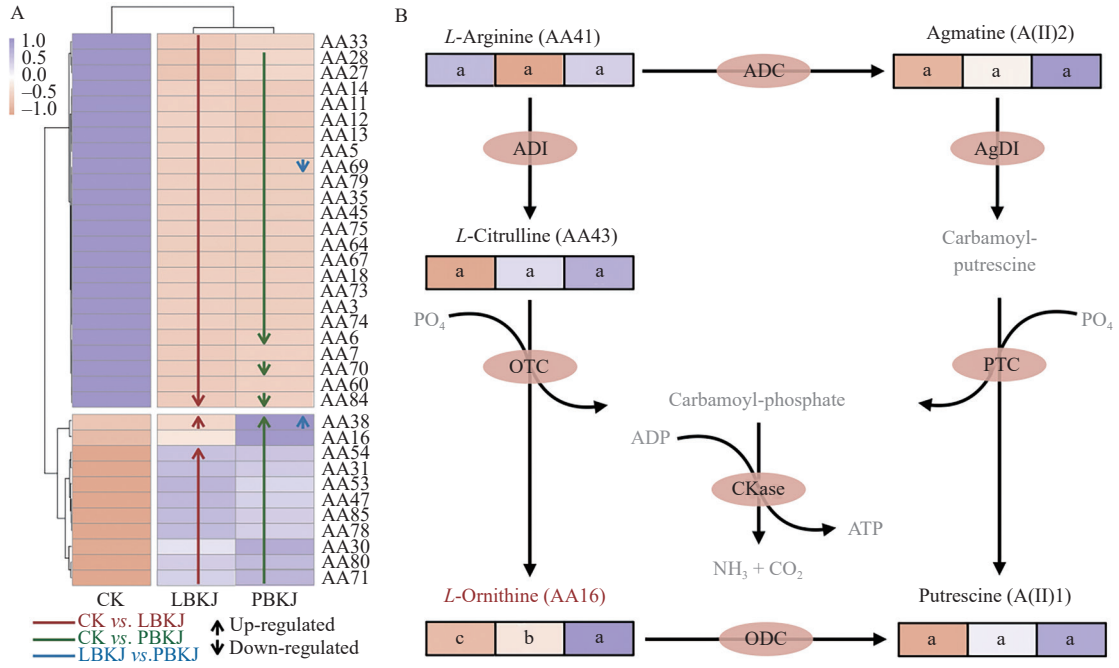

Fig. 4 Heatmap of (A) DAMs of AAs and (B) ADI pathway. The red words: significantly up-regulated DAMs; black words: non-DAMs; grey words: undetected substances and the pink oval: enzyme. The shifting shades from orange to purple represent peak areas changing from small to large, and different lowercase letters indicate significant differences ($P < 0.05$) among samples. ADC, arginine decarboxylase; ADI, arginine deiminase; AgDI, agmatine deiminase; ODC, ornithine decarboxylase; OTC, ornithine carbamoyltransferase; PTC, putrescine carbamoyltransferase.

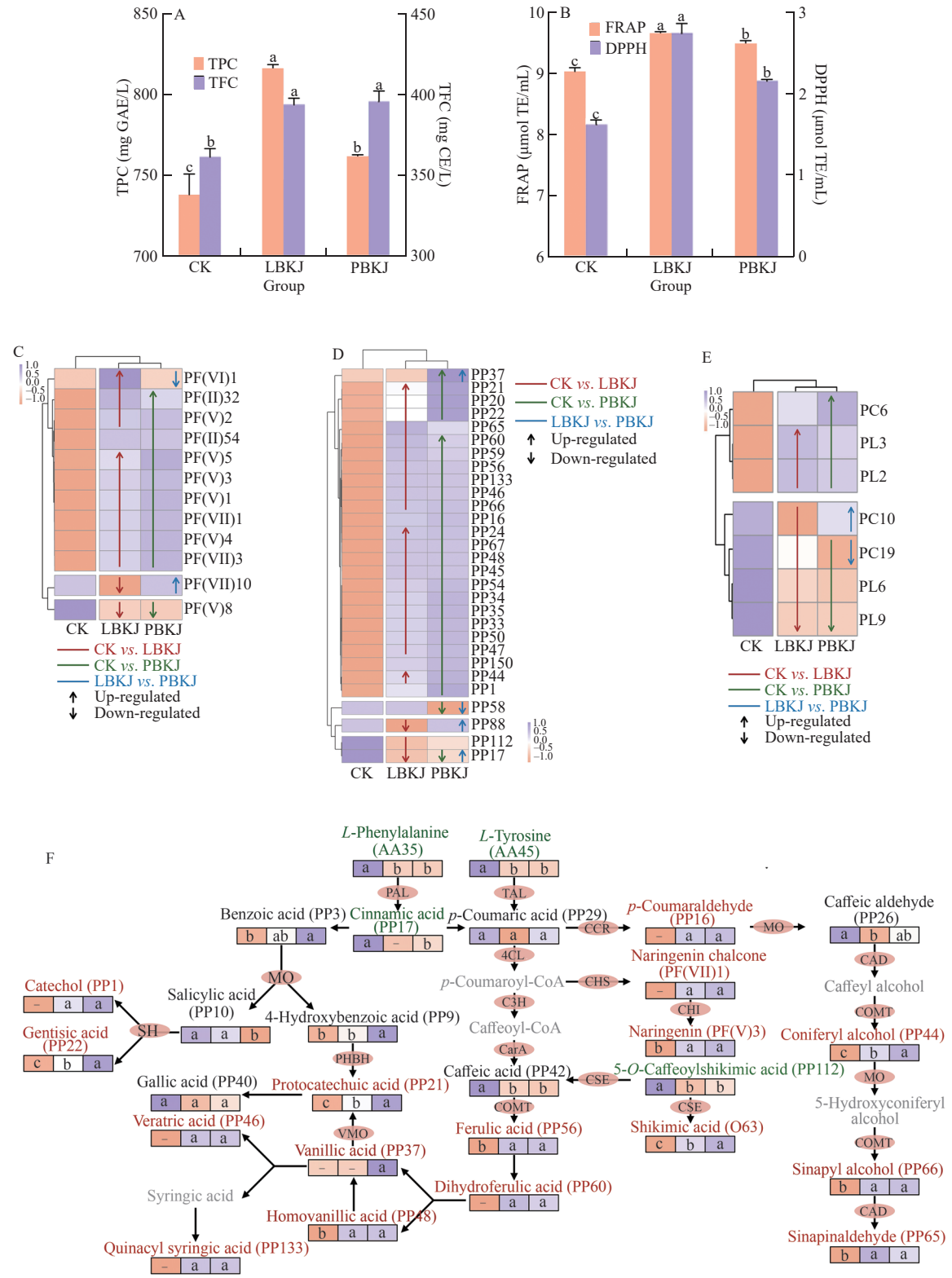

Fig. 5 (A) TPC and TFC of juice samples; (B) the DPPH and FRAP of juice samples; the heatmaps of DAMs of (C) PFs, (D) PPs, and (E) PLs and PCs; and (F) phenylalanine/tyrosine metabolism pathway. The red words: significantly up-regulated DAMs; green words: significantly down-regulated DAMs; black words: non-DAMs; grey words: undetected substances and the pink oval: enzyme. The shifting shades from orange to purple represent peak areas changing from small to large, and different lowercase letters indicate significant differences ($P < 0.05$) among samples. 4CL, 4-coumarate-CoA ligase; CAD, cinnamyl-alcohol dehydrogenase; CarA, caffeate CoA-transferase; CCR, cinnamoyl CoA reductase; COMT, caffeate *O*-methyltransferase; MO, monooxygenase; PAL, phenylalanine ammonia-lyase; PHBH, 4-hydroxybenzoate-3-hydroxylase; SH, salicylate hydroxylase; TAL, tyrosine ammonia-lyase; VMO, vanillate monooxygenase.

3.7.1.2 PF(V)s

Six PF(V)s were identified as DAMs in the CK *vs*. LBKJ and CK *vs*. PBKJ comparisons. Pinocembrin (PF(V)1), butin (PF(V)2), naringenin (PF(V)3), isookanin (PF(V)4), and eriodictyol (PF(V)5) were significantly up-regulated, while only pinocembrin-7-*O*-glucoside (PF(V)8) was significantly down-regulated (Fig. 5C). The flavanone aglycones in KJ increased significantly after fermentation, possibly due to the deglycosylation of specific bacterial glycosyl hydrolases[32]. Previous studies have shown that the biological activities of these flavanone aglycones are higher than those of their precursor glycosides in the human body. Consider PF(V)8 and PF(V)1 as examples, which were significantly down-regulated and up-regulated after fermentation, respectively. PF(V)1 exhibits better anti-apoptosis, antioxidant, and anti-inflammatory effects compared with those of PF(V)8[33], which might be one of the reasons for the enhanced antioxidant capacity in fermented KJ. Moreover, the PF(V)5 content in PBKJ was significantly higher than that in LBKJ (Table S2), which could be due to the mixed fermentation favoring the release of PF(V)5 from eriodictyol glycosides. Studies have proven the various health benefits of PF(V)5, including neuroprotective, anti-inflammatory, antioxidant, anticancer, antidiabetic, and antiobesity effects[34].

3.7.1.3 PF(VI)s

Most of the flavonoid glycosides detected were *O*-glycosides and only a few were *C*-glycosides, with the *C*-glycosylation of PFs mainly catalyzed by *C*-glycosyltransferase. Recent studies have shown that PF(VI)s have various health benefits, including anti-neuroinflammatory, liver protective, and anti-oxidative effects. They also have excellent *in vivo* stability and bioavailability in terms of acid and enzymolysis resistance[35]. Luteolin-8-*C*-arabinoside (PF(VI)1) was significantly up-regulated in CK *vs*. LBKJ but showed no significant difference between CK and PBKJ (Fig. 5C and Table S2), suggesting that PF(VI)1 may be a characteristic product of LBKJ.

3.7.1.4 PF(VII)s

As natural precursors in the biosynthesis of PFs, PF(VII)s exhibit various biological activities, including analgesic, anti-inflammatory, antibacterial, and anticancer effects[36]. Naringenin chalcone (PF(VII)1) and okanin (PF(VII)3) were produced and significantly up-regulated in KJ after fermentation (Fig. 5C). PF(VII)1 was mainly produced through chalcone synthase (CHS) catalysis and could be further isomerized by chalcone isomerase (CHI) to form PF(V)3 (Fig. 5F). Moreover, monoculture fermentation resulted in the complete degradation of 3,4,2′,4′,6′-pentahydroxychalcone-4′-*O*-glucoside (PF(VII)10), with no significant change observed during mixed fermentation (Fig. 5C and Table S2), which could be associated with the type of hydrolase in each strain.

3.7.2 PPs

In this study, PPs showed an overall up-regulatory trend during fermentation. As shown in Fig. 5D, of the 29 PPs identified as DAMs, 25 were significantly up-regulated and only 4 were significantly down-regulated, namely cinnamic acid (PP17), 3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin (PP58), 6-*O*-acetyl arbutin (PP88), and 5-*O*-caffeoylshikimic acid (PP112). As an intermediate of various PPs, PP17 was massively metabolized during fermentation to form other PPs, which led to the up-regulation of PPs (Fig. 5F). Furthermore, PP112 deacylation by caffeoylshikimate esterase (CSE) produced caffeic acid (PP42) and shikimic acid (O63) (Fig. 5F). Notably, only mixed fermentation completely degraded PP58 and its content in LBKJ showed no significant difference from that in CK; conversely, monoculture fermentation completely metabolized PP88 and its content in PBKJ showed no significant difference from that in CK (Fig. 5D and Table S2), which may be associated with the different combinations and functions of hydrolytic enzymes produced by the two strains.

Among the 25 up-regulated DAMs of PPs, 10 were produced during fermentation and were more significantly up-regulated during mixed fermentation (Fig. 5D and Table S2). Seven of the remaining significantly up-regulated DAMs of PPs, namely 2,3-dihydroxybenzoic acid (PP20), protocatechuic acid (PP21), gentisic acid (PP22), 2,6-dimethoxybenzaldehyde (PP33), 3-(4-hydroxyphenyl) -propionic acid (PP34), 2-hydroxy-3-phenylpropanoic acid (PP35), and coniferyl alcohol (PP44), were not only significantly up-regulated in both the CK *vs*. LBKJ and CK *vs*. PBKJ comparisons, but also had significantly higher contents in PBKJ compared with LBKJ ($P < 0.05$) (Fig. 5D and Table S2). Moreover, 4 PPs were significantly up-regulated only in the CK *vs*. PBKJ comparison, namely catechol (PP1), *p*-coumaraldehyde (PP16), vanillic acid (PP37), and verbasoside (PP150), of which PP37 was only produced in PBKJ, suggesting the dependence of PP37 production on LP enzymes. Compared with PFs, the metabolic pathway of PPs was clearer, mainly synthesized by Phe (AA35) and Tyr (AA45) through the phenylalanine and tyrosine metabolic pathway. As shown in Fig. 5F, KJ fermentation resulted in the formation of several PPs through PP17 deamination, hydroxylation, and methylation, as well as the actions of various enzymes to produce functional components such as PFs, PLs and PCs. In conclusion, fermentation produced large quantities of PPs, with the mixed fermentation strategy involving LB and LP favoring metabolism and PP production.

3.7.3 PLs and PCs

PLs and PCs are bioactive substances with known antibacterial, antioxidative, anticancer, and antiviral activities[37-38]. Studies have shown that bacterial fermentation improves the PL content in foods[39]. In this study, the production and significant up-regulation of epipinoresinol (PL2) and pinoresinol (PL3) were observed during fermentation (Fig. 5E and Table S2), probably formed through the polymerization of PP44 (http://www. genome. jp/kegg/). Regarding PCs, isoscopoletin (PC6) showed a significant up-regulation in the CK *vs*. PBKJ comparison (Fig. 5E and Table S2). Moreover, both esculetin-7-*O*-glucoside (PC10) and fraxetin-7,8-*di*-*O*-glucoside (PC19) were degraded to different degrees under both fermentation strategies. In particular, PC10 and PC19 degraded completely when fermented with a monoculture and mixed culture, respectively (Fig. 5E and Table S2). This could be due to the strain specificity for the deglycosylation of different substances.

3.8 Differentially accumulated Vs

As shown in Fig. 6A and Table S2, 18 Vs were detected in all KJ samples, including B vitamins and vitamins C, K_1, and K_2.

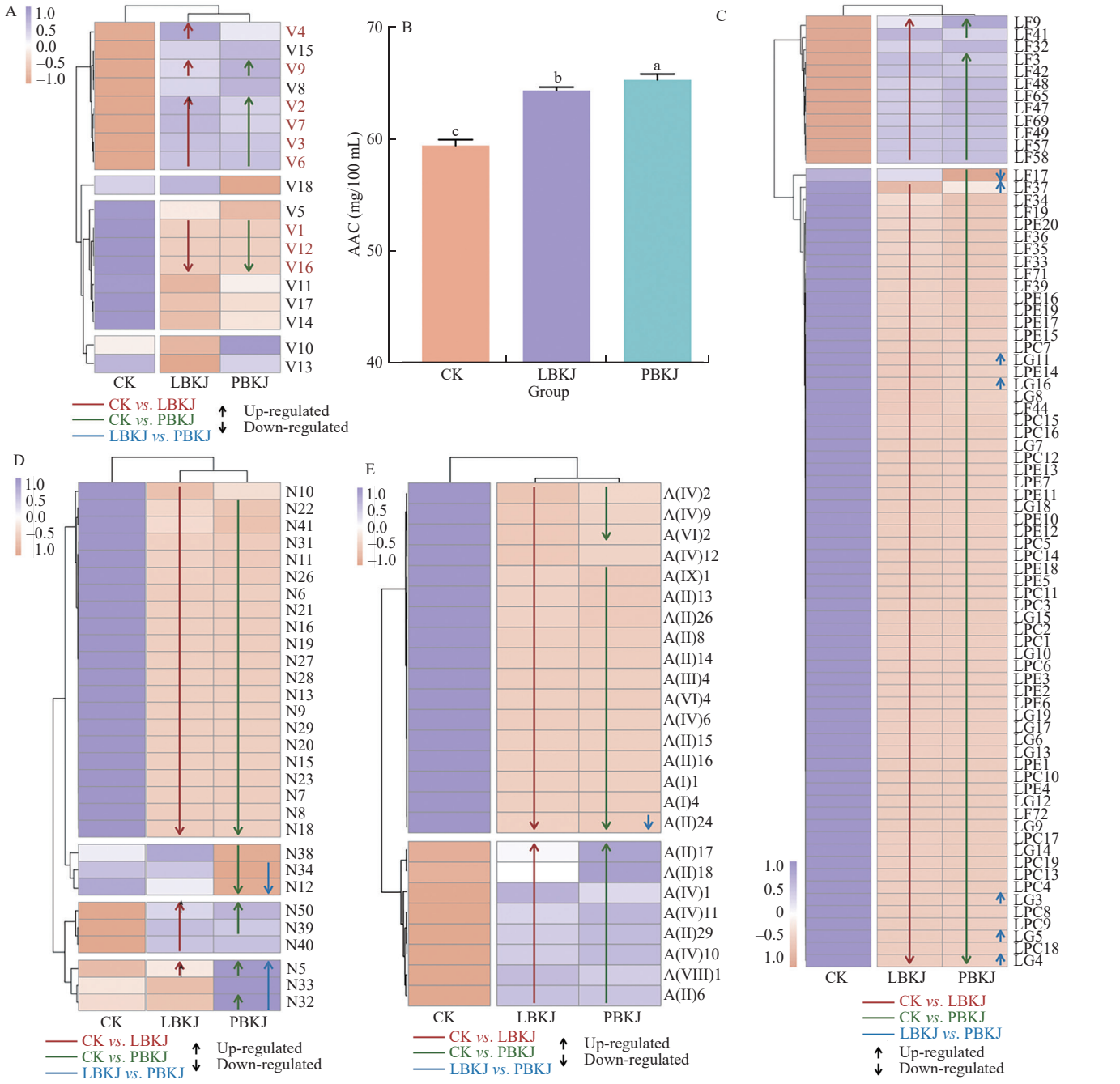

Fig. 6 (A) Heatmap of Vs of juice samples and the DAMs of them are marked as red; (B) the AAC of juice samples; and the heatmaps of DAMs of (C) Ls, (D) Ns and (E) As. The shifting shades from orange to purple represent peak areas changing from small to large, and different lowercase letters indicate significant differences ($P < 0.05$) among samples.

3.8.1 *B vitamins*

B vitamins were the richest Vs in KJ, with 13 B vitamins and their derivatives detected in all KJ samples. LAB fermentation did not significantly affect *D*-pantothenic acid (vitamin B_5, V10), biotin (vitamin B_7, V11), and thiamine (vitamin B_1, V13) (Table S1). However, fermentation did impact other B vitamins. Nicotinic acid (V3) and nicotinamide (V1), collectively referred to as vitamin B_3, are precursors to the coenzyme nicotinamide adenine dinucleotide and cofactor nicotinamide adenine dinucleotide phosphate and participate in lipid and sugar metabolism[40]. Fermentation significantly up-regulated and down-regulated V3 and V1, respectively. The production of V3 may occur *via* the following pathways: 1) V1 metabolism by nicotinamide deaminase and 2) nicotinate *D*-ribonucleoside (V12) phosphorylation (http://www.genome.jp/kegg/), both of which were confirmed by the significant down-regulation of V1 and V12 during fermentation. Moreover, 4 forms of vitamin B_6 and their derivatives were detected in all KJ samples, namely pyridoxal (V5), pyridoxine (V6), pyridoxine-5′-*O*-glucoside (V14), and 4-pyridoxic acid-*O*-glucoside (V15) (Fig. 6A and Table S1), with only V6 significantly up-regulated following fermentation (Fig. 6A). Additionally, orotic acid (vitamin B_{13}, V4), a key intermediate in the pyrimidine biosynthesis pathway that is widely used for clinically

treating hyperuricemia, anemia, and heart disease[41], was also significantly up-regulated after fermentation (Fig. 6A). However, riboflavin (vitamin B_2 and V16), which was significantly down-regulated (Fig. 6A), is mainly involved in bacterial energy metabolism, thereby providing energy for carbohydrate, fat, and protein metabolism[42]. Similar results were obtained in a previous study on yogurt fermentation by Elmadfa et al.[43].

3.8.2 Vitamin C

Vitamin C, or ascorbic acid, is a well-known nutrient in kiwifruit[2]. Fig. 6B shows the AAC of KJ determined by physiochemical testing. The AAC of LBKJ and PBKJ were significantly higher than that of CK and the AAC of PBKJ was significantly higher than that of LBKJ ($P < 0.05$), which was consistent with the metabolomics results.

In particular, *L*-ascorbic acid (V9), isoascorbic acid (V8), and dehydroascorbic acid (V7) were detected in KJ, with V8 and V7 being the stereoisomeric and oxidative forms of V9, respectively. The levels of these three Vs in LBKJ and PBKJ were significantly higher than those in CK ($P < 0.05$) (Table S1) and V9 and V7 were significantly up-regulated in both the CK *vs*. LBKJ and CK *vs*. PBKJ (Fig. 6A). The CK sample in this study was subjected to the same conditions as the fermented juice but without LAB inoculation. Thus, the up-regulation of vitamin C was attributed more to the high retention of the original vitamin C levels in KJ under LAB fermentation conditions, rather than being produced during fermentation. Vitamin C is highly unstable during processing and is easily oxidatively degraded by factors such as pH, metal ions, dissolved oxygen, and light[44-45]. The lower pH values and oxygen consumption levels during LAB fermentation improved the stability of vitamin C in the juice matrix[14], resulting in a higher retention rate of vitamin C in the fermented juice.

3.9 Differentially accumulated Ls

In all the KJ samples, 77 Ls were identified as DAMs, accounting for 27.8% of the total DAMs and making them the most abundant species of DAMs. As shown in Fig. 6C and Table S2, fermentation either significantly downregulated or completely degraded most glycerol esters (LGs), lysophosphatidylethanolamines (LPEs), and all lysophosphatidylcholines (LPCs). This may be because LGs are metabolized into free fatty acids (LFs) by lipase, while LPEs and LPCs are further degraded by lysophospholipase to produce phosphoric acids, bases, and glycerol. LPCs participate in an important fatty acid transport pathway during brain development and growth, while LPEs exhibit antiapoptotic effects[46]. Therefore, fermentation could negatively impact the functional properties of KJ due to LPCs and LPEs degradation.

LFs are the most abundant Ls in KJ, with various physiological functions and health benefits[47]. In this study, 23 LFs were identified as DAMs, of which 12 and 11 were significantly up-regulated and down-regulated, respectively (Fig. 6C). Most of the significantly up-regulated LFs showed varying degrees of hydroxylation, possibly due to the hydroxylation of fatty acids by fatty acid hydratases or cytochrome P450 monooxygenase in LAB. Hydroxy fatty acids have proven health benefits, including improved inflammatory responses and regulated gut flora[48]. Meanwhile, the production and significant up-regulation of 10-hydroxy-2-decenoic acid (10-HDA, LF3) was observed during fermentation (Fig. 6C and Table S2). While the formation of 10-HDA could proceed *via* cytochrome P450 monooxygenase hydroxylation after forming *trans*-2-decenoic acid from decanoic acid, this metabolic pathway has only been confirmed for *Escherichia coli*. Current studies have demonstrated the various pharmacological effects of 10-HDA, including antibacterial and antioxidative activities, enhanced immune functions, and lower blood Ls[49]. In summary, LAB fermentation of KJ leads to the significant loss and degradation of Ls, but also produces various LFs, which positively affect the health functions of KJ.

3.10 Differentially accumulated As

As protect plants from other organisms through their toxicity, despite their antioxidant activities, they are considered potentially harmful toward humans due to their toxicities and anti-nutritional properties at low concentrations[50]. Studies have shown that some LAB such as *L. plantarum* are capable of degrading As[51]. This study identified 25 As as DAMs, of which 17 were significantly down-regulated after fermentation, indicating that both fermentation strategies were capable of efficiently degrading As (Fig. 6E). However, 8 As were still significantly up-regulated during fermentation. Among these, guanidinoacetate (A(II)6), *L*-tyramine (A(II)17), phenylethanolamine (A(II)18), and 4-hydroxyquinoline (A(VIII)1) were produced during fermentation, of which A(II)6 was a direct precursor of creatine and highly involved in its metabolism[52]. Meanwhile, plumeranes (A(IV)s) were abundant and significantly up-regulated during fermentation, including indole (A(IV)1), indole-3-lactic acid (A(IV)10), and 3-hydroxy-3-acetonyloxindole (A(IV)11) (Fig. 6E); these are mainly produced by tryptophan metabolism and possess antibacterial, anti-inflammatory, and antitumor activities[53]. Notably, most up-regulated As DAMs (5 of 8) in PBKJ were significantly higher than those in LBKJ (Table S2) ($P < 0.05$), suggesting a higher activity of As metabolism during mixed fermentation.

3.11 Differentially accumulated Ns

During LAB fermentation, bacteria require large nucleotide counts to meet their proliferation needs. As nucleotide synthesis is an energy-intensive anabolic process, including nucleotides in the food matrix benefits cell proliferation[54]. In total, 30 Ns were identified as DAMs in this study (Fig. 6D). Among these, 24 Ns DAMs, including adenine (N7), guanine (N12), cytidine (N18), adenosine (N23), and guanosine (N27), were significantly down-regulated after fermentation (Fig. 6D), indicating the numerous of Ns participated in cell proliferation and various regulatory and metabolic activities during KJ fermentation. Nucleotide catabolism includes purine and pyrimidine metabolism. Purine nucleotides decompose into purines, phosphates, and pentoses when catalyzed by enzymes such as nucleotidase, nucleotide hydrolase, and nucleotide phosphorylase; pyrimidine nucleotides typically decompose into acetyl-coenzyme A (CoA) and succinyl-CoA, which participate in multiple metabolic activities[55-56].

In summary, LAB fermentation significantly impacts the phytochemicals in KJ. Specifically, large quantities of AAs, Ls, Ns, some sugars and acids in KJ are consumed during fermentation, while Os, Ps, LFs, Vs, and some As with biological activities were produced and significantly up-regulated and some anti-nutritional factors were degraded.

These results demonstrated that fermentation effectively improves the nutritional and functional properties of KJ, thereby improving its performance in healthcare. Compared with the monoculture fermentation, the mixed fermentation strategy generally enhanced the production of more biologically active phenols and As, of which PP37 was considered the characteristic metabolite of the mixed fermentation by LP and LB. Moreover, PF(VI)1 was identified as a characteristic metabolite of LB fermentation, while *D*-glucose 1,6-bisphosphate (SA56), *L*-citramalic acid (O50), and decanoic acid (O61) were exclusively utilized and degraded during monoculture fermentation. These observations explain the superior fermentation characteristics of LB under low pH stress, as it enables the more efficient metabolism of acids.

4. Conclusions

This study used widely targeted metabolomics to investigate the metabolite profiles of KJ under different LAB fermentation strategies as well as the impact of each strategy on the nutritional and functional properties of KJ. LAB fermentation effectively improved the nutritional and functional properties of KJ. As a good matrix for LAB fermentation, KJ provided LAB with various nutrients essential for fermentation, including sugars, acids, amino acids, and nucleotides. This resulted in a large number of active probiotics (> 9 (lg (CFU/mL))) in the fermented KJ, ensuring its ability to offer health benefits to the host. Furthermore, fermentation promoted the production of many bioactive substances such as Ps, Os, LFs, and Vs and inhibited anti-nutritional factors such as As. A mixed fermentation involving LP and LB produced more phenols and As compared with LB alone, among which vanillic acid (PP37) was considered the characteristic metabolite of PBKJ, while luteolin-8-*C*-arabinoside (PF(VI)1) was considered the characteristic metabolite of LBKJ. Based on the dynamic metabolite changes during fermentation, some metabolic pathways of key metabolites were predicted, including the EMP and PK pathways that primarily utilize carbohydrates, the organic acid metabolic and ADI pathways that improve LAB acid resistance, and the phenylalanine and tyrosine metabolic pathway that is based on PPs. These results will likely provide strong support for developing high-quality fermented KJ and guide future research on the metabolic rules and pathways of typical phytochemical components in fermented juice matrices.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by the National Nature Science Foundation Project (32372261), and the Innovation Capacity Support Plan of Shaanxi Province (2024NC-BSLD-01, 2024NC-ZDCYL-04-22, 2024NC-LSTD-001, 2024QCY-KXJ-083).

Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250257.

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