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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Improving flavor metabolism of *Saccharomyces cerevisiae* by mixed culture with *Bacillus licheniformis* for Chinese *Maotai*-flavor liquor making

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Abstract Microbial interactions could impact the metabolic behavior of microbes involved in food fermentation, and therefore they are important for improving food quality. This study investigated the effect of *Bacillus licheniformis*, the dominant bacteria in the fermentation process of Chinese *Maotai*–flavor liquor, on the metabolic activity of *Saccharomyces cerevisiae*. Results indicated that *S. cerevisiae* inhibited the growth of *B. licheniformis* in all mixed culture systems and final viable cell count was lower than 20 cfu/mL. Although growth of *S. cerevisiae* was barely influenced by *B. licheniformis*, its metabolism was changed as initial inoculation ratio varied. The maximum ethanol productions were observed in *S. cerevisiae* and *B. licheniformis* at 10^6:10^7 and 10^6:10^8 ratios and have increased by 16.8 % compared with single culture of *S. cerevisiae*. According to flavor compounds, the culture ratio 10^6:10^6 showed the highest level of total concentrations of all different kinds of flavor compounds. Correlation analyses showed that 12 flavor compounds, including 4 fatty acids and their 2 corresponding esters, 1 terpene, and 5 aromatic compounds, that could only be produced by *S. cerevisiae* were significantly correlated with the initial inoculation amount of *B. licheniformis*. These metabolic changes in *S. cerevisiae* were not only a benefit for liquor aroma, but may also be related to its inhibition effect in mixed culture. This study could help to reveal the microbial interactions in Chinese liquor fermentation and provide guidance for optimal arrangement of mixed culture fermentation systems.

Keywords *Bacillus licheniformis* · Ethanol · Flavor compounds · *Maotai*-flavor liquor · Metabolic behavior · *Saccharomyces cerevisiae*

Introduction

Traditional food fermentation has been carried out in a spontaneous way and multiple species are involved. Microbial communities are the major drivers of fermentation processes [1]. Their stability is important for the success and safety of food fermentation. The study on microbial communities includes identifying the diversity of species, the functions of individual organisms, microbial interactions, and predictions at the community scale [2]. Microbial interactions are the fulcrum of communities. It would influence the growth and fermentative behavior of microbes, thereby influencing structures and functions of the microbial community [3, 4]. Studying them is essential to understand community function, but that study is challenging because of the microbial diversity and the complexity of spontaneous fermentation processes. Over the past decades, microbial interaction between functional microorganisms in wine, such as yeast–yeast and yeast–lactic acid bacteria, has been studied extensively [5–9]. These have driven an explosion of knowledge that will be successfully applied in

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improving the stability and sensory property of fermented foods like wine and dairy. However, relatively little is known about how the microbes interact with each other in Chinese liquor fermentation. This leads to confusion about the production mechanism of the flavor compound, which makes it difficult to understand the fermentation process of Chinese liquor, and then hinder the standardization and optimization of Chinese liquor production.

Chinese *Maotai*-flavor liquor is a symbolic drink in China and is famous for its soy sauce-like and roasted aroma style [10, 11]. The fermentation of Chinese *Maotai*-flavor liquor is a typical spontaneous process, which includes Daqu making, stacking fermentation, and alcoholic fermentation in pits [11]. Daqu is a mixture of milled wheat and a complex microbial community. It undergoes a 30-day spontaneous fermentation and stored for 3–4 months to mature. The mature Daqu powder was then mixed with sorghum that was steamed before and stacked as a cone on the ground for 2–7 days stacking fermentation. After that, the fermented grain from stacking fermentation is put into pits for alcoholic fermentation under anaerobic conditions.

The complex microbial community involved in *Maotai*-flavor liquor making process has been studied for decades [12]. More than 300 microorganisms have been isolated and characterized in the whole fermentation process [13]. *B. licheniformis* was found to be the dominant bacteria during the whole fermentation process. It could produce C4 compounds (mainly pyrazines, volatile acids, aromatic and phenolic compounds) with a high yield which will influence the aroma of *Maotai*-flavor [14]. *S. cerevisiae* was the most important group of microorganisms in the fermentation process, since they contribute significantly to the fermentation rate, product flavor, and quality. The two strains lived together and shared the same environment. It is critical, then, to be able to study their behavior in mixed culture. In this study, we compared the metabolic profiles of *S. cerevisiae* in mixed culture with different amounts of *B. licheniformis* on a laboratory scale. Sorghum extract was used as a fermentation medium. The starch material in it was hydrolyzed into fermentable sugars through α-amylase and glucoamylase. Flask-shaking fermentation instead of solid-state fermentation was used to ensure optimal connection between both strains. This study may help reveal the microbial interaction in fermentation of *Maotai*-flavor liquor and understand the production mechanism of flavor compounds.

Materials and methods

Strains and growth medium

Two indigenous strains, *S. cerevisiae* CCTCC M2014463 and *B. licheniformis* CGMCC 3962 were used in this study. Both strains were previously isolated from samples of Daqu from *Maotai*-flavor liquor and deposited in China Center for Type Culture Collection and China General Microbiological Culture Collection Center, respectively. Stock cultures of *S. cerevisiae* and *B. licheniformis* strain were kept frozen at −80 °C in YEPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and LB medium (20 g/L NaCl, 20 g/L peptone, 10 g/L yeast extract) containing 50 % glycerol at 40 % (v/v), respectively.

Glutinous sorghum named "hongyingzi" from *Maotai* wine factory was used for making sorghum extract. It was prepared according to the methods described below. 500 g of ground sorghum was added with 2 L of deionized water plus moderate thermostable α-amylase solution (2×10^7 U/L). The mixture was cooked for 2 h, followed by saccharification at 60 °C for 4 h with the addition of glucoamylase (5×10^7 U/L). Finally, the above mixture was centrifuged for 10 min at 8000 rpm and the supernatant was collected as sorghum extract. The sugar content was measured on a Leica refractometer (Fisher Scientific, Pittsburg, PA, USA). Sorghum extract was diluted with water to give a final sugar concentration of 10°B× (75 ± 5 g/L) and were sterilized at 121 °C for 20 min before use.

Fermentations

Fermentations were carried out for the single cultures of *S. cerevisiae* and *B. licheniformis*, respectively, as well as for their mixed cultures with different inoculation ratios.

S. cerevisiae and *B. licheniformis* were pre-cultured, respectively, in a YPD liquid medium at 30 °C and LB liquid medium at 37 °C, for 16 h. Appropriate pre-cultures were sucked and centrifuged for collecting cells and then inoculated into the fermentation medium after washing by saline. Aliquots of 50 mL of sorghum extract were fermented in 250 mL flasks at 30 °C, 200 rpm/min. Sorghum extract was inoculated with cells to obtain a cellular population of 1×10^6 cfu/mL. Mixed culture fermentations with *S. cerevisiae*:*B. licheniformis* at ratios of 10^6:10^5, 10^6:10^6, 10^6:10^7, and 10^6:10^8 were tested. Samples were taken from each flask throughout the fermentation process to enumerate viable cell counts, analyze sugar consumption, and produce ethanol. After fermentation, the fermented liquid was centrifuged (8000 rpm, 10 min) to remove cells for the analysis of flavor compounds in both single and mixed cultures. Each experiment was performed in triplicate.

Analytical determinations

Microscope was used to determine the cell numbers of *S. cerevisiae* and *B. licheniformis* in pre-cultures. Viable cell counts were determined by plate count during fermentation.

YEPD agar plates provided *S. cerevisiae* count, while LB agar plates yielded *B. licheniformis* count. Samples were analyzed for ethanol by high-performance liquid chromatography with a refractive index detector (Varian 355 RI). A BioRad 87H column (0.05 mM H_2SO_4 as a mobile phase, flow rate 0.6 mL/min, column temperature 60 °C, and injection volume of 10 μL) was used to determine ethanol content. Reducing sugars were assayed by DNS (dinitrosalicylic acid) method [15]. The pH was determined by a pH meter. Flavor compounds were analyzed by HS-SPME (headspace solid-phase microextraction) and GC–MS (gas chromatography–mass spectrometry) method according to previous studies [16].

Statistical analysis

One-way Analysis of Variance (ANOVA) and Tukey honest significant difference (HSD) tests with $\alpha = 0.05$ were performed by mean comparison. Principal component analysis (PCA) was carried out on the concentration of flavor compounds in order to visualize relationships between samples. Correlation analysis was used to make an expression of the concentrations of flavor compounds produced by *S. cerevisiae* varied with different initial inoculation ratios of *B. licheniformis*. Data applied to correlation analysis were first normalized to the final *S. cerevisiae* population (μg/10^6 cells) and then to correlation analysis. Statistical analyses were performed with SPSS 9.0 software.

Results and discussion

Influence of microbial interaction on cell growth

Cell growth and reducing sugar consumption were monitored in single and mixed cultures. Figure 1a shows viable cells of *S. cerevisiae* in single and mixed cultures. In all cultures tested, *S. cerevisiae* reached a maximum population (1.5–2.4 × 10^8 cfu/mL) within 24 h and then kept stable as fermentation progressed to completion. The growth and survival of *S. cerevisiae* in mixed cultures were kept nearly the same as that obtained in single cultures (Fig. 1a), which demonstrated that *S. cerevisiae* growth was barely influenced by mixed culture with *B. licheniformis*. However, *S. cerevisiae* presented a negative effect on *B. licheniformis* growth. As shown in Fig. 1b, single culture of *B. licheniformis* indicated a maximal population (2.2 × 10^8 cfu/mL) within 24 h. But when in mixed culture with *S. cerevisiae*, *B. licheniformis* population constantly decreased, no matter what the initial inoculation ratio was. Its final viable cell count was lower than 20 cfu/mL.

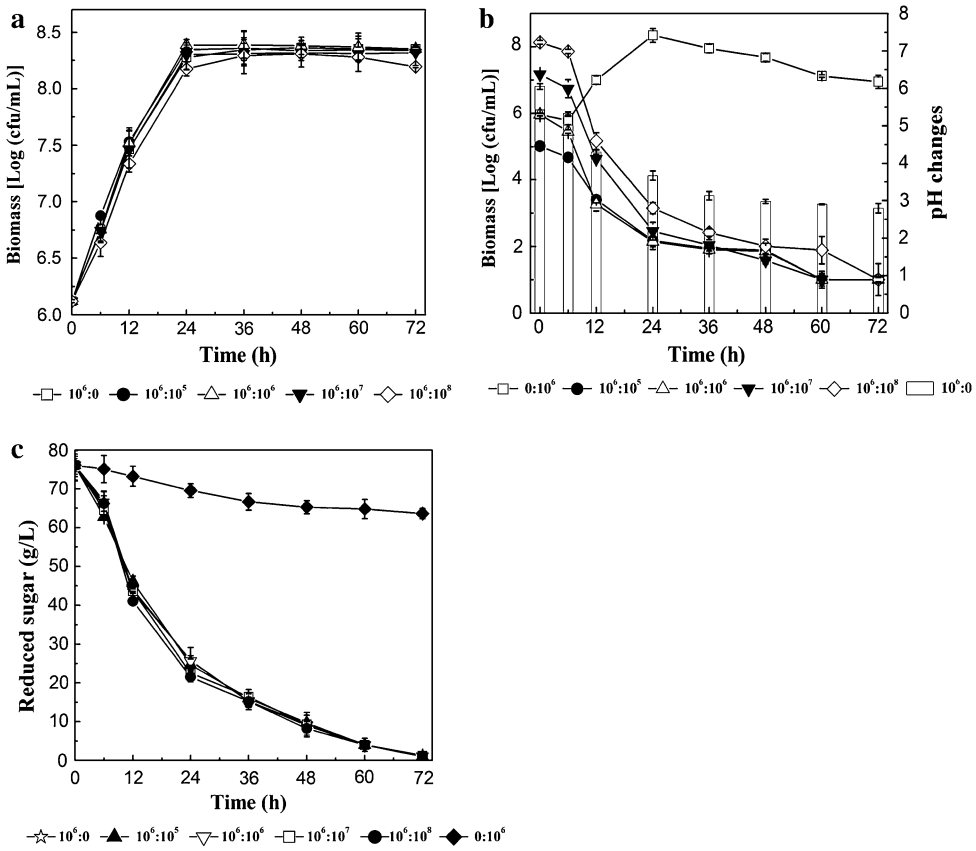

Fig. 1 Viable biomass and sugar consumption in single and mixed cultures of *S. cerevisiae* and *B. licheniformis* with different inoculation ratios (*S. cerevisiae*:*B. licheniformis*). **a** Viable biomass of *S. cerevisiae* in single and mixed cultures; **b** viable biomass of *B. licheniformis* in single and mixed cultures; **c** sugar consumption of different cultures

Similar results have also been described for *S. cerevisiae* and malolactic bacteria in both single and mixed cultures [17]. Traditionally, the disappearance of these bacteria during fermentation has been associated with their low tolerance to ethanol, lower pH, depletion of nutrients, or antipeptide. As shown in Fig. 1b, *S. cerevisiae* could lower the pH of the fermented broth below 4.0 within 12 h and the pH was about 2.8 at the end of fermentation. It has been reported that growth of *B. licheniformis* was delayed at acidic pH and was arrested at pH 4.0 [18]. Thus, this antagonistic effect was probably due to the lower pH. Fermentation profiles were measured by reduced sugar consumption (Fig. 1c). *S. cerevisiae* in single and mixed fermentation went to completion and, at day 3, each system contained less than 2 g/L of reduced sugar. No significant difference was observed in reducing sugar consumption of *S. cerevisiae* in single and mixed fermentation. In single culture of *B. licheniformis*, reducing sugar was consumed slowly compared with *S. cerevisiae* and about 10.0 g of reducing sugar was consumed in 3 days.

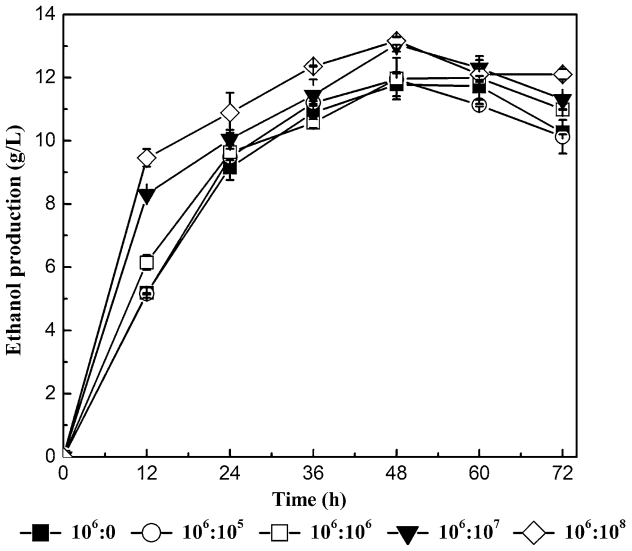

Fig. 2 The concentration of ethanol produced by *S. cerevisiae* with different initial inoculation ratios of *B. licheniformis* (*S. cerevisiae*:*B. licheniformis*)

Impact of *B. licheniformis* on ethanol production of *S. cerevisiae*

Ethanol production was different among different culture fermentation systems (Fig. 2). Mixed cultures with *S. cerevisiae*:*B. licheniformis* at 10^6:10^7 and 10^6:10^8 ratios produced more ethanol than other ratios during fermentation (Fig. 2). Remarkably, they almost have produced approximately two times higher ethanol (8.3–9.5 g/L) than other ratios at 12 h (5.2–6.1 g/L). And the maximum ethanol (13.1 and 13.2 g/L) they produced during fermentation has increased about 16.8 % compared with *S. cerevisiae* single culture (11.3 g/L). As viable counts and sugar consumption of *S. cerevisiae* in single and mixed culture were roughly the same (Fig. 1a, c), this increase may be due to the microbial interaction between yeast and bacteria. Annan et al. have also shown an enhancement of ethanol content with *S. cerevisiae*/*Lactobacillus fermentum* in mixed culture [19]. It has been reported that ethanol could play a role in interspecies interaction or might be as a signaling compound [6, 20]. However, further research was needed to find the convincing answer.

Impact of *B. licheniformis* on flavor compounds production by *S. cerevisiae* during mixed cultures

In order to assess the influences of interaction on the metabolism of flavor compounds, the results of GC analysis of the flavor fraction in different inoculation ratios were compared. A total of 29 flavor compounds were detected as presented in Supplementary Table 1. Samples showed significant differences for all the aroma compounds analyzed. The flavor compounds were used as variable vectors for chemometric analysis of samples to obtain more detailed information on metabolic activity changes in single and mixed cultures. Two principal components namely Component 1 and Component 2 were extracted that accounted for 74.43 and 18.32 % of the variance, respectively, in six variable systems.

In PCA plot, the relative orientation among the attribute vectors reflects the attribute correlations. It appears that mixed fermentations produced more flavor compounds compared with single fermentation by *S. cerevisiae* or *B. licheniformis* (Fig. 3). Especially fermentation was conducted with the culture ratio 10^6:10^6. It showed the highest level of total concentrations of all different kinds of flavor compounds (Table 1). Approximately, 2 times higher amounts of these compounds were produced by this mixed culture ratio than by *S. cerevisiae* alone. *B. licheniformis* in single culture (0:10^6) was characterized by 2,3-butanediol and tetramethylpyrazine (Fig. 3), which was consistent with previous studies [14]. An interesting finding is that the PCA plot unravels the interactions that occurred in mixed cultures. While *S. cerevisiae* alone was characterized by nothing, the aromatic profile changed completely in the presence of *B. licheniformis*. Flavor compounds variables plotted in the right, bottom quadrant of the biplot contributed largely to the samples located in the same region (Fig. 3). Variables plotted close to the axes had lower contributions to the position of samples than those further away from the axes. The mixed culture ratios, 10^6:10^6 and 10^6:10^7 had higher Component 1 values than ratio 10^6:10^5 (Fig. 3) and they produced the same higher levels of total

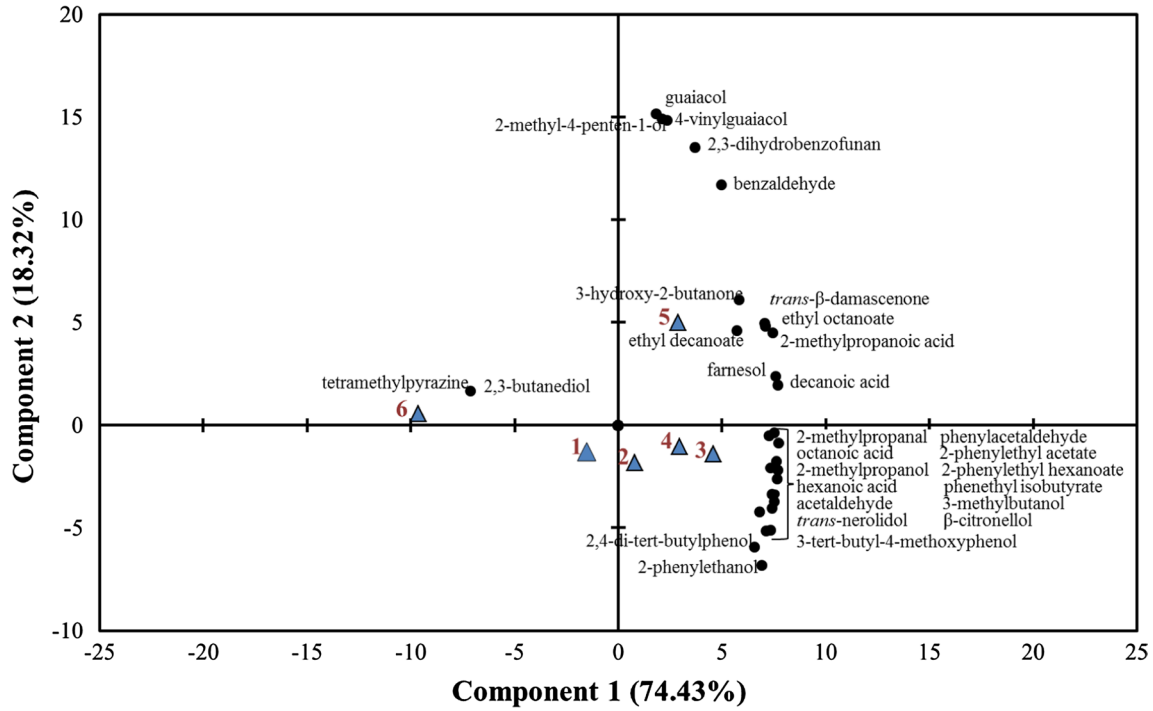

Fig. 3 Biplot of the principal components analysis (PC 1 vs. PC 2) of metabolite profiles from single and mixed culture fermentations: Numbers 1–6 refer to *S. cerevisiae*:*B. lcheniformis* culture ratios: 1, 10^6:0; 2, 10^6:10^5; 3, 10^6:10^6; 4, 10^6:10^7; 5, 10^6:10^8; 6, 0:10^6

Table 1 Total concentration of flavor compounds (μg/L) produced by pure and mixed cultures of *S. Cerevisiae* and *B. Licheniformis*

Compounds	Mixed culture (*S. cerevisiae*:*B. licheniformis*)					
	10^6:0	10^6:10^5	10^6:10^6	10^6:10^7	10^6:10^8	0:10^6
∑Aldehydes***	187.2^{b}	238.5^{b}	323.6^{c}	318.0^{c}	354.1^{c}	31.9^{a}
∑Alcohols***	819.9^{b}	1088.4^{bc}	1533.7^{c}	1242.3^{bc}	1047.4^{bc}	29.0^{a}
∑Aromatic compounds***	4403.5^{bc}	6398.1^{bc}	7008.9^{c}	5854.5^{bc}	3900.6^{b}	9.2^{a}
∑Esters***	13.4^{bc}	11.1^{b}	28.0^{d}	22.6^{cd}	29.9^{d}	nd^{a}
∑Ketones and Furans**	161.6^{a}	163.5^{a}	289.9^{b}	244.3^{ab}	308.3^{b}	151.5^{a}
∑Acids***	141.3^{b}	212.9^{bc}	295.7^{c}	248.3^{c}	255.4^{c}	nd^{a}
∑Terpenes and Pyrazines***	157.5^{b}	180.9^{bc}	239.5^{d}	208.6^{cd}	166.4^{b}	7.0^{a}

Values with the same letters are not significantly different according to the Tukey test (95 %)

*** Significance level of the one-way ANOVA (* $0.05 > p > 0.01$;** $0.01 > p > 0.001$;*** $p < 0.001$), nd: not detected

amounts of aldehydes and fatty acids (Table 1). The mixed culture with *S. cerevisiae*:*B. licheniformis* at a 10^6:10^8 ratio appeared to be far from the other cultures (Fig. 3). This culture ratio was characterized by higher production of guaiacol, 4-vinylguaiacol, benzaldehyde, 2-methyl-4-penten-1-ol, and ethyl decanoate (Supplementary Table 1).

These results demonstrated a positive effect that *B. licheniformis* had on flavor compounds production in mixed culture and may provide a new opportunity to alter the aroma and flavor perception of *Maotai*-flavor liquor.

For further study on the positive effect of *B. licheniformis* on flavor metabolism of *S. cerevisiae* in mixed culture, correlation analyses were used to reveal how the metabolism of flavor compounds produced by *S. cerevisiae* varied with different initial inoculation ratios of *B. licheniformis*. In order to assess whether differences in biomass production rather than the influence of *B. licheniformis* that were responsible for the observed differences in flavor compounds, we normalized compound concentrations to the final biomass before correlation analyses.

Correlation analyses showed 16 flavor compounds that were significantly correlated with the initial inoculation ratio of *B. licheniformis* ($r \geq 0.5$ and $p \leq 0.01$) (Table 2). Among them, 2 compounds were negatively correlated and another 14 compounds were positively correlated. As shown in Table 1, 2-phenylethanol, 3-hydroxy-2-butanone, 2,3-dihydrobenzofuran, and *trans*-β-damascenone could be produced by both *S. cerevisiae* and *B. licheniformis*. However, except 3-hydroxy-2-butanone, their production in single cultures of *B. licheniformis* was significantly lower than that in single culture of *S. cerevisiae*. The other 12 flavor compounds could only be produced by *S. cerevisiae*.

Fatty acids and their corresponding esters were positively correlated with the initial inoculation ratio of *B. licheniformis* in mixed culture, including decanoic acid, hexanoic acid, octanoic acid, ethyl octanoate, and ethyl decanoate (Table 2). Fatty acids are the main components in Chinese liquor. They could eliminate the bitter taste, accelerate the liquor aging process, or act as precursor of esters which provide fruit, flower, and sweet aromas for Chinese liquor [21]. Moreover, medium chain fatty acids and their corresponding esters have been reported to have inhibition effects on bacteria [5, 22–24]. Within these perspectives, the increased levels of fatty acids may not only benefit for liquor aroma, but also could help *S. cerevisiae* to improve its competitiveness during fermentation.

For aromatic compounds, 2-phenylethanol (rose-like odor) and 2-phenylethyl acetate (honey, rose-like) were negatively correlated with the initial inoculation ratio of *B. licheniformis* (Table 2). 2-Phenylethyl hexanoate (honey-scented, fruity, flower, and floral) and phenethyl isobutyrate (green, fruity, and rose-like) were improved as the initial inoculation ratio of *B. licheniformis* increased in mixed culture. The decreased concentration of 2-phenylethyl acetate can be explained by the low content of acetic acid which was not detected in the fermented liquid. In addition, 2-phenylethyl hexanoate ($r = 0.997$) exhibited a closely linear relationship with initial inoculation ratio of *B. licheniformis*, which meant that its metabolism in *S. cerevisiae* multiplied with the initial *B. licheniformis* population. The closely linear relationship was also seen between benzaldehyde ($r = 0.999$) and phenylacetaldehyde ($r = 0.940$) with initial inoculation of *B. licheniformis*. Benzaldehyde and phenylacetaldehyde are integral parts of *Maotai*-flavor liquor. Phenylacetaldehyde was considered as aroma-active compounds in Daqu and presented a concentration in liquor which could influence the liquor aroma [25].

Terpenes are known as functional factors in Chinese liquor that have antimicrobial, antiviral, analgesic, digestive, and anticarcinogenic activities [26–28]. In mixed cultures, *trans*-β-damascenone and farnesol levels were increased as the initial population of *B. licheniformis* increased (Table 2). *Trans*-β-damascenone has a strong rose-like flavor with good diffusion, and farnesol has a cloves-like flavor. Moreover, farnesol is a 15-carbon isoprenoid and has been shown to act as signaling molecules in microbial interactions [29]. Further investigations are needed to study the potential role of increased metabolism of farnesol by *S. cerevisiae* in yeast–bacteria interactions.

Table 2 Flavor compounds and their linearity with respect to the increased abundance in *B. licheniformis*

Compound	Linearity	*R* Value	*P* value
Aromatic compounds			
2-Phenylethanol	Negative	−0.952	<0.0001
Benzaldehyde	Positive	0.999	<0.0001
Phenylacetaldehyde	Positive	0.940	<0.0001
2-Phenylethyl acetate	Negative	−0.700	<0.0001
2-Phenylethyl hexanoate	Positive	0.997	<0.0001
Phenethyl isobutyrate	Positive	0.583	<0.0001
Esters			
Ethyl octanoate	Positive	0.923	<0.0001
Ethyl decanoate	Positive	0.519	<0.0001
Fatty Acids			
2-Methylpropanoic acid	Positive	0.936	<0.0001
Hexanoic acid	Positive	0.890	<0.0001
Octanoic acid	Positive	0.707	<0.0001
Decanoic acid	Positive	0.838	<0.0001
Terpenes			
trans-β-damascenone	Positive	0.829	<0.0001
Farnesol	Positive	0.955	<0.0001
Ketones and others			
3-Hydroxy-2-butanone	Positive	0.821	<0.0001
2,3-Dihydrobenzofuran	Positive	0.999	<0.0001

Compounds are selected with $r \geq 0.5$ and $p \leq 0.05$

3-Hydroxy-2-butanone, also known as acetoin, was a common metabolite of both strains. Its concentration was positively correlated with the initial inoculation ratio of *B. licheniformis* (Table 2). Acetoin offers a pleasant yogurt odor and has been detected in Chinese liquor [30, 31]. Moreover, it was an important physiological metabolite of microbes that could serve as cellular storage energy, and help microbes against acidic environment [32]. The changed metabolism of acetoin has been reported before in mixed culture of *S. cerevisiae* and *Hanseniaspora uvarum* compared with their single culture [33]. However, the mechanism behind was unknown and needed further research.

Conclusions

This work gained an insight into the interaction between *S. cerevisiae* and *B. licheniformis*, which were the dominant

yeast and bacteria in Chinese *Maotai*-flavor liquor making process. *S. cerevisiae* had an inhibition effect on *B. licheniformis*; however, *B. licheniformis* impacted positively on the metabolic activity of *S. cerevisiae*. More ethanol and flavor compounds were produced in mixed cultures. Correlation analyses gave a deep understanding of the interaction between *S. cerevisiae* and *B. licheniformis*. A total of 16 flavor compounds were significantly correlated with the initial amount of *B. licheniformis*, and 12 flavor compounds produced by *S. cerevisiae* were independent of biomass production, including 4 fatty acids and their 2 corresponding esters, 1 terpene, and 5 aromatic compounds. These metabolic changes in *S. cerevisiae* not only play important roles in the formation of liquor aroma, but also benefit for keeping its dominant status. Further studies including the mechanism of metabolic changes in S. *cerevisiae* and microbial interactions between other yeast–bacteria pairs during *Maotai*-flavor liquor making process are in progress.

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