UPLC-Q-TOF-MS based metabolomics and chemometric analyses for green tea fermented with *Saccharomyces boulardii* CNCM I-745 and *Lactiplantibacillus plantarum* 299V

Rui Wang [a], Jingcan Sun [b], Benjamin Lassabliere [b], Bin Yu [b], Shao Quan Liu [a,c,*]

[a] *Department of Food Science and Technology, National University of Singapore, Science Drive 2, Singapore, 117542, Singapore*
[b] *Mane SEA PTE LTD, Biopolis Drive 3, 138623, Singapore*
[c] *National University of Singapore Research Institute, No. 377 Linquan Street, Suzhou Industrial Park, Suzhou, Jiangsu, 215123, China*

ABSTRACT

UPLC-Q-TOF-MS was employed to analyse the non-volatile components of green teas fermented with probiotic yeast (*Saccharomyces boulardii*) and lactic acid bacteria (LAB, *Lactiplantibacillus plantarum*). The flavone glycosides in yeast-fermented and stored tea decreased significantly, together with the increases of flavone aglycones and other simple flavone glycosides. LAB-fermented tea presented different flavone glycoside profiles; in which, both C-glycosides and O-glycosides decreased and the flavone aglycones were further degraded. The profiles of flavone glycosides and aglycones in co-cultured tea differed from that in yeast- or LAB-fermented tea; less glycosides were degraded but a greater number of aglycones were produced. Two unique LAB metabolites with bioactive and antifungal properties, D-phenyllactic acid (PLA) and *p*-OH-PLA, were found in both *L. plantarum* and co-cultured teas, and the co-fermentation showed a synergic effect on the production of these two compounds that would enhance the quality and preservation of fermented teas.

1. Introduction

Biotransformation of tea with various microorganisms can impact the chemical components of tea, as these microorganisms would metabolize these compounds through different pathways (Ge et al., 2019; Li et al., 2017). As a consequence, numerous secondary metabolites or novel compounds can be produced after microbial fermentation (Wang et al., 2015). Lactic acid bacteria (LAB) and yeasts are the most commonly used microorganisms in food fermentation. Some species of LAB such as *Limosilactobacillus fermentum, Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum* and yeast *Saccharomyces boulardii* showed probiotic potentials in inhibiting the growth of pathogenic bacteria, neutralising enterotoxins and resisting antibiotics (Kakisu et al., 2012; Nayak, 2011).

To date, dairy-based products have been the most used carriers for probiotics, however, there are some concerns about lactose intolerance, high cholesterol levels, allergy to dairy protein for dairy-based probiotic products. Meanwhile, the demand for new flavor or taste, increasing health awareness and veganism has stimulated the trend in non-dairy matrices as potential carriers of probiotics. In addition, the low pH

resulted from LAB fermentation is a challenge of the dairy-based probiotic products. The high acidity that originates from the organic acids released by metabolic activities of LAB reduces the survival of probiotic bacteria during the processing and shelf-life of fermented dairy-based products (Aspri et al., 2020). Therefore, there is a strong interest in finding new matrices and novel processing methods to enhance the survival and validity of probiotics.

There are numerous novel vegan-based probiotic products, such as probiotic vegetables, soy, and fruits. However, tea as a novel carrier for probiotics has not been explored so far. From the previous study (Chan et al., 2021), co-culturing of yeast and LAB enhanced the survival of LAB and modulated the flavor of coffee brews. We therefore conducted probiotic tea fermentation with monocultures of *S. boulardii*, *L. plantarum*, and their combination.

High-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) or mass spectrometry (MS) detectors are commonly used for qualitative and quantitative analyses of tea (Tao et al., 2016; Zhang et al., 2011). However, most of these studies were conducted with analysis of a small number of targeted components, which limited the discovery of novel compounds. In recent years, ultra-performance

---

LC-quadrupole time-of-flight MS (UPLC-Q-TOF-MS) has been receiving more attention due to its high sensitivity, high-throughput and efficiency (Jing et al., 2017). UPLC-Q-TOF-MS together with a chemometric approach enables a comprehensive analysis of chemical components in tea (Dai et al., 2015; Zhou et al., 2019). Chemometric methods have been widely used in food analysis. Principal component analysis (PCA), projection to latent structures discriminate analysis (PLS-DA), variable importance for prediction (VIP) and hierarchical cluster analysis (HCA) have been employed to extract the key metabolites from overwhelming data sets obtained from high-throughput analysis (Bevilacqua et al., 2017).

Therefore, in this study, a non-targeted UPLC–Q-TOF-MS approach was applied to investigate the variations in the metabolite profiles of fermented and stored teas obtained by fermentation with probiotic *S. boulardii*, *L. plantarum* and the combination of *S. boulardii* and *L. plantarum*. The differentiation among probiotic teas fermented with starter culture variations was explored with the aid of chemometrics. Furthermore, the changes of tea components during storage were also evaluated.

2. Materials and methods

2.1. Sample preparation

Green tea (Guizhou, China) fermentation was conducted by inoculating probiotic yeast *S. boulardii* CNCM I-745 (Biocodex, Inc., California, USA), *L. plantarum* 299V (Probi, Singapore) and their combination into tea infusions. The tea infusions were obtained by brewing tea leaves (5% w/w) in hot water (95 °C) for 10 min, then removing tea leaves. Before inoculation, the tea infusions supplemented with 0.5% glucose and 0.06% Opti-White® yeast extract (Lallemand, Edwardstown, Australia) were sterilized at 121 °C for 15 min. The sterilized tea infusions were cooled down to room temperature, then inoculated with washed yeast and LAB cells (10 mL of 0.9% (w/v) NaCl solution × 3) to achieve an initial cell count about 5–7 log CFU/mL. The inoculated tea infusions were incubated at 30 °C for 2 days, followed by storing at 25 °C for 4 weeks. Uninoculated sterilized green tea infusions were incubated under the same conditions and served as the control. All the fermentations were done in triplicate and samples were withdrawn periodically for analysis.

The samples for UPLC-Q-TOF-MS analysis were prepared as follows: 700 μL of acetonitrile were added into 700 μL of tea infusion and mixed by using a Vortex Mixer at 272×g for 10 s, then the mixture was centrifuged at 7200×g for 10 min; afterwards, it was filtered through an 0.20-μm regenerated cellulose (RC) membrane. One hundred μL of filtered tea infusion were added into 600 μL of deionised (DI) water, vortexed at 272×g for 10 s, then centrifuged at 7200×g for 10 min. The supernatant was filtered through an 0.22 μm Polytetrafluoroethylene (PTFE) filter and diluted with 0.1% aqueous formic acid solution, then subjected to UPLC-Q-TOF-MS analysis.

2.2. UPLC-Q-TOF-MS analysis

The separation of non-volatile compounds was carried out by using Nexera X2 UPLC system (Shimadzu, Tokyo, Japan), with a Kinetex 2.6-μm Biphenyl column (2.1 mm × 100 mm,100 Å) (Phenomenex, USA) at 40 °C. The flow rate was 0.5 mL min^{-1} and the injection volume was 5 μL. The mobile phase A was 0.1% aqueous formic acid solution and mobile phase B was methanol/acetonitrile (v/v=30/70) with gradient elution. Mass spectrometry (MS) analysis was performed on a QTOF AB Sciex Triple TOF™ 5600 (AB SCIEX, USA), which was calibrated every five samples in a highly sensitive mode as recommended. The electrospray ionization (ESI) source was operated in negative mode, and spectra were obtained in full-scan mode. Declustering potential (DP), ion release delay (IRD) and ion release width (IRW) were set at 80 V, 67 V and 25 V, respectively. IDA MS/MS was conducted at the same collision energy (CE) of 40 V for all ions (intensity≥100 cps, ion tolerance≤10 ppm). All analyses were carried out in triplicate. Analyst 1.7.1 (Framingham, MA, USA), PeakView 2.2 and MultiQuant 3.0.2 were used for data acquisition and processing (He et al., 2020).

2.3. Data processing and statistical analysis

Raw data files acquired from the UPLC-Q-TOF-MS analysis were imported into the MS-DIAL ver. 4.38 software (RIKEN, Yokohama, Japan) to generate a peak table that included information on retention time, mass-to-charge ratio (m/z), and MS intensity of the metabolites in the sample. The signal-to-noise threshold for peak detection was set to 10. The retention time tolerance, MS 1 and MS 2 tolerances for the peak alignment were set to 0.2 min, 0.01 and 0.05 Da, respectively. PCA and partial least-squares discriminant analysis (PLS-DA) were performed using MS-DIAL after gap filling and normalization to investigate the overall tea metabolome variations caused by fermentation. Variable importance for prediction (VIP) analysis, fold change and ANOVA *p*-value were conducted with MS-DIAL ver. 4.38 software. Volcano plots were performed using Origin 2019 (OriginL. plantarum Corporation, Northampton, Massachusetts, USA).

2.4. Identification of compounds based on MS/MS spectra

The identification of metabolites was performed based on the accurate mass and mass spectrometric fragmentation patterns guided by the Human Metabolome Database (HMDB; http://www.hmdb.ca), MS-DIAL metabolomics MSP spectral kit (MSMS-Public-Neg-VS15 database), MassBank (https://massbank.eu), National Library of Medicine, National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/), related literatures (Dai et al., 2015; Zhou et al., 2019) and in-house databases based on commercial standards and theoretical MS2 fragments.

3. Results and discussion

3.1. Non-targeted analysis of tea samples by UPLC-Q-TOF-MS

Teas fermented and stored with *S. boulardii* CNCM I-745, *L. plantarum* 299V and a co-culture of both microorganisms were sampled and analysed for the comparison of substrate and metabolite changes in relation to the different fermentation treatments. A total of 1680 peaks were tentatively extracted and used for statistical analysis after alignment and normalization. The metabolites were identified as flavonoids, amino acids, organic acids, sugars, alkaloids, glycosides and fatty acids, etc.

In order to evaluate the metabolic differences between fermented and stored teas with different microorganisms, unsupervised PCA method based on the UPLC-Q-TOF-MS data sets was applied to obtain an overview of the difference in the metabolite phenotypes among the tea samples. Fig. 1A shows the PCA score plot with PC1 and PC2 explaining 42.7% of the total variance (26.7% and 16.0%, respectively). It could be observed that the *L. plantarum* fermented tea can be completely separated from the unfermented tea, *S. boulardii* and co-culture fermented teas based on the metabolic profiles, which further confirmed that microbial fermentations had significant effects on the tea metabolome during fermentation. Furthermore, the clear separation of the fermented tea samples from the stored tea samples in the score plot indicated a significant effect of the storage time on the tea metabolome.

A supervised PLS-DA analysis and variable importance for prediction (VIP) were employed to investigate the metabolites that showed the greatest difference. In total, 589 metabolites with VIP > 1 were filtered to do further analysis. As shown in the PLS-DA plot (Fig. 1B), the starter culture inoculated tea samples were separated from the unfermented sample by latent variable 1, and the stored samples were separated from the 2-day fermented samples by latent variable 2, which indicate different metabolite phenotypes for the teas fermented in different ways.
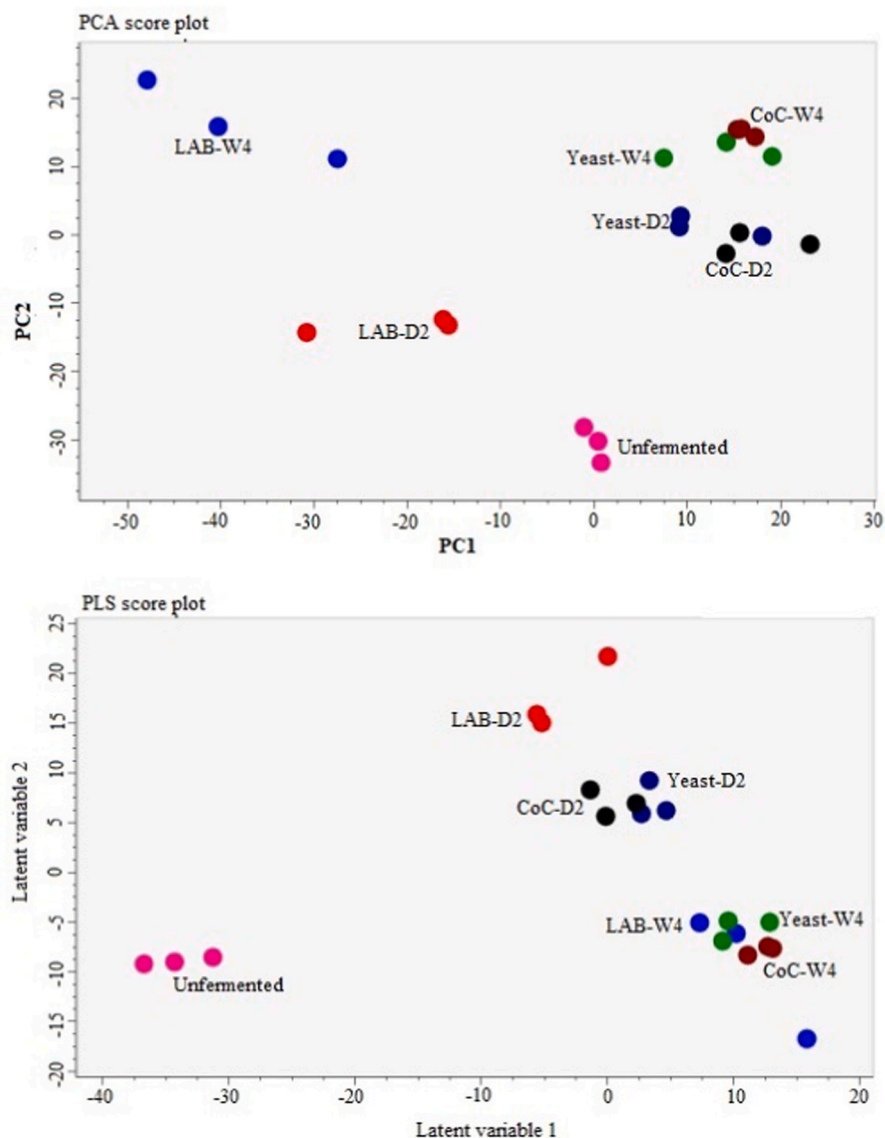
Fig. 1. Multivariate statistical analysis of unfermented (pink), *L. plantarum* fermented (red), *S. boulardii* fermented (dark blue), coculture fermented (black), *L. plantarum* stored (light blue), *S. boulardii* stored (green), co-culture stored (brown) tea samples: (A) PCA score plot; (B) PLS-DA score plot, R2Y =57.8%, Q2 = 59.2%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Influence of fermentation and storage on tea metabolome

3.2.1. Influence of S. boulardii fermentation

To further investigate the effects of *S. boulardii* on tea metabolome during fermentation and storage, a volcano-plot was employed to extract the key metabolites that contributed to the differentiation of pairwise samples (unfermented & *S. boulardii* fermented; 2-day fermented & 4-week storage teas). The number and degree of deviations of metabolites in each comparison group are shown in the form of volcano plots (Figs. 2–1: unfermented & 2-day fermented, Fig. 2–: 2-day fermented & 4-week storage). In total, 1680 metabolites in negative ion mode were evaluated in these two pairwise groups. The volcano plot analysis was performed with fold change > 2 and $p < 0.05$ using the ANOVA: 331 metabolites including organic acids, catechins, alkaloids and glycosides, etc. were significantly different in *S. boulardii* fermented and unfermented teas; further, 161 of 331 metabolites showed significantly lower levels in *S. boulardii* fermented tea and 170 metabolites exhibited higher levels. For the pair of 2-day fermented and 4-week stored teas (Fig. 2–), the number of down and up regulated

metabolites was 110 and 123 respectively, which were less than that in Figs. 2–1.

Table 1 shows the tentatively identified compounds with VIP > 1, fold change ≥ 2 and $p < 0.05$ in unfermented, *S. boulardii* fermented and stored teas. These metabolites were confirmed by comparing the RT and MS/MS fragment patterns with authentic standards and metabolomics databases. From Table 1, glucose, sucrose, fumaric acid, malic acid and most of the flavonol or flavone glycosides such as quercetin 3-O-galactosyl-rutinoside, kaempferol 3-O-galactosyl-rutinoside, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, gallic acid hexoside and aroma glycoside (2-phenylethyl-6-O-β-D-xylopyranosyl-β-D-glucopyranoside) were reduced significantly during the two days fermentation, while the cellular metabolic intermediates including guanosine 5′-monophosphate, adenosine 3′-monophosphate, adenosine 3′:5′-cyclic-monophosphate, and 2′-deoxyguanosine 5′-monophosphate were clearly generated at higher levels during fermentation and storage. The highest contents of these monophosphates appeared at the day-2 fermentation time. Meanwhile, some flavone aglycones such as eriodictyol, luteolin, naringenin, apigenin, kaempferol and flavone
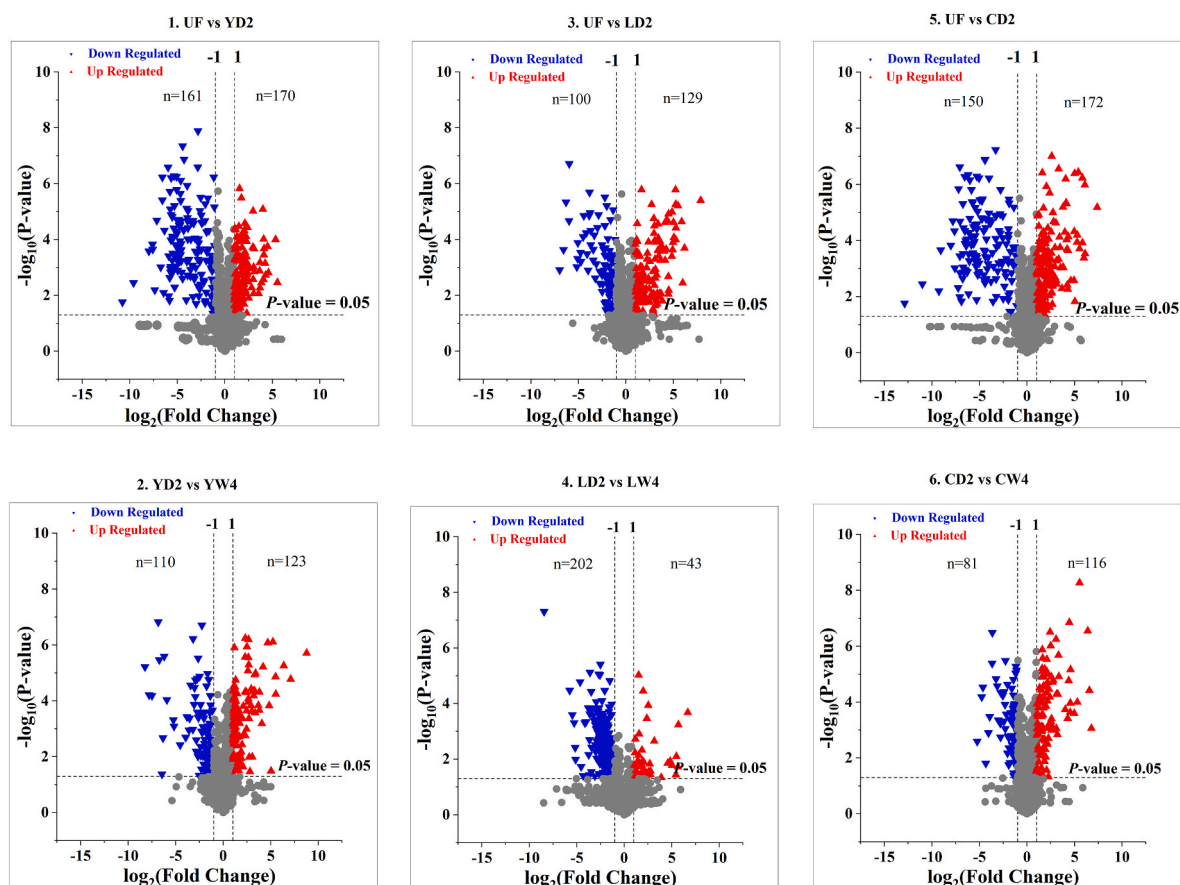
Fig. 2. Volcano-plots of unfermented, fermented and stored tea samples; (2–1)- unfermented & 2-day *S. boulardii* fermented; (2-2)-2-day *S. boulardii* fermented & 4-week *S. boulardii* storage; fermented and stored tea samples, (2–3)-unfermented & 2-day *L. plantarum* fermented; (2–4)-2-day *L. plantarum* fermented & 4-week *L. plantarum* storage; (2–5)-unfermented & 2-day co-culture fermented; (2–6)-2-day co-culture fermented & 4-week co-culture storage.

glycosides increased during fermentation and storage.

Sugars (sucrose and glucose) as the major energy sources were nearly used up in the exponential stage to support *S. boulardii* growth. When the available nutrients were exhausted by proliferating *S. boulardii* cells, they entered a stationary phase (Herman, 2002). The biochemical characteristics of the stationary phase *S. boulardii* cells would change since the cellular energy-consuming metabolic activity decreased to save ATP for energy-dependent stress responses (Zakrajšek et al., 2011). The volcano plot of fermented and stored teas (Fig. 2–) showed that the number of metabolites with significant changes was far less than that in the pair of unfermented and 2-day fermented tea samples. This result was in line with the decline of cell metabolism during the stationary phase (Zakrajšek et al., 2011). The decreases of flavone glycosides and the increases of flavone aglycones could be due to the *S. boulardii* glycosidase that broke the glycosidic bond to generate corresponding secondary metabolites (Wilkowska and Pogorzelski, 2017). Besides, several flavone glycosides were also accumulated during the long-time storage and these flavone glycosides could originate from other flavone di- and/or tri-glycosides that lost one or more glycosyl groups via glycosidase-mediated hydrolysis (Fig. 3) (Dai et al., 2015). From the UPLC-Q-TOF-MS analysis, the glycoside of 2-phenylethanol (2-phenyl-ethyl-6-O-β-D-xylopyranosyl- β-D-glucopyranoside) was also found (Fig. 3), which further verified the results discussed in our previous study that the key flavor compound 2-phenylethanol was not only from phenylalanine metabolism but also from glycoside precursor hydrolysis (Wang et al., 2020).

3.2.2. Influence of L. plantarum fermentation

The effects of *L. plantarum* fermentation and storage were evaluated

in the same way as *S. boulardii*. A total of 1680 peaks obtained from UPLC-Q-TOF-MS were subjected to multivariate analysis to extract the key differences among unfermented, fermented and stored teas. The pairwise comparisons of two groups (unfermented & 2-day fermented teas - Figs. 2–3; 2-day fermented & 4-week storage teas - Figs. 2–4) were performed by volcano plots to unearth the critical compounds. In total, 229 compounds were significantly different in *L. plantarum* fermented and unfermented teas; 129 of 229 metabolites showed significantly higher levels and 100 metabolites showed lower levels in *L. plantarum* fermented tea (Figs. 2–3). For the pair of 2-day fermented and 4-week stored teas (Figs. 2–4), there were 202 metabolites that decreased and only 43 metabolites increased in 4-week stored tea. The significantly changed (fold change ≥ 2 and $p < 0.05$) metabolites in unfermented, *L. plantarum* fermented and stored teas are presented in Table 2. These metabolites were tentatively identified by comparing the MS/MS data of standards and public data base.

As expected, *L. plantarum* exerted different effects on tea metabolome from *S. boulardii*, and the differences could be summarized into three points. First, flavone glycosides (quercetin-3,4′-O-di-β-glucoside, quercetin 3-O-galactosyl-rutinoside, myricetin 3-galactoside, luteolin-6-C-glucoside, vitexin (apigenin-8-C-glucoside), kaempferol 7-O-glucoside) (Table 2) decreased significantly in *L. plantarum* fermented tea infusion; however, the individual flavone glycosides impacted by *L. plantarum* differed from that in *S. boulardii* fermentation. The significantly reduced flavone glycosides in *L. plantarum* fermented tea included both C-glycosides and O-glycosides, whereas only O-glycosides were affected by *S. boulardii* fermentation (Fig. 3). Microbes can influence the flavonoid glycosides in three ways during fermentation: cell wall absorption, flavonoid glycoside hydrolysis and formation of new

Table 1

Compounds with VIP > 1, fold change ≥ 2 and *p* < 0.05 in unfermented, *S. boulardii* fermented and stored teas.

RT	M/Z	Compounds	ANOVA *p*-value	Fold change (Max/Min)
Down regulated				
0.45	275.0157	6-Phosphogluconic acid	0.001	2.72
0.46	165.0412	3-Methylxanthine	0.001	2.54
0.46	280.1030	1-Methyladenosine	<0.001	28.00
0.46	179.0565	Glucose	<0.001	3.48
0.47	341.1086	Sucrose	<0.001	20.05
0.49	115.0041	Fumaric acid	<0.001	2.63
0.49	133.0146	Malic acid	<0.001	3.60
0.53	331.0678	Gallic acid hexoside	0.001	2.02
0.70	455.0980	Riboflavin-5′-monophosphate	<0.001	38.50
0.73	290.0898	N-Fructosyl pyroglutamate	<0.001	2.90
0.76	441.1104	(−)-Catechin gallate (CG)[a]	0.01	2.69
0.83	202.1114	N6-(delta2-isopentenyl)-adenine	<0.001	78.67
0.84	292.1400	N-Fructosyl isoleucine	<0.001	285.83
1.47	451.1260	Aspalathin	0.001	76.05
1.59	462.0967	7-Methylthioheptyl glucosinolate	0.001	228.43
8.32	337.0910	4-O-p-Coumaroylquinic acid	0.005	2.00
9.22	577.1315	Procyanidin B1	0.001	2.08
10.18	635.0922	1,3,6-tri-O-Galloylglucose	0.001	2.45
10.33	491.1418	4-Acetyl-2-hydroxyphenyl 2-O-beta-D-Fructofuranosyl-beta-D-xylopyranoside	<0.001	2.00
10.35	649.1392	Flavonoid-7-O-glucuronides	<0.001	16.45
10.39	461.1643	2-Phenylethyl-6-O-β-D-xylopyranosyl- β-D-glucopyranoside	<0.001	2.00
10.62	771.2018	Quercetin 3-O-galactosyl-rutinoside	0.001	1.98
11.07	755.2063	Kaempferol 3-O-galactosyl-rutinoside	0.001	1.97
11.61	739.2065	Kaempferol-3-galactoside-6″-rhamnoside-3‴-rhamnoside	<0.001	1.98
11.66	593.1509	Kaempferol-3-O-rutinoside[a]	0.002	1.96
11.79	447.0937	Kaempferol-3-O-glucoside[a]	<0.001	2.25
13.70	306.0534	2-Deoxycytidine-5-monophosphoric acid (dCMP)	<0.001	76.24
Up regulated				
0.46	235.0626	Austadiol	<0.001	38.54
0.48	362.0484	Guanosine 5′-monophosphate (GMP)	0.014	1.98
0.48	346.0538	Adenosine 3′-monophosphate (AMP)	0.001	1.97
0.50	173.0940	Gly-Val	0.001	49.87
0.69	328.0457	Adenosine 3′:5′-cyclicmonophosphate	<0.001	7.69
0.69	346.0545	2′-Deoxyguanosine 5′-monophosphate (dGMP)	<0.001	19.32
0.75	117.0198	Succinic acid	<0.001	4.10
9.34	416.1340	Sorbicillactone A	0.004	3.07
11.76	767.1836	Unknown	0.001	4.48
11.78	303.0503	Haemoventosine	0.002	27.37
12.49	389.0842	5,7-dihydroxy-2-(3-hydroxy-4,5-dimethoxyphenyl)-3,6-dimethoxy-4H-chromen-4-one	<0.001	3.92
13.00	287.0566	Eriodictyol[a]	<0.001	18.72
13.10	301.0333	Quercetin[a]	<0.001	2.04
13.15	285.0409	Luteolin[a]	0.004	1.98
13.84	271.0619	Naringenin[a]	<0.001	5.84
13.85	269.0428	Apigenin[a]	<0.001	2.25
13.88	285.0410	Kaempferol[a]	0.001	9.68
14.89	593.0840	Biflavonoids	<0.001	5.82
14.96	425.0784	Epiafzelechin 3-gallate	0.001	11.26
15.50	577.2098	Apigenin-8-C-glucoside-2′-rhamnoside	0.001	9.40
15.66	595.0853	Phenolic glycosides	0.001	7.87
17.13	543.1536	4,5-Diferuloylquinic acid	0.046	3.66

Note.

[a] Confirmed with authentic standards.

metabolites. Both yeast and LAB cell walls can absorb glycosides, but the absorption is species-dependent, which could partially contribute to the variations of flavonoid glycosides in *L. plantarum* and *S. boulardii* fermented tea infusions (Devi, Aiyappaa & Waterhouse, 2019; Božič et al., 2020). Glycosidase hydrolysis plays a part in flavonoid glycosides during fermentation and the various changes of flavonoid glycosides in *L. plantarum* and *S. boulardii* cultures suggested that the enzyme active sites of glycosidases in *L. plantarum* and *S. boulardii* could be originally different or impacted by pH, since the pH of *L. plantarum* fermented tea was lower (pH ≈ 3.7) than *S. boulardii* fermented tea (pH ≈ 4.8) (Michlmayr and Kneifel, 2014). In addition, the changes of flavone aglycones exhibited opposite trends to that in *S. boulardii* fermented tea. For example, myricetin, luteolin, apigenin, kaempferol, and quercetin decreased during *L. plantarum* fermentation and storage. Following the hydrolysis of flavone glycosides, the flavone aglycones could be further degraded via B-ring and C-ring fission, thus leading to the reduction of flavone aglycones (Fig. 3); this result concurred with the research that LAB could metabolize flavone aglycones during fermentation (Guo et al., 2021).

Second, *L. plantarum* degraded gallic acid and quinic acid derivatives (3,4-di-O-caffeoylquinic acid and 4,5-dicaffeoylquinic acid), which were not found in *S. boulardii* fermentation. *L. plantarum* possesses a decarboxylase that decarboxylates gallic acid to another antibacterial agent - pyrogallol (Reverón, de las Rivas, Matesanz, Muñoz, & López de Felipe, 2015), and pyrogallol was also found increasing during the fermentation and storage (Table 2). The quinic acid derivatives could be converted into caffeic acid and quinic acid by specific esterases of *L. plantarum*, then caffeic acid and quinic acid could be further oxidized or reduced to corresponding secondary products (Naranjo Pinta et al., 2018).

Third, two food-borne pathogen inhibitors D-phenyllactic acid (PLA) and *p*-hydroxy-D-phenyllactic acid (OH-PLA) increased by 125-fold and 97-fold after 4 weeks storage in *L. plantarum* fermented tea. PLA and *p*-OH-PLA were generated in *L. plantarum* from the degradation of phenylalanine and tyrosine (Tyr), respectively (Chesters, Walker, O'Hagan and Floss, 1996). These two compounds are known to possess effective antimicrobial activity and have the potential to be used as novel antimicrobial agents in food preservation. As such, *L. plantarum* fermentation may provide probiotic tea with natural and self-originated preservatives (Dao et al., 2019).

3.2.3. Influence of co-culture fermentation

Both *S. boulardii* and *L. plantarum* respectively exerted various influences on the tea metabolome as discussed above. A co-culture of both yeast and LAB was also investigated to uncover the interactive effects of the two microorganisms on tea components during fermentation and storage. Figs. 2–5 and 2-6 illustrate the significantly changed metabolites in co-cultured teas (Figure 2-5-unfermented & 2-day fermented teas, Figs. 2-6-2-day fermented & 4-week stored teas). In general, the number of metabolites which were significantly regulated was far greater in the fermentation stage (Figs. 2–5)than in the storage stage (Figs. 2–6). In total, 322 metabolites with significant changes were revealed in co-culture fermented tea; 150 of them showed lower levels and the other 172 metabolites presented higher levels after 2-day fermentation. For the storage stage (Figs. 2–6), 81 of 197 metabolites were down regulated and 116 metabolites were up regulated in the stored tea. More information on these key metabolites was extracted and is presented in Table 3. The metabolites were identified by comparing the MS/MS data with authentic standards and public data base.

Co-cultured tea shared the metabolite profiles with that of both *S. boulardii* and *L. plantarum* to some degree; meanwhile, it also showed specific metabolic characteristics. The conversion of sugars and organic acids and the production of cellular metabolic intermediates (the monophosphates) in co-cultured teas showed similar trends to that in the monocultures of *S. boulardii* and *L. plantarum*. The flavone or flavonoid glycosides were more stable in the co-culture than in the monocultures, since less glycosides decreased significantly in the co-
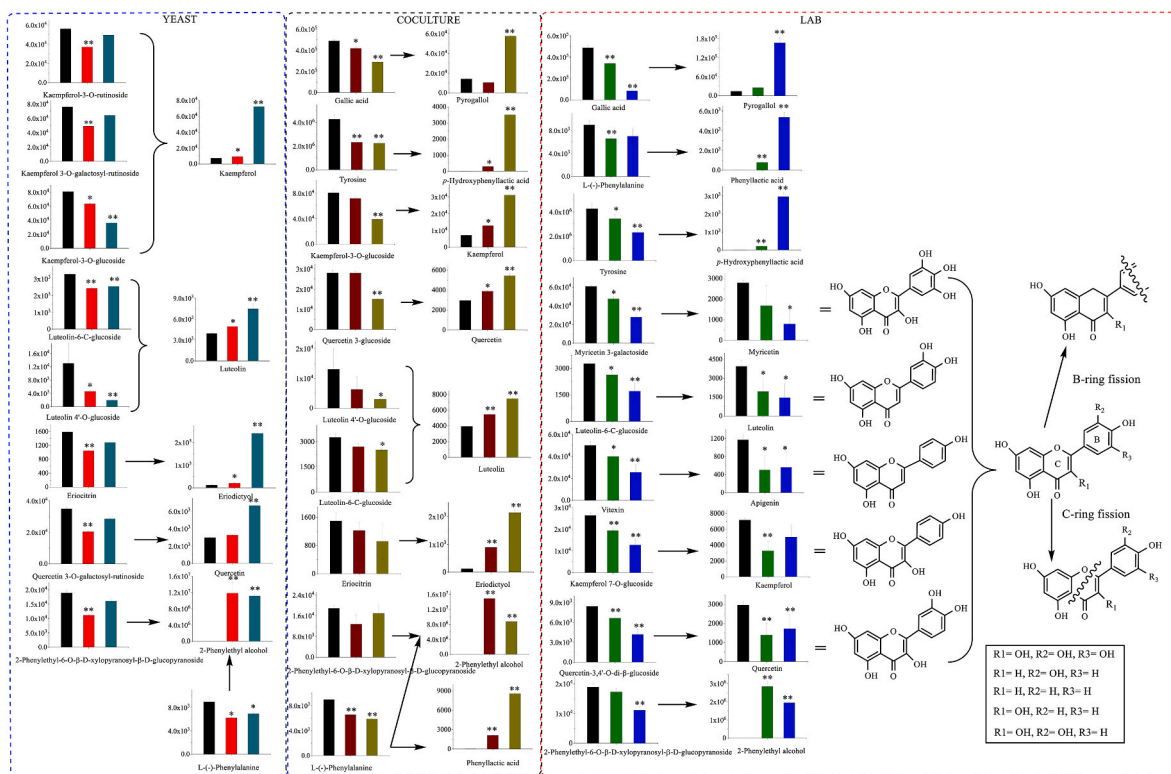
Fig. 3. Metabolic pathway changes in metabolites caused by tea fermentation. The black, red, dark cyan, wine, dark yellow, green and blue columns represent the mass intensity of metabolites in yeast, coculture, and LAB fermented tea samples, respectively; (*$p < 0.05$; ** < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

culture. In addition, several flavone aglycones including eriodictyol, quercetin, luteolin, naringenin, apigenin and kaempferol accumulated in the co-cultured tea (Fig. 3). As discussed in Sections 3.2.1 and 3.2.2, both yeast and LAB can change the flavonoid profile by cell wall absorption, glycosidase hydrolysis and generation of new metabolites. Besides, LAB can also degrade flavone aglycones by breaking B-ring and C-ring (Guo et al., 2021). In the co-culture, less flavonoid glycosides were down-regulated than in yeast and LAB monocultures, but more flavone aglycones were up-regulated than in the *L. plantarum* monoculture. Flavonoid glycosides could be absorbed by microbe cell walls or hydrolyzed by glycosidases to release flavone aglycones and sugar. The changes of flavonoid glycosides in the co-culture suggested that the cell wall absorption and glycosidase activities could be inhibited during co-culture fermentation. Unlike the decreases in *L. plantarum* monoculture, flavone aglycones accumulated in the co-culture even *L. plantarum* existed, suggesting that the flavones-degrading enzymes in *L. plantarum* could be inhibited in the co-culture.

D- Phenyllactic and *p*-hydroxy-D-phenyllactic acids are the products of amino acids decomposition (Phe and Tyr) of *L. plantarum* fermentation (Dao et al., 2019). These two aromatic acids were also found increased by 187 and 113-fold in the co-cultured tea. As discussed in *L. plantarum* fermentation, the increase of PLA and *p*-OH-PLA was 125-fold and 97-fold, respectively. Hence, the production of PLA and *p*-OH-PLA was enhanced significantly in the co-culture. The higher contents of PLA and *p*-OH-PLA in the co-culture could be due to more substrates (phenylalanine and tyrosine) provided by yeast, as *S. boulardii* cells contain abundant amino acids that can be released, which can support the metabolism of *L. plantarum* (Ponomarova et al., 2017). Most importantly, *L. plantarum* survived much longer in the co-culture and this would enable production of more PLA and *p*-OH-PLA.

Another interesting finding was the gallic acid changes in different fermentation systems. Unlike the remarkable decrease of gallic acid in *L. plantarum* monoculture, the gallic acid content was not significantly

influenced by co-culturing. Meanwhile, the decarboxylated product of gallic acid - pyrogallol showed no significant change in the co-culture, which may suggest that the expression of decarboxylase related genes or decarboxylase activity could be inhibited in the co-culture (Reverón et al., 2015) and further research is needed to investigate the mechanism of decarboxylase expression.

In summary, this metabolomics study on tea revealed the significant impact of different cultures and their combination on the tea constituents with the discovery of numerous previously unreported bioactive compounds, especially flavonoids and amino acid derivatives. These findings would lay the foundation for developing novel fermented functional tea beverages, particularly in the emerging field of probiotics and postbiotics.

4. Conclusion

Compounds from teas fermented with different probiotic microorganisms were obtained using UPLC-Q-TOF-MS analysis, through which the differential metabolites contributing to their special metabolic profiles were identified, such as PLA and *p*-OH-PLA in *L. plantarum* associated samples, flavone glycosides and aglycones in all tea infusions. Some flavone glycosides in *S. boulardii* fermented tea decreased significantly and flavone aglycones and other flavone glycosides were generated during fermentation and storage. In L. *plantarum* fermented tea, the metabolic profile of flavone glycosides was different from that in *S. boulardii* fermented tea; both C-glycosides and O-glycosides were hydrolyzed and the flavone aglycones were further degraded in *L. plantarum* monoculture. The profiles of flavone glycosides and aglycones in co-cultured tea differed from that in *S. boulardii* or *L. plantarum* fermented tea; less glycosides were degraded but a greater number of aglycones produced in the co-culture. In general, UPLC-Q-TOF-MS analysis coupled with chemometrics presented great potential for the untargeted metabolic analysis of fermented teas.

Table 2

Compounds with VIP > 1, fold change ≥ 2 and *p* < 0.05 in unfermented, *L. plantarum* fermented and stored teas.

RT	M/Z	Compound	ANOVA *p*-value	Fold change (Max/Min)
Down regulated				
0.45	275.0157	6-Phosphogluconic acid	<0.001	13.06
0.46	165.0412	3-Methylxanthine	0.001	4.20
0.46	179.0565	Glucose	<0.001	3.15
0.46	195.0530	Gluconic acid	0.007	2.00
0.47	191.0565	Citric acid	<0.001	6.50
0.49	115.0041	Fumaric acid	0.001	3.54
0.72	331.0672	Gallic acid hexoside	0.02	2.32
0.81	282.0844	Guanosine	<0.001	33.46
0.84	292.1400	N-Fructosyl isoleucine	<0.001	4.78
0.85	169.0150	Gallic acid[a]	<0.001	5.10
0.86	484.1389	Uridine 5-triphosphate	0.024	19.97
8.19	319.0434	Unknown	<0.001	8.00
8.57	633.0711	Hydrolyzable tannins	0.001	71.10
9.01	337.0926	5-O-*p*-Coumaroylquinic acid	0.001	2.59
9.22	577.1315	Procyanidin B1	0.001	2.46
9.43	451.1244	Aspalathin	0.001	7.19
10.26	625.1407	Quercetin-3,4′-O-di-beta-glucoside	0.001	1.95
10.28	479.0825	Myricetin 3-galactoside	0.008	2.27
10.37	447.0920	Luteolin-6-C-glucoside	0.045	1.97
10.49	635.0885	1,3,6-tri-O-galloylglucose	0.001	2.86
10.50	291.1266	Curvularin	<0.001	388.94
10.62	771.2018	Quercetin 3-O-galactosyl-rutinoside	0.037	1.96
11.16	431.0196	Vitexin[a]	0.016	2.26
11.19	463.0895	Hirsutrin	0.034	1.96
11.60	447.0917	Kaempferol 7-O-glucoside	0.020	2.03
11.89	515.0772	3,4-di-O-Caffeoylquinic acid[a]	0.036	33.13
14.28	517.0979	Unknown	0.001	126.73
15.30	515.1200	4,5-Dicaffeoylquinic acid[a]	0.015	61.77
15.37	467.1353	Unknown	0.001	81.39
17.10	393.1712	Aloesin	<0.001	7.41
Up regulated				
0.50	173.0940	Gly-Val	0.001	40.32
0.54	187.0424	1-Hydroxy-2-naphthoic acid	<0.001	234.66
0.70	455.0980	Riboflavin-5′-monophosphate	0.023	2.15
0.96	125.0251	Pyrogallol	<0.001	14.20
2.10	181.1510	*p*-Hydroxy-D-phenyllactic acid	0.008	97.01
7.96	165.0558	D-Phenyllactic acid	<0.001	124.78
7.97	233.0434	Anhydrobrazilic Acid	<0.001	122.29
9.31	186.1138	3-(2-amino-2-oxoethyl)-5-methyl-Hexanoic acid	<0.001	9.20
9.87	416.1343	Sorbicillactone A	<0.001	2.31
17.05	337.2048	Bavachinin A	<0.001	13.96
17.14	303.0378	Thymidine-3′,5′-cyclic monophosphate	0.01	4.55

Note.

[a] Confirmed with authentic standards.

Table 3

Compounds with VIP > 1, fold change ≥ 2 and *p* < 0.05 in unfermented, coculture fermented and stored teas.

RT	M/Z	Compound	ANOVA *p*-value	Fold change (Max/Min)
Down regulated				
0.46	165.0412	3-Methylxanthine	0.001	2.57
0.46	179.0650	Glucose	<0.001	3.75
0.47	341.1086	Sucrose	<0.001	23.48
0.49	115.0041	Fumaric acid	0.001	4.17
0.47	191.0565	Citric acid	<0.001	3.31
0.49	133.0146	Malic acid	<0.001	6.63
0.70	455.0980	Riboflavin-5′-monophosphate	<0.001	17.29
0.73	290.0898	N-Fructosyl pyroglutamate	<0.001	2.97
0.81	282.0844	Guanosine	0.006	9.20
0.83	202.1114	N6-(Delta2-Isopentenyl)-adenine	<0.001	64.66
0.84	292.1400	N-Fructosyl isoleucine	<0.001	146.88
9.22	577.1315	Procyanidin B1	<0.001	2.09
9.43	451.1244	Aspalathin	<0.001	42.13
9.78	387.1655	12:4+3O Fatty acyl hexoside	0.003	2.51
10.49	635.0885	1,3,6-tri-O-Galloylglucose	<0.001	9.73
11.11	463.0892	Quercetin 3-glucoside	0.05	1.95
11.31	477.0622	Indolylmethyl glucosinolate + 1MeO	0.004	2.07
11.61	739.2065	Kaempferol-3-galactoside-6″-rhamnoside-3‴-rhamnoside	0.001	1.95
11.79	447.0937	Kaempferol-3-O-glucoside[a]	<0.001	1.95
13.7	306.0534	2′-Deoxycytidine 5′-monophosphate (dCMP)	<0.001	107.51
Up regulated				
0.44	241.0832	Thymidine	0.001	4.01
0.48	346.0532	Adenosine 3′-monophosphate	0.001	2.04
0.48	362.0484	Guanosine 5′-monophosphate	0.001	2.29
0.50	173.0940	Gly-Val	<0.001	58.93
0.69	328.0457	Adenosine 3′:5′-cyclicmonophosphate	<0.001	10.72
0.69	346.0545	Deoxyguanosine monophosphate (dGMP)	<0.001	23.46
0.75	117.0198	Succinic acid	<0.001	4.98
2.10	181.0510	*p*-Hydroxyphenyllactic acid	<0.001	112.57
7.96	165.0558	Phenyllactic acid	<0.001	186.94
7.97	233.0434	Anhydrobrazilic Acid	<0.001	210.08
9.31	186.1138	3-(2-Amino-2-oxoethyl)-5-methyl-hexanoic acid	<0.001	11.30
9.34	416.134	Sorbicillactone A	0.001	3.41
11.78	303.0503	Haemoventosine	<0.001	16.11
12.49	389.0842	6-O-methylated Flavonoids	<0.001	2.08
13.00	287.0566	Eriodictyol[a]	<0.001	17.88
13.10	301.0333	Quercetin[a]	<0.001	2.00
13.15	285.0409	Luteolin[a]	0.001	2.02
13.29	449.0735	Myricetin-3-O-xyloside	0.002	6.75
13.84	271.0619	Naringenin[a]	<0.001	5.21
13.85	269.0425	Apigenin[a]	<0.001	2.00
13.88	285.0410	Kaempferol[a]	<0.001	3.89
14.89	593.0840	Biflavonoids	<0.001	9.47
14.96	425.0784	Epiafzelechin 3-gallate	<0.001	14.91

Note.

[a] Confirmed with authentic standards.

CRediT authorship contribution statement

Rui Wang: Formal analysis, Writing – original draft, conceived and designed the experiments, performed the experiments, analysed the data wrote the paper. **Jingcan Sun:** Formal analysis, performed the analysed the dataexperiments. **Benjamin Lassabliere:** conceived and designed the experiments. **Bin Yu:** performed the experiments.. **Shao Quan Liu:** Writing – original draft, conceived and designed the experiments, wrote the paper.

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.

References

Aspri, M., Papademas, P., Tsaltas, D., 2020. Review on non-dairy probiotics and their use in non-dairy based products. Fermentatio 6 (1), 30. https://doi.org/10.3390/fermentation6010030.

Bevilacqua, M., Bro, R., Marini, F., Rinnan, Å., Rasmussen, M.A., Skov, T., 2017. Recent chemometrics advances for foodomics. Trac. Trends Anal. Chem. 96, 42–51. https://doi.org/10.1016/j.trac.2017.08.011.

Božič, J.T., Butinar, L., Albreht, A., Vovk, I., Korte, D., Vodopivec, B.M., 2020. The impact of *Saccharomyces* and non-*Saccharomyces* yeasts on wine colour: a laboratory

study of vinylphenolic pyranoanthocyanin formation and anthocyanin cell wall adsorption. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 123, 109072. https://doi.org/10.1016/j.lwt.2020.109072, 0023–6438.

Chan, A.M.Z., Toh, M., Liu, S.Q., 2021. Growth, survival, and metabolic activities of probiotics *Lactobacillus rhamnosus* GG and *Saccharomyces cerevisiae* var. *boulardii* CNCM-I745 in fermented coffee brews. Int. J. Food Microbiol. 350, 109229. https://doi.org/10.1016/j.ijfoodmicro.2021.109229.

Chesters, N.C.J.E., Walker, K., O'Hagan, D., Floss, H.G., 1996. The biosynthesis of tropic acid: a reevaluation of the stereochemical course of the conversion of phenyllactate to tropate in *Datura stramonium*. J. Am. Chem. Soc. 118 (4), 925–926. https://doi.org/10.1021/ja9531485.

Dai, W., Qi, D., Yang, T., Lv, H., Guo, L., Zhang, Y., Lin, Z., 2015. Nontargeted analysis using ultraperformance liquid chromatography-time-of-mass spectrometry uncovers the effects of harvest season on the metabolites and taste quality of tea (*Camellia sinensis* L.). J. Agric. Food Chem. 63 (44), 9869–9878. https://doi.org/10.1021/acs.jafc.5b03967.

Dao, Y., Zhang, K., Lu, X., Lu, Z., Liu, C., Liu, M., Luo, Y., 2019. Role of glucose and 2-oxoglutarate/malate translocator (OMT1) in the production of phenyllactic acid and *p*-hydroxyphenyllactic acid, two food-borne inhibitors. J. Agric. Food Chem. 67 (20), 5820–5826. https://doi.org/10.1021/acs.jafc.9b01444.

Ge, Y., Bian, X., Sun, B., Zhao, M., Ma, Y., Tang, Y., Wu, J.L., 2019. Dynamic profiling of phenolic acids during pu-erh tea fermentation using derivatization liquid chromatography-mass spectrometry approach. J. Agric. Food Chem. 67 (16), 4568–4577. https://doi.org/10.1021/acs.jafc.9b00789.

Guo, X., Guo, A., Li, E., 2021. Biotransformation of two citrus flavanones by lactic acid bacteria in chemical defined medium. Bioproc. Biosyst. Eng. 44 (2), 235–246. https://doi.org/10.1007/s00449-020-02437-y.

He, G.Y., Han, X., Han, M., Qiu, S.T., Li, Y., Zhang, S.l., Chen, X., 2020. UPLC-QTOF-MS analysis of 13 isomers of polyphenols. Chin. J. Synth. Chem. 28 (10), 9.

Herman, P.K., 2002. Stationary Phase in Yeast, 5. Elsevier Ltd, London, pp. 602–607.

Jing, J., Shi, Y., Zhang, Q., Wang, J., Ruan, J., 2017. Prediction of Chinese green tea ranking by metabolite profiling using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF/MS). Food Chem. 221, 311–316. https://doi.org/10.1016/j.foodchem.2016.10.068.

Kakisu, E., Abraham, A.G., Tironi Farinati, C., Ibarra, C., De Antoni, G.L., 2012. *Lactobacillus plantarum* isolated from kefir protects vero cells from cytotoxicity by type-II shiga toxin from *Escherichia coli* O157:H7. J. Dairy Res. 80 (1), 64–71. https://doi.org/10.1017/S0022029912000659.

Li, Q., Huang, J., Li, Y., Zhang, Y., Luo, Y., Chen, Y., Liu, Z., 2017. Fungal community succession and major components change during manufacturing process of Fu brick tea. Sci. Rep. 7 (1), 6947–6949. https://doi.org/10.1038/s41598-017-07098-8.

Michlmayr, H., Kneifel, W., 2014. β-Glucosidase activities of lactic acid bacteria: mechanisms, impact on fermented food and human health. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 352 (1), 1–10. https://doi.org/10.1111/1574-6968.12348.

Naranjo Pinta, M., Montoliu, I., Aura, A.M., Seppänen-Laakso, T., Barron, D., Moco, S., 2018. In vitro gut metabolism of [U-^{13}C]-quinic acid, the other hydrolysis product of chlorogenic acid. Mol. Nutr. Food Res. 62 (22), 1800396. https://doi.org/10.1002/mnfr.201800396.

Nayak, S.K., 2011. Biology of eukaryotic probiotics. In: Liong, M.T. (Ed.), Probiotics: Biology, Genetics and Health Aspects. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 29–55.

Ponomarova, O., Gabrielli, N., Sévin, D.C., Mülleder, M., Zirngibl, K., Bulyha, K., Patil, K. R., 2017. Yeast creates a niche for symbiotic lactic acid bacteria through nitrogen overflow. Cell Systems 5 (4), 345–357. https://doi.org/10.1016/j.cels.2017.09.002 e346.

Reverón, I., de las Rivas, B., Matesanz, R., Muñoz, R., López de Felipe, F., 2015. Molecular adaptation of *Lactobacillus plantarum* WCFS1 to gallic acid revealed by genome-scale transcriptomic signature and physiological analysis. Microb. Cell Factories 14 (1). https://doi.org/10.1186/s12934-015-0345-y, 160-160.

Tao, W., Zhou, Z., Zhao, B., Wei, T., 2016. Simultaneous determination of eight catechins and four theaflavins in green, black and oolong tea using new HPLC-MS-MS method. J. Pharmaceut. Biomed. Anal. 131, 140–145. https://doi.org/10.1016/j.jpba.2016.08.020.

Wang, Q., Gong, J., Chisti, Y., Sirisansaneeyakul, S., 2015. Fungal isolates from a Pu-erh type tea fermentation and their ability to convert tea polyphenols to theabrownins. J. Food Sci. 80 (4), M809–M817. https://doi.org/10.1111/1750-3841.12831.

Wang, R., Sun, J., Lassabliere, B., Yu, B., Liu, S.Q., 2020. Biotransformation of green tea (*Camellia sinensis*) by wine yeast *Saccharomyces cerevisiae*. J. Food Sci. 85 (2), 306–315. https://doi.org/10.1111/1750-3841.15026.

Wilkowska, A., Pogorzelski, E., 2017. Aroma enhancement of cherry juice and wine using exogenous glycosidases from mould, yeast and lactic acid bacteria. Food Chem. 237, 282–289. https://doi.org/10.1016/j.foodchem.2017.05.120.

Zakrajšek, T., Raspor, P., Jamnik, P., 2011. *Saccharomyces cerevisiae* in the stationary phase as a model organism-characterization at cellular and proteome level. J. Proteomics 74 (12), 2837–2845. https://doi.org/10.1016/j.jprot.2011.06.026.

Zhang, L., Li, N., Ma, Z.Z., Tu, P.F., 2011. Comparison of the chemical constituents of aged pu-erh tea, rpened pu-erh tea, and other teas using HPLC-DAD-ESI-MSn. J. Agric. Food Chem. 59 (16), 8754–8760. https://doi.org/10.1021/jf2015733.

Zhou, P., Hu, O., Fu, H., Ouyang, L., Gong, X., Meng, P., Wang, Y., 2019. UPLC-Q-TOF/MS-based untargeted metabolomics coupled with chemometrics approach for tieguanyin tea with seasonal and year variations. Food Chem. 283, 73–82. https://doi.org/10.1016/j.foodchem.2019.01.050.