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Effects of *Saccharomyces cerevisiae* strains on the metabolomic profiles of Guangan honey pear cider

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ABSTRACT

In recent years, the interest in cider produced from pear has considerably increased as a way to add value to fruits unsuitable for direct consumption or to valorize pear varieties grown in restricted regions. In the current study, with the China National Geographic Indication Products Guangan honey pear as raw material, four commercial *Saccharomyces cerevisiae* strains, namely RW, SY, DV10 and Drop Acid Yeast (DAY), were used to produce pear cider, comparing their taste characteristics and metabolomic profiles changes through the combination of GC-MS, ^{1}H-NMR and E-tongue. The results showed that there was a clear classification from the data obtained according to different yeast strains fermented Guangan honey pear cider, represented by a high discrimination index. Several pathways were altered during fermentation due to the distinct yeast strains, namely arginine and proline metabolism, glycine and serine metabolism, urea cycle, alanine metabolism, citric acid cycle, amino sugar metabolism, carnitine synthesis and warburg effect. This study could help improve the quality of Guangan honey pear cider by selecting different *Saccharomyces cerevisiae* yeasts and, in turn, it could shed lights on the large-scale production of pear cider.

1. Introduction

Pear cider, also known as pear wine or perry, is made by fermenting pear juice, with an ethanol concentration that does not exceed 8% (v/v) (Jarvis, 1996). In each country where pears are grown, pear cider is traditionally produced from specific varieties, leading to the product that can be strictly called perry (https://www.nationalperrypearcentre.org.uk/. Accessed December 10, 2022). Anyway, pear producers recently explored the possibility to produce pear cider as a tool for adding value to pear varieties intended for fresh consumption, when defects like below-standards fruit size make them unsuitable for direct consumption (Guerrini et al., 2022; Kieliszek et al., 2018), or when overproduction reduces margins for pears growers (Khalifa et al., 2020; Wang et al., 2011). Perry makers have also noted that consumption of their product is well suited to the spread desire to consume local products, developing niche brands based on varieties produced in restricted geographic areas. These factors, together with an evolution of consumer's preferences towards drinks less alcoholic than grape wine, have contributed to the steady growth of pear cider market across the world (Merwin et al., 2008).

Yeasts are primarily responsible for ethanol fermentation during the cider production process. While spontaneous fermentation can be still traced as a mean of production (Wang et al., 2011), selected strains of *Saccharomyces cerevisiae* are ubiquitously applied as a starter to control the fermentation process and improve the overall quality (García-Llobodanin et al., 2008; Y.Yang et al., 2020). As a large part of the metabolites of cider depends directly on yeast fermentation, pear ciders with markedly different flavor profiles can be obtained when fermenting the same pear with different strains of *Saccharomyces cerevisiae*. Liu et al. found that different yeast strains could affect the total volatiles, especially esters, acids and phenols of fermented Jinchuan pear wine (Liu et al., 2022a). Yang et al. found that Dangshan pear wine fermented by *Saccharomyces cerevisiae* SY was characterized by a short fermentation cycle, a suitable sugar-acid ratio and the highest total amount of flavor

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compounds, comparing to the other two commercial *Saccharomyces cerevisiae* strains (H. Yang et al., 2019).

Yeast action is expected to influence simultaneously wide portions of pear cider molecular profile. Therefore, metabolomics, the study of the ensemble of a food's low weight molecules, seems a very well-suited tool to observe the consequences of *Saccharomyces cerevisiae* metabolism on the cider's overall characteristics. In this respect, confirmations come from the production of wine, where metabolomics has been often applied as a hypothesis-generating tool, in an unbiased and non-targeted way. For example, it has been used to characterize wine's geographical origin (*terroir*) and wine's yeast metabolic traits. In addition, it has been used to determine biomarkers of sensory characteristics, and to follow the grapes' ripening (Beckner Whitener et al., 2016; Son et al., 2009; W. Zhang et al., 2019).

To the best of our knowledge, there are few comprehensive papers on the metabolomic characteristics of pear ciders, especially when fermented by distinct strains of *Saccharomyces yeast*. Moreover, studies on the combination of GC-MS, ^{1}H-NMR and E-tongue to analyze the flavor profile of fruits' wine products are rare. In this work, with the China National Geographic Indication Product of Guangan honey pear as raw material, four commercial *Saccharomyces cerevisiae* strains, namely RW, SY, DV10 and Drop Acid Yeast (DAY), were used to produce pear cider, comparing their taste characteristics and metabolomic profiles. The outcome of this study could help to select *Saccharomyces cerevisiae* yeasts for further investigations into enhancing the quality of Guangan honey pear cider, providing a theoretical basis for the large-scale production of pear cider. In addition, the present work could be important and may be beneficial for producers of Guangan honey pear fruit, to reduce financial losses due to the seasonality and high perishability of this fruit.

2. Materials and methods

2.1. Materials and solvents

In the current study, we selected Guangan honey pear as raw material, which grows at altitudes from 600 to 1000 m in Guangan, Sichuan China (30°01′-30°52′ N, 105°56′-107°19′ E). It has been recognized as China National Geographic Indication Product in 2013.

Guangan honey pears (harvested in 2022, with a weight of approximately 400 g) were bought from a planting base in Guangan, China. *S. cerevisiae* strain DAY (AN) was purchased from Yantai Dibosh CO., LTD (China); *S. cerevisiae* strain DV10 (BN) was purchased from Lallemand CO., LTD (China); *S. cerevisiae* strain RW (CN) and SY (DN) was purchased from Angel Yeast CO., LTD (China). All chemicals used in the analysis were of analytical grade.

2.2. Pear cider samples

After picking, fresh Guangan honey pears were sent to the laboratory immediately by cold chain transportation. The fermentation process of Guangan honey pear was implemented according to Yang et al. (H. Yang et al., 2020). We washed, peeled and removed the core of Guangan honey pears. Then, we cut the pears into small pieces and pressed them by means of a laboratory pilot press. The initial sugar content was adjusted to 23° Brix by sucrose, and samples were treated with potassium metabisulfite (70 mg/L) and citric acid to prevent browning. Next, pectinase (0.02 g/L, 100,000 U/g, SAS SOFRALAB, France) was added to accelerate the release of cells' content, followed by water bath at 40 °C for 2 h. Subsequently, the pH of the liquid was adjusted to 4 by adding citric acid and calcium carbonate. Lastly, yeast (0.2 g/L) was added to the mixture and maintained at 25 ± 1 °C for 30 days. Eight replicate Guangan honey pear juice fermentations were carried out with each yeast strain. Apart from yeast strains, any other fermentation condition was identical. Immediately after the fermentation process, samples were collected and filtered by triple layer sterile gauze. All the obtained samples were moved to −80 °C refrigerator prior to further analysis.

2.3. Analytical methods

2.3.1. Electronic tongue analysis

An α-ASTREE electronic tongue (Alpha MOS, France), equipped with seven Ag/AgCl inner reference electrodes and an auto-sampler, was used for electronic tongue analysis. Eighty milliliters of each Guangan honey pear cider was introduced in specific electronic tongue beakers of the auto-sampler, separated by beakers with bidistilled water. The testing time was set to 120 s for each sample. So as to minimize possible errors linked to memory of the electrodes, all samples were measured five times in random order.

2.3.2. GC-MS analysis

Samples of 0.1 mL of each Guangan honey pear cider were added with 1 mL extractant (isopropanol: acetonitrile: water = 3:2:2, v/v/v) and 5 μL myristic acid-d27 solution (3 mg/mL). Subsequently they were vortex mixed for 1 min and centrifuged for 5 min at 13000 g and 4 °C. Supernatant (0.8 mL) were dried by means of nitrogen blowing. A two-step derivatization was then applied: first, 20 μL methoxyamine hydrochloride/pyridine (40 mg/mL) was added to the powder at 30 °C for 90 min; second, 90 μL MSTFA (N-methyl-N-(trimethylsily)trifluoroacetamide) containing 1% of TMCS (trimethylchlorosilane) was mixed with the content at 37 °C for 30 min. Finally, all the samples were centrifuged at the above conditions.

GC-MS analysis was set up on an Agilent 7890A gas chromatography system equipment with an Agilent 5975C inert MSD system (Agilent Technologies Inc., CA, USA). An Agilent DB5-MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm) was applied to separate the derivatives. A constant flow rate of Helium (>99.999%) was set to 1.1 mL/min, which was used as the carrier gas. Injection volume and solvent delay time was 1 μL and 5.9 min respectively. The initial oven temperature was held at 60 °C for 1 min, increased to 300 °C at a rate of 10 °C/min, and finally held at 300 °C for 10 min. The temperatures of injector, transfer line, and electron impact ion source were set to 250 °C, 230 °C, and 250 °C, respectively. The impact energy was 70 eV, and data was obtained in a full scan mode (m/z 50–500).

For molecules' identification and relative quantification, firstly the raw data were transformed into general format (NetCDF) through Agilent Chrom Station software (Agilent Technologies, USA). Then, peak extraction, retention time alignment and automatic integration were performed using "erah" package in R software platform. MS spectra of all components were matched in accordance with Golm Metabolome Database (GMD).

2.3.3. ^{1}H-NMR analysis

The samples were analyzed by ^{1}H-NMR according to the method described by Zhu et al. (Zhu et al., 2022). In brief, 0.5 mL of each pear cider sample were centrifuged for 15 min at 18630 g and 4 °C, to remove solid residues. Each supernatant (0.35 mL) and the same amount of bidistilled water were taken to a new Eppendorf tube. Then, a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP) (10 mmol/L) was added, which could be used as NMR chemical-shift reference. The mixture was buffered at pH 7.00 ± 0.02 by means of phosphate buffer (1 mol/L). Ten microliters of NaN_3 (2 mmol/L) were added to avoid microbial proliferation. Prior to moving to NMR tubes, all the samples were centrifuged again at the above conditions.

^{1}H-NMR spectra were obtained at 298 K by means of an AVANCE III spectrometer (Bruker, Wuhan, China) operating at a frequency of 600.13 MHz. Broad resonances from large molecules were suppressed by a CPMG (Carr-Purcell-Meiboom-Gill) filter of 330 ms, which included 400 echoes with a τ of 400 μs and a 180° pulse of 24 μs. Presaturation was used to suppress the heavy water (HOD) residual signal, by employing the cpmgpr1d sequence, belonging to the standard pulse sequence library. Each spectrum was acquired with an acquisition time of 2.28 s, by summing up 256 transients using 32 K data points over a 7184 Hz spectral window. In order to apply NMR as a quantitative

technique (Herbert-Pucheta et al., 2019; Zhu et al., 2021), the recycle delay was set to 5 s, considering the relaxation time of the protons under investigation.

^{1}H-NMR spectra phase was manually adjusted by means of Topspin (ver. 4.2), with any subsequent adjustment performed through in-house R computational language scripts. After the residual water signal removal, we adjusted the baseline of the spectra by means of peak detection, in accordance with the "rolling ball" principle (Kneen & Annegarn, 1996) implemented in the baseline R package (Liland et al., 2010). Differences in water and protein content among samples were taken into consideration by probabilistic quotient normalization (PQN) (Dieterle et al., 2006), applied to the entire spectra array. We assigned the signals by means of comparisons of their chemical shift and multiplicity with Chenomx software library (Chenomx Inc., Canada, ver 8.4). Rectangular integration was used to calculate integration of the signals.

2.4. Statistical analysis

All the data reported in the tables are expressed as mean values, followed by standard, deviations of eight biological replicates. Statistical analysis was conducted in R computational language (R Development Core Team, 2011). Prior to univariate and multivariate analyses, the distribution of the concentration of the molecules was transformed to normality according to Box and Cox (Box & Cox, 2018). Following Zhu et al. (Zhu et al., 2020), we looked for molecules variations connected to fermentation by different yeast strains by means of ANOVA, followed by Tukey HSD post hoc test ($P < 0.05$). In order to obtain an overall view of the trends characterizing the metabolomic profiles of the samples, robust principal component analysis (rPCA) models were employed (Hubert et al., 2005). The result of each rPCA model was constituted by a scoreplot, the projection of the samples in the PC space, which allowed to highlight the overall structure of the data. Moreover, a Pearson's correlation plot was calculated, which highlighted the relations between the concentration of each variable and its importance over each component of the model. A discrimination index was also calculated, based on the ratio between groups variance and total variance. To evidence variations encompassing the metabolome's profile of the samples, heatmap and enrichment analysis were applied by means of online data analysis platform MetaboAnalyst 5.0 (https://www.metaboanalyst.ca, Accessed December 8, 2022).

3. Results

3.1. Electronic tongue in Guangan honey pear cider

To investigate from a descriptive point of view the effects of the four yeast strains on the taste features of Guangan honey pear cider, an electronic tongue was applied and its results were summarized by a radar chart and by a PCA model, as show in Fig. 1.

The radar chart evidences that the fermentations by the strains AN and DN led to taste profiles more balanced than the others, especially for sourness. The scoreplot of the PCA model calculated on the same data is shown in Fig. 1 (B), where the Guangan honey pear cider samples fermented by the four yeast strains are represented by different colors. This unsupervised procedure evidenced a clear-cut separation of the data obtained from the different yeast strains, as summarized by the high discrimination index of 83. The first and second principal components accounted for as much as 87.24% of the total variance of the original information of the sensor, thus reflecting the overall information of the samples. The most interesting information was described by PC1, with BN and AN samples appearing at high and low PC1 scores, respectively, and CN and DN appearing at intermediate values.

3.2. Pear cider metabolome characterized by GC-MS and ^{1}H-NMR

In the study, we were able to obtain the absolute quantification of 66 molecules by means of ^{1}H-NMR. In parallel, 104 molecules were relatively quantified through GC-MS. Typical GC-MS and ^{1}H-NMR spectra from Guangan honey pear cider are reported in Figures S1 and S2. Moreover, supplemental material reports, for each characterized molecule by ^{1}H-NMR, superimpositions of spectra registered and simulated for pure compounds (Figures S3−S12). The complete list of molecules and their concentrations are reported in Tables S1 and S2. Among the metabolites characterized, 22 were detected by both techniques. They pertain to amino acids, peptides and analogues (10), carbohydrates and derivates (3), organic acids and derivates (6), nucleosides, nucleotides and analogues (2) and miscellaneous (1). Interestingly, no alcohol was detected by both approaches. The performances of the two techniques are highlighted in the circular barplot shown in the panels (B) and (C) of Fig. 2. OA, AA and CA were mainly quantified by GC-MS. In contrast, AL, ML and OA represented the dominant categories characterized by means of ^{1}H-NMR.

3.3. How yeast strains affect the metabolome of Guangan honey pear cider

Focusing on ^{1}H-NMR, twenty-eight of the quantified molecules were significantly different among the four groups, namely malate, taurine, cytidine, arabinose, isovalerate, *sn*-glycero-3-phosphocholine, pyruvate, ethanolamine, fumarate, carnosine, carnitine, butyrate, uridine, isocaproate, tartate, uracil, cytosine, acetone, proline, propylene glycol, N, N-dimethylglycine, fucose, succinate, tyramine, acetate, creatinine, valine and ornithine. Their concentrations in each group are shown in Table 1. To highlight the overall trend of the characterized molecules, an

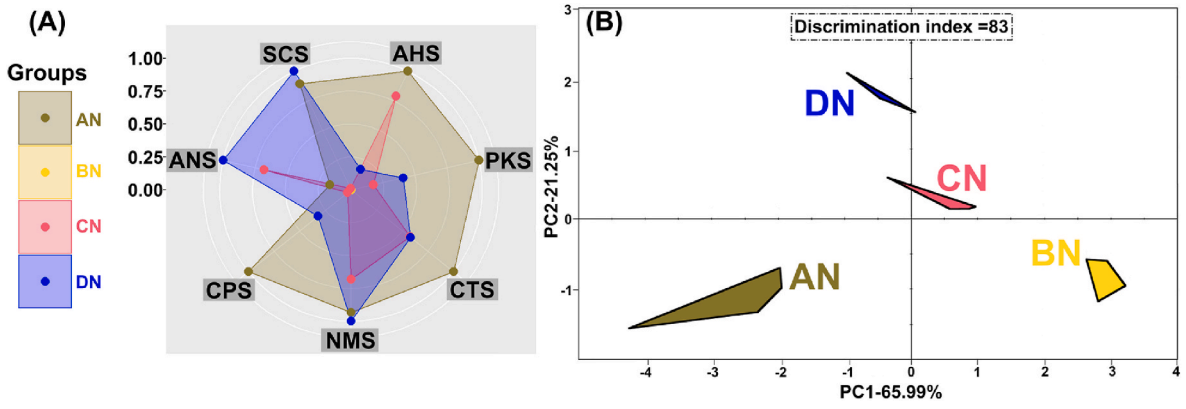

Fig. 1. (A) Radar chart of scores obtained by different sensors of four yeast strains fermented Guangan honey pear ciders. All the sensors are common sensors except for AHS, CTS and NMS, which are specifically sensitive to sourness, saltiness and umami, respectively. (B) PCA model built on sensors scores.

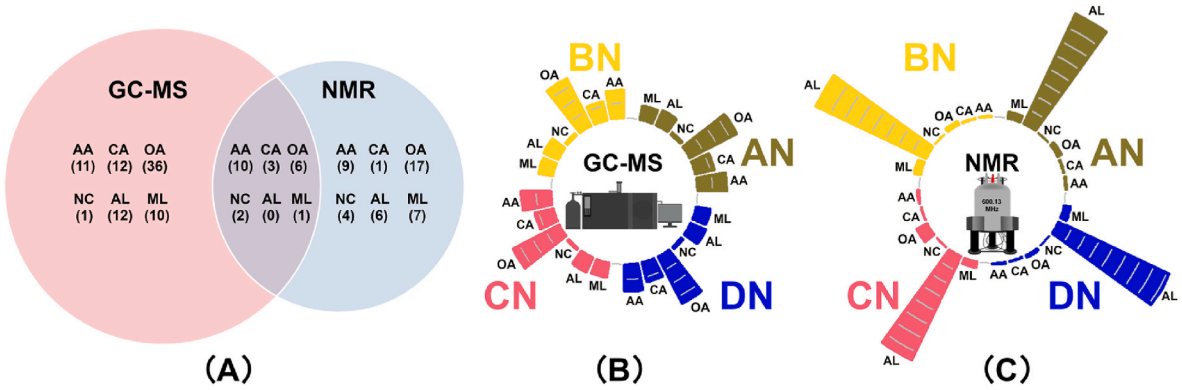

Fig. 2. (A) Venn diagram showing unique and shared metabolites characterized by GC-MS and ^{1}H-NMR. AA stands for amino acids, peptides and analogues, CA stands for carbohydrates and derivates, OA stands for organic acids and derivates, NC stands for nucleosides, nucleotides and analogues, AL stands for alcohols and ML stands for miscellaneous. Each number in brackets shows the number of molecules shared in the specified classification. Relative abundance of the molecule classes assigned by GC-MS (B) and ^{1}H-NMR (C). In (B) and (C), the lines inside the bars exhibited 10% steps, as a reference.

Table 1
Molecules whose concentrations (mmol/L, mean ± sd) showed significant differences among the four groups of samples (8 samples per group), by means of ^{1}H-NMR.

Amino Acids, Peptides and Analogues	AN	BN	CN	DN
Asparagine	$2.34 \pm 1.99^{ab,a}$	$6.53x10^{-1}\pm5.35x10^{-1\ b}$	4.00 ± 2.59^{a}	1.89 ± 1.56^{ab}
Aspartate	$7.66x10^{-1}\pm4.32x10^{-1\ a}$	$3.11x10^{-1}\pm3.40x10^{-1\ b}$	$3.76x10^{-1}\pm2.61x10^{-1\ ab}$	$3.24x10^{-1}\pm1.99x10^{-1\ ab}$
Carnitine	$1.37x10^{-2}\pm6.57x10^{-4\ a}$	$1.32x10^{-2}\pm9.45x10^{-4\ ab}$	$1.18x10^{-2}\pm1.71x10^{-3\ b}$	$1.34x10^{-2}\pm1.66x10^{-3\ ab}$
Carnosine	$1.44x10^{-1}\pm1.29x10^{-2\ a}$	$1.32x10^{-1}\pm1.51x10^{-2\ a}$	$7.52x10^{-2}\pm3.10x10^{-2\ b}$	$1.27x10^{-1}\pm1.39x10^{-2\ a}$
Creatinine	$1.60x10^{-2}\pm6.94x10^{-3\ ab}$	$1.56x10^{-2}\pm6.20x10^{-3\ ab}$	$6.82x10^{-2}\pm5.38x10^{-2\ a}$	$9.29x10^{-3}\pm3.08x10^{-3\ b}$
N,N-Dimethylglycine	$8.77x10^{-4}\pm3.19x10^{-4\ c}$	$1.52x10^{-3}\pm6.00x10^{-4\ b}$	$4.95x10^{-3}\pm3.43x10^{-3\ a}$	$1.52x10^{-3}\pm5.39x10^{-4\ b}$
Ornithine	$3.24x10^{-2}\pm1.90x10^{-2\ ab}$	$3.31x10^{-2}\pm1.33x10^{-2\ ab}$	$9.93x10^{-2}\pm7.37x10^{-2\ a}$	$2.17x10^{-2}\pm6.94x10^{-3\ b}$
Proline	$3.58x10^{-1}\pm4.63x10^{-2\ b}$	$3.66x10^{-1}\pm4.25x10^{-2\ b}$	$6.32x10^{-1}\pm1.17x10^{-1\ a}$	$3.07x10^{-1}\pm7.00x10^{-2\ b}$
Taurine	$1.58x10^{-1}\pm3.16x10^{-2\ ab}$	$1.73x10^{-1}\pm2.45x10^{-2\ a}$	$1.08x10^{-1}\pm5.63x10^{-2\ b}$	$1.85x10^{-1}\pm2.20x10^{-2\ a}$
Tyramine	$1.68x10^{-2}\pm3.51x10^{-3\ b}$	$1.63x10^{-2}\pm4.48x10^{-3\ b}$	$7.92x10^{-2}\pm7.83x10^{-2\ a}$	$1.56x10^{-2}\pm3.75x10^{-3\ b}$
Valine	$2.06x10^{-1}\pm4.86x10^{-2\ ab}$	$1.98x10^{-1}\pm7.57x10^{-2\ b}$	$3.30x10^{-1}\pm8.85x10^{-2\ a}$	$1.62x10^{-1}\pm3.70x10^{-2\ b}$
Carbohydrates and Derivates				
Arabinose	$4.12 \pm 6.00x10^{-1\ a}$	3.58 ± 1.66^{a}	1.40 ± 2.49^{b}	$4.13 \pm 6.15x10^{-1\ a}$
Fucose	$3.03x10^{-1}\pm4.25x10^{-2\ ab}$	$2.97x10^{-1}\pm6.12x10^{-2\ b}$	$5.00x10^{-1}\pm1.85x10^{-1\ a}$	$3.05x10^{-1}\pm7.47x10^{-2\ b}$
Organic Acids and Derivates				
2-Hydroxyisovalerate	$9.85x10^{-3}\pm6.86x10^{-3\ ab}$	$8.72x10^{-3}\pm6.35x10^{-3\ ab}$	$1.89x10^{-2}\pm1.18x10^{-2\ a}$	$7.19x10^{-3}\pm4.72x10^{-3\ b}$
Acetate	7.40 ± 3.00^{ab}	5.52 ± 3.52^{b}	9.21 ± 4.22^{a}	$4.70 \pm 6.97x10^{-1\ ab}$
Butyrate	$5.43x10^{-1}\pm7.08x10^{-2\ a}$	$5.45x10^{-1}\pm1.14x10^{-1\ a}$	$3.73x10^{-1}\pm3.58x10^{-2\ b}$	$5.04x10^{-1}\pm8.90x10^{-2\ a}$
Fumarate	$5.06x10^{-2}\pm3.19x10^{-2\ ab}$	$9.22x10^{-2}\pm5.99x10^{-2\ a}$	$3.14x10^{-2}\pm4.24x10^{-2\ b}$	$1.21x10^{-1}\pm9.23x10^{-2\ a}$
Isocaproate	$9.94x10^{-2}\pm1.74x10^{-2\ c}$	$1.21x10^{-1}\pm2.60x10^{-2\ bc}$	$1.35x10^{-1}\pm6.89x10^{-3\ ab}$	$1.59x10^{-1}\pm2.71x10^{-2\ a}$
Isovalerate	$3.57x10^{-2}\pm8.90x10^{-3\ b}$	$2.86x10^{-2}\pm4.98x10^{-3\ b}$	$3.44x10^{-2}\pm1.25x10^{-2\ b}$	$7.76x10^{-2}\pm1.21x10^{-2\ a}$
Malate	4.07 ± 3.16^{ab}	3.69 ± 2.16^{ab}	1.67 ± 2.53^{b}	$5.42 \pm 9.51x10^{-1\ a}$
Pyruvate	2.17 ± 2.87^{ab}	1.44 ± 1.78^{ab}	$5.17x10^{-1}\pm8.94x10^{-1\ b}$	1.97 ± 1.73^{a}
Succinate	$2.06 \pm 7.09x10^{-1\ b}$	2.98 ± 1.25^{ab}	$3.31 \pm 7.23x10^{-1\ a}$	$2.18 \pm 5.22x10^{-1\ b}$
Tartrate	$1.18x10^{-2}\pm4.49x10^{-3\ a}$	$1.66x10^{-2}\pm9.79x10^{-3\ a}$	$3.56x10^{-3}\pm3.39x10^{-3\ b}$	$1.38x10^{-2}\pm3.75x10^{-3\ a}$
trans-Aconitate	$4.61x10^{-2}\pm1.80x10^{-2\ a}$	$8.61x10^{-2}\pm9.32x10^{-2\ a}$	$2.39x10^{-2}\pm1.16x10^{-2\ b}$	$6.54x10^{-2}\pm3.20x10^{-2\ a}$
Nucleosides, Nucleotides and Analogues				
Cytidine	$5.80x10^{-2}\pm1.61x10^{-2\ a}$	$5.77x10^{-2}\pm1.39x10^{-2\ a}$	$3.45x10^{-2}\pm1.38x10^{-2\ b}$	$6.88x10^{-2}\pm1.09x10^{-2\ a}$
Cytosine	$2.94x10^{-2}\pm9.30x10^{-3\ ab}$	$3.49x10^{-2}\pm2.27x10^{-2\ ab}$	$5.17x10^{-2}\pm1.92x10^{-2\ a}$	$2.50x10^{-2}\pm8.58x10^{-3\ b}$
Dimethylamine	$2.27x10^{-2}\pm2.30x10^{-2\ b}$	$3.14x10^{-2}\pm1.00x10^{-2\ a}$	$2.39x10^{-2}\pm6.01x10^{-3\ a}$	$1.62x10^{-2}\pm3.71x10^{-3\ b}$
Uracil	$1.41x10^{-1}\pm3.32x10^{-2\ ab}$	$1.30x10^{-1}\pm3.14x10^{-2\ b}$	$1.56x10^{-1}\pm1.75x10^{-2\ a}$	$1.08x10^{-1}\pm1.85x10^{-2\ b}$
Uridine	$1.28x10^{-1}\pm2.62x10^{-2\ a}$	$1.58x10^{-1}\pm8.91x10^{-2\ a}$	$4.80x10^{-2}\pm4.43x10^{-2\ b}$	$1.49x10^{-1}\pm3.45x10^{-2\ a}$
Miscellaneous				
Acetone	$2.36x10^{-2}\pm2.74x10^{-3\ b}$	$2.86x10^{-2}\pm4.36x10^{-3\ ab}$	$3.36x10^{-2}\pm6.81x10^{-3\ a}$	$2.81x10^{-2}\pm5.23x10^{-3\ ab}$
Allantoin	$1.04x10^{-2}\pm3.86x10^{-3\ ab}$	$1.10x10^{-2}\pm4.35x10^{-3\ a}$	$8.88x10^{-3}\pm1.55x10^{-3\ b}$	$1.20x10^{-2}\pm3.53x10^{-3\ a}$
Ethanolamine	$2.76x10^{-1}\pm1.45x10^{-2\ a}$	$2.87x10^{-1}\pm1.58x10^{-2\ a}$	$2.47x10^{-1}\pm2.94x10^{-2\ b}$	$2.96x10^{-1}\pm1.97x10^{-2\ a}$
Hypoxanthine	$9.80x10^{-2}\pm2.39x10^{-2\ a}$	$1.22x10^{-1}\pm3.85x10^{-2\ a}$	$6.08x10^{-2}\pm1.31x10^{-2\ b}$	$1.01x10^{-1}\pm2.12x10^{-2\ a}$
sn-Glycero-3-phosphocholine	$4.68x10^{-2}\pm3.23x10^{-3\ ab}$	$4.82x10^{-2}\pm5.47x10^{-3\ a}$	$2.86x10^{-2}\pm1.80x10^{-2\ b}$	$4.76x10^{-2}\pm2.44x10^{-3\ ab}$
xanthine	$8.42x10^{-2}\pm2.74x10^{-2\ a}$	$6.26x10^{-2}\pm1.43x10^{-2\ a}$	$3.72x10^{-2}\pm1.11x10^{-2\ b}$	$5.98x10^{-2}\pm9.57x10^{-3\ a}$
Alcohols				
Propylene glycol	5.13 ± 5.15^{b}	5.95 ± 8.97^{b}	8.09 ± 3.28^{a}	3.38 ± 1.07^{b}

[a] For each molecule, the comparisons among the groups are represented by a compact letter display, where sd values followed by a common superscript identify no significant differences.

rPCA model was performed on the basis of their concentrations, as shown in Fig. 3.

As shown in the scoreplot (Fig. 3A), PC1 accounted for as much as 81.8% of the entire samples' variance, therefore nicely summarizing the differences among the samples from the four groups. In detail, CN was characterized by the highest levels of uracil, cytosine, acetone, proline, propylene glycol, N,N-dimethylglycine, fucose, succinate, tyramine, acetate, creatinine, valine and ornithine. At the opposite, samples fermented by DN strains showed the highest concentrations of malate, taurine, cytidine, arabinose, isovalerate, *sn*-glycero-3-phosphocholine,

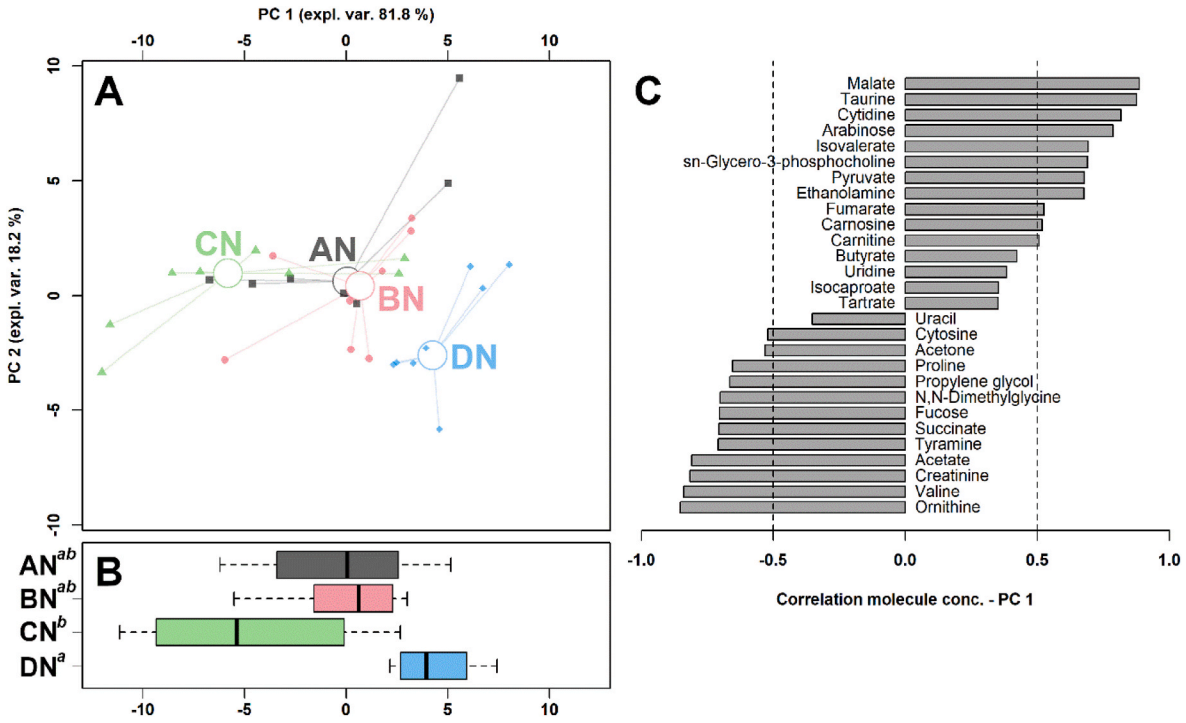

Fig. 3. rPCA model built on the concentration of the molecules detected by ^{1}H-NMR that showed significant differences among the four groups. In the scoreplot (A), samples from the four groups are represented with squares (AN), circles (BN), triangles (CN) and diamonds (DN) respectively. The median of each samples' group was indicated as wide and empty circles. Boxplot (B) summarized the position of the samples along PC1. The comparisons among the groups are represented by a compact letter display, where group names followed by a common superscript identify no significant differences. The loading plot (C) showed the significant correlations ($P < 0.05$) between the concentration of each substance and its importance along PC 1.

pyruvate, ethanolamine, fumarate, carnosine, carnitine, butyrate, uridine, isocaproate and tartate.

As for GC-MS, the concentrations of thirteen molecules showed statistically significant differences among the four groups, thus providing a different set of potential biomarkers to distinguish Guangan honey pear cider fermented by the different yeast strains, such as N-acetylglucosamine, 2-(4-hydroxyphenyl)-ethanol, glutamic acid, methyl 3,6-anhydro-alpha-D-galactopyranoside. Similarly to ^{1}H-NMR, an rPCA model

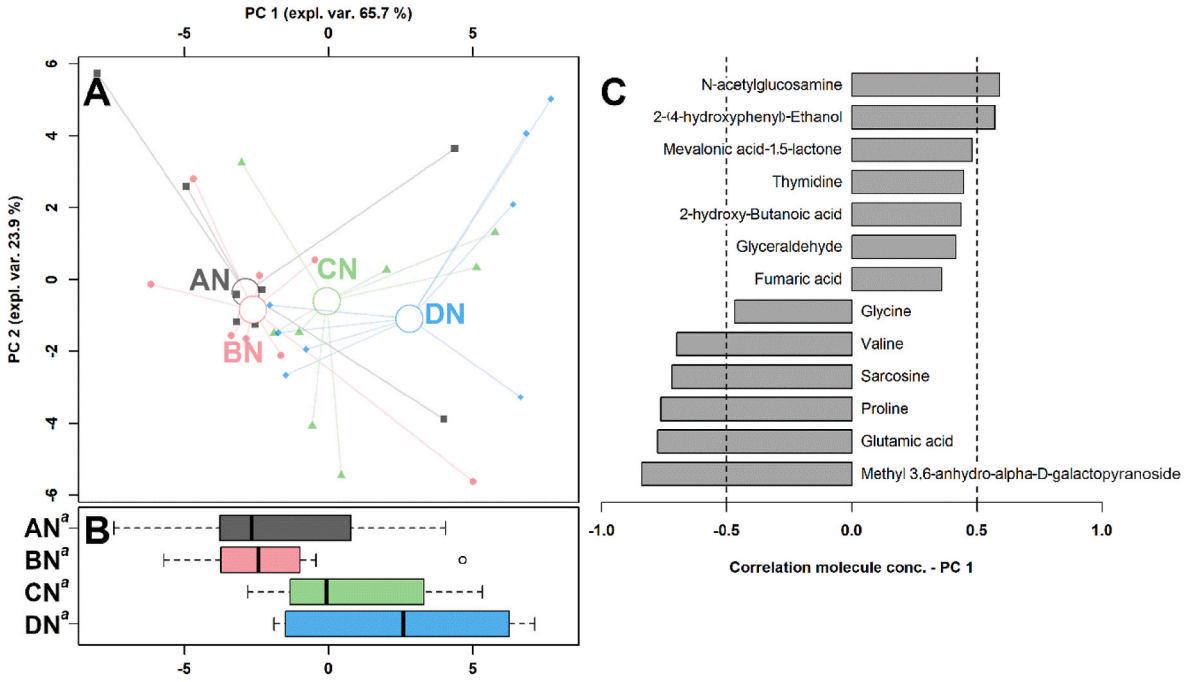

Fig. 4. rPCA model built on the concentration of the molecules observed by GC-MS that showed a significant difference among the four groups. In the scoreplot (A), samples from the four groups are represented with squares (AN), circles (BN), triangles (CN) and diamonds (DN). The median of each samples' group was indicated as wide and empty circles. Boxplot (B) summarized the position of the samples along PC1. The loading plot (C) showed the significant correlations (*$P < 0.05$) between the concentration of each substance and its importance along PC 1.

built on the basis of the thirteen molecules concentrations is shown in Fig. 4.

As shown in the scoreplot (Fig. 4A), PC1 accounted for 65.7% of the entire samples' variance, thus effectively summarizing the differences among the samples from the four groups. Samples fermented by AN and BN strains showed the highest levels of N-acetylglucosamine, 2-(4-hydroxyphenyl)-ethanol, mevalonic acid-1,5-lactone, thymidine, 2-hydroxy-butanoic acid, glyceraldehyde and fumaric acid. The highest amounts of glycine, valine, sarcosine, proline, glutamic acid and methy 3,6-anhydro-alpha-D-galactopyranoside were exhibited by CN and DN groups.

In order to represent the entire metabolomic features of Guangan honey pear cider affected by the fermentation by different yeast strains, a heatmap was generated from the molecules quantified by untargeted ^{1}H-NMR and GC-MS, as shown in Fig. 5.

The heatmaps allowed to appreciate that samples fermented by CN strain differed from the others, showing positive correlations among several amino acids (such as tyramine, creatine and creatinine) and negative correlations among a few sugars and alcohols, namely arabinose, galactose and *myo*-inositol. It's worth noting that metabolomic data obtained from ^{1}H-NMR had a higher ability to distinguish Guangan honey pear cider fermented by different yeast strains, in comparison with GC-MS.

In order to find out the most relevant pathways differentiating the groups, a pathway enrichment analysis was performed on the basis of the molecules whose concentrations were significantly different among the samples. Eight pathways were highlighted (*P $<$ 0.05), namely arginine and proline metabolism, glycine and serine metabolism, urea cycle, alanine metabolism, citric acid cycle, amino sugar metabolism, carnitine synthesis and warburg effect, as shown in Fig. 6.

3.4. Discussion

As a good source for low-alcohol beverages, pears are very popular by the consumers thanks to their sweetness, slight aroma, crispness and characteristic fragrance. China, as one of the main pears producing nations in the world, has abundant pear cultivars with distinct compositional characteristics (Chen et al., 2007). However, only a few pear

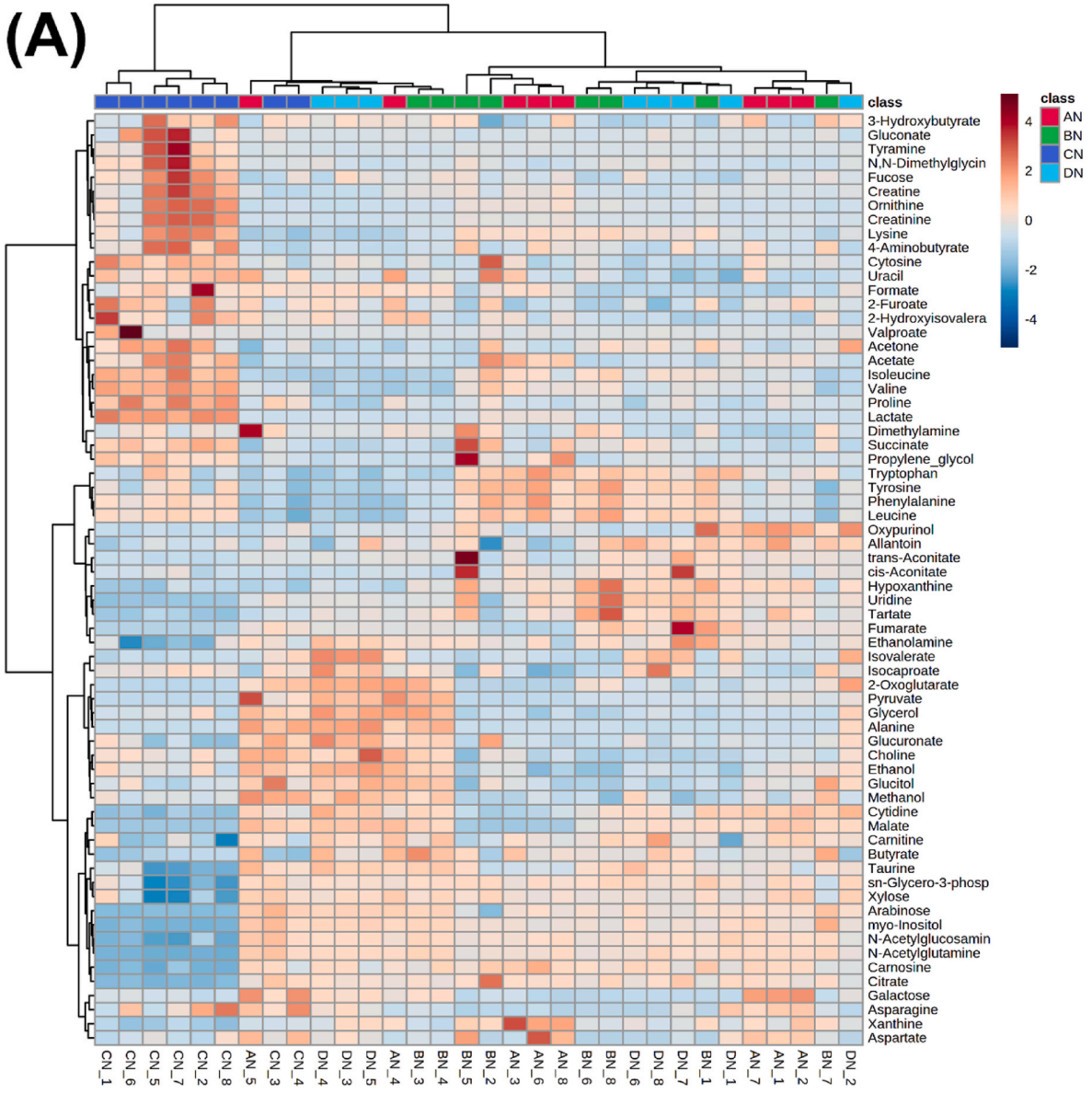

Fig. 5. Heatmaps of the concentration of the molecules characterized by ^{1}H-NMR (A) and GC-MS (B) in Guangan honey pear cider fermented by the four commercial yeast strains tested.

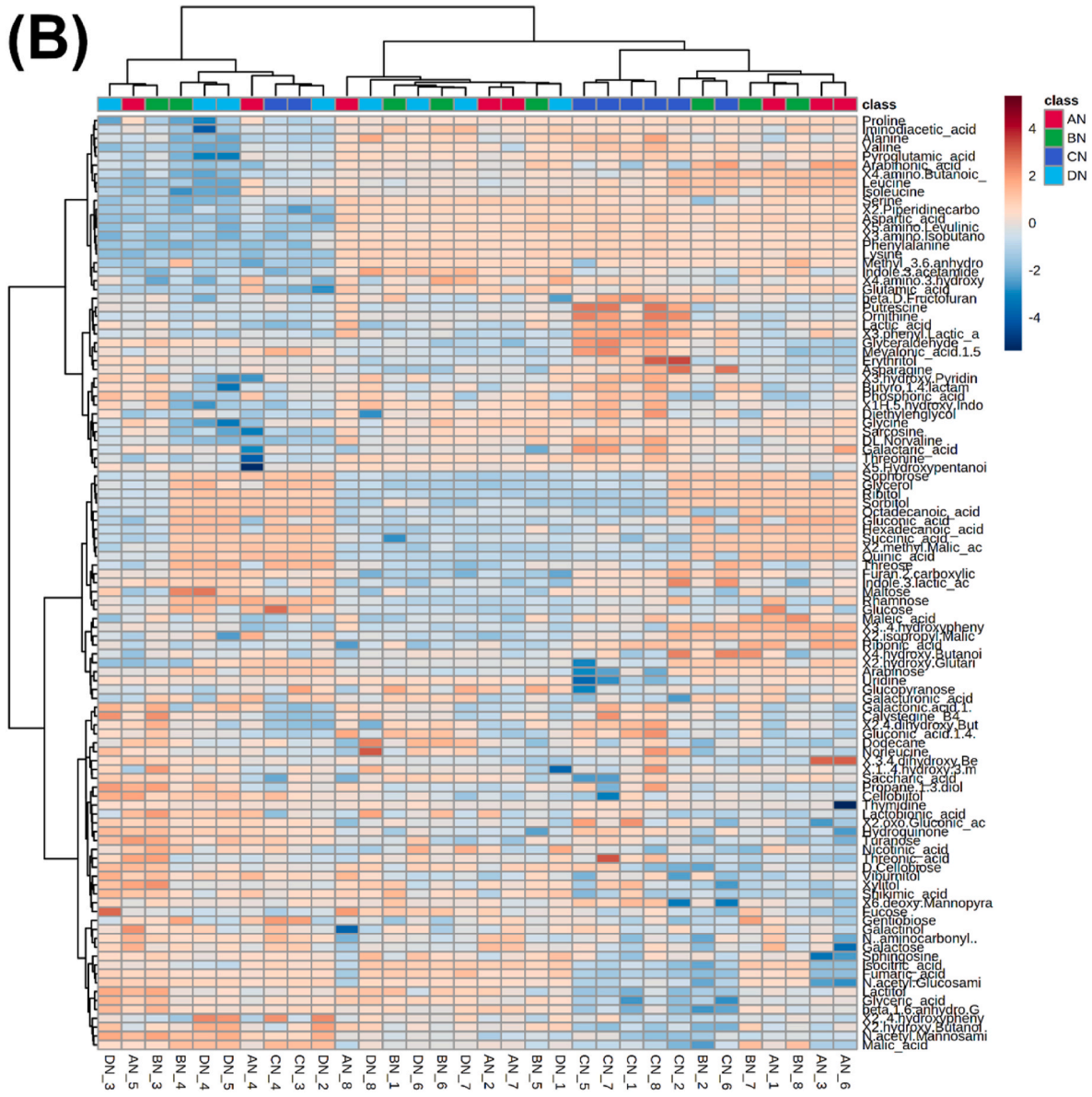

Fig. 5. (*continued*).

cultivars have been used for pear cider making, such as Zaosu pear, Jinchuan pear, Dongli pear and Dangshan pear, while the others have received limited attention (Liu et al., 2022b; Ndayambaje et al., 2021; H. Yang et al., 2019; X. Yang et al., 2022).

The fermentation of fruit cider is considered a consequence of the biochemical processes of microbial metabolism, where yeasts play an important role in two successive fermentation processes, namely alcoholic fermentation and lactic acid fermentation. During fermentation the sugars can be converted into alcohol, esters and other low weight molecules (Li et al., 2020; Oliveira et al., 2011).The sensory and quality features of fruit ciders can be significantly altered by these metabolites. Therefore, it is urgent to obtain a comprehensive view of the metabolic mechanisms in the fermentation process. Among the omics techniques, metabolomics has been confirmed to fulfill the above requirements and has entered wine science such as wine chemistry and yeast metabolism (Ai et al., 2021; X. K. Zhang et al., 2021), to acquire a further insight into the genotype-phenotype connections (García et al., 2018).

Till now, research has been focused on the optimization of pear cider fermentation procedures, while the corresponding comprehensive metabolomic profiles have been rarely investigated. To the best of our knowledge, this is the first attempt to systematically characterize the metabolome of Guangan honey pear cider fermented by different yeast strains through high throughput platforms like ^{1}H-NMR and GC-MS. Moreover, an electronic tongue was employed to obtain descriptive features of the corresponding overall taste.

Taking advantage of ^{1}H-NMR and GC-MS, a total of 148 molecules were characterized in Guangan honey pear cider samples. Among them, 22 molecules were quantified through both techniques, while 82 and 44 molecules were detected exclusively by GC-MS and ^{1}H-NMR, respectively, covering the molecular classes of amino acids, peptides and analogues, carbohydrates and derivates, organic acids and derivates, nucleosides, nucleotides and analogues and miscellaneous. It is worth noting that GC-MS was more sensitive to organic acids, while alcohols were the most abundant compounds quantified by ^{1}H-NMR. Therefore, the combination of GC-MS and ^{1}H-NMR appeared as a key factor to provide a more comprehensive metabolomic fingerprint than single techniques.

Amino acids are one of the main classes detected in different fruit wines, such as grape wine (Malagoli et al., 2022), orange wine (Xu et al., 2013) and pear wine (H. Yang et al., 2019).

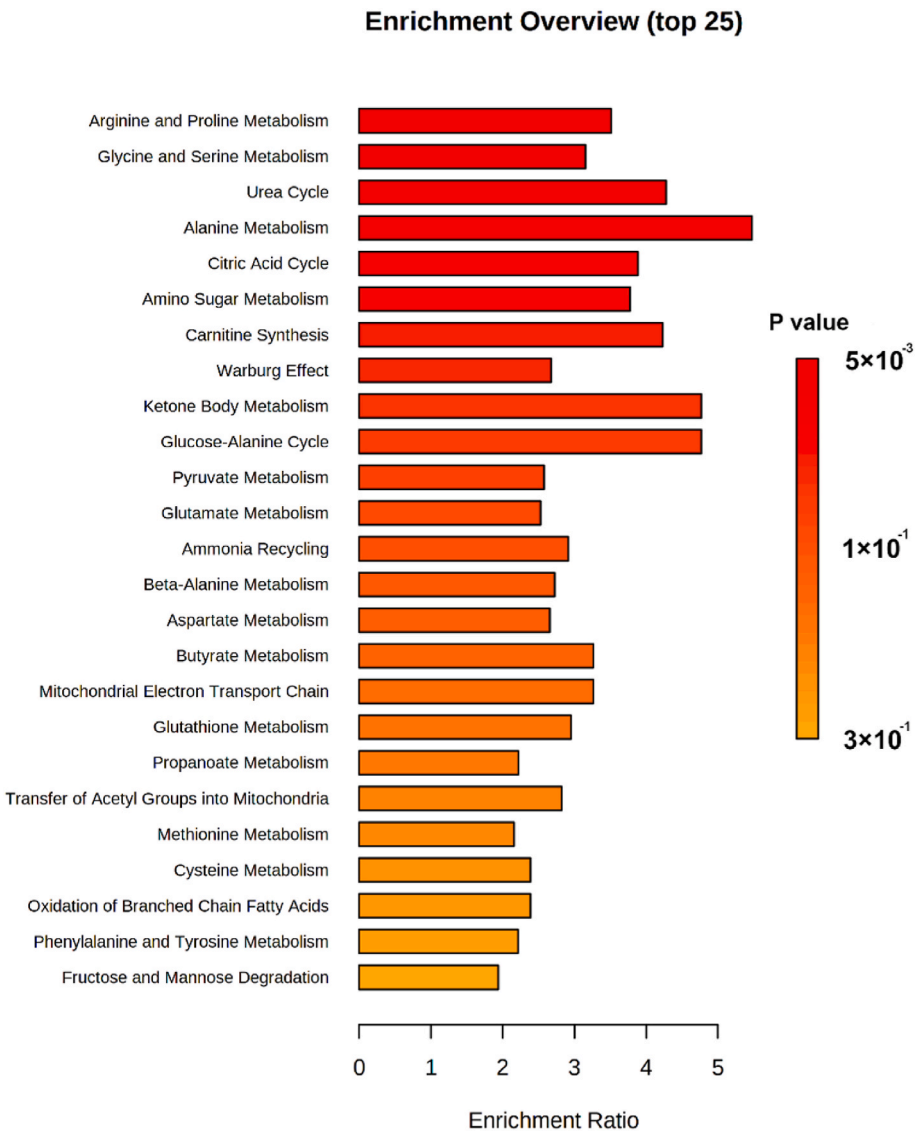

Fig. 6. Enrichment analysis of the molecules whose concentrations were significantly altered by the four commercial yeast strains in the process of Guangan honey pear cider fermentation.

Amino acids, especially aromatic ones, not only play a fundamental role on fruit aroma (Chen et al., 2007), but are also considered as precursors of important wines' volatile compounds (Bell & Henschke, 2005). Moreover, they are regarded as nitrogen sources for the growth of yeast in the must. However, it's worth noting that the presence of amino acids could be considered as one of the main factors causing pear cider browning, through the non-enzymatic mechanisms of the Maillard reactions (H. Yang et al., 2020). In the present study, thirteen amino acids exhibited significant differences among the four groups. Among them, only three amino acids exhibited higher levels in DN group, namely taurine, carnosine and carnitine, while the others of their derivatives showed lower amounts, namely creatinine, N,N-dimethylglycine, ornithine, proline, tyramine, valine, glycine, sarcosine and glutamate. Carnosine is a dipeptide constituted by *beta*-alanine and histidine, with antioxidant, buffering, chelating and anti-glycation activities (Kwolek-Mirek et al., 2016). Significantly higher concentrations of carnosine could be linked to beneficial effects of DN fermented Guangan honey pear cider on health. In wine fermentation, carnosine is thought to affect the growth of *Saccharomyces cerevisiae* in a metabolism-dependent manner linked to carbon source (Cartwright et al., 2012). In addition, Mirek et al. found that L-carnosine was able to enhance the replicative potential of the *Saccharomyces cerevisiae*, when growing on a medium containing glucose as a source of carbon (Kwolek-Mirek et al., 2016). In our study, considering that glucose was found to be the dominant carbohydrate in pear cider by means of GC-MS, higher level of carnosine in DN group could enhance DN yeast fermentation ability, with positive consequences on cider's overall quality. Carnitine can be involved in the lipids' metabolism in yeasts, notably as an indispensable cofactor of fatty acid intracellular trafficking (Jacques et al., 2018). The wine yeast *Saccharomyces cerevisiae* plays a central role in the production of aroma compounds during fermentation. Esters are regarded as some of the most important yeast-derived aroma compounds. The esters ethyl acetate and isoamyl acetate are formed from alcohols and acetyl-CoA in a reaction catalyzed by alcohol acetyltransferase. Acetyl-CoA available in yeast cells plays a key role in the production of ester aromas. Acetylcarnitine and free CoA can be formed by carnitine acetyltransferase by catalyzing the reversible reaction between carnitine and acetyl-CoA. This reaction is essential for transferring activated acetyl groups to the mitochondria and in regulating the acetyl-CoA/CoA pools within the cell. Carnitine acetyltransferase expression could potentially be used successfully to modulate wine flavour (Cordente et al., 2007). In the current study, higher concentrations of carnitine could indicate a lower

acetyltransferase expression in DN strain. In turn, this could lead to a higher synthesis of esters, with positive consequences on the flavor of DN fermented pear cider. In *S. cerevisiae*, the genes involved in sulfur metabolism are thought to be regulated by the sulfur pool, consisting mainly of what constitutes cysteine (Hansen & Francke Johannesen, 2000). Sulfur excess seems to be directly linked to hypotaurine and taurine synthesis, which maintains a steady content of intermediates of glutathione synthesis (Hébert et al., 2013). Valine, one of the main branched amino acids, is considered as a nitrogen source for yeast fermentation. In parallel, yeast itself is a valine producer (Takpho et al., 2018). Works on beer's wort fermentation established a link between the concentration of branched amino acids and aroma-active substances. In fact, Procopio et al. found that the most important explanatory variables affecting the synthesis of aroma-active substances of *S. cerevisiae* are the concentrations of leucine, isoleucine, valine, histidine, glutamine and proline in this matrix (Procopio et al., 2013). In addition, Krogerus et al. found that the modification of the amino acid's profile of wort, especially with respect to valine and the other branched-chain amino acids, may effectively decrease the amount of diacetyl formed during fermentation (Krogerus & Gibson, 2013). Moreover, it's worth noting that during wort fermentation valine and isoleucine biosynthesis can produce undesirable butter-tasting vicinal diketones as by-products. One promising way to decrease diacetyl production is related to the control of the level of valine in wort, because valine is involved in the inhibition of enzymes controlling diacetyl precursors formation (Krogerus & Gibson, 2013). Lower contents of valine and proline could therefore decrease undesirable compounds and enhance the presence of odorous substances in DN fermented pear cider. N,N-Dimethylglycine, as one of glycine betaine analogues, is commonly found in pears (de Zwart et al., 2003). Linder found that glycine, sarcosine and N, N-dimethylglycine supplementation caused a decrease in growth efficiency, that appeared to be proportional to the concentration of N, N-dimethylglycine. On the other hand, inhibition effects of yeast growth were reduced when sodium L-glutamate was the sole source of nitrogen (Linder, 2020). Ornithine plays a central role in the urea cycle, which allows for the disposal of excess nitrogen. Tyramine, one of the most toxic biogenic amines produced by the decarboxylation of tyrosine during fermentation, is commonly found in alcoholic beverages and fruits, such as pear and grape (Gao et al., 2023; Tarján & Jánossy, 1978). However, the ability to produce biogenic amines is not a constant characteristic of *S. cerevisiae*, but it seems to be related to yeast species and nitrogen sources during wine alcoholic fermentation (Bordiga et al., 2020).

Sugars are some of the most intensively studied nutrients in fruit ciders. They play important roles not only in the metabolism and maintenance of the normal physiological state of yeast, but they are also directly linked to the taste of pear cider (Gao, Feng, Liu, et al., 2021; Gao, Feng, Sheng, et al., 2021). In the study, sixteen sugars were characterized by means of GC-MS and ^{1}H-NMR. Among them, glucose was found to be the most abundant sugar in the samples, followed by arabinose. On one side, glucose could come from pear itself. On the other side, glucose could also be generated through sucrose splitting, together with fructose, and then partially metabolized. Therefore, the level of glucose could exhibit vital roles, linked to biosynthetic capabilities, reproductive potential and ethanol production of yeast. A high level of glucose could not only affect yeast cells metabolism by elevating the amount of reactive oxygen through extramitochondrial pathways, but also have adverse effects on the color of the product through caramelization and Maillard reactions (H. Yang et al., 2021).

Organic acids play essential roles in fruit flavor and nutrition. Pear is characterized by a variety of organic acids, the most concentrated of which are malic acid and citric acid, followed by quinic acid, oxalic acid and shikimic acid (Wu et al., 2022). Organic acids are indispensable for wine quality, with specific reference to the aroma and taste, with important effects on wines classification (Milovanovic et al., 2019). Importantly, concerns about the use of synthetic chemical antimicrobials and antioxidants are leading to a renewed interest of consumers for natural, and potentially safer, alternatives (Perumalla & Hettiarachchy, 2011). This has increased the importance of the studies about organic acids in wine, not only from a quality control point of view, but also for their beneficial properties to human health. From this point of view, the potentially adverse effects of organic acids shouldn't be neglected, with particular reference to their action as gastric acid stimulants (Liszt et al., 2012). In the current study, nine of the 58 quantified organic acids showed significant differences among the four groups, namely acetate, butyrate, fumarate, isocaproate, malate, pyruvate, succinate, tartrate and 2-hydroxy-butanoic acid. Acetate is one of the most important volatile acids produced by yeasts, resulting from the irreversible oxidation of acetaldehyde to acetate by Ald6. The latter is a cytosolic enzyme activated by Mg^{2+}, that uses $NADP^{+}$ as the preferred coenzyme, and is active during fermentation. Malate, fumarate, succinate and pyruvate can be involved in Krebs tricarboxylic acid (TCA) cycle. Malate can be formed from pyruvate through one of the anaplerotic reactions, only in the presence of glucose or other assimilable carbon sources. Fumarate is formed by the oxidation of succinic acid by succinate dehydrogenase in the TCA cycle. It is also considered as a precursor of malate, taking advantage of the enzyme fumarase. Succinate is formed by *S. cerevisiae* both through the oxidative or the reductive directions of the TCA cycle, depending on the fermentation stage and the nitrogen source. Pyruvate is an essential precursor of many fermentation byproducts. In *S. cerevisiae*, three pyruvate decarboxylases isoforms (PDC1, PDC5, PDC6) are implicated in the decarboxylation of pyruvate to acetaldehyde and CO_2.

Tyrosol (2-(4-hydroxyphenyl)-ethanol) is a phenolic compound with antioxidant properties that is commonly present in wine (Gris et al., 2011). Tyrosol was regarded as a secondary metabolite from tyrosine, formed by yeasts in alcoholic fermentation process (Dickinson et al., 2003). In addition, the synthesis of tyrosol has been thought as directly proportional to the fermentation temperature, alcoholic degree and amino acids concentrations (Bordiga et al., 2016; Garde-Cerdán & Ancín-Azpilicueta, 2008). Higher levels of tyrosol in DN fermented pear cider could be linked to beneficial effects at the cardiovascular level, in part by modulating blood lipid profile (Rodríguez-Morató et al., 2021). Acetyl-CoA is the main substance for acetone synthesis. In detail, two molecules of acetyl-CoA are condensed to one acetoacetyl-CoA by acetoacetyl-CoA synthase, where acetoacetate is formed by acetoacetyl-CoA transferase. Finally, acetoacetate is decarboxylated to acetone by acetoacetate decarboxylase (Walther & François, 2016).

There are molecules, that went undetected in the present investigation, that have been found to be related to technological treatments of the ciders. As an example, aldehydes and esters can form in spirit drinks derived from cereals as a consequence of rectification (He et al., 2020). Single platforms, such as ^{1}H-NMR, and/or single injection methods are unlikely to grant the sufficient sensitivity to quantify or even detect all of them. Anyway, pleiotropic phenomena could give rise to fingerprints of their formation in the molecules we here describe. A tempting future perspective with respect to the present work could be represented by the combination of metabolomics information from platforms based on different principles and therefore with different sensitivities.

4. Conclusions

To the best of our knowledge, this is the first time that the China National Geographic Indication Products Guangan honey pear was observed as a raw material for cider, fermented by four commercial strains of *Saccharomyces cerevisiae*, namely RW, SY, DV10 and Drop Acid Yeast (DAY). The taste characteristics and metabolomic profiles peculiarities were investigated by means of the combination of GC-MS, ^{1}H-NMR and E-tongue. The taste characteristics of Guangan honey pear cider fermented by the different yeast strains were clearly distinguished by E-tongue. A total of 148 molecules were characterized, which provided a more comprehensive metabolomic fingerprint than single

techniques. The importance of several pathways was highlighted during fermentation by the distinct yeast strains, namely arginine and proline metabolism, glycine and serine metabolism, urea cycle, alanine metabolism, citric acid cycle, amino sugar metabolism, carnitine synthesis and warburg effect. This study could facilitate the improvement of the quality of Guangan honey pear cider, by selecting different *Saccharomyces cerevisiae* yeasts, and in turn it could shed lights on the large-scale production of pear cider.

Authors' declaration of interests

No competing interests have been declared.

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CRediT authorship contribution statement

Chenglin Zhu: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Writing – original draft, Preparation, Writing – review & editing. **Zhibo Yang:** Formal analysis, Writing – review & editing. **Xuan Lu:** Formal analysis, Writing – review & editing. **Yuwen Yi:** Formal analysis, Writing – review & editing. **Qing Tian:** Writing – review & editing. **Jing Deng:** Writing – review & editing. **Dan Jiang:** Writing – review & editing. **Junni Tang:** Conceptualization, Funding acquisition, Writing – review & editing. **Luca Laghi:** Conceptualization, Methodology, Writing – original draft, Preparation, Writing – review & editing.

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.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lwt.2023.114816.

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Note: The references indicated by an * in front of the reference in the reference section can be considered as key references. Such references exhibit that metabolomics seem a very well-suited tool to observe the consequences of *Saccharomyces cerevisiae* metabolism on the cider's overall characteristics. Moreover, these references provided us so many interesting hints when we discussed our data and results.