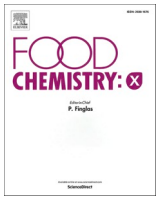
Food Chemistry: X 21 (2024) 101116

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



# Effect of sugar content on characteristic flavour formation of tomato sour soup fermented by *Lacticaseibacillus casei* H1 based on non-targeted metabolomics analysis

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ARTICLE INFO

*Keywords:*
Tomato sour soup
*Lacticaseibacillus casei* H1
Metabolomics
Organic acids
Flavour metabolites

ABSTRACT

To reveal the formation mechanism of the characteristic flavour of tomato sour soup (TSS), metabolomics based on UHPLC–Q–TOF/MS was used to investigate the effect of sugar addition on TSS metabolomics during fermentation with *Lacticaseibacillus casei* H1. A total of 254 differentially abundant metabolites were identified in the 10% added-sugar group, which mainly belonged to organic acids and derivatives, fatty acyls, and organic oxygen compounds. Metabolic pathway analysis revealed that alanine aspartate and glutamate metabolism, valine leucine and isoleucine metabolism and butanoate metabolism were the potential pathways for the flavour of TSS formation. Lactic acid, acetic acid, Ala, Glu and Asp significantly contributed to the acidity and umami formation of TSS. This study showed that sugar regulation played an important role in the formation of the characteristic TSS flavour during fermentation, providing important support for understanding the formation mechanism of organic acids as the main characteristic flavour of TSS.

## 1. Introduction

Tomato sour soup (TSS) is a famous regional characteristic food cuisine with a spontaneously fermented tomato originating from southwestern China, which is prepared by anaerobic fermentation containing raw materials tomatoes and red peppers, among other ingredients such as ginger and glutinous rice flour (Lin, Zeng, Tian, Ding, Zhang, & Gao, 2022; Wang, Song, Li, He, Wang, & Zeng, 2023). TSS is popular with consumers owing to its unique taste and a variety of bioactive ingredients. The unique flavour of TSS stems from its rich organic acids and aroma composition. Fermentation leads to the production of bioproducts enriched with organic acids, short-chain fatty acids, minerals and other nutrients, lycopene and capsaicin, which contribute to intestinal health (Lin, Du, Zeng, Liang, Zhang, & Gao, 2020). According to the fermentation technology used in the preparation, TSS is classified into natural fermentation and dominant strain-enhanced fermentation. Fermentation starters can effectively shorten the fermentation cycle, are conducive to industrial production and ensure the consistent quality of the produced fermented product, which are considered to offer advantages to promote improvements in the flavour and quality of TSS (Li et al., 2022). Previous studies have established that TSS prevents and treats hyperlipidaemia and improves the intestinal flora to facilitate weight loss and lipid reduction in obese rats (Zhou, Qu, Wang, Liu, Yang, & Wang, 2023).

Tomatoes are rich in organic acids such as citric acid and succinic acid, which contribute to their acidic taste (Otify et al., 2023). Lactic acid bacteria (LAB) are the most common and predominant microorganisms in fermented fruit and vegetable products. Sucrose, cellulose and other monosaccharides from tomatoes provide fermentative substrates for LAB to produce organic acids. Organic acids play a crucial role in determining the flavour and aroma of fermented foods and are irreplaceable in the overall flavour and tartness formation for TSS. (Nie et al., 2022).

In the process of fruit and vegetable fermentation, carbohydrates are an important energy source for microbial fermentation. Starches varying in amylose content exerted discernible influences on the microbial community and metabolic products throughout the kimchi fermentation process (Park et al., 2023). The addition of pickled rice paste and fish sauce to kimchi modified its quality and taste (Baek, Kim, Han, Lee, & Jeon, 2023). Although glutinous rice flour was added to TSS in the

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https://doi.org/10.1016/j.fochx.2024.101116
Received 26 October 2023; Received in revised form 24 December 2023; Accepted 1 January 2024
Available online 2 January 2024

traditional fermentation process, it may mainly affect its taste and flavour metabolites. Marianna et al. (2020) reported that the addition of exogenous glucose during oat flour fermentation resulted in better bacterial growth and lactic acid production. The fermentation of a blend of oat and rice flours supplemented with a glucose solution at a concentration of 2 % w/v was assessed as the minimum threshold for eliciting a substantial enhancement in bacterial proliferation. In addition, carbohydrate stress is one of the most common conditions during fermentation. Therefore, the types and levels of carbon sources are critical for tomato sour soup fermentation. However, the biological mechanism underlying the effect of carbohydrates on the formation of sour soup fermentation flavour is poorly understood.

Although considerable progress has been made in the production of TSS, the mechanism of flavour metabolite formation during fermentation remains largely unexplored. Ultra-high performance liquid chromatography coupled with quadrupole time of flight mass spectrometry (UHPLC–Q–TOF/MS) has been widely employed for the identification and differential analysis of flavour metabolites with high stability, resolution, selectivity and quality precision among other advantages (Shi et al., 2020). In addition, the types and levels of organic acids and characteristic flavour metabolites determine the quality of TSS. Therefore, the metabolomics method based on UHPLC–Q–TOF / MS is a powerful tool to identify the characteristic flavour metabolites in TSS, which is of great significance to analyse the formation mechanism of TSS characteristic flavour.

The primary objective of this study was to analyse the differentially abundant metabolites of added-sugar TSS and no-added-sugar TSS by UHPLC–Q–TOF/MS, and to further elucidate the probable influence mechanism of *Lacticaseibacillus casei* H1 inoculation on the flavour and quality of fermented TSS. The goal of this study is to highlight the contribution of sugar and *Lacticaseibacillus casei* H1 to flavour formation, provide a new understanding of the formation mechanism of TSS flavour substances characterized by organic acid metabolism, and provide a possibility for the application of flavour quality control in TSS production.

## 2. Materials and methods

### 2.1. Materials and reagents

Tomatoes, salt, sucrose and other materials were purchased from Wal-Mart Stores (Huaxi, Guiyang, China). Chromatography-grade solvents such as methanol were obtained from Tedia Company, Inc. (Fairfield, USA), and phosphoric acid and organic acid standards were acquired from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). De Man Rogosa Sharpe (MRS) broth was purchased from Bo Microbial Technology Co., Ltd. (Shanghai, China). The remaining reagents utilized in this study were of analytical grade, such as sodium hydroxide and potassium dihydrogen phosphate, which were procured from Chengdu Jinshan Chemical Reagent Co., Ltd. (Chengdu, China).

### 2.2. Preparation of the starter

The strain of *Lacticaseibacillus casei* H1 (CCTCC NO: M2016524) was previously screened from fermented food of Dong and Miao nationalities in Guizhou Province, China, which holds certain acidogenesis and antioxidant capacity (Zheng et al., 2022). *Lacticaseibacillus casei* H1 was inoculated into MRS broth at 37 °C for 24 h. The cells were centrifuged at 10,000 rpm for 10 min and washed twice with 0.85 % (w/v) saline. The cells were resuspended in 0.85 % saline to obtain a concentration of approximately $10^9$ CFU/mL for starter inoculation in tomato sour soup and stored at 4 ℃ for subsequent fermentation (Zheng et al., 2022).

### 2.3. Fermentation of tomato sour soup

The tomato sour soup method was performed as described by Li et al. (2022). First, tomatoes were rinsed with distilled water and beaten with a blender. Beaten tomatoes were mixed with salt (2 %, $w/w_0$, $w_0$ is the mass of beaten tomatoes) and white wine (3 %, $w/w_0$), and the mixture was sterilized at 90 ℃ for 10 min (LS-B75L-I, BINJIANG, Jiangsu, China). Then, the mixture was put into a sterile glass fermenter (3 L) on a clean bench. Sucrose was added to 0 %, 2 %, 5 % and 10 % ($w/w_1$, $w_1$ is the mass of mixture), and these groups were named 'A0′, 'A2′, 'A5′ and 'A10′, respectively. *Lacticaseibacillus casei* H1 prepared as above was inoculated into the crushed tomato at a rate of 2 % ($v/w_1$, $w_1$ is the mass of mixture). Finally, the tomato sour soup was fermented at 25 °C, and the pH was tested every day. When the pH reached 3.4, it was considered the end point of fermentation and was sampled for subsequent experiments. Six replicates were carried out for each sample. After the fermentation process of tomato sour soup was completed, the final product was immediately frozen in liquid nitrogen and kept at − 80 ℃ until further analysis.

### 2.4. Physicochemical properties

The pH values of the tomato sour soup were determined with a digital pH meter (PHS-3C, LEICI, Shanghai, China). Total titratable acidity was measured using the titration method previously described by Qiu et al. (2022) with some minor modifications. The acidity was determined via titration to neutrality (pH 8.2 ± 0.2) using NaOH (0.1 mol/L) in the presence of phenolphthalein. The results were expressed as grams of lactic acid per litre.

### 2.5. Organic acids

Organic acids were analysed as described by Li et al. (2022), with some modifications. The organic acid analysis was carried out by HPLC (1260 VWD, Agilent, Santa Clara, CA, USA) coupled with a Z0RBAX SB-Aq column (4.6 × 250 mm, 5 μm, Agilent, Palo Alto, CA, USA) and kept at 35 ℃. The mobile phase conditions were as follows: Isocratic elution was performed with 0.02 mol/L phosphoric acid solution and methanol (95:5, v/v) at a flow rate of 0.8 mL/min. Every sample was repeated three times.

### 2.6. Untargeted metabolomics analysis

#### 2.6.1. Extraction of metabolites

The extraction procedure was based on Chen et al. (2023) with some modifications. For the tomato sour soup, 25 mg of the sample was combined with 800 μL of precooled precipitant (methanol:acetonitrile: pure water = 2:2:1) in a 1.50 mL sample vial with a lid. Initially, two steel balls were added to a grinder, and the mixture was ground at 60 Hz for 4 min. Subsequently, the steel balls were removed, and the mixture was sonicated in ice water for 10 min (80 Hz). The mixture was then maintained at − 20 ℃ for 2 h and subsequently centrifuged for 15 min (25,000 × g, 4 °C). The resulting supernatant was collected and dried in a freeze dryer at − 18 °C for 2 h. The lyophilized product was reconstituted by adding 600 μL of 10 % methanol solution and sonicated for 10 min (80 Hz) in an ice bath. After that, it was centrifuged for 15 min (25,000 × g, 4 °C), and the supernatant was used for metabolomic analysis. Quality control (QC) samples were prepared by combining equal aliquots (50 μL) of the metabolite extracts from all samples.

#### 2.6.2. Untargeted metabolomic analysis

The UHPLC–Q–TOF/MS system was coupled with a 2777C UPLC system (Waters, UK) and a Xevo G2-XS QTOF system (Waters, UK). The UHPLC system utilized an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm × 1.8 μm, Waters, UK) with a temperature oven set at 50 ℃. Mobile phase A consisted of water with 0.1 % formic acid, while mobile phase B was methanol with 0.1 % formic acid. The gradient elution proceeded as follows: 0–2 min, 100 % A; 2–11 min, 0 % to 100 % B; 11–13 min, 100 % B; 13–15 min, 0 % to 100 % A; then reverting to 40 %

B at 20–20.5 min; finally, reducing to 35 % B at 20.5–22 min. The injection volume was set at 5 μL, and the flow rate was maintained at 400 μL/min.

The Q–TOF MS operated in both positive and negative ion modes, with optimized MS conditions as previously reported by Shi et al. (2020). The TOF mass was acquired in full-scan mode over the mass range of 50–1200 Da with a scan time of 0.2 s. Capillary voltages and cone voltages were set at 3.0 kV and 40.0 V in positive ionization mode, and 2.0 kV and 40.0 V in negative ionization mode, respectively. All precursors were fragmented at a collision energy of 30 eV, and MS/MS spectra acquisition was performed with a scan time of 0.2 s. The LE signal was acquired every 3 s to calibrate the mass accuracy during acquisition. To ensure sequence analysis stability throughout the acquisition, a quality control sample (QC) was acquired after every 6 samples.

### 2.7. Statistical analysis

The compounds identified through qualitative methods underwent a screening process based on qualitative result scoring. Subsequently, the data from both positive and negative ion modes were amalgamated into an all-encompassing data matrix table, establishing the fundamental cornerstone for further research (Liu et al., 2023). Bioinformatic analysis was conducted using the OECloud tools (https://cloud.oebiotech.com). Statistical analysis and graphical representation between groups were generated using Origin (Version 2021, OriginLab Corp., Northampton, MA, USA) and TBtools software. All data are expressed as the mean ± standard deviation (SD). The pH and titratable acid were determined by two-way analysis of variance in IBM SPSS 26.0, and the organic acid content was analysed by Duncan's multiple range test in one-way analysis of variance. Multivariate analyses were carried out using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

## 3. Results

### 3.1. Influence of different sugar additions on the level of titratable acidity, pH and organic acids in fermentation

Titratable acidity and pH are important indices to evaluate the fermentation level of sour soup. As shown in Fig. 1, the value of titratable acidity and pH will increase and decrease during fermentation, respectively. The amplitude range of the titratable acidity and pH value will increase with the amount of added sugar. In addition, the final pH value (approximately 3.4) after the fermentation of lactic acid bacteria is usually considered to be the end point of the fermentation of sour soup, and it is also an important factor affecting the final acceptance of TSS. A prolonged fermentation period can result in an excessively sour taste of TSS, potentially diminishing consumer acceptance. Titratable acidity mainly originates from the accumulation of various organic acids such as lactic acid. Organic acids are crucial components of tomato sour soup, playing pivotal roles in shaping the organoleptic properties and flavour quality of TSS. The main organic acids in tomato sour soup and their contents are shown in Table 1. The most commonly detected organic acids were lactic acid, citric acid, succinic acid, acetic acid, malic acid, tartaric acid and oxalic acid, which were consistent with the results reported by others (Li et al., 2022; Lin et al., 2020). Meanwhile, lactic acid, acetic acid, citric acid and succinic acid were the main acids found to be in the 6.20–12.71, 5.33–6.00, 2.50–2.87, and 1.47–1.72 mg/mL biomass ranges, respectively. The organic acid content showed different trends with the augmentation of added sugar ($P < 0.05$), among which lactic acid increased significantly by nearly twofold. The elevated content of lactic acid can be attributed to the accelerated carbohydrate breakdown by *Lacticaseibacillus casei* H1 in a carbohydrate-enriched environment, resulting in an augmented production of lactic acid. These results indicated that the fermentation strain responded differently to the stress caused by sugar, resulting in variations in the production of primary and secondary fermentation metabolites (Malacrinò, Tosi, Caramia, Prisco, & Zapparoli, 2005). These results suggested that lactic acid and acetic acid were the main predominant taste contributors to TSS. Notably, the addition of sugar significantly inhibited the production of acetic acid, citric acid and succinic acid, while there was no significant relationship with the sugar content. The escalated rate of carbohydrate glycolysis contributes to the enhanced generation of oxalic acid and tartaric acid. Moreover, under conditions of limited carbohydrate availability, lactobacilli can harness organic acids such as lactic acid as alternative sources of energy. This observation aptly elucidates the enrichment of these organic acids' concentrations in the high-sugar group. Acetic acid has a pungent odour, and reducing the content of acetic acid helps to improve the off-flavour of sour soup. These organic acids are also highly plausible factors influencing the gustatory attributes and quality enhancement of the final TSS product. Furthermore, glycolysis is a process that must be carried out under neutral or weakly alkaline conditions, and the production of lactic acid leads to acidification of the environment. When the environment is acidified to a

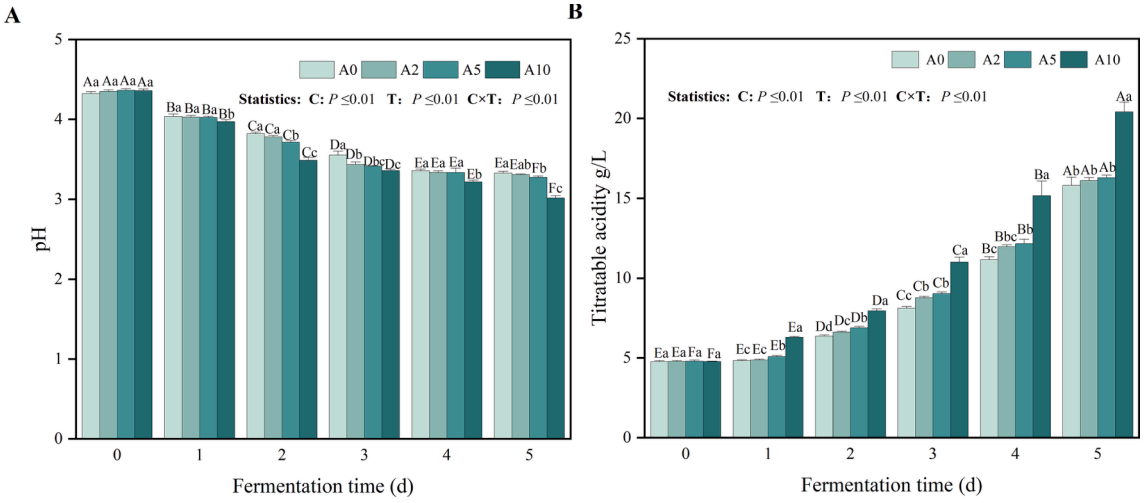


**Fig. 1.** Change in pH (A) and titratable acidity (B) in tomato sour soup during fermentation. A0, A2, A5, A10 represent the fermentation groups with 0 %, 2 %, 5 % and 10 % sugar, respectively. [a-c] Different superscripts in the graphs indicate statistical differences between added sugar groups ($P < 0.05$). [A-F] Different superscripts in the graphs indicate statistical differences between fermentation time ($P < 0.05$). *C*: Concentration of sugar; *T*: Fermentation time; $C \times T$: Interaction between concentration and time.

**Table 1**
Change in the content of different organic acids in tomato sour soup during fermentation.

| Samples[1] | Oxalic acid | Tartaric acid | Malic acid | Lactic acid | Acetic acid | Citric acid | Succinic acid |
|---|---|---|---|---|---|---|---|
| A0(mg/mL) | 0.08 ± 0.00$^{c}$ | 0.24 ± 0.01$^{b}$ | 0.48 ± 0.01$^{a}$ | 6.20 ± 0.20$^{d}$ | 6.00 ± 0.09$^{a}$ | 2.87 ± 0.02$^{a}$ | 1.72 ± 0.05$^{a}$ |
| A2(mg/mL) | 0.19 ± 0.01$^{b}$ | 0.28 ± 0.03$^{b}$ | 0.46 ± 0.03$^{ab}$ | 7.63 ± 0.71$^{c}$ | 5.43 ± 0.50$^{b}$ | 2.66 ± 0.20$^{b}$ | 1.47 ± 0.05$^{b}$ |
| A5(mg/mL) | 0.29 ± 0.05$^{a}$ | 0.27 ± 0.02$^{b}$ | 0.41 ± 0.02$^{c}$ | 8.80 ± 0.18$^{b}$ | 5.33 ± 0.26$^{b}$ | 2.50 ± 0.04$^{b}$ | 1.49 ± 0.05$^{b}$ |
| A10(mg/mL) | 0.34 ± 0.00$^{a}$ | 0.34 ± 0.01$^{a}$ | 0.43 ± 0.01$^{bc}$ | 12.71 ± 0.29$^{a}$ | 5.38 ± 0.06$^{b}$ | 2.59 ± 0.00$^{b}$ | 1.48 ± 0.06$^{b}$ |

[1]A0, A2, A5 and A10 represent the tomato sour soup fermentation groups added with 0 %, 2 %, 5 % and 10 % sugar, respectively. $^{a-d}$ Different superscripts in the graphs indicate statistical differences between added sugar groups ($P < 0.05$).

certain extent, the rate of glycolysis is inhibited, which can also explain the decrease in citric acid and succinic acid. Therefore, it is essential to control the sugar content of the fermentation process. According to the results of the previous experiment, we found that the difference in fermentation results during the period of 10 % added sugar was obvious, and the end point of fermentation could be reached after 4 d. Therefore, the 10 %-added sugar-group was chosen as the experimental group, and the group with no added sugar was chosen as the control group for the subsequent experiments.

### 3.2. Identification of metabolites

To better understand flavour formation during TSS fermentation, we performed untargeted metabolomic UHPLC–Q–TOF/MS, in which a total of 10,343 peaks were identified (including 5131 negative ion peaks and 5212 positive ion peaks) in samples. Furthermore, 8306 metabolites were identified according to the metabolomics database and MS2 spectra, which included a wide variety of metabolites likely to contribute to flavour, such as organic acids and derivatives, organic oxygen compounds, fatty acyls, phenylpropanoids and polyketides. Nonvolatile metabolite profiles were analysed utilizing UHPLC–Q–TOF/

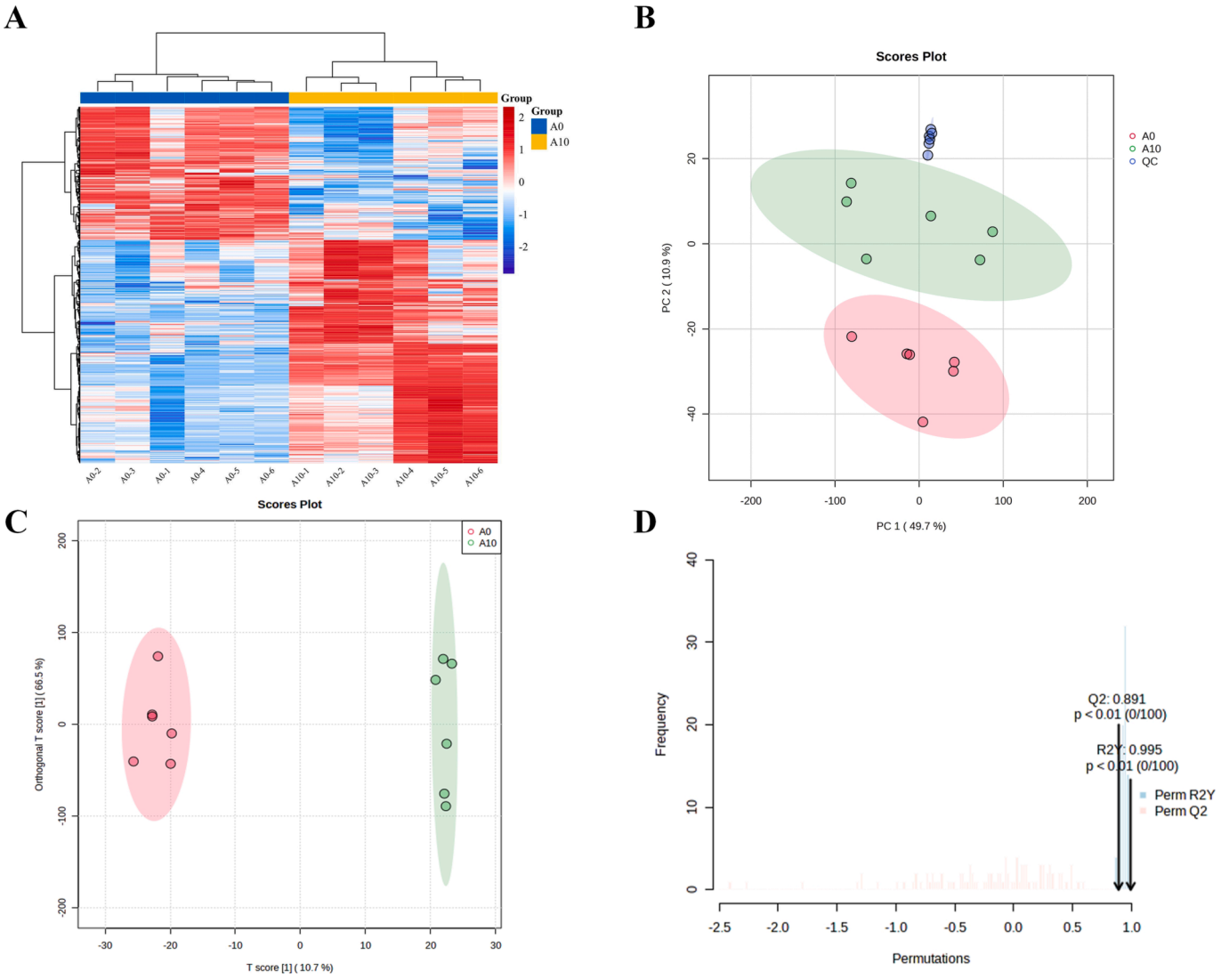


**Fig. 2.** Heatmap of all metabolites (A). PCA scores plot of metabolites (B). OPLS-DA scores plot of metabolites (C). Response permutation test under 100 times for OPLS-DA (D).

MS metabolomics during the fermentation process of *Lacticaseibacillus casei* H1, and the majority of compounds exhibited upregulation or downregulation compared to those in the no-added-sugar sample. The heatmap (Fig. 2A) shows the relative abundance of differentially abundant metabolites, where the 10 %-added-sugar group metabolites were higher than those of the no-added-sugar group after fermentation, illustrating the important role of sugar stress in TSS fermentation.

### 3.3. Multivariate analysis of identified metabolites

Unsupervised principal component analysis (PCA) was applied to intuitively demonstrate metabolite differences in fermented TSS, and all samples were separated. The degree of aggregation of the QC samples is better, indicating that the good stability and reproducibility of the test instrument, the higher the quality of the data collected. As shown in Fig. 2B, the PCA results indicated that all samples fell within 95 %, indicating that the results were credible. The first and second principal components jointly accounted for 60.06 % of the total variance between samples. The first principal component explained 49.70 % of the difference between the samples, distinctly segregating the two sample groups. The PCA results showed obvious differences among the groups with different sugar additions, indicating that sugar addition affected TSS metabolites during fermentation.

To better filter out the noise irrelevant to the classification information and to further obtain ameliorative and preferable differentiation on the differences between groups, the metabolites were further analysed by supervised orthogonal partial least squares discriminant analysis (OPLS-DA). Based on the OPLS-DA plot (Fig. 2C), samples were clearly segregated into two groups, signifying substantial differences in metabolites among them. To further verify the validity of the model and defend against overfitting, 100 response permutation tests were applied. $R^2$ and $Q^2$, two essential parameters are commonly employed to evaluate the accuracy and predictive capability of OPLS-DA models and their values exceeding 0.5 and approaching 1.0 indicate an excellent model The fitting test results further illustrated the goodness of fit and predictive ability of the models without overfitting, as presented in Fig. 2D ($R^2Y = 0.995$, $Q^2 = 0.891$, and $P < 0.01$). The PCA and OPLS-DA models exhibited strong stability and representativeness. The results presented above further suggest that the levels of secondary metabolites underwent changes during TSS fermentation induced by sugar.

### 3.4. Differentially abundant metabolites and correlation analysis

To further investigate and identify the differentially abundant metabolites in the fermentation process with varying sugar content, the criteria for screening differentially abundant metabolites were set as

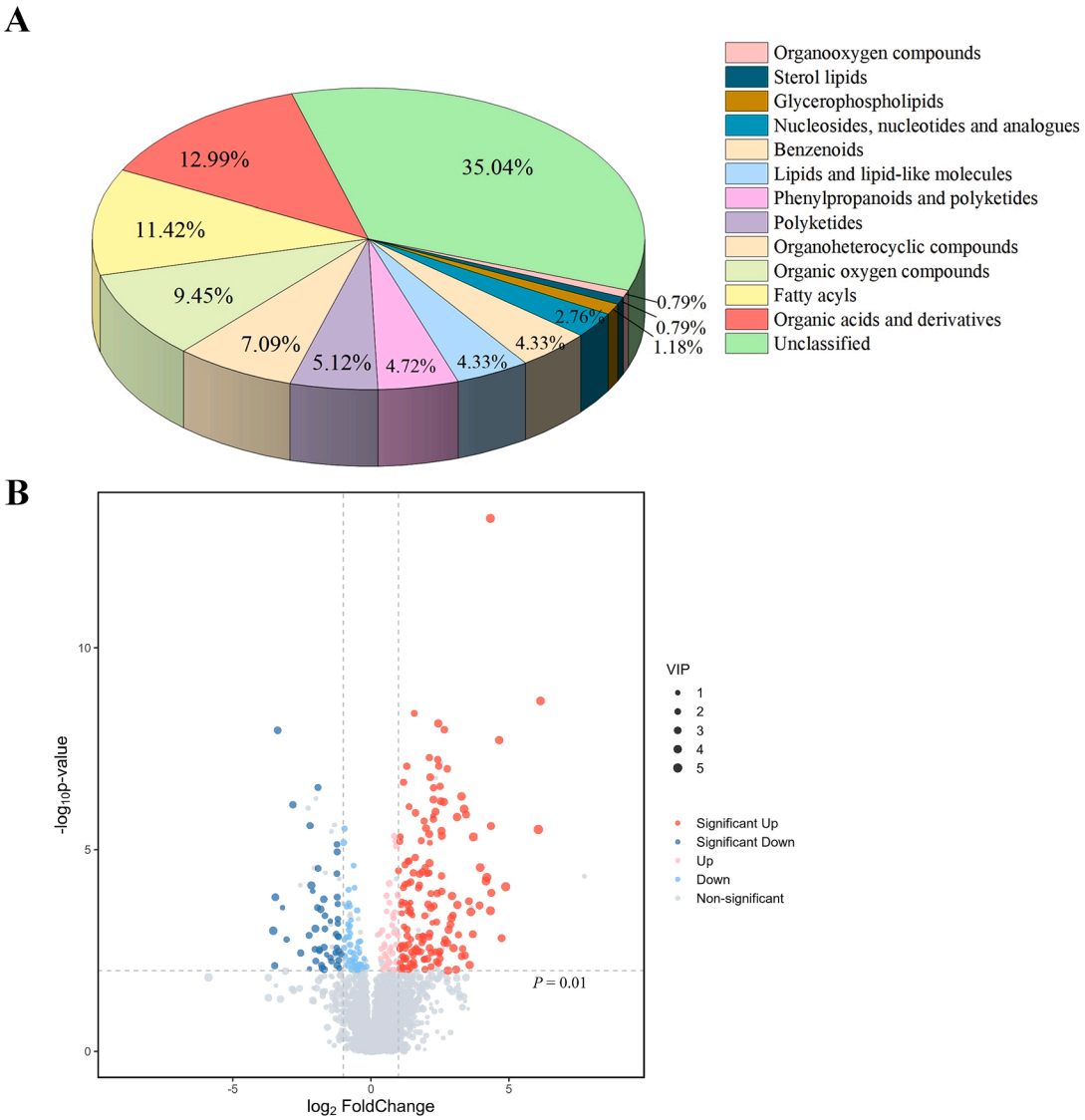


**Fig. 3.** Super class of differentially abundant metabolites (A). Volcano plot of differentially abundant metabolites (B).

follows: $P$ value ($P < 0.05$), fold changes (FC < 0.5 or FC > 2), and variable importance in projection of the first principal component in the OPLS-DA model (VIP > 1.5). These parameters were instrumental in pinpointing key metabolites contributing to the metabolic variations among the distinct TSS samples. With the assistance of this model, 254 differentially abundant metabolites were identified (Fig. 3A), including 33 organic acids and derivatives, 29 fatty acyls, 24 organic oxygen compounds, 18 organoheterocyclic compounds, 13 polyketides, 12 lipids and lipid-like molecules, 11 phenylpropanoids and polyketides, 11 benzenoids, 7 nucleosides, nucleotides and analogues, 3 glycerophospholipids, 2 organooxygen compounds, 2 sterol lipids and 89 unclassified compounds (Fig. S1-6).

The volcano plot was used to visualize the $P$ value, VIP and FC values and the statistical significance of the differences between the two groups, which was helpful in screening for different metabolites. Each dot in the figure corresponds to an individual metabolite, with the dot's dimensions reflecting the magnitude of the VIP value. The significantly upregulated and downregulated metabolites are represented by red and blue dots, respectively, and the grey dots indicate that the differentially abundant metabolites are not significant. According to Fig. 3B, compared to the non-sugar group, 132 differentially abundant metabolites were upregulated and 39 differentially abundant metabolites were downregulated in the 10 % sugar group. Sugar is an important energy source in the metabolic process of lactic acid bacteria and facilitates fermentation by acting as a nutrient source for microorganisms during the TSS fermentation process (Park et al., 2023).

### 3.5. Metabolite analysis

#### 3.5.1. Metabolism of organic acids and derivatives

The type and content of organic acids are regarded as the principal determinants responsible for the flavour quality of sour soup. As shown in Supplemental Fig. S1, TSS was fermented with or without the addition of sugar, and the levels of different metabolites were significantly different. Compared to the non-sugar group, lactic acid, glutamylglycine, aspartyl-histidine, hydroxypyruvic acid, (R)-β-aminoisobutyric acid, 1-aminocyclopropane-1-carboxylate, *N*-lactoyl-glycine, medicanine, 3-(cystein-S-yl) acetaminophen, methotrexate, diethyl tartrate, 3-hydroxy-2-isobutyrate, *N*-methyl-D-aspartate and acetyl phosphate were significantly upregulated by the addition of 10 % sugar to fermented TSS. Lactic acid has a soft and refreshing taste, and is the major organic acid present in TSS. Lactic acid and succinic acid that primarily feed short-chain fatty acidproduction (Tudela, Claus, & Saleh, 2021). Within the carbon metabolism pathway, pyruvate can oxidize succinic acid to produce hydrogen ions and acetic acid, and a small amount of lactic acid can also be generated. The subsequent catabolism of pyruvate has the potential to generate volatile compounds, including 2,3-butanediol, diacetyl, and acetylacetone, which could exert a significant impact on the aroma of fermented comestibles (Wang, Zhang, Liu, Jin, & Xia, 2022). Glutamylglycine and aspartyl-histidine are peptide molecules connected by peptide bonds. Glutamylglycine is considered to have a certain antioxidant capacity, which can help reduce cellular oxidative stress and protect cells from damage by oxygen free radicals. Previous studies have shown that β-aminoisobutyric acid may affect energy metabolism by regulating fatty acid oxidation and glucose metabolism (Roberts et al., 2014). Acetylphosphoric acid plays an important role in the metabolic pathway of bacteria. It can be used as an energy source or metabolic intermediate to participate in a variety of chemical reactions. Hydroxypyruvate can also appear as an intermediate product in the glycolysis pathway, which is related to glucose metabolism and energy production. The upregulation of acetyl phosphoric acid and hydroxypyruvate further clarified that the addition of exogenous sugars promoted the metabolic rate. Diethyl tartrate is tartaric acid with a fruity aroma and flavour that can add specific flavour characteristics to the product. Additionally, γ-aminobutyric acid (GABA) is byproduct of lactic acid bacterial fermentation (Pannerchelvan et al., 2023), and these organic acids and derivatives contribute significantly to the aroma profile of TSS, among which lactic acid contributes to acid odour, heptyl 2-methylpropanoate and caryophyllene alcohol acetate contribute to fruity odours, and tartaric acid plays an indispensable role in the characteristic flavour of TSS. Increased sugar concentrations resulted in improved growth performance and organic acid accumulation of *Lacticaseibacillus casei* H1, which subsequently enhanced the metabolism of organic acids, producing more flavour compounds.

#### 3.5.2. Metabolism of fatty acyls

A total of 29 types of fatty acyls were identified, of which five subclasses of fatty acids and conjugates, octadecanoids, eicosanoids, fatty aldehydes and fatty amides were mainly involved (Fig. S1). Among these metabolites, 22 were upregulated, and 7 were downregulated. Lipolysis and the oxidation of fatty acids are widely regarded as two pivotal biochemical processes intricately linked to the flavour profile of fermented goods (Wang et al., 2022)0.12-Oxo phytodienoic acid (12-oxo-PDA) constitutes a pivotal precursor within the biosynthetic route of jasmonic acid (JA), which has a wide range of physiological regulation in plants and can be used as a signalling molecule to participate in the regulation of plant growth and development (Lally, Murphy, & Horgan, 2023). α-Acetyl lactic acid was involved in butyric acid metabolism and the biosynthesis of pantothenic acid and acetyl-CoA. S-acetyldihydrolipoamide is an intermediate in the metabolism of alanine, aspartate and pyruvate metabolism and glycolysis/gluconeogenesis, which is converted from lipoamide and 2-hydroxyethyl-THPP by the enzyme pyruvate dehydrogenase and then converted to acetyl-CoA via dihydrolipoamide acetyltransferase (Song & Jordan, 2012). Excessive pyruvate accumulation leads to its conversion into α-acetolactate via α-acetolactate synthase, and subsequently into diacetyl (Robert & Gregory, 2000).

#### 3.5.3. Metabolism of organic oxygen compounds

A total of 24 types of organic oxygen compounds were identified, primarily encompassing three subclasses: carbohydrates and carbohydrate conjugates, carbonyl compounds, and alcohols and polyols (Fig. S2). L-gulose is an L-hexose sugar and serves as an intermediary compound in the biosynthetic pathway of L-ascorbate. Isobiflorin is a naphthoquinone compound used in traditional Chinese medicine that has a wide range of biological properties, such as anti-inflammatory, antibacterial and anticancer properties (Mariza et al., 2019). Erythritol is produced by certain bacteria as a byproduct during the regeneration of NADPH via the phosphoketolase pathway (Rzechonek, Dobrowolski, Rymowicz, & Mirończuk, 2018) and as a low-calorie sweetener. Compared to other sugars, erythritol cannot be fermented by bacteria and can inhibit the growth of some microorganisms. It is an ideal multifunctional healthy sweetener that can improve the taste of TSS after fermentation. *N*-Acetylneuraminic acid is widely used as a nutritional adjunct for enhancing cognitive well-being and bolstering immune function (Zhang et al., 2021). Compared to other organic oxygen compounds, 5-sulfoxymethylfurfural, (R)-humulone and acetoxyacetone were more easily vested to a specific flavour in TSS fermentation production.

### 3.6. Analysis of KEGG enrichment and metabolism

KEGG pathway enrichment analysis was conducted based on the annotated results. It filtered the top metabolic pathways of differentially abundant metabolites based on the $P$ value, ranked from smallest to largest. A total of 254 metabolites were annotated using the KEGG database to acquire their corresponding pathway information. Pathways with a $P$ value $\leq 0.05$ were deemed significantly enriched among the differentially abundant metabolites. The analysis revealed that during the fermentation of TSS, 254 metabolites might be involved in 7 pathways, including alanine aspartate and glutamate metabolism, valine

leucine and isoleucine metabolism, butanoate metabolism, tyrosine metabolism, pyruvate metabolism, propanoate metabolism, and lysine degradation metabolism (Fig. 4A). According to the bubble diagram (Fig. 4B), the metabolites enriched in alanine aspartate and glutamate metabolism, valine leucine and isoleucine metabolism and butanoate metabolism were the largest group during fermentation. Overall, these pathways whose changes were altered at a variety of different levels are interconnected and play an important role in the flavour formation of TSS. By understanding and exploring these pathways, we may further clarify mechanisms with unique and desirable flavour profiles during TSS fermentation.

### 3.6.1. *Alanine aspartate and glutamate metabolism*

Alanine (Ala), aspartate (Asp), and glutamate (Glu) are derived from intermediates of central metabolism, primarily from the citric acid cycle. Asp and Glu are amino acids that give the product a fresh taste and play an important role in promoting the formation of the final flavour. In contrast to the large increases in succinate and derivatives during fermentation, Asp is reduced. Ala is the product of degradation of aspartic and glutamic (Pisarenko, Solomatina, & Studneva, 1986)and produced from pyruvic acid through transamination. A previous report demonstrated that alanine was the main flavour amino acid in TSS (Li et al., 2021). Glu and Asp are the two primary free amino acids that contain umami flavour in food. Both belong to the group of glutamic acid umami compounds and are significant contributors to the umami taste of broad bean paste (Sun et al., 2019). Aspartate is also a precursor of volatile aroma components in fermented foods, such as aspartate transaminase, which can metabolize aspartate to produce oxaloacetate by *Lb.paracasei*, which is further decomposed into diacetyl, acetoin and 1,3-butanediol (Thage et al., 2004). Furthermore, free amino acids may function as substrates for subsequent flavour generation, encompassing processes such as amino acid aminotransferases and elimination reactions (Wang et al., 2022).

### 3.6.2. *Valine leucine and isoleucine metabolism*

Amino acid metabolism plays a pivotal role in generating the metabolites that contribute to the flavour profile of fermented foods. For instance, leucine (Leu) can participate in the biosynthesis of 2-methyl butyric acid as a precursor (Zhou et al., 2022). The degradation and metabolism of amino acids make significant contributions to the flavour of food products. (R/S)-3-Aminoisobutyric acid and 3-hydroxy-2-isobutyrate were primarily enriched in pathways associated with amino acid synthesis or degradation, particularly in the valine leucine and isoleucine metabolism pathway. The transamination of leucine forms alpha-ketoisocaproic acid, which is decarboxylated to 3-methylbutanal by nonoxidative decarboxylation under the action of alpha-ketoacid dehydrogenase (Afzal et al., 2017). Valine, leucine and isoleucine are amino acids that possess a bitter taste. Degradation of these amino acids can to some extent reduce the bitter taste of TSS products. 3-Hydroxy-2-isobutyrate is commonly found as an intermediate in the degradation of valine and isoleucine.

In addition, 5-hydroxy-$_L$-tryptophan levels were upregulated, and tryptophan intake had important effects on mood, sleep, appetite and other neurophysiological functions. Sugar concentration is a pivotal fermentation factor profoundly affecting the biosynthesis of citric acid. A sugar concentration within the range of 120 to 180 g/L can inhibit the oTCA (acetyl-CoA) pathway by suppressing α-ketoglutarate dehydrogenase (KGDH), which may promote the accumulation of citric acid (Zhou, Ding, Han, & Deng, 2023). Citrulline and aspartate generate $_L$-arginosuccinate, which is subsequently cleaved into $_L$-arginine and fumaric acid during the fermentation process (Zhang et al., 2015). It is widely recognized that flavonoids present in plant-derived foods exert anti-inflammatory and antioxidative properties and are linked to a myriad of health advantages, including compounds such as hibiscetin, avicularin, 3,5-dihydroxy-6,7-methylenedioxyflavanone, and hirsutin. As shown in Fig. 5, based on KEGG analysis, the metabolic pathways of *Lacticaseibacillus casei* H1 that may be involved in the formation of flavour substances were further explored, including glycolysis, organic acid and amino acid metabolism, and the metabolites, including organic acids and their derivatives, fatty acyls, and amino acid derivatives, were significantly altered when TSS was fermented at 10 % sugar, indicating that exogenous sugar significantly promoted lactic acid levels and enhanced the generation of unique aroma compounds.

## 4. Discussion

To investigate the mechanism of TSS flavour formation under the influence of sugar, we analysed the characteristic differentially abundant metabolites and established a metabolic network model of the main organic acids and amino acids (Fig. 5). TSS flavour is predominantly derived from the raw material and the metabolites generated by LAB fermentation. Lactic acid accumulation is an important index to evaluate

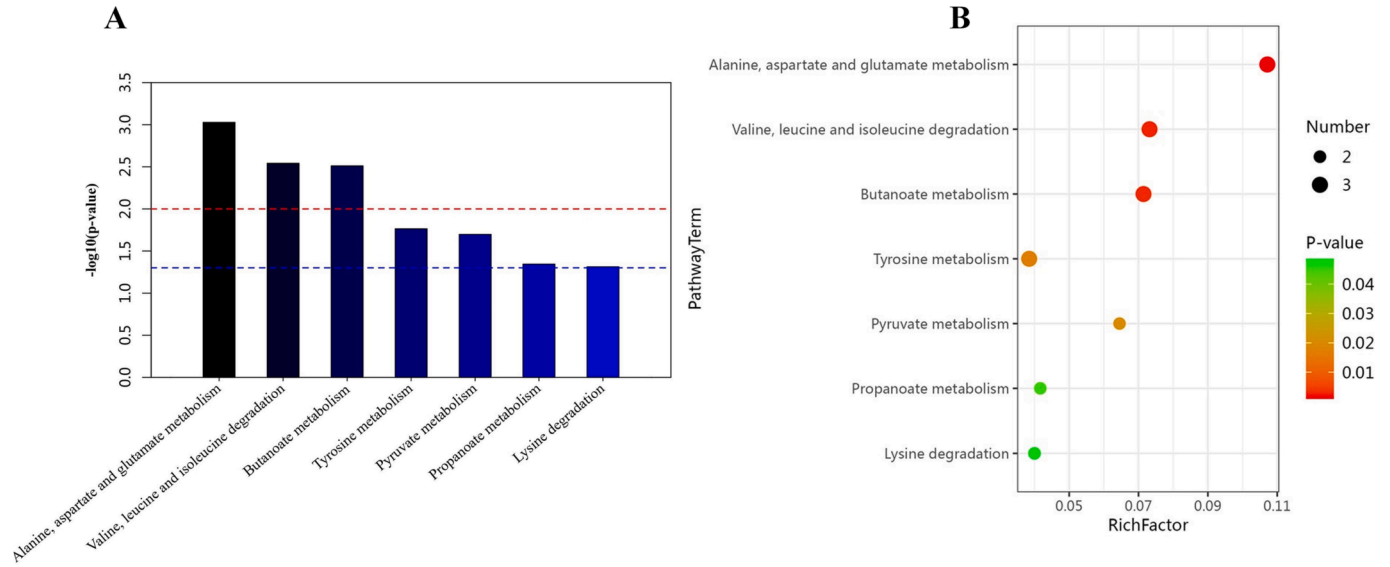


**Fig. 4.** Enriched pathways of differentially abundant metabolites (A), red and blue dotted lines indicate *P* value = 0.01 and *P* value = 0.05, respectively. Bubble diagram (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

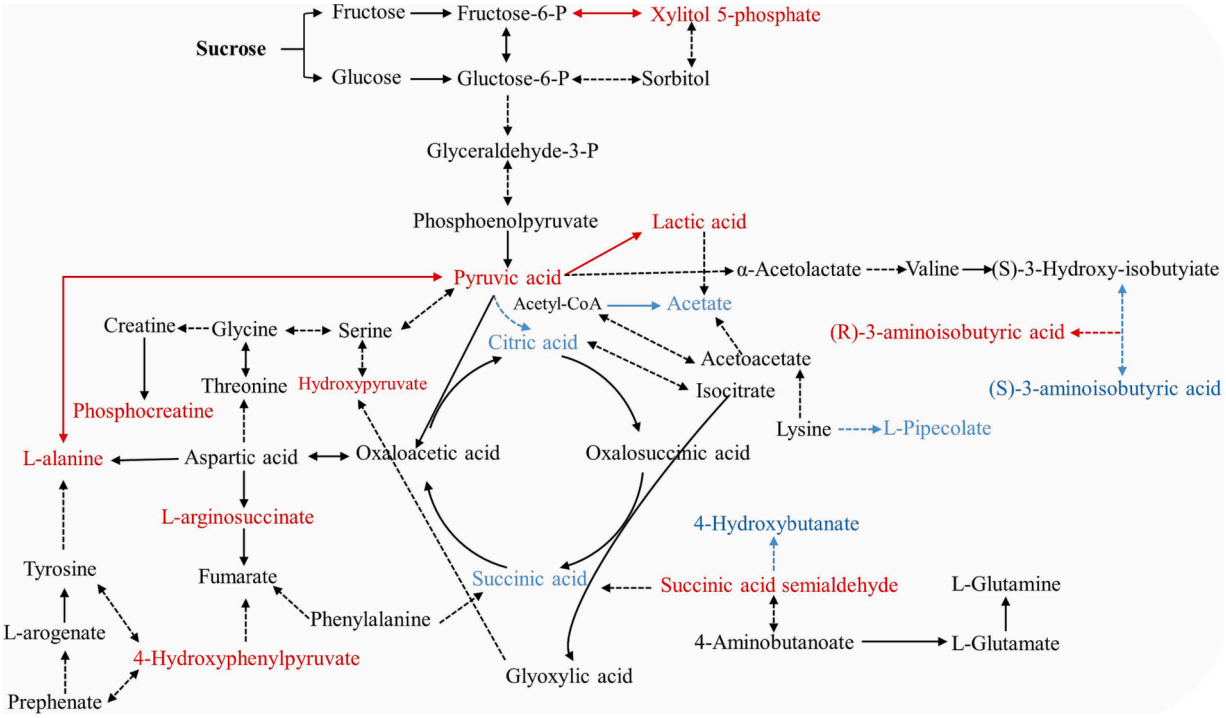


**Fig. 5.** The pathway map of different KEGG differentially abundant metabolites under the influence of sugar. Red highlighted metabolites represent up-regulated differentially abundant metabolites and blue highlighted metabolites represent down-regulated differentially abundant metabolites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the fermentation quality of fruits and vegetables (Gao et al., 2019). An appropriate sugar content can promote fermentation and improve the taste of fermented products. In the glycolytic pathway, glucose and fructose are converted into pyruvate. This process results in the production of a variety of flavourful aldehydes, ketones, acids, and alcohols through various metabolic pathways, such as citric acid metabolism. Meanwhile, tomato is rich in organic acids such as malic acid and citric acid, and LAB can utilize these substances to produce a variety of flavour substances (Wang, Zhang, He, & Li, 2020).

Through its rich sugar metabolic pathways, *Lactobacillus paracasei* has the ability to ferment various monosaccharides and disaccharides, including glucose, fructose, galactose, mannose, lactose and maltose (Cui & Qu, 2021). Fructose and glucose are hydrolysed products of sucrose and are considered to be the main soluble sugars in tomatoes, and they are converted to phosphoenolpyruvate (PEP) via glycolysis and fructose metabolism pathways and are involved in a variety of other metabolic reactions, which is why we chose sucrose as a carbohydrate, while also considering the convenience of actual production. Hexose and pentose are metabolized to produce lactic acid, acetic acid, propionic acid and other organic acids through catabolism, which can be directly used as flavour substances or precursors of important flavour substances (Lorenzo, Emanuele, & Elke, 2016). LAB metabolism can produce nucleic acid, adenosine, guanine, and hypoxanthine, which can impart unique flavours. We also found D-allulose in the fermented product, which has been reported to possess beneficial effects, such as suppressing postprandial blood glucose elevation in humans, and it can serve as a low-calorie substitute for sucrose in food ingredients (Kimoto-Nira et al., 2017). Studies have confirmed the natural concentrations of cellobiose and gentiobiose in both fresh and fermented cucumbers and further evaluated the capacity of selected LAB associated with cucumber fermentation to metabolize these sugars (Ucar, Pérezdíaz, & Dean, 2020). In this work, it was found that adding exogenous glucose can enhance TSS fermentation, along with improving the quality and flavour of its fermented products. Of course, further study is needed to determine whether adding more sugar will adversely affect the quality and metabolites of TSS.

In addition to carbohydrate metabolism, the central metabolic pathways of LAB encompass proteolysis and amino acid catabolism, along with lipolysis and fatty acid metabolism (Wang et al., 2022). In the process of protein metabolism, proteases break down proteins into various metabolites such as peptides and amino acids. Some small molecules such as amino acids also form volatile aromatic compounds, and the formation of this series of volatile byproducts plays a crucial role in the taste and flavour of fermented products. Biosynthesis and metabolism of amino acids contributed to TSS flavor and taste compounds. Ala, Glu, and Asp play a pivotal role in driving flavour development during the fermentation of various foods (Wang et al., 2022). Meanwhile, the formation of free amino acids by TSS during fermentation and their participation in biochemical reactions such as transamination, deamination and desulfurization decarboxylation in subsequent reactions play a crucial role in the flavour and quality of TSS. Furthermore, *Lacticaseibacillus casei* H1 produces organic acids and antibacterial substances, including benzoic acid and antibiotic GR 95647X, aiming to suppress the growth of both pathogens and spoilage microorganisms throughout the fermentation process. These substances also enhance the stability of the fermentation process and improve the quality of tomato sour soup. These results were consistent with those of (Li et al., 2022; Zheng et al., 2022).

## 5. Conclusion

In this study, we investigated the changes in TSS metabolites in *Lacticaseibacillus casei* H1 during fermentation with or without sugar addition. A total of 254 differentially abundant metabolites were identified compared to the no-added-sugar group. Among them, 132 were upregulated, and 39 were downregulated. Organic acids and derivatives, fatty acyls and organic oxygen compounds are important differentially abundant metabolites that affect the flavour of TSS. The predominant metabolic pathways during TSS fermentation were involved in alanine aspartate and glutamate metabolism, valine leucine

and isoleucine metabolism and butanoate metabolism. This study showed that *Lacticaseibacillus casei* H1 was able to accumulate more organic acids and amino acid derivatives in the 10 % sugar addition group, which in turn promoted the formation of TSS characteristic flavour substances and effectively improved and modified the quality and flavour of TSS. The results revealed that under the influence of exogenous sugars, *Lacticaseibacillus casei* H1 significantly influenced the flavour profile of fermented TSS, primarily through metabolic pathways associated with amino acids and the liberation of aromatic compounds bound to organic acids. This study provides insight into the influence mechanism of sugar content on TSS characteristic flavour formation and provides support for flavour regulation and quality control in TSS industrial production.

## CRediT authorship contribution statement

**Huaisheng Zheng:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jingzhu Jiang:** Resources, Formal analysis. **Chaobing Huang:** Validation, Formal analysis. **Xiaoyu Wang:** Writing – review & editing, Funding acquisition. **Ping Hu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## Declaration of competing 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.

## Data availability

Data will be made available on request.

## Acknowledgments

This work was funded by National Natural Science Foundation of China (No. 31960485, 31260379) and the Science and Technology Support Program of Guizhou Province, China (QKHZC-2021-178).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101116.

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