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Regulation of ethyl carbamate by *BTN2* of *Saccharomyces cerevisiae* during mixed-culture Huangjiu fermentation with *Pediococcus pentoses*

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Abstract

Ethyl carbamate (EC) is a naturally occurring potential carcinogen in fermented foods. Our prior research indicated that *BTN2* knockout affected arginine metabolism in *Saccharomyces cerevisiae*, and the inhibitory effect was more pronounced in mixed cultures with *Pediococcus pentoses* (PP). Consequently, in this study, we investigated the potential mechanisms of EC regulation by *BTN2* knockout strains in single- and mixed-culture fermentation systems in Huangjiu and determined their fundamental qualities. The findings revealed that the *BTN2* knockout strain could reduce the EC content by decreasing the amount of EC precursors and that mixed fermentation with PP could impede the reaction between urea and ethanol, thereby exerting a more favorable EC abatement effect. The *BTN2* knockout strain had a positive effect on free amino acids in Huangjiu, thus improving the flavor. Additionally, transcriptome analysis demonstrated that the knockout of *BTN2* and the addition of PP affected gene expression levels, paticularly genes associated with transcription factor activity and amino acid transport in *S. cerevisiae*, which subsquently affected the metabolic pathways during the fermentation process.

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Introduction

Ethyl carbamate (EC) is a natural product produced in fermented foods such as cheese, bread, soy sauce and alcoholic beverages[1,2]. Owing to its carcinogenic and genotoxic properties, it is categorized as a Group 2A carcinogen[3,4]. Huangjiu, known as one of the world's three ancient wines[5], is a traditional fermented alcoholic beverage in China. It is rich in amino acids, vitamins, active peptides and other biologically active ingredients[6], and thus has a vast consumer market in southeast China[7]. However, a large amount of EC is generated during the production and storage of Huangjiu[8]. As a result, it is necessary to find ways to reduce the EC content.

During the fermentation of Huangjiu, EC is mainly produced by *S. cerevisiae* and lactic acid bacteria (LAB) through the reaction of EC precursors, which are produced by arginine metabolism and ethanol[9]. The metabolism of arginine can be divided into urea cycle pathway[10] and the arginine deiminase (ADI) pathway[11,12]. *S. cerevisiae* degrades arginine into urea and ornithine via the urea cycle pathway[2,13], whereas LAB generates citrulline through the ADI pathway[14]. Urea and citrulline are the most important EC precursors[12], and reducing their contents can effectively inhibit EC production[15]. Enzymatic degradation of EC has the advantages of precision, high efficiency and environmental friendliness. Liu[16] isolated an EC enzyme from *Alicyclobacillus pomorum* with high salt and high ethanol tolerance, which not only reduces EC in soy sauce but also shows high activity against acrylamide, another Class 2A carcinogen in foods. In addition, Liu[17] also identified a heat- and high-acid-tolerant EC enzyme from *Thermoflavimicrobium dichoditicum*, which was also highly active against EC and acrylamide.

Numerous studies have revealed that mixed fermentation can diminish the EC content[18–19] and non-brewer's yeasts can produce aromatic substances such as higher alcohols, glycerols, volatile phenols, and aromatic ketones[20–21].

BTN2, encoded by Btn2p, could interact with Rhb1p, which inhibits the activity of Can1p arginine permease[22]. Knockout of *BTN2* leads to the loss of arginine uptake by Can1p arginine permease, thereby preventing arginine metabolism as well as the production of urea and citrulline. In our previous study[23], it was discovered that the reduction in EC in Huangjiu through mixed fermentation was accompanied by a significant downregulation of *BTN2* in *S. cerevisiae*. This implies that different fermentation systems may influence the transcriptional expression of *BTN2*, resulting in EC regulation.

Building upon our prior research, we isolated a strain of *Pediococcus pentosaceus* (PP) from Chinese koji provided by a Huangjiu company. This strain is among the most prevalent LAB strains in Chinese koji. Despite its ability serve as a fermenter during wine production[9], studies on PP are infrequently reported. There is also no reported research on investigating the reduction in EC content through mixed fermentation with PP. However, we found that mixed-culture fermentation of *BTN2* knockout strains with PP exhibited a more favorable inhibitory effect on EC precursors[24], especially urea and citruline. Consequently, we applied a *BTN2* knockout strain to different Huangjiu fermentation systems to investigate the regulation mechanism of EC and the alterations of basic qualities. Meanwhile, transcriptomic analysis was utilized to identify the key genes involved in single- and mixed-culture systems. Herein, we aim to provide novel perspectives for reducing the EC content in Huangjiu fermentation.

Materials and methods

Strains and chemicals

Wild-type *BTN2* was obtained from Hangzhou Medical College. The *BTN2* knockout strain was obtained from Shanghai Jiao Tong University. The PP used in this study was isolated from Chinese koji supplied by a Huangjiu company in Zhejiang Province. L-citrulline (> 98%), L-arginine (> 98.5%), L-ornithine (> 98%), urea (> 98%), EC (> 99%), Trizol, Avian Myeloblastosis Virus Reverse Transcriptase and SYBR® Green Premix Pro Taq HS qPCR Kit II were purchased from Sangon Biotech Corporation (Shanghai, China). Methanol and acetonitrile (HPLC grade) were obtained from China National Pharmaceutical Group Corporation (Shanghai, China).

Huangjiu fermentation process

All three groups of Huangjiu (SC, *S. cerevisiae*; SK, *BTN2* knockout strain; SPK, *BTN2* knockout strain with PP) were produced according to the method described by Fang et al.[23] (Fig. 1). PP was added on Day 10 of fermentation.

Determination of EC, extracellular arginine, citrulline, ornithine and urea

EC was determined by high-performance liquid chromatograpy with fluorescence detection (HPLC-FLD)[14]. Extracellular arginine, citrulline and ornithine were detected by high-performance liquid chromatography–ultraviolet (HPLC-UV) according to the method of Li & Chen[25]. Urea was quantified following the methods of Fu et al.[4].

Key enzyme activity assays

The urease activity was measured using a urease assay kit purchased from Boxbio Science & Technology Corporation (Beijing, China, Catalog Number: AKNM003M). One urease unit was defined as the amount of enzyme required to catalyze the degradation of urea to 1 μmol of ammonia per minute. ADI and ornithine transcarbamoylase (OTC) activity levels were expressed as one unit of enzyme required to hydrolyze 1 μg of protein to yield 1 μmol of citrulline and ornithine per minute, respectively[26,27].

RNA extraction and transcriptome sequencing

In total, 50 mL of Huangjiu samples fermented for 20 d were rapidly centrifuged at 4 °C and 10,000 rpm for 5 min. Subsequently, the samples were washed with diethyl pyrocarbonate (DEPC) and water and centrifuged twice. The supernatant was discarded, and samples were frozen in liquid nitrogen for 15 min, then the samples were stored at −80 °C. Comparative RNA sequencing analysis was conducted by Guangzhou Magigene Co., Ltd.

The analysis system employed was the Illumina HiSeq 2500 sequencing system, and the raw data were filtered using Fastq to remove low-quality readings. Subsequently, the filtered data were compared with the reference genome GCA_003086655.1 (NCBI database) for the statistical matching ratio and the reading distribution on the reference sequence. Gene expression levels, Gene Ontology (GO) functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (metabolism, etc.,) were investigated.

Verification by reverse transcription–quantitative polymerase chain reaction

Ten differentially expressed genes (DEGs) were selected for reverse transcription–quantitative polymerase chain reaction (RT-qPCR) analysis, and the primers designed for this are listed in Supplementary Table S1. The mRNA expression level of genes was calculated using the $2^{-\Delta\Delta C_T}$ method.

Determination of the basic quality of Huangjiu

The amino acid concentration was determined with an automatic amino acid analyzer (L-8900; Hitachi Co., Tokyo, Japan), and volatile flavor compounds were detected by gas chromatography–mass spectrometry (GC-MS) with mass selective detection. The ethanol content was determined according to the methods described by Zhou et al.[14]. The color variation and sugar–acid ratio of Huangjiu were measured with a colorimeter (CR400, Japan) and a refractometer (PAL-BX/ACID, Tokyo, Japan), respectively.

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate significant differences ($p < 0.05$), and Origin 2022 software was used for creating graphics.

Results and discussion

Single- and mixed-culture fermentaion of *S. cerevisiae* to reduce EC levels

EC formation in different fermentation systems

As shown in Fig. 2a, EC content displayed an upward trend throughout the fermentation period. SK and SPK exhibited lower EC content compared with control group. Notably, the SPK group exhibited the lowest and most stable EC content. Therefore, it could be concluded that the *BTN2* knockout strain could effectively reduce EC, and that mixed fermentation with PP exerted a more favorable effect on reducing EC, which is consistent with previously reported research[23,24].

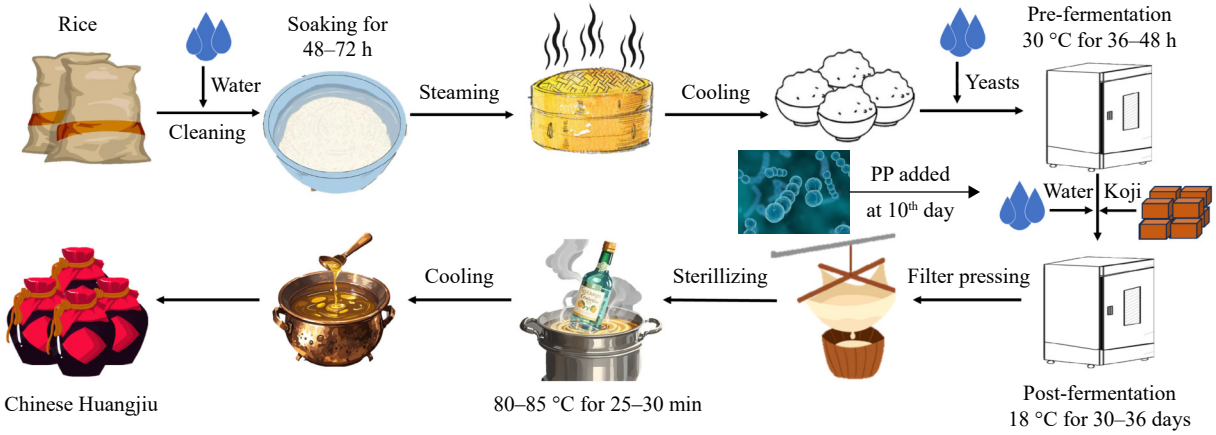

Fig. 1 Huangjiu fermentation process.

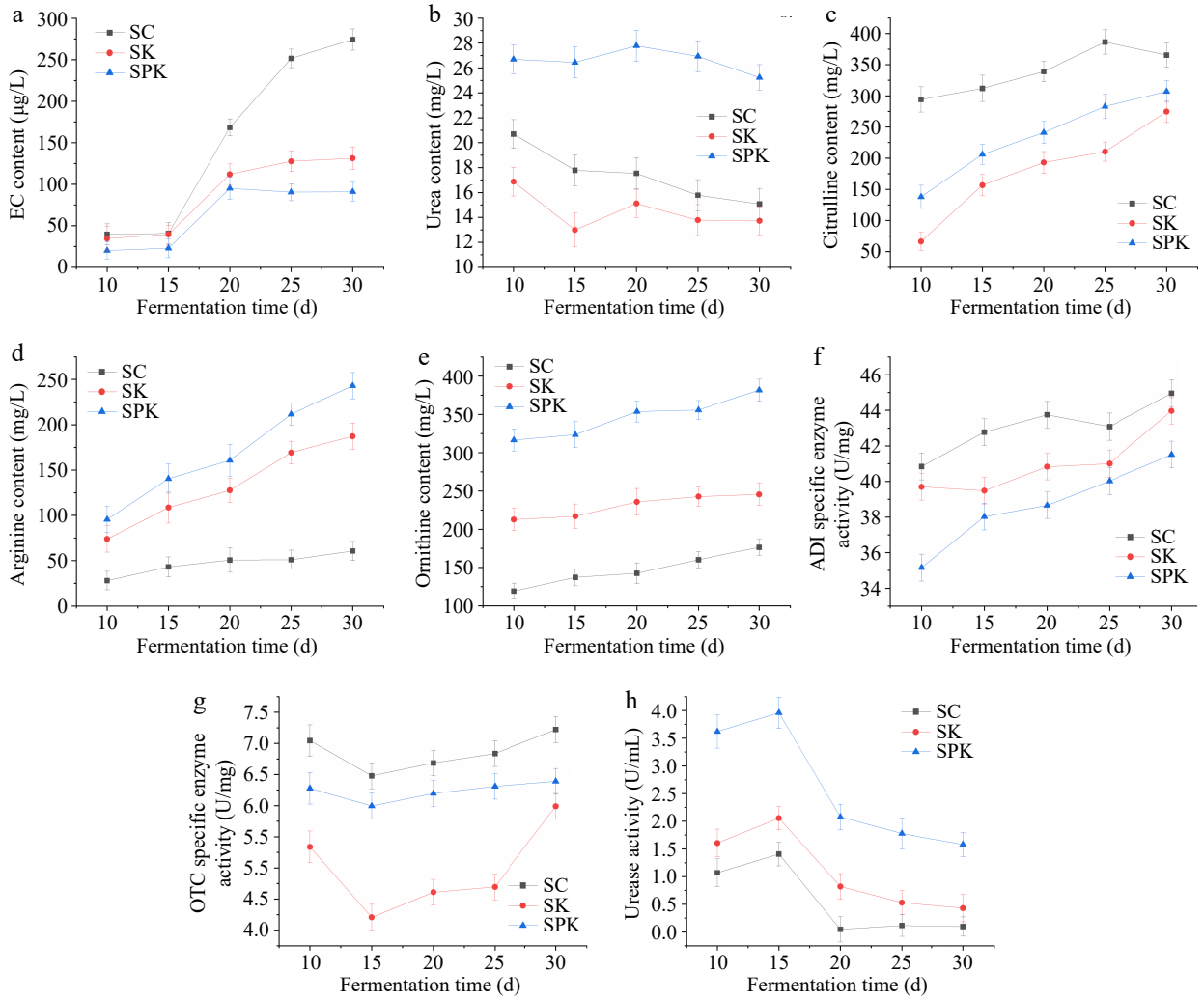

Fig. 2 Variation in EC, arginine-related metabolites and enzymes in the processing of Huangjiu under different fermentation systems. (a) Effect of different fermentation systems on EC formation. (b) Urea content. (c) Citrulline content. (d) Arginine content. (e) Ornithine content. (f) ADI-specific activity. (g) OTC-specific activity. (h) Urease activity. SPK, *BTN2* knockout strain with PP; SC, *S. cerevisiae*; SK, *BTN2* knockout strain.

Contributions of urea and citrulline to EC formation

Urea and citrulline served as the primary precursors of EC[28]. Consequently, one of the crucial approaches to eliminate EC was to regulate their generation. As shown in Fig. 2b, c, SK exhibited a lower content of urea and citrulline, resulting in reduced EC production. SPK had a higher urea content but a lower citrulline content. It was hypothesized that the pathway for EC synthesis from urea was inhibited during the fermentation of SPK, and thus EC was mainly produced by the reaction of citrulline with ethanol. This also accounted for the lower EC production in SPK.

The variations in arginine and ornithine are shown in Fig. 2d, e. The arginine and ornithine contents in SPK and SK were higher than those in SC and were on the rise, indicating that amino acids were constantly produced by cellular metabolism during the fermentation of Huangjiu. By integrating the results in Table 1 and Fig. 3, it was found that many of the upregulated DEGs in SPK and SK were associated with amino acid transport. This suggested that the knockout of *BTN2* influenced amino acid transport in *S. cerevisiae*, which, in turn, affected the amino acid content during the fermentation of Huangjiu.

In conclusion, the *BTN2* knockout strain could reduce EC formation by inhibiting the precursors, and mixed fermentation with PP may inhibit the reaction between urea and ethanol, leading to reduced EC in SPK.

Comparative analysis of key enzyme activity

Citrulline is primarily generated by PP through the degradation of ADI[14]. Subsequently, it could be subsequently degraded to ornithine and carbamoyl phosphate by OTC. Urease could decompose to urea directly[29,30]. Therefore, it was necessary to study the activities of these key enzymes involved in the fermentation process.

As can be seen from Fig. 2f, g, the ADI activities of SK and SPK were reduced compared with the control group. This reduction would result in a greater consumption of arginine in SC, thereby generating more citrulline and EC, which is consistent with the fingdings presented in Fig. 2a, c. According to Fig. 2g, mixed fermentation could increase OTC activity. This might accounted for the lower EC content in the mixed-culture group compared with the single-culture group.

The urease activities of both SK and SPK were higher than that of SC, with SPK exhibiting the highest activity (Fig. 2h). This indicates that the *BTN2* knockout strain could increase urease activity. This phenomenon may be due to the accumulation of urea stimulating an increase in urease activity.

Table 1. DEGs in *S. cerevisiae* and their functions.

Genes	Function	Log_2(Fold change)
DEGs and their functions in SK		
Ard1	Predicted to enable DNA-binding transcription factor activity and RNA Polymerase II *cis*-regulatory region sequence-specific DNA-binding activity.	3.7122
Ntc20	Predicted to enable reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase activity. Involved in NADH oxidation and glycolytic fermentation to ethanol.	3.563
Shq1	Involved in the cellular response to amino acid stimuli and transcription factor catabolic processes. Located in the inner nuclear membrane	3.3911
Erv15	Enables organic acid transmembrane transporter activity. Involved in organic acid transport.	3.3432
Mum3	Enables protein kinase activity. Involved in several processes	3.3293
Mga1	Predicted to enable DNA-binding transcription factor activity and sequence-specific DNA-binding activity.	2.909
Pex21	Enables adenosine triphosphate synthase (ATPase) activator activity and unfolded protein binding activity.	2.6193
Mud1	Enables mannosyltransferase activity. Involved in glycosylphosphatidylinisotol (GPI) anchor biosynthetic process.	2.4528
Cab1	Enables adenosine triphosphate (ATP)-dependent peptidase activity.	2.3866
Rrg8	Predicted to enable amino acid transmembrane transporter activity.	2.285
Asi2	Enables DNA-binding activity, bending and centromeric DNA-binding activity.	2.2283
Kip2	Involved in DNA repair. Located in the cytosol and nucleus.	2.1517
Rps9	Enables cyclin-dependent protein serine/threonine kinase regulator activity	2.1481
Gzf3	Enables cyclin-dependent protein serine/threonine kinase regulator activity.	2.1396
Kar1	Enables 3′–5′ DNA helicase activity and enzyme activator activity.	2.064
Yar1	Enables protein kinase inhibitor activity. Involved in fungal-type cell wall organization and negative regulation of protein kinase activity.	2.0084
Mak16	Predicted to enable oxidoreductase activity. Involved in protein targeting to membrane and protein transport into membrane rafts.	1.9673
Gar1	Predicted to enable DNA-binding activity. Involved in regulation of fungal-type cell wall biogenesis and regulation of the mitotic cell cycle.	1.9195
Osw1	Enables dicarboxylic acid transmembrane transporter activity and sulfate transmembrane transporter activity.	−2.9978
Aro80	Enables protein transporter activity and unfolded protein binding activity.	−2.4876
Rec104	Enables DNA secondary structure binding activity.	−2.2241
Shy1	Enables cyclin-dependent protein serine/threonine kinase regulator activity.	−1.5477
Bud27	Enables protein kinase activity. Involved in several processes, including DNA recombination, positive regulation of DNA-dependent DNA replication and protein phosphorylation.	−1.2341
Lif1	Enables DNA replication origin binding activity and adenylate kinase activity.	−1.2149
Sip4	Enables sequence-specific DNA-binding activity.	−1.2094
Lam5	Enables DNA-binding transcription factor activity, RNA Polymerase II-specific activity and RNA Polymerase II cis-regulatory region sequence-specific DNA-binding activity	−1.1971
Pib2	Involved in DNA recombinase assembly and maintenance of rDNA.	−1.1861
Atg5	Enables RNA Polymerase I general transcription initiation factor activity.	−1.165
Apl5	Enables sequence-specific DNA-binding activity.	−1.1553
Vac17	Enables DNA-binding transcription factor activity. Involved in the response to xenobiotic stimulus.	−1.1399
Fet3	Involved in positive regulation of transcription by RNA Polymerase II.	−1.1133
Lre1	Enables DNA-binding transcription activator activity, RNA Polymerase II-specific.	−1.046
DEGs and their functions in SPK		
Sbh1	Enables guanyl nucleotide exchange factor activity and protein transmembrane transporter activity. Contributes to protein-transporting ATPase activity.	3.0518
Utp4	Involved in maturation of small subunit (SSU) rRNA from tricistronic rRNA transcripts and positive regulation of transcription by RNA Polymerase I.	2.6671
Dpm1	Enables dolichyl-phosphate beta-D-mannosyltransferase activity.	1.5603
Gar1	Enables Box H/ACA small nucleolar (snoRNA) binding activity.	1.5532
Tif11	Enables RNA binding activity, ribosomal small subunit binding activity and translation initiation factor binding activity.	1.5054
Adk1	Enables DNA replication origin binding activity and adenylate kinase activity.	1.2293
Git1	Enables glycerol-3-phosphate transmembrane transporter activity and glycerophosphodiester transmembrane transporter activity.	1.2141
Dic1	Enables dicarboxylic acid transmembrane transporter activity.	1.1481
Egt2	Predicted to enable cellulase activity.	1.1403
Mcm4	Enables DNA replication origin binding activity and single-stranded DNA-binding activity.	1.129
Hxt6	Enables glucose transmembrane transporter activity and pentose transmembrane transporter activity.	1.1233
Mot3	Enables DNA-binding transcription factor activity	1.0971
Cin5	Enables DNA-binding transcription factor binding activity and sequence-specific DNA-binding activity.	1.0629
Kti12	Enables chromatin binding activity.	1.0457
Hop1	Enables four-way junction DNA-binding activity.	−1.0204
Tah11	Enables DNA replication origin binding activity.	−1.0225
Cbf2	Enables DNA-binding activity, bending and centromeric DNA-binding activity.	−1.0359
Bur2	Enables cyclin-dependent protein serine/threonine kinase regulator activity.	−1.0365
Lpx1	Enables triglyceride lipase activity. Involved in the triglyceride catabolic process.	−1.054
Stn1	Enables single-stranded telomeric DNA-binding activity and translation elongation factor binding activity.	−1.0597
Pcl10	Enables cyclin-dependent protein serine/threonine kinase regulator activity.	−1.1792
Ast1	Predicted to enable oxidoreductase activity. Involved in protein targeting to the membrane and protein transport into membrane rafts.	−1.1986

Table 1. (continued)

Genes	Function	Log_2(Fold change)
Rog3	Enables ubiquitin protein ligase binding activity.	−1.2246
Ctf3	Involved in the initiation of DNA replication, establishment of mitotic sister chromatid cohesion and mitotic spindle assembly checkpoint signaling.	−1.2298
Pga2	Involved in protein transport. Located in the endoplasmic reticulum and the nuclear envelope.	−1.2993
Faa4	Enables long-chain fatty acid–coenzyme A (CoA) ligase activity. Involved in long-chain fatty acid import into the cell	−1.4328
Tda6	Predicted to be involved in protein transport. Located in the cell periphery and fungal-type vacuoles	−1.5558
Cyk3	Enables enzyme regulator activity. Involved in secondary cell septum biogenesis.	−1.7224
Sps1	Enables protein kinase activity. Involved in several processes, including ascospore wall assembly and meiotic spindle disassembly	−1.9151

Transcriptome analysis of *S. cerevisiae* in single and mixed fermentation cultures

Transcriptome analysis of S. cerevisiae in different fermentation systems

The level of difference ($|\log_2(\text{Fold change})| > 1$) and significance level ($p < 0.05$) were employed to obtain the number of DEGS in Huangjiu under different fermentation systems[31]. As shown in Fig. 3, the red and green dots represented a significant decrease or increase, respectively, in the DEGs compared with the control group, and the gray points indicate nonsignificant DEGs. In total, 320 DEGs were found in the single-culture system; among these, 200 DEGs were upregulated and 120 DEGs were downregulated (Fig. 3a). Meanwhile, there were 36 upregulated DEGs and 23 downregulated DEGs during mixed-culture fermentation (Fig. 3b).

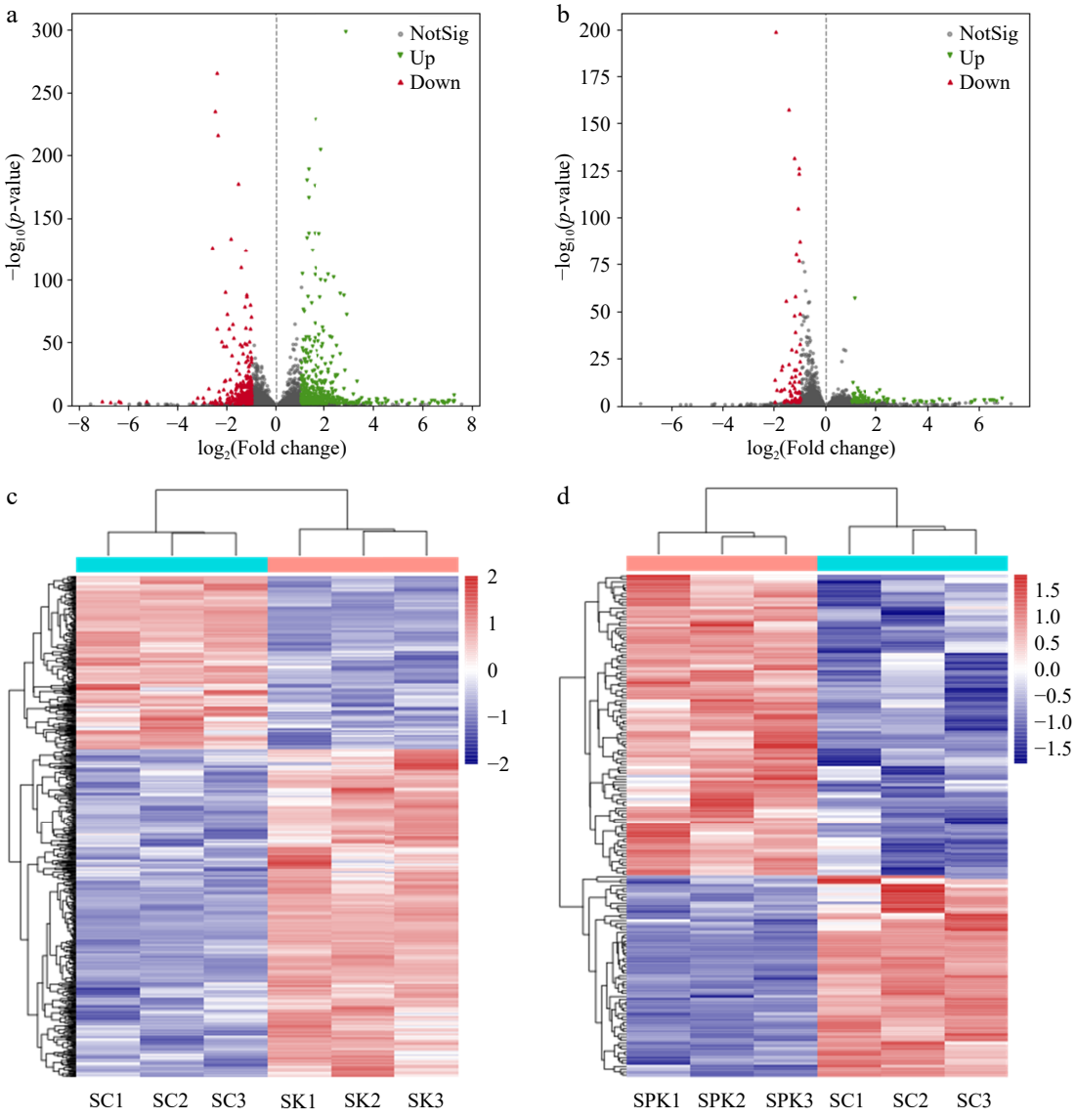

Fig. 3 Heatmap and volcano plot of DEGs. (a) DEGs in SK. (b) DEGs in SPK. (c) Volcano plot of DEGs in SC and SK. (d) Volcano plot of DEGs in SC and SPK. SPK, *BTN2* knockout strain with PP; SK, *BTN2* knockout strain.

The datasets of significant DEGs were analyzed via the DESeq2 database to gain a more in-depth understanding of how different fermentation systems affected the overall regulatory network of *S. cerevisiae*[32]. The activity of each transcription factor was predicted according to the number of documented targets[33], and the results are presented in Fig. 3c, d.

To better clarify the DEGs and their functions, several examples were selected and the results are shown in Table 1. A positive (negative) multiplicity of difference indicates an increase (decrease) in gene expression compared with SC.

Transcriptional profiling of S. cerevisiae in different fermentation systems

To gain a more intuitive understanding of how *BTN2* knockout strains regulate the metabolic pathways of *S. cerevisiae* in different fermentation systems, KEGG and GO analysis were performed[34], and the results areshown in Fig. 4.

It can be seen in Fig. 4a that the DEGs in SK were associated with 30 cell functions, which were categorized into three major parts: molecular functions, cellular components and biological processes. Fourteen molecular functions were involved, including translational regulator activity, transcriptional regulatory activity, molecular function regulator activity, etc. Fifteen functions were related to biological processes, including bio-regulation, reproductive processes and cellular processes. A substantial varity of genes related to metabolic processes, cellular processes and catalytic activities were identified. These finding implied that knockout of *BTN2* exerted a significant influence on molecular functions and biological processes in *S. cerevisiae*.

Figure 4b indicates that the DEGs in SPK participated in a total of 25 molecular functions. Compared with the single-culture fermentation group, the genes involved in molecular functions decreased. Most genes were associated with biological processes, such as bio-regulation, cellular processes and metabolic processes. This suggested that mixed-culture fermentation has a greater impact on the biological processes of *S. cerevisiae*.

According to Figs 3 and 4, it was observed that a large number of genes were upregulated in both SK and SPK. Genes with an

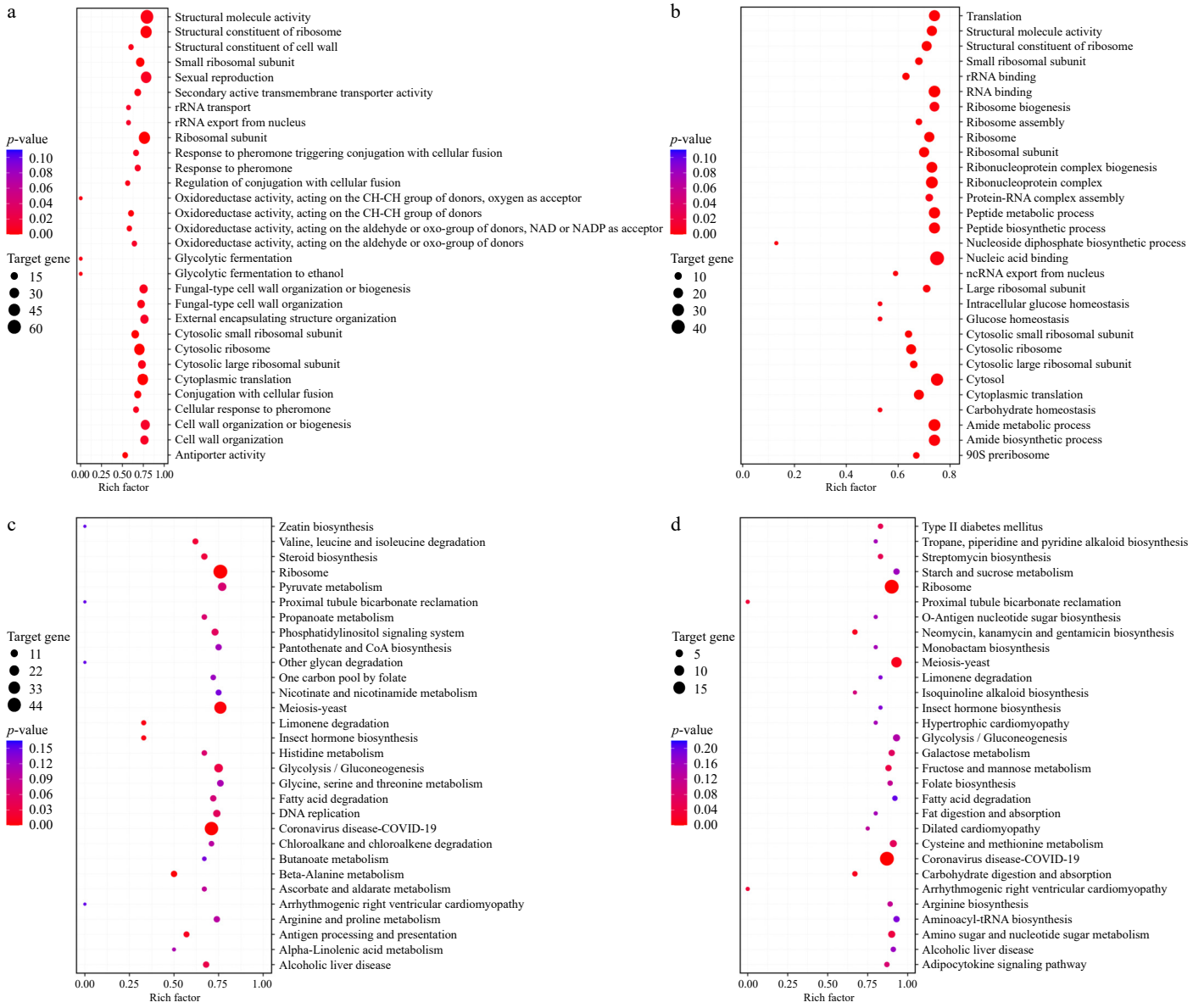

Fig. 4 GO and KEGG analysis. (a) GO analysis of SK. (b) GO analysis of SPK. (c) KEGG analysis of SK. (d) KEGG analysis of SPK. SPK, *BTN2* knockout strain with PP; SK, *BTN2* knockout strain.

increased expression level were detected in SK, especially *Prp38* (transcription), *Sbh1* (folding, sorting and degradation; transport and catabolism), *Nej1* (replication and repair), *Pop7* (translation), *Rdh54* (replication and repair), *Pdc5* (carbohydrate metabolism), *Izh4* (signal transduction), *Leu9* (amino acid metabolism), *Dib1* (transcription), *Dpm1* (glycan biosynthesis and metabolism), *Cab1* (metabolism of cofactors and vitamins), *Hxt6* (cell growth and death), *Sok2* (cell growth and death), *Ccz1* (transport and catabolism) and *Iqg1* (cell motility).

The following genes were identified as being upregulated in SPK: *Prp38* (transcription), *Sbh1* (folding, sorting and degradation; infectious disease: bacterial; transport and catabolism), *Utp4* (translation), *Fmp30* (nervous system), *Dpm1* (glycan biosynthesis and metabolism), *Adk1* (metabolism of cofactors and vitamins; nucleotide metabolism), *Dic1* (excretory system), *Mcm4* (cell growth and death; replication and repair), *Hxt6* (cell growth and death) and *Rog1* (signal transduction).

Our research concentrated on three genes, namely *Lso2*, *Shq1*, and *Kti12*. *Lso2* is associated with DNA-binding activity. It participates in the catabolic processes of aromatic amino acids and the positive regulation of transcription by RNA polymerase II. *Shq1* is involved in the cellular response to amino acid stimuli and transcription factor catabolic process. *Kti12* is related to the transmembrane transport of amino acids and was predicted to be involved in amino acid transmembrane transport. All three genes are involved in amino acid transport and transcription factor expression, suggesting that *BTN2* knockout affected the regulation of other transcription factors and amino acid transport, which, in turn, affected EC formation in Huangjiu.

In total, 35 identical DEGs were identified between SK and SPK, including *Ard1*, *Csi1*, *Lso2*, *Mrx7*, etc. The expression levels of these genes were dramatically upregulated in both SK and SPK, and all of them are related to the activities of protein transporters and DNA-binding transcription factors. In addition, there were eight DEGs with significant differences, namely *Ast1p*, *Cbf2p*, *Pac1p*, *Pcl10p*, *Pga2p*, *Rog3p*, *Sps1p* and *Tda6p*. Among them, *Pac1p* is associated with DNA-binding activity, histone-binding activity, and lipid metabolism. *Sps1p* is associated with lipid-binding activity, sequence-specific mRNA binding activity, and cell growth and apoptosis.

In order to verify the results of RNA-seq, 10 DEGs were selected for RT-qPCR analysis and the results areshown in Fig. 5. There are six upregulated DEGs (e.g., *Lso2, Nct20* and *Shq1*) and three downregulated DEGs (such as *Osw1, Shy1* and *Sps1*) in the RT-qPCR results, consistent with the results of RNA-seq. This indicates that the results of RNA-seq were reliable.

In summary, *BTN2* knockout and PP significantly affected gene expression in *S. cerevisiae*. A large number of genes upregulated in SK and SPK were involved in multiple processes such as transcription, metabolism, and cell growth. Genes involved in molecular functions were reduced in SPK compared with SC. Most of the genes were related to biological processes, suggesting that mixed-culture fermentation had a greater impact on biological processes in *S. cerevisiae,* and *BTN2* knockout affected the expression of genes related to amino acid transport and transcription factor metabolism, which, in turn, affected EC formation.

Effect of single- and mixed-culture fermentaion of *S. cerevisiae* on the basic quality of Huangjiu

Effects of single- and mixed-culture fermentaion on amino acids

The decomposition of proteins in the raw materials of Huangjiu could produce various free amino acids, which served as the precursors of flavor substances and are among the important criteria for evaluating the quality of Huangjiu[35].

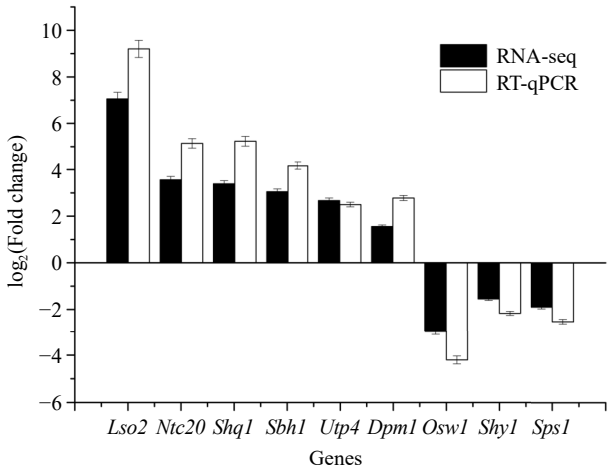

Fig. 5 The result of verification by RT-qPCR.

As shown in Fig. 6a, 18 free amino acids were detected. They could be classified into two categories: positively (aspartate [Asp], threonine [Thr], serine [Ser], glycine [Gly], proline [Pro], isoleucine [Ile], leucine [Leu]) and negatively (histidine [His], arginine [Arg], methionine [Met]) correlated with the flavor of Huangjiu[14]. The total amount of amino acids in SPK was greater than that in SK, indicating that mixed-culture fermentation could increase the amino acid concentration. In comparison with SC, the amount of positively correlated amino acids in SK was higher, while that of negatively correlated amino acids was reduced, implying that the *BTN2* knockout strain had a positive effect on free amino acids and thus could improve the flavor of Huangjiu.

We employed the variable importance in projection (VIP) method to identify the key compounds in different Huangjiu samples[35]. Compounds with VIP > 1 can be considered as significant discriminators. Aspartic acid, glutamic acid, and arginine were key discriminators of SPK (Fig. 6b).

The correlation analysis of the amino acids is presented in Fig. 6c. Most of the amino acids exhibited a high degree of correlation. The absolute values of the correlation coefficients were greater than 0.5, indicating a strong correlation among the quality indicators of free amino acids.

Effects on volatile flavor compounds

The flavor profile of Huangjiu is predominantly characterized by mellowness, bitterness and freshness, accompanied by acidity and sweetness[36]. In Fig. 6d, 20 volatile flavor compounds were detected, mainly consisting of alcohols and esters, with alcohols being the most abundant. Isoamyl alcohol and phenylethanol were the most dominant alcohols in the samples. SK exhibited the highest content of isoamyl alcohol and phenylethanol. Although the content of the main alcohols in SPK was lower than that in SK, the variety was more diverse, including octanol, isopropyl alcohol and isobutyl alcohol. These alcohols are also important flavor-contributing substances.

Esters were the most diverse volatile flavorings in Huangjiu[37]. The content of some esters in SK and SPK was relatively high, such as ethyl acetate, ethyl oleate, ethyl linoleate and ethyl palmitate, all of which are the main contributors to the aromatic character of Huangjiu. The VIP method was also employed to identify the key compounds in different samples. Six volatile flavor substances could be regarded as significant indicators (Fig. 6e). Among them, isopropanol, diethyl succinate and ethyl myristate were the key discriminatory compounds for SC, while phenyl ethanol and

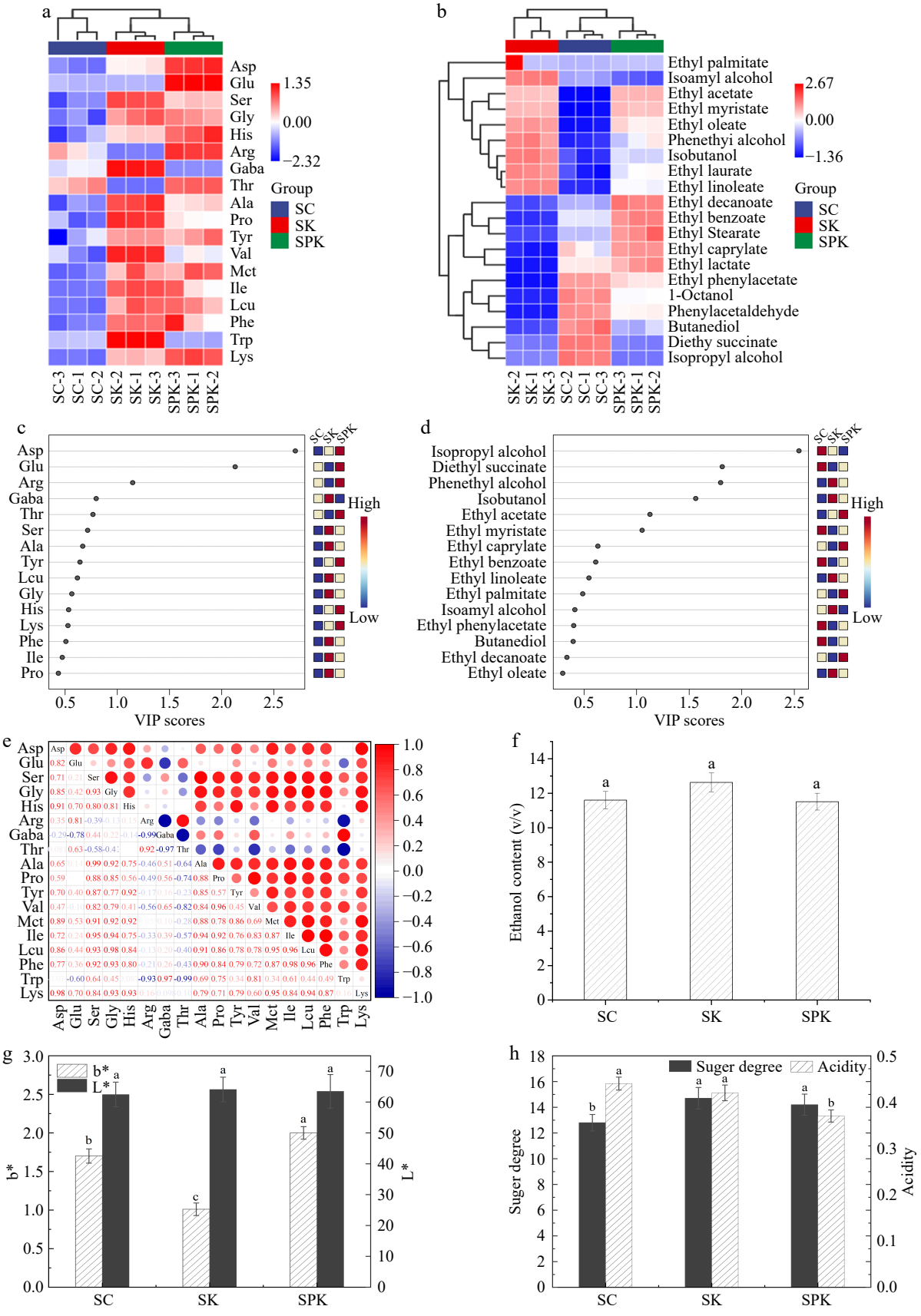

Fig. 6 Analysis of amino acids and volatile flavor substances in Huangjiu under different fermentation systems. (a) Cluster heat map analysis of free amino acids in Huangjiu. (b) variable importance inprojection (VIP) scores of free amino acids under different fermentation systems. (c) Heat map of free amino acid correlations. (d) Cluster heat map analysis of volatile flavor substances (e) VIP score of volatile flavor substances under different fermentation systems. (f) Alcohol content. (g) b* and L* values. (H) Sugar–acid ratio. SPK, *BTN2* knockout strain with PP; SC, *S. cerevisiae*; SK, *BTN2* knockout strain.

isobutanol were the dominant flavor substances for SK, and ethyl acetate was the most important flavor-contributing compound for SPK.

In conclusion, mixed-culture fermentation facilitated the formation of various volatile flavor compounds during the fermentation of Huangjiu, thereby endowing Huangjiu with a more favorable flavor.

Effect on ethanol content

In accordance with the methods proposed by Zhou et al.[14], the ethanol content in Huangjiu should be no less than 8% (v/v). As shown in Fig. 6f, all samples conformed to this standard. Compared with SC, the alcohol content of SK increased slightly, whereas the difference between SPK and SC was not statistically significant.

Effects on the color and sugar–acid ratio of Huangjiu

The color of Huangjiu is one of the most important indicators of the overall quality[36]. According to Fig. 6g, SK and SPK exhibited higher L* values, which can be attributed to the relatively high content of phenylalanine and tyrosine in these two samples. Phenylalanine and tyrosine are important color-producing amino acids that participated in the meladic reaction during the decoction process, resulting in the production of more nigrosome-like substances[38,39]. Therefore, *BTN2* knockout led to the accumulation of color-producing amino acids, and mixed-culture fermentation had no significant impact on lightness. The b* values indicate the yellow–blue color tones. In Huangjiu samples, the b* value of SPK was higher than that of SK, suggesting that PP may significantly improve the yellow colour.

As depicted in Fig. 6h, the Brix values of SPK and SK were significantly higher than those of SC. Meanwhile, the acid concentration of SPK was lower than that of the single-culture fermentation samples, which was also associated with the content of free amino acids and volatile flavoring substances, as previously mentioned.

Conclusions

This study investigated the mechanism of EC regulation by the *BTN2* knockout strain in different Huangjiu fermentation systems. The findings indicated that the *BTN2* knockout strain could diminish the EC content by reducing EC precursors during the fermentation process. Moreover, mixed fermentation with PP could inhibit the reaction between urea and ethanol, resulting in a reduction in EC.

Transcriptomic analysis revealed that the knockout of *BTN2* and the addition of PP affected the gene expression of *S. cerevisiae*. *BTN2* knockout exerted a more substantial influence on molecular function and biological processes, while the mixed-culture fermentation had a greater effect on biological processes. The *BTN2* knockout affected the regulation and amino acid transport processes of other transcription factors, thereby influencing the formation of EC. For instance, the expression of genes related to transcription factor activity and amino acid transport, such as *Lso2*, *Shq1* and *Kti12*, was significantly upregulated.

Regarding the basic quality of Huangjiu, both the mixed-culture fermentation and *BTN2* knockout strain could increase the amino acid content, thereby improving the flavor of Huangjiu. SK and SPK exhibited the highest lightness values, which may be attributed to the color-producing amino acids, such as phenylalanine and tyrosine.

In conclusion, this study provides a practical foundation for the regulation of EC by *BTN2* in different fermentation systems. It contributes to a deeper understanding of EC's formation mechanism in Huangjiu and provides a novel strategy for reducing EC. Consequently, this study holds important theoretical value for the Huangjiu industry.

Author contributions

The authors confirm their contributions to the paper as follows: conceptualization: Fang R; methodology: Fang R, Hu J; writing – original draft: Fang R, Xu H; writing – review & editing: Chen T, Tang X, Xiao G, Zhou A; funding acquisition: Fang R, Tang X; software: Zhou H, Hu J; formal analysis: Zhou H, Xu H; visualization: Zhou H, Lin W, Xu H; data curation: Zhou H, Lin W; investigation: Shi C, Lin W; validation: Shi C, Hu J; Zhou A; resources: Shi C, Tang X; project administration, supervision: Chen T, Xiao G. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All relevant data are within the manuscript and its supplementary files.

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Conflict of interest

The authors declare that they have no conflict of interest.

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