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Transcriptional Analysis of Mixed-Culture Fermentation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* for Natural Fruity Sour Beer

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Abstract: Increasingly high interest in yeast–yeast interactions in mixed-culture fermentation is seen along with beer consumers' demands driving both market growth and requests for biotechnological solutions that can provide better sensory characteristics. In this study, *Lachancea thermotolerans* and *Saccharomyces cerevisiae* with a cell population ratio of 10:1 were inoculated for sour beer fermentation while the process conditions within the brewing industry remained unchanged. With *L. thermotolerans* producing lactic acid (1.5–1.8 g/L) and bringing down the pH to 3.3–3.4 whilst adding no foreign flavors herein, this study revealed a new natural, fruity sour beer with a soft, sour taste. In this study, the double-yeast mixed-culture fermentation produced more flavor substances than a single-culture process, and plenty of isobutyl acetate and isoamyl acetate enhanced the fruit aroma and balanced the sour beer with a refreshing taste. While playing a positive role in improving the beer's quality, the double-yeast mixed-culture fermentation developed in this study helps to offer an alternative mass production solution for producing sour beer with the processes better controlled and the fermentation time reduced. The stress responses of the *L. thermotolerans* during the fermentation were revealed by integrating RNA sequencing (RNA-Seq) and metabolite data. Given that the metabolic flux distribution of the *S. cerevisiae* during the fermentation differed from that of the non-*Saccharomyces* yeasts, transcriptional analysis of non-*Saccharomyces* yeast and *S. cerevisiae* could be suitable in helping to develop strategies to modulate the transcriptional responses of specific genes that are associated with the aroma compounds released by *S. cerevisiae* and non-*Saccharomyces* yeasts. In the case of some non-*Saccharomyces* yeast species/strains, the diversion of alcoholic fermentation and the formation of a great number of secondary compounds may, in part, account for the low ethanol yield.

Keywords: *Lachancea thermotolerans*; *Saccharomyces cerevisiae*; double-yeast mixed-culture fermentation; lactic acid; transcriptomics; yeast interaction

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1. Introduction

In the world of beer, brewers' quests for unique flavors often face significant technical hurdles. The use of mixed starters, namely selected strains of S. cerevisiae and non-*Saccharomyces* yeast, suggests a constructive strategy to improve beer with an aromatic complexity and desirable characteristics while reducing off flavors as compared with spontaneous fermentation [1,2]. Non-*Saccharomyces* yeasts are typically found in spontaneous fermentation and, in some cases, are used as starter cultures for brewing. Most of them belong to the following genera: *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*, *Ka-zachstania*, *Kluyveromyces*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Pichia*, *Rhodotorula*, *Star-merella*, *Saccharomycodes*, *Saccharomycopsis*, *Torulaspora*, *Trichosporon*, *Wickerhamomyces*, *Wil-liopsis*, *Yarrowia*, *Zygoascus*, and *Zygosaccharomyces* [3–9]. Brewers usually use *Lachancea*

together with *Saccharomyces* in fermentations to produce a rapid drop in pH [10]. On the other hand, studies have shown that only three non-*Saccharomyces* (*T. delbrueckii*, *S. pombe*, and *L. thermotolerans*) possess enough fermentative power to properly ferment beer with ethanol levels of up to nearly 100%, and *Lachancea* is uniquely characterized by its ability to produce lactic acid, thus influencing both flavor and mouth feel.

Traditionally, crafting sour beers involves a complex interaction of multiple microbes and lengthy aging [11] while at the same time having to deal with finicky lactic acid bacteria that are sensitive to hops [12] and demand cross-contamination control. It is therefore of particular interest that strains producing high levels of lactic acid could potentially be used to make sour beer without adding bacteria, thus simplifying and shortening the process. Winemakers have found success with the mixed-culture fermentation technique, using *Lachancea thermotolerans* in combination with *Saccharomyces cerevisiae* and resulting in enhanced quality and a layered character. Could this approach be replicated in beer brewing, particularly on an industrial scale? This opens a new door. In this study, a combination of *L. thermotolerans* and *S. erevisiae* were used in the fermentation process without the addition of foreign flavors, and the process resulted in a broader spectrum of flavor compounds compared to single-culture processes.

In addition, the authors employed RNA high-throughput sequencing technology to unravel the transcriptome-level gene expression of *S. cerevisiae* and *L. thermotolerans* in a mixed fermentation environment. While delving deeper into the intriguing realm of yeast–yeast interactions, this technology can help to illuminate the research on the cellular adaptive responses of both yeasts, building upon previous genetic analyses of expression variations during beer fermentation [12–14]. Therefore, this study provides a framework for establishing the process and procedure of mixed-culture fermentation, which is expected to inspire the production of natural, fruity sour beer from industrial wort.

2. Materials and Methods

2.1. Strains and Media

Saccharomyces cerevisiae 68obg, *Lachancea thermotolerans* FBA-2, and *Brettanomyces bruxellensis* WDB24 were provided by the Laboratory Strain Preservation Center. *Kluyveromyces marxianus* 1695, *Kluyveromyces marxianus* 1042, and *Lachancea thermotolerans* 1548 were provided by the China Common Microbial Species Preservation and Management Center.

Expansion culture medium: wort was produced by the saccharification workshop of a company and was sterilized at 115 °C for 20 min.

Fermentation medium: 2% fructose syrup was added to the wort to increase the available sugar content of the non-*Saccharomyces* yeast, which was sterilized at 115 °C for 20 min.

2.2. Beer Fermentation Experiment

After activation, the bacterial solution was inoculated into a 500 mL triangular flask containing 250 mL of wort and incubated at 25 °C for 24 h to prepare the seed solution. The inoculum was inoculated into the wort fermentation medium at an inoculum size of 1–1.2 $\times$ 10^7 CFU/mL and incubated at 20 °C for 7 days. Then, 600 mL of the inoculum was fermented. The fermentation broth was injected into sterile clamp bottles, and the beer was stored at 0 °C for 7 days after capping (3 in parallel for each sample).

2.3. Analysis of Quantity Change of Different Yeast Species in Double-Yeast Mixed Fermentation System

qPCR and ethidium bromide azide were used to quantitatively analyze each strain in the double-yeast mixed-culture fermentation to characterize the dynamics of each yeast quickly.

Correlation analysis was performed using methylene blue staining method for micro-monitoring analysis of cell mortality [12], and the yeast mortality Color process was determined by the methylene blue staining method. The EMA pretreatment conditions suit-

able for the two yeast strains were determined as EMA concentration of 20 μmol/L upon placed in the dark for 10 min. Samples were exposed at a distance of 10 cm from a 500 W halogen lamp for 15 min, which could effectively inhibit the amplification of dead bacteria DNA and thus exclude dead bacteria Effect of DNA amplification from quantification of qPCR strains. The known concentration of yeast pure culture liquid, 10 times gradient dilution, and then EMA-qPCR was performed respectively, with the two yeast-specific primers being *S. cerevisiae* CESP-F: ATCGAATTTTTGAACGCACATTG, SCER-R: CGCAGAGAAACCTCTCTTTGGA [13]; *L. thermotolerans* LTH2-F CGCTCCTTGTGGGTGGGGAT, LTH2-R CTGGGCTATAACGCTTCTCC [14]. The 30 μL qPCR reaction system consisted of 12 μL Sybrgreen Mix (Tien root), 1 μL upstream and downstream primers, 1 μL mode Plate DNA, and 15 μL of sterilized water. The qPCR reaction program includes 95 °C for 5 min and 40 cycles, with each cycle at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. Finally, the standard curves of 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/mL viable cells were obtained to determine the correlation between the corresponding cycle threshold (Ct) of qPCR and plate count, completing the quantitative analysis of each strain. At last, EMA-qPCR monitoring technology was used to characterize instantly the growth status of each single bacteria in the double-yeast mixed-culture fermentation.

2.4. Analytical Determinations

Gas chromatograph was used to determine the flavor substances in the fermented liquor after distillation [12]. The real fermentability and alcohol were determined according to GB/T 4928-2008 (Beer Analysis Method) [15]. High performance liquid chromatraphy (HPLC, Agilent 6890, Santa Clara, CA, USA)) was used to determine the main sugars in the fermentation broth [16].

Organic acids greatly influence the flavor and taste of beer and are important indicators in sour beer evaluation. The organic acids (lactic acid, acetic acid, formic acid, propionic acid, pyruvate, succinic acid, tartaric acid, oxalic acid, fumaric acid and citric acid) in liquor were identified by ion chromatography (DX-320, Dionex, Sunnyvale, CA, USA). The mixed standard solution and the sample to be tested were injected onto the ion chromatograph respectively, then the response value (peak area) of each organic acid was measured. The concentration of organic acid content of the sample to be tested was determined by comparing the response value (peak area) with the standard solution chromatogram. The pH was determined using an FE28 pH meter (Mettler Toledo Shanghai, Shanghai, China).

For sensory evaluation of beer, quantitative descriptive analysis (QDA) is a descriptive method widely performed in the beer industry nowadays, and a group of 10 beer sensory evaluation attributes is used to describe the texture, taste and aroma to score beer on a scale of flavor intensity.

2.5. RNA Extraction Samples and RNA-Seq Analysis

Cell samples for RNA-sequencing were obtained from both single and mixed culture fermentations at 24 h, 72 h and 120 h respectively. Total RNAs were extracted using the hot phenol method [17]. Concentration and purity of RNA were determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RNA integrity number (RIN) over 8, and 280:260 ratios of over 2 were further used for the RNA-sequencing purpose. Complementary DNA (cDNA) library was generated using TruSeq® Library Prep Kit v2. Paired-end reads were sequenced on the Illumina NextSeq platform. RNAseq data processing Low quality reads (<Q20), polyA-reads as well as ambiguous reads (containing N) were removed using FastX 0.0.13 [18]. Furthermore, reads less than 35 bp were removed with Short Read [19] and adapters on the remaining reads were trimmed using cutadapt [20].

Annotation of genomic features was performed using the reference genomes of *S. cerevisiae* S288c and *L. thermotolerans* CBS6340. In the case of *L. thermotolerans*, unknown genes were identified by the homology with the *S. cerevisiae S288c* genome. Reads from

L. thermotolerans and *S. cerevisiae* monoculture fermentation samples were aligned with the reference genomes of the two yeasts with TopHat v2.0.13 [21]; and reads that were non-primary mapping or had a mapping quality ≤ 20, were removed. Subsequently, cross-mapping between *S. cerevisiae* S288c and *L. thermotolerans* was evaluated to determine the effect of genomes merging. Cross mapping between the two yeasts was found to be less than 1%; consequently, pre-processed reads of all fermentations were aligned with the reference genome of S288cplusLT. The obtained bam files were converted into gff files for further data analysis. Reads in the alignments that overlap with gene features were counted using htseq-count 0.6.1p1 [22]. Genes which all samples had and are less than 1 count-per-million were removed, and the full quantile normalization using the EDASeq package from Bioconductor was applied to correct sample-specific variation typically arising out of differences in library size and RNA composition. Transcript abundance was measured in Fragments Per Kilobase of exon per Million mapped reads (FPKM).

2.6. Statistical Analysis

To evaluate the statistical significance of the chemical and organoleptic data of the beer, these were subjected to one-way analysis of variance using the SuperANOVA software (version 1.1, for Macintosh OS 9.1). The significant differences among the data were determined using the Duncan test, at $p < 0.05$.

3. Results and Analysis

3.1. Non-Saccharomyces Yeast to Saccharomyces cerevisiae in Mixed-Culture Beer Fermentation

Non-*Saccharomyces* yeasts of both certain acid capacity and wort fermentation properties were analyzed with reference to relevant studies [23–25]. The results showed that, in contrast to *S. cerevisiae* 68obg, in most cases non-*Saccharomyces* yeasts only used the glucose, fructose and sucrose in the wort, while not metabolizing maltose-trisaccharides such as maltose and maltotriose. An exception is *L. thermotolerans* FBA-2, which used a small amount of maltose, and delivered a metabolic rate that was significantly lower than that of *S. cerevisiae*.

As shown in Table 1, analysis of the organic acid species and yield of the five non-Non-*Saccharomyces* yeast strains, *L. thermotolerans* FBA-2 presented the highest lactate yield at 2.573 g/L. Given the lactic acid and organic acid concentrations higher than those in the other strains, the sensory evaluation showed that the *L. thermotolerans* FBA-2, which presented a better balanced and drinkable taste, was the best choice for brewing sour beer.

Table 1. Organic acid production from wort fermented by 5 non-*Saccharomyces* yeast strains.

Organic Acid mg/L	Yeast Strains					
	***K. marxianus* 1695**	***K. marxianus* 1042**	***L. thermotolerans* 1548**	***L. thermotolerans* FBA-2**	***B. bruxellens* WDB24**	***S. cerevisiae* 68obg**
Lactic acid	539.6 ± 2.31^{d}	966.83 ± 6.13^{c}	1517.57 ± 6.75^{b}	2573.18 ± 3.66^{a}	254.66 ± 4.18^{e}	156.37 ± 3.63^{f}
Acetic acid	168.12 ± 3.81^{d}	239.23 ± 0.45^{b}	226.6 ± 1.42^{c}	121.92 ± 2.14^{f}	324.17 ± 2.43^{a}	104.31 ± 1.59^{e}
Formic acid	3.75 ± 0.44^{cd}	4.3 ± 0.10^{c}	4.15 ± 0.07^{cd}	11.54 ± 0.57^{a}	8.87 ± 0.52^{b}	3.64 ± 0.33^{d}
Pyruvic acid	106.68 ± 4.16^{d}	132.15 ± 0.78^{c}	98.59 ± 2.52^{e}	145.94 ± 1.95^{b}	130.74 ± 1.78^{c}	157.38 ± 2.73^{a}
Succinic acid	249.27 ± 4.43^{c}	249.83 ± 1.69^{c}	312.49 ± 0.50^{b}	209.69 ± 2.33^{d}	212.37 ± 3.29^{d}	513.41 ± 10.21^{a}
Oxalic acid	26.22 ± 0.45^{c}	29.52 ± 1.22^{b}	22.7 ± 1.25^{d}	37.33 ± 0.92^{a}	24.86 ± 0.84^{cd}	29.54 ± 2.71^{b}
Fumaric acid	2.08 ± 0.17^{c}	2.59 ± 0.07^{c}	2.45 ± 0.29^{c}	2.68 ± 0.33^{c}	3.31 ± 0.08^{b}	5.36 ± 0.75^{a}
Citric acid	195.50 ± 1.11^{c}	194.17 ± 0.56^{c}	212.52 ± 0.88^{b}	193.26 ± 2.99^{c}	188.97 ± 1.29^{d}	293.41 ± 1.23^{a}

Data are means ± standard deviations of three separate replicates. Values with different superscript letters (a,b,c,d,e,f) within each row are significantly different, according to the Duncan test ($p > 0.05$).

Single-culture fermentation experiments were performed with *S. cerevisiae* 68obg and *L. thermotolerans* FBA-2. *S. cerevisiae* 68obg resulted efficient fermentation and rich esters with a strong clove flavor [26]. As shown in Figure 1A, *L. thermotolerans* FBA-2 exhibited low rate of maltose use, zero rate of maltose oligosaccharides use such as maltotriose,

delivering higher residual sugar content. As shown in Table 1, *L. thermotolerans* FBA-2 presented the highest yields of lactic acid and total acid, and yields of major hetero-alcohols such as isobutanol and isoamyl alcohols significantly lower than *S. cerevisiae* 68obg as the capacity of using carbon sources of the single strain limits the flavor substances, resulting in lower total alcohol but higher ester content compared with *S. cerevisiae* 68obg.

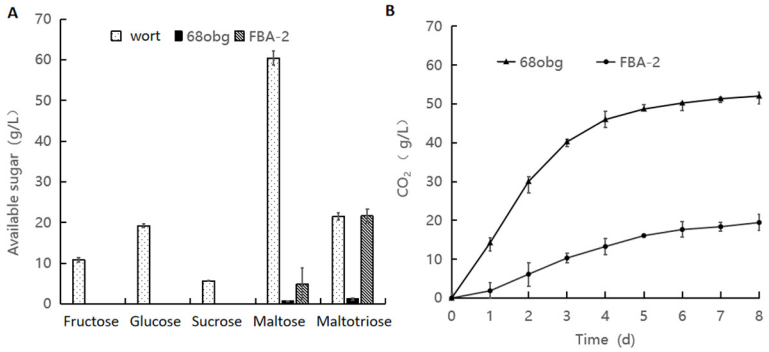

Figure 1. Available sugars content and CO_2 release of pure-culture wort fermentation. (**A**) available sugars content of pure-culture wort fermentation. (**B**) CO_2 release of pure-culture wort fermentation.

Figure 1B shows the weight loss in the fermentation process. *L. thermotolerans* FBA-2 presented a slower fermentation rate than *S. cerevisiae* 68obg and delivered 16.65 g/L (2.11%vol) alcohols in fermentation broth versus 53.26 g/L (6.75%vol) in fermentation broth of *S. cerevisiae* 68obg. The sensory evaluation of *L. thermotolerans* FBA-2 revealed bold acidity, on top of little other flavors, which reflected on the balanced and drinkable taste of beer body and exhibited flavors significantly different from standard beer out of *S.* cerevisiae 68obg fermentation. The study of wine [27] also showed that fermentation by mixing heat-resistant *L. thermotolerans* and *S. cerevisiae* increased the total acidity and yields of glycerol, polysaccharide and 2-phenyl ethanol, while reducing the volatile acidity of wine. In conclusion, using *S. cerevisiae* 68obg as the base yeast and *L. thermotolerans* FBA-2 as the featured yeast, the dual-yeast mixed-culture fermentation will brew sour beer with acidity acting as a balanced flavor element.

Interaction of different yeasts looks obvious in the mixed fermentation. Many studies have revealed [28,29] differences in metabolic activities between fermentation of mixing non-*Saccharomyces* yeast and *S. cerevisiae* and the single-culture fermentation, and the ratio of strains in the mixed fermentation plays a decisive role in the final taste of beer.

L. thermotolerans FBA-2 and *S. cerevisiae* 68obg were inoculated at 1:100, 1:50, and 1:10 for the mixed-culture fermentation, *S. cerevisiae* 68obg presented higher growth rates than those of *L. thermotolerans* FBA-2, and *S. cerevisiae* 68obg was the dominant strain. In the case of wort supplemented with high fructose syrup, the featured yeast *L. thermotolerans* FBA-2 also failed to dominate the growth due to competing with the base yeast *S. cerevisiae* 68obg for glucose, fructose, and sugar, resulting in insignificant change to the composition of the final beer product.

However, the featured strain *L. thermotolerans* FBA-2 was found to dominate the growth when *L. hermotolerans* FBA-2 and *S. cerevisiae* 68obg were inoculated at 100:1 and 50:1, and *S. cerevisiae* 68obg demonstrated constant weakness in yeast population even at late fermentation stages. In contrast, upon mixing the *L. thermotolerans* FBA-2 and *S. erevisiae* 68obg at 10:1, the *L. thermotolerans* FBA-2 started fermentation, declined in the middle stage and finally failed in fermentation while *S. cerevisiae* 68obg started to dominate to allowing continuous fermentation until completion.

3.2. Wort with L. thermotolerans and S. cerevisiae Fermentation to Produce New Fruity Sour Beer

Taking account of the flavor analysis and sensory evaluation results, we chose *L. thermotolerans* FBA-2 and *S. cerevisiae* 68obg with an inoculation ratio of 10:1 and a total number of yeast in wort in a controlled range of 1~1.2 $\times$ 10^7 CFU/mL, and applied the

EMA-qPCR monitoring technology to characterize the growing dynamics of each single fungus during the double-yeast mixedculture fermentation.

Standard curves between viable cell content and *Ct* values were established using EMA-qPCR for *L. thermotolerans* FBA-2 and *S. cerevisiae* 68obg, respectively. *Ct* values showed a linear relationship with viable concentration. The standard curve equation for *L. thermotolerans* FBA-2 was y = −3.118x + 38.952, and the correlation coefficient was R^2 = 0.9883 and the standard curve equation for *S. cerevisiae* 68obg was: y = −3.222x + 37.235, the correlation coefficient was R^2 =0.9938, showing healthy linear relationships. EMA−qPCR is able to shorten the detection time to 3~4 h, compared with 36 h of the traditional plate counting method, a marked improvement in detection efficiency.

EMA−qPCR technology was used to characterize the growth status of single bacteria during the dual-yeast mixed-culture fermentation. Figure 2 showed that *L. thermotolerans* FBA-2 presented a significant increase in yeast population after 1 day in the tank, and became the dominant strain. the pH of the fermentation broth decreased from 5.42 to 3.51 after 1 day and was 3.33 after 2 days, which remained until Day 7. Upon fermentation of 2 days, *L. thermotolerans* FBA-2 demonstrated declined activity due to depletion of available sugars, while *S. cerevisiae* 68obg continued to increase its yeast population by utilizing fermentable sugars left in the fermentation solution such as maltose and maltotriose. As greatly inhibited in the early stage when *L. thermotolerans* FBA-2 was dominant, *S. cerevisiae* 68obg showed limited growth of yeast population of up to 1.38 × 10^7 CFU/mL compared with single-culture fermentation of *S. cerevisiae* 68obg. Therefore, the fermentation liquor came with a relatively content of alcohols produced by *S. cerevisiae* 68obg, and alcohol of 43.79 g/L (5.55% vol).

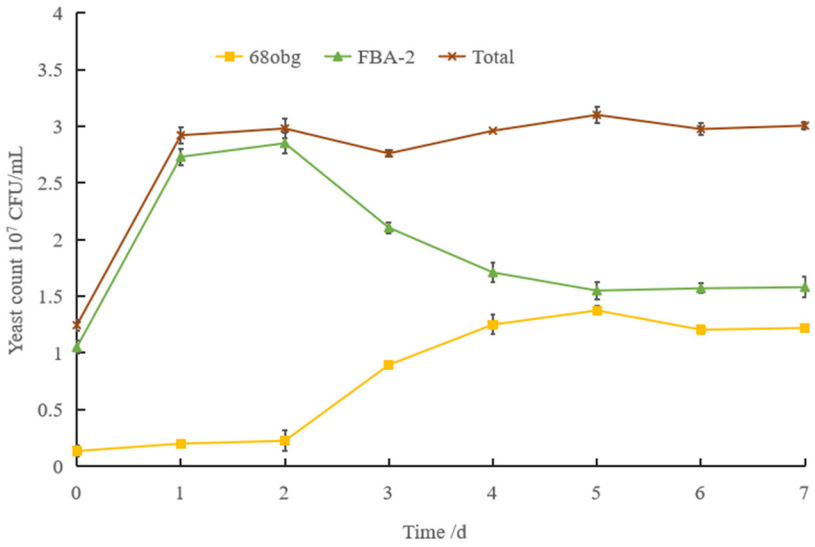

Figure 2. Quantitative analysis of different strains during double-yeast mixed fermentation by EMA-qPCR.

Scaling up the effective fermentation volume from the laboratory conditions to a wort fermentation experiment of 100 L fermenter, monitoring of relevant key parameters in the fermentation process showed that the lactic acid content was 1.5–1.8 g/L, the pH value of the liquor was 3.3–3.4, and the alcohol was about 39.45 g/L (5%vol). It can be concluded that the double-yeast mixed-culture fermentation consisting of non-*Saccharomyces* yeast and *S. cerevisiae* has a certain stability to function.

Interaction between non-*Saccharomyces* yeast and *S. cerevisiae* [30] may help to yield more enzymes and volatile compounds. In addition to lactic acid and a lower pH, more aromas of great complexity may be also brought by the *L. thermotolerans* FBA-2. Double-yeastmixed fermentation delivered more flavored substances by producing more isobutyl acetate and isoamyl acetate ester, enhancing the fruity aroma of sour beer. The sensory

evaluation also showed a combination of attributes of two yeasts in the resulting sour beer, such as instantly recognizable fruity flavors and positive influence on the beer's quality (Figure 3).

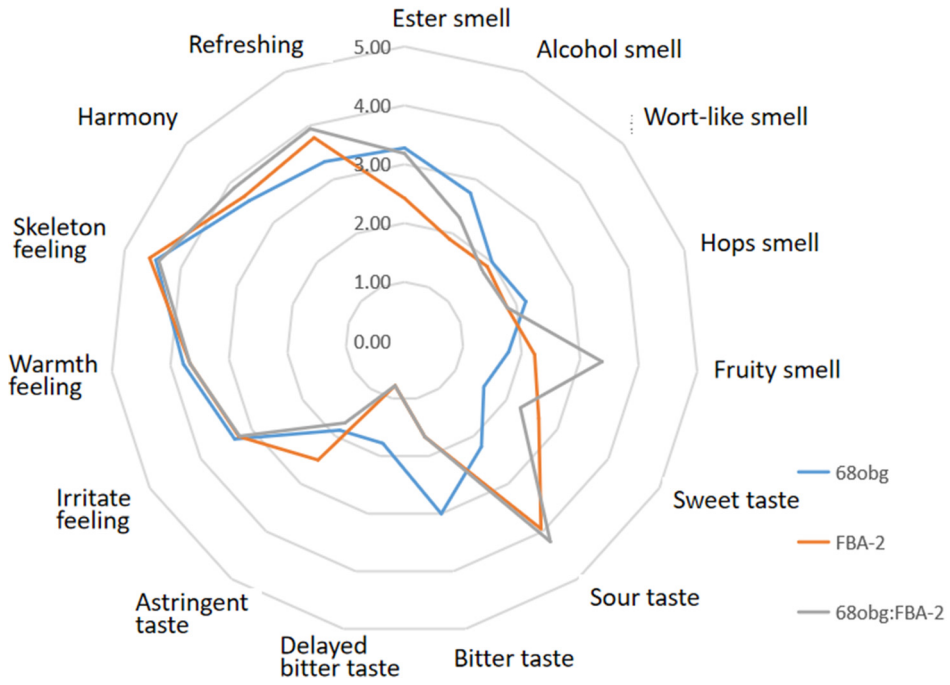

Figure 3. Sensory properties for the three different beer produced by pure and double-yeast mixed fermentation.

3.3. Overview of Transcriptional Response in Single- and Mixed-Culture Fermentations

Effect of the mixing (MIX) on the gene transcription in *S. cerevisiae* and *L. thermotolerans* was assessed by comparing the transcriptome of the mixed cultures to the monocultures under beer fermentations (Figure 4). Genes uniquely and differentially expressed in the mixed fermentations compared to monocultures were identified. In the initial global analysis, the differentially expressed genes (DEGs) were visualized by overlaying the data on the Biocyc Omics dashboard. Generally, the interaction between the single-and mixed-culture fermentations elicited a stronger response in *L. thermotolerans* than in *S. cerevisiae*. Compared with the single fermentation, the transcription level of the two yeasts changed significantly during the mixed fermentation. In order to better adapt to the environment, *S. cerevisiae* enhanced the ribosome-related functions, up-regulated the expression of genes related to the integrity of cell wall and membrane structure, and enhanced the level of cell competition by absorbing more carbon and nitrogen sources. However, genes related to cell aggregation, death and response to osmotic stress were significantly up-regulated in *L. thermotolerans*. The results showed that the response of *L. thermotolerans* than in *S. cerevisiae* was different during double-yeast mixed fermentation.

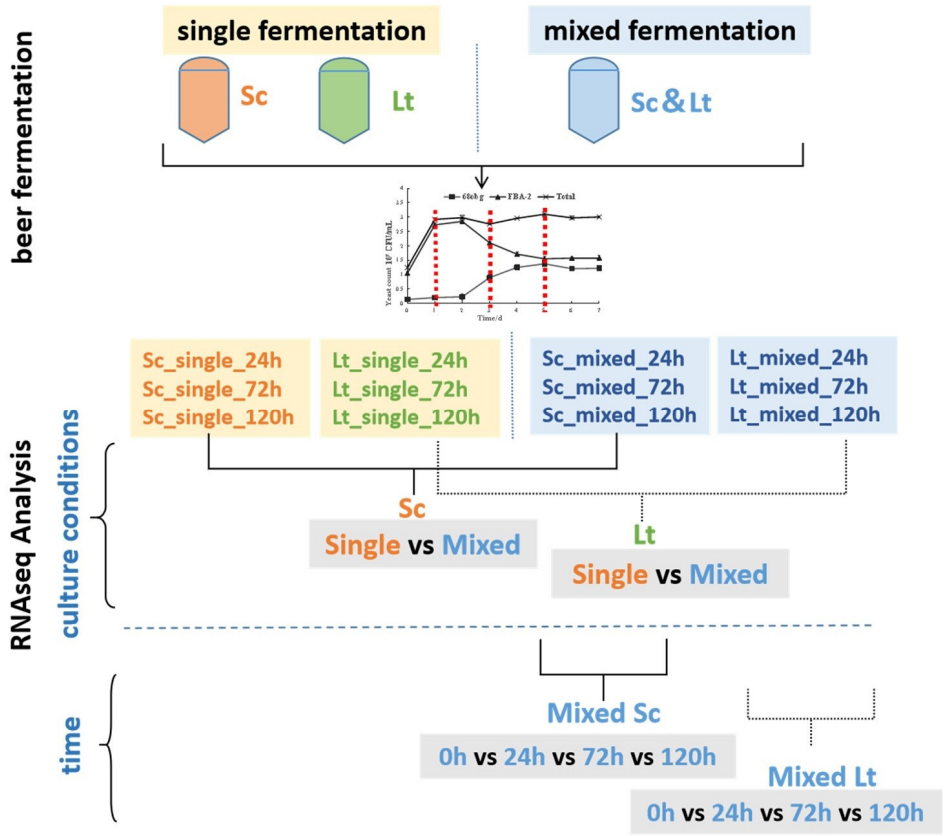

Figure 4. Simplified scheme of the strategy followed in the transcriptomic.

3.4. Transcriptional Profiling of S. cerevisiae in Single- and Mixed-Culture Fermentations

The transcriptomic profiling of the mixed-culture fermentation was performed at three time-points, the mid-exponential growth phase (24 h), the early stationary-phase (72 h), and the late stationary growth-phase (120 h). Principal Component Analysis (PCA) was used to check the data obtained from the microarray's experiments, in order to get a global view on how the presence of *S. cerevisiae* impacted the transcriptome of *S. cerevisiae* throughout the mixed-culture fermentation in Figure 5. Notably, the *S. cerevisiae* genomic expression indicated the maximal variation at later fermentation stages, in agreement with the much higher number of genes that was found to be differentially in the pair-wise comparisons performed between the two fermentations at the same time-point. As denoted by Maligoy et al. [31], caution should be taken when analyzing transcriptome data from two parallel cultures, since the observed variance of transcript levels could be either specific to the comparison of the two culture conditions or linked to a difference in the dynamics of the two cultures. To assure that the observed changes in the expression of *S. cerevisiae* genes truly reflects the influence of the presence of *L. hermotolerans*, rather than being attributable to different fermentation stages of the mixed and single cultures, the expression of a given gene in a given fermentation stage was compared to its mean expression (calculated taking the average of the expression levels obtained in the three time points analyzed). Although the mean expression value of each gene along the fermentation is merely an arbitrary reference point, such way of analyzing gene expression mitigates the influence exerted by fermentation dynamics, while maintaining the aptitude to identify expression differences [32].

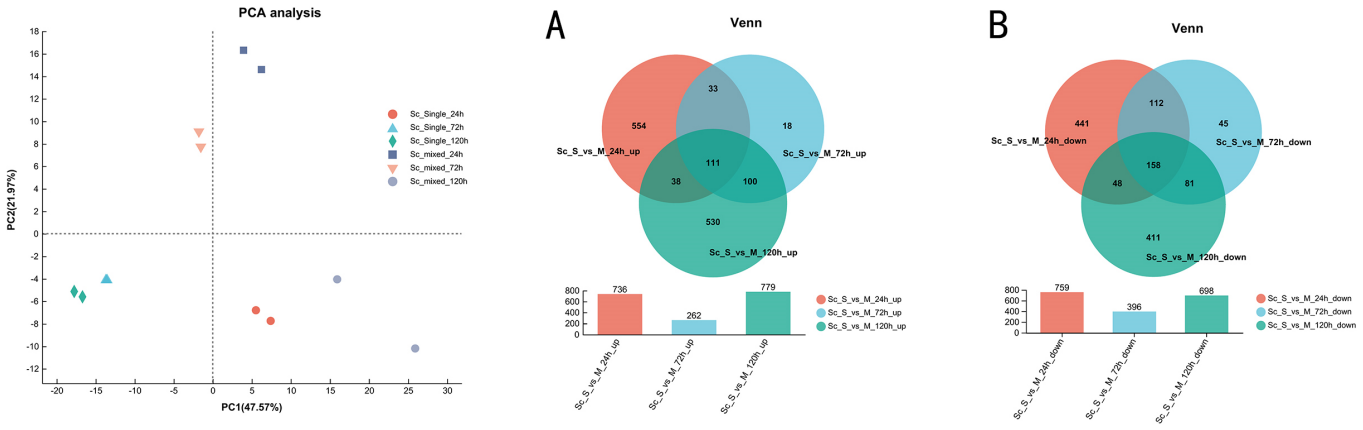

Figure 5. Principal Component Analysis (PCA) plot of the biological replicates of transcripts for each time point in mixed ande single beer fermentation of *S. cerevisiae* (Sc). (**A**,**B**) Venn diagram showing up-regulated and down-regulated significant genes (DEGs) commonly regulated for each time point in mixed ande single beer fermentation of *S. cerevisiae* (Sc).

Compared with the pure breed fermentation of a single strain, the strain *S. cerevisiae* based on the double-yeast mixed fermentation system had 1495 DEGs at the initial 24 h of fermentation, of which 736 DEGs were up-regulated and 759 DEGs were down-regulated. This is because the inoculation ratio of the strain *S. cerevisiae* in the early stage of fermentation was only one tenth of that of *L. thermopolerans*, which was greatly affected by the dominant strain *L. thermopolerans* in the mixed fermentation system. The differentially expressed genes of *S. cerevisiae* were significant in the mixed fermentation system. With the middle stage of fermentation, the strain *S. cerevisiae* gradually formed growth advantages in the mixed fermentation system. Therefore, compared with single-culture fermentation, *S. cerevisiae* had gene differential expression decreased at 72 h, only 658 DEGs, of which 262 DEGs were up-regulated and 396 DEGs were down-regulated, and 1477 genes were differentially expressed at 120 h in the late stage of fermentation, of which 779 DEGs were up-regulated and 698 DEGs were down-regulated. It can be seen that in the process of mixed fermentation, *S. cerevisiae* was significantly affected by *L. thermopolerans* in the double-yeast mixed fermentation system, which caused a huge disturbance in the level of gene expression, and a large number of genes had regulatory changes. It can be seen that the mixed fermentation mode had a significant impact on the fermentation of *S. cerevisiae*.

A closer look into the functional categories of genes included in each cluster revealed that the herein observed alterations of the *S. cerevisiae* transcriptome along beer fermentation, either in single or in mixed-culture, are consistent with the results reported in other studies carried out with different *S. cerevisiae* strains and/or exploring different fermentation conditions [23,25–27,33].

Through the GO function enrichment analysis of the differentially expressed genes in single culture and mixed culture beer fermentation of *S. cerevisiae*, it was found that among the 111 up-regulated genes, the decomposition of cellular protein complex, the translation termination, the peptide metabolism process, the maturation of small ribosomal RNA subunits of SSU-rRNA. The positive regulation enrichment rate of GO items such as cytoplasmic translation, translation translation, protein-containing complex decomposition, cellular amide metabolic process is the highest. The translation process of *S. cerevisiae* is significantly up-regulated in the double-yeast mixed fermentation system, which is consistent with the KEGG enrichment analysis result that the expression of ribosome-related genes is significantly enriched. As shown in the Figure 6, *S. cerevisiae* can improve its translation level in the double-yeast mixed fermentation system to form growth advantages.

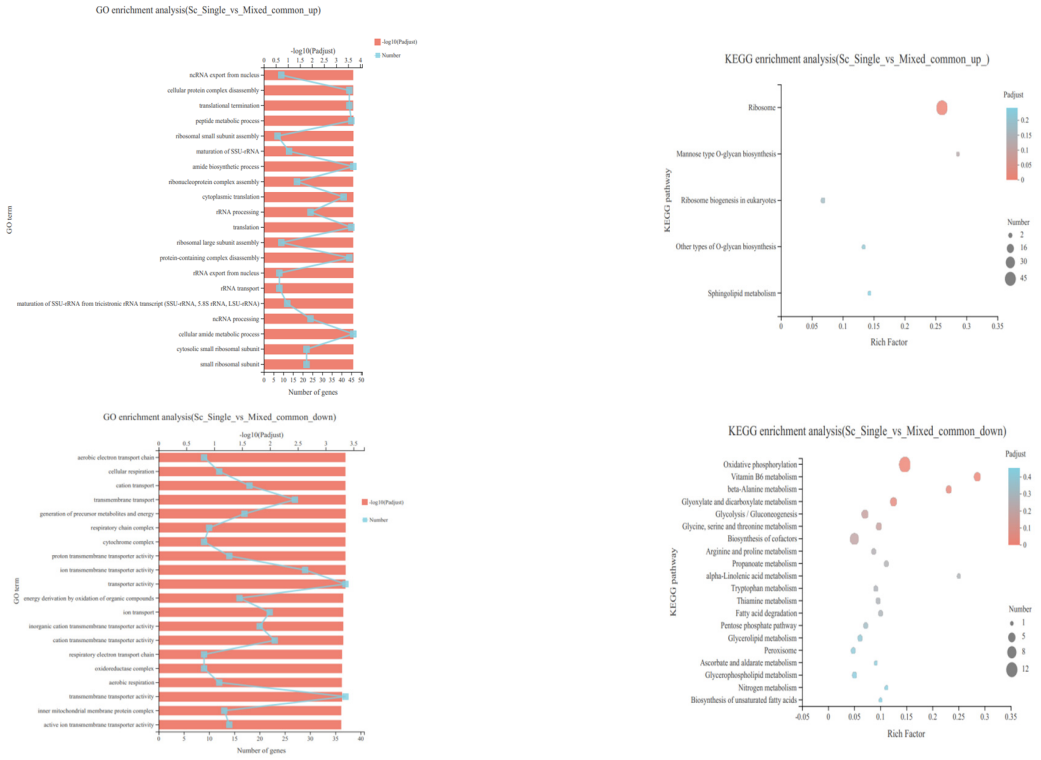

Figure 6. DEGs associated with the enrichment of the cellular component GO terms in *S. cerevisiae*.

Among the 158 down-regulated genes, a large number of GO entries related to transmembrane protein and transmembrane transport process were significantly enriched and down-regulated. The results of KEGG enrichment analysis of 158 down-regulated genes showed that the genes related to oxidative phosphorylation, carbon source metabolism and nitrogen source metabolism were significantly enriched and down-regulated. In conclusion, in the double-yeast mixed fermentation system, *S. cerevisiae* was competitive with *L. thermopolerans*. The expression of related energy synthesis and carbon and nitrogen source metabolism regulation is affected, which is also consistent with the production of main products of mixed fermentation lower than that of pure yeast.

3.5. Transcriptional Profiling of L. thermotolerans in Single- and Mixed-Culture Fermentations

In order to clarify the mechanism of changes in *L. thermotolerans* during the double-yeast mixed fermentation, Venn analysis was performed on the up- and down-regulated genes based on the expression matrix, and the co- and specifically expressed genes/transcripts of samples could be obtained, as shown in Figure 7. A total of 7 up-regulated genes and 10 down-regulated genes were found in *L. thermotolerans* in the mixedfermentation, and were further analyzed for functional enrichment.

GO function enrichment analysis of differentially co-expressed genes in single and mixed culture beer fermentation of *L. thermotolerans*, indicated that among the 101 up-regulated genes, carbon utilization and negative regulation of transcription from RNA polymerase II promoter by pheromone regulation Synthesis ones, negative regulation of transcription by synthesis ones, negative regulation of molecular function), autophagy regulation macroautophagy, regulation of mitophagy, regulation of autophagy of mitochondrion, negative regulation of hydrolytic enzymes and catalytic activities Hydrolase activity, negative regulation of catalytic activity), modified amino acid biosynthesis (cellular modified amino acid biosynthetic process) and other GO items have the highest positive regulation enrichment rate, which is consistent with KEGG enrichment analysis results, as shown in Figure 8. In the study of beer mixed-culture fermentation [34], *L. termotolerans* usually declines in the early stage of fermentation. The fatal factors include its own low

ethanol tolerance, low concentration of dissolved oxygen in the fermentation system and the interaction between different strains. Such as nutrient competition between strains, accumulation of harmful metabolites, contact between cells and antimicrobial peptides secreted by other strains, etc. Usually, these factors do not exist separately, but act on the whole fermentation process. The results showed that *L. termotolerans* constantly adjusted its gene expression in order to adapt to the harsh environment such as nutrient competition and accumulation of harmful metabolites during the middle and later stages of mixed fermentation, as the growth advantage of *S. cerevisiae* gradually formed in the mixed fermentation.

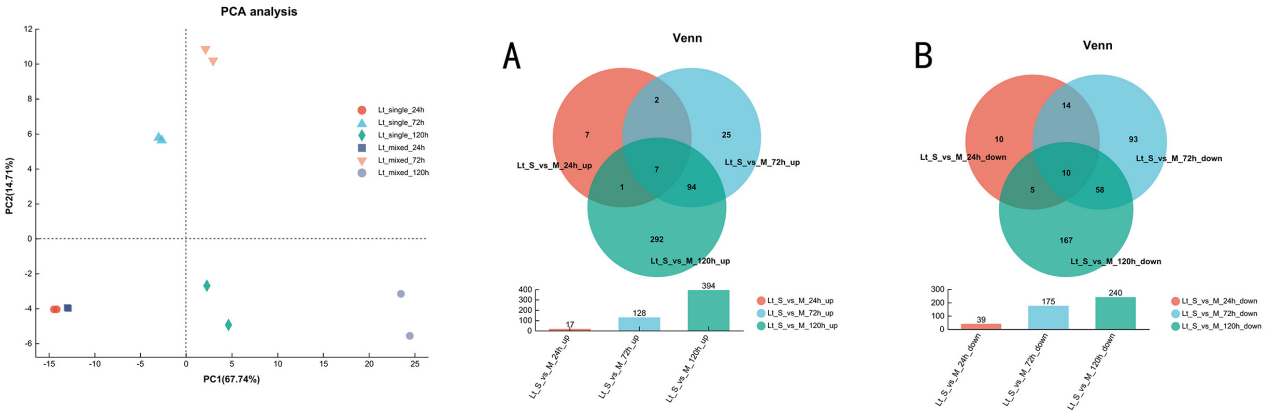

Figure 7. Principal Component Analysis (PCA) plot of the biological replicates of transcripts for each time point in mixed ande single beer fermentation of *L. thermotolerans*. (**A**,**B**) Venn diagram showing up-regulated and down-regulated significant genes (DEGs) commonly regulated for each time point in mixed and single beer fermentation of *L. thermotolerans*.

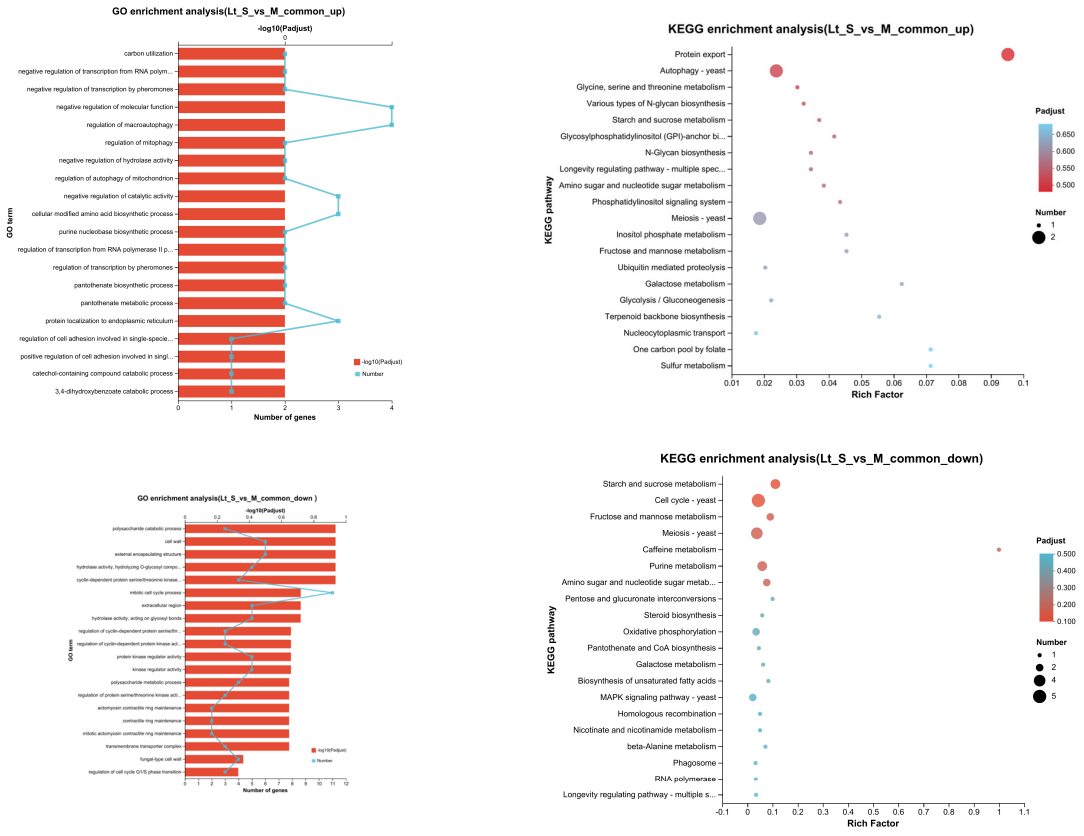

Figure 8. DEGs associated with the enrichment of the cellular component GO terms in *L. thermotolerans*.

4. Conclusions

This study explored a novel mixed-culture fermentation strategy for sour beer production using a 10:1 ratio of *L. thermotolerans* and *S. cerevisiae* under standard industrial brewing conditions. *L. thermotolerans* produced lactic acid (1.5–1.8 g/L), lowered the pH to 3.3–3.4 and produced a naturally fruity sour beer with a gentle tartness, all without the addition of foreign flavoring agents. Compared to single-yeast fermentation, this double-yeast mixed fermentation yielded an enhanced fruity aroma and a more refreshing taste, as isobutyl acetate and isoamyl acetate contribute to an augmented fruity aroma, effectively balancing the sour notes and giving the beer a refreshing taste. Beyond its positive impact on beer quality, this mixed-culture fermentation method explored the potential of mixed-culture fermentation with *L. thermotolerans* and *S. cerevisiae* for the production of sour beer, while remaining harmonious with the established framework of traditional brewing practices.

Although co-fermentation remains uncommon in beer brewing due to technical challenges, this study used RNA high-throughput sequencing technology to reveal distinct shifts in gene expression at the transcriptome-level for *S. cerevisiae* and *L. thermotolerans* in mixed fermentation. The data gained from RNA sequencing can provide valuable insights into yeast behavior in mixed cultures. *S. cerevisiae*, to better adapt to the mixed environment, increased ribosome-related functions, up-regulated genes associated with cell wall and membrane integrity, and increased carbon and nitrogen uptake. In contrast, *L. thermotolerans* exhibited significant upregulation of genes linked to cell aggregation, cell death, and osmotic stress response. These findings suggest divergent adaptive strategies: *S. cerevisiae*, stimulated by the competitive pressure of being outnumbered, accelerated nutrient uptake and became the dominant strain by mid-fermentation, surpassing *L. thermotolerans* in biomass. The elevated ethanol and hypoxia conditions generated by *S. cerevisiae* posed a stress challenge to *L. thermotolerans*, as evidenced by their weaker adaptation and up-regulation of stress-related genes, highlighting their vulnerability in this mixed-culture system.

This study provides new insights into the role of RNA-seq in analyzing changes in the transcriptome of mixed cultures. Combining RNA sequencing (RNA-Seq) and metabolite data helps to better understand the distribution of metabolic fluxes in *S. cerevisiae* and non-*Saccharomyces* yeasts, potentially leading to strategies to modulate transcriptional responses associated with aroma compounds.

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