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Analysis of yeast diversity during spontaneous fermentation of red and white pitaya

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Abstract

Yeast communities during spontaneous fermentation of red and white pitaya were characterised and compared to provide a reference for the isolation of pitaya yeast strains. Using Illumina MiSeq high-throughput sequencing technology, we investigated the composition and diversities of yeast communities during five stages of spontaneous fermentation (1, 3, 5, 7, and 15 d), and the results showed 584,055 and 699,120 valid sequences from red and white pitaya, respectively. The sequences were classified into 86 and 42 operational taxonomic units, then assigned to 69 species in 49 genera (red pitaya) and 37 species in 32 genera (white pitaya). Taxonomic composition and diversity analysis results showed high yeast diversity during the early stage of spontaneous fermentation (RF1) for red pitaya, and during the middle stage (WF5) for white pitaya fermentation. The dominant yeast species was unclassified_o_*Saccharomycetales* in red pitaya, and *Clavispora opuntiae* in white pitaya, and these species showed opposite trends during red and white pitaya fermentation. One-way ANOVA showed highly significant differences in unclassified_o_*Saccharomycetales* ($p \leq 0.01$) in red pitaya, and in *Clavispora opuntiae* ($p \leq 0.001$) in white pitaya across the five spontaneous fermentation stages. In addition, phylogenetic analysis revealed that unclassified_o_*Saccharomycetales* and *Clavispora opuntiae* were positioned farthest from other yeast species. Overall, yeast community diversity during spontaneous fermentation was higher in red rather than white pitaya.

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Introduction

Pitaya, also known as dragon fruit, belongs to the Cactaceae family, and has great nutritional and medicinal applications (Wang *et al*., 2014; Li *et al*., 2015). The fruits can be classified as red pitaya (*Hylocereus polyrhizus*) or white pitaya (*Hylocereus undatus*) based on the colour of their flesh (Gao *et al*., 2014; Shen *et al*., 2015). Pitaya grows in China over approximately 42,000 hectares, and the fruit can be fermented to make pitaya wine and vinegar (Zhu *et al*., 2014; Sun *et al*., 2019). Currently, commercial active dry yeast for winemaking is used for pitaya wine brewing (Liu *et al*., 2021; Yu *et al*., 2021). However, due to its poor adaptability and limited strains, only few pitaya wine products are available on the market, and quality improvement is needed. Furthermore, studies on yeast diversity for pitaya products have not yet been reported.

Most yeast used in pitaya winemaking has been isolated from commercial strains (Yan *et al*., 2008; Li *et al*., 2015; Lin *et al*., 2016; Guo *et al*., 2017), and there are few reports on the isolation and characterisation of yeast strains specific to pitaya wine brewing. Xie *et al*. (2013) isolated brewing yeast adapted to the pH of red pitaya juice from the spontaneously fermented pulp of red pitaya. Yuan *et al*. (2016) compared spontaneous strains and commercially available wine yeast to the quality of red pitaya wine, and the authors showed significantly higher initial fermentation and sugar conversion rates with natural yeast. Huang *et al*. (2019) analysed changes in the number of yeast during pitaya drink fermentation using inoculated and natural yeasts, and showed that the number of yeast cells increased throughout fermentation, and yeast count was lower in inoculated fermentation as compared to spontaneous fermentation. Additionally, unique metabolic characteristics of indigenous yeast in microenvironments are responsible for the special flavours of local fruit wines (Massera *et al*., 2012). Therefore, the isolation of suitable native yeast strains

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from local fruits suitable for brewing fruit wine is of great significance in order to utilise the microbial resources, and to develop unique and high-quality fruit wine (Santamaría *et al*., 2008). In the present work, high-throughput sequencing was used to analyse the diversity and dynamic changes of yeast communities during spontaneous red and white pitaya fermentation. Our results offered significant insight into isolating yeast, and controlling the quality of wine made from different varieties of pitaya.

Materials and methods

Materials

Red and white pitaya were purchased from a local market in Guiyang city, Guizhou province (Figures 1A and 1B, respectively). The mean weight, diameter, and sugar content of red and white pitaya were 596.53 ± 29.18 g, 9.80 ± 0.20 cm, and 16.87 ± 0.68 Brix; and 525.47 ± 18.90 g, 8.93 ± 0.35 cm, and 15.10 ± 0.44 Brix, respectively.

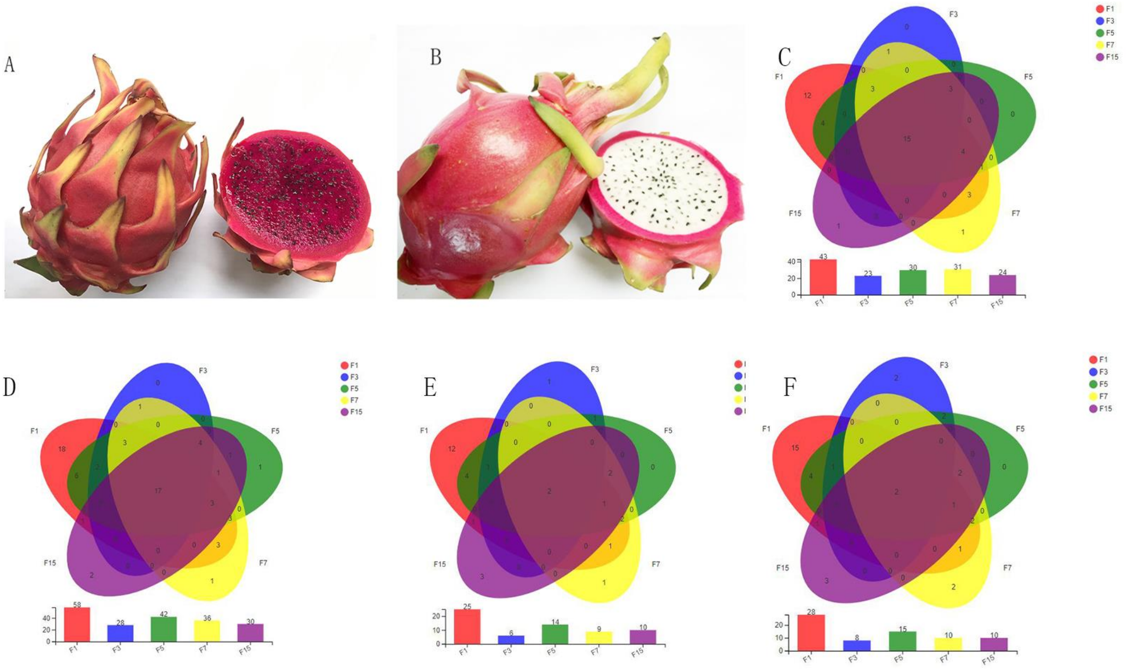

Figure 1. *Saccharomyces* yeast community distributions at different spontaneous fermentation stages by **(A)** *Hylocereus polyrhizus* [**(C)**: genus level; **(D)**: species level] and **(B)** *Hylocereus undatus* [**(E)**: genus level; **(F)**: species level).

Chemical reagents

The present work utilised a DNA extraction kit (MP Biomedicals, USA), Pfu high-fidelity DNA polymerase (100 U/tube), TransGen Biotech (Beijing, China), agarose (Biowest, Spain), DNA markers (Sangon Biotech, Shanghai, China), and primers namely ITS1F_ITS2R 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) (Majorbio, Shanghai, China).

Instruments

The present work utilised N13462C pipette and a 5430 R centrifuge (ABSON MiFly-6 microcentrifuge, Eppendorf, Germany; respectively), an Abson scientific instrument (Hefei, China), a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA), a GeneAmpR 9700 PCR system (ABI, USA), and an Illumina MiSeq sequencer (Illumina, USA).

Methods

Spontaneous fermentation of pitaya

Fresh, ripe red and white pitaya were rinsed with sterilised water to remove any residual soil on the fruit surface. Then, the fruit was chopped and placed in a sterile 1-L Erlenmeyer flask. Static spontaneous fermentation was carried out at 28°C, and samples (1 mL) were obtained at 1, 3, 5, 7, and 15 d, and denoted as F1, F3, F5, F7, and F15, respectively. Each sample consisted of three replicates, and was stored at -80°C for subsequent high-throughput sequencing.

High-throughput sequencing and statistical analysis of bioinformatics

Genomic DNA was extracted from the fermentation broth of red and white pitaya following the kit instructions. DNA purity and concentration were measured using a NanoDrop2000 spectrophotometer, and DNA integrity was detected by agarose gel electrophoresis. An appropriate amount of DNA template was amplified using ITS2F and ITS2R primers, and PCR products were detected using agarose gel and subsequently purified. Library construction and MiSeq sequencing were performed by Majorbio.

The obtained sequences were subjected to bioinformatics analysis using the Majorbio I-Sanger Cloud Platform (Liu *et al.*, 2020). The samples were divided into five groups (F1, F3, F5, F7, and F15) based on the sampling time and method. Species composition was compared between the samples, and phylogenetic analysis was performed at 97% similarity after subsampling the lowest number of sequences among the samples. The QIIME2 dada2 plugin was applied to control the quality of sequencing raw data, trim, denoise, splice, and remove chimeras. Then, the QIIME2 feature classifier plugin was used to compare the representative sequences with the GREENGENES database, and to obtain a species classification information table. Later, the Alpha diversity index was calculated using the QIIME2 core diversity plugin. Venn diagrams was applied to analyse the number of common and unique species in multiple samples, which can intuitively display the similarity and overlap of species composition in different environmental samples. The species richness of each sample was counted at different taxonomy levels, and the community composition was visually analysed through the column chart. The differences in species composition was compared using One way ANOVA analysis, and by using FastTree software, and a phylogenetic evolution tree of species was constructed based on the Maximum Likelihood (ML) method.

Results and discussion

Data sequencing of white and red pitaya

Red and white pitaya broth samples were sequenced during five stages of spontaneous fermentation (F1, F3, F5, F7, and F15). Specifically, 584,055 and 699,120 valid sequences were obtained after quality control. After subsampling, 86 and 42 operational taxonomic units (OTUs) were obtained with 97% similarity. The OTUs of red pitaya belonged to nine classes, 17 orders, 33 families, 49 genera, and 69 species, while the OTUs of white pitaya belonged to eight classes, 14 orders, 23 families, 32 genera, and 37 species.

Alpha diversity of yeast communities during spontaneous fermentation of red and white pitaya

Alpha diversity refers to the diversity within a particular region or ecosystem, and is usually measured by Shannon, Simpson, Ace, and Chao indices. Shannon and Simpson indices measure species diversity in a sample, while bigger Shannon and smaller Simpson indices indicate bigger and smaller species diversity, respectively (Delgado-Baquerizo *et al*., 2016; Santini *et al*., 2017). Ace and Chao indices measure the number of species in a sample, with larger values indicating higher number of species (Swamy and Gayathri, 2021). If inconsistencies among the four indices are found, the Shannon index should prevail, and followed by the Simpson index.

The alpha diversity results of red and white pitaya during spontaneous fermentation are shown in Table 1.

Table 1. Diversity indices of yeast communities at different stages of spontaneous red and white pitaya fermentation.

	Red pitaya					**White pitaya**				
	RF1	**RF3**	**RF5**	**RF7**	**RF15**	**WF1**	**WF3**	**WF5**	**WF7**	**WF15**
Shannon	2.13	0.61	0.73	1.24	1.51	0.06	0.03	0.34	0.22	0.33
Simpson	0.20	0.73	0.67	0.46	0.28	0.98	0.99	0.82	0.89	0.85
Ace	37.81	32.61	40.80	39.18	26.47	22.28	14.99	16.01	10.36	7.09
Chao	35.83	31.36	39.73	37.40	25.83	19.31	11.00	12.83	7.00	8.33

Red pitaya had the highest species diversity (largest Shannon and smallest Simpson indices) during the initial stage of fermentation (RF1). By contrast, white pitaya had the highest diversity of yeast species (highest Shannon, Ace, and Chao indices, and lowest Simpson index) during the middle stage of fermentation (WF5). Comparative analysis of species diversity changes of the yeast communities between the red and white pitaya revealed that the F3 stage during spontaneous fermentation had the lowest diversity. This indicated that during spontaneous fermentation, the third day was a critical point for the evolution of the yeast community, and the diversity of yeast species composition for the red and white pitaya was significantly different before and after F3. The Shannon index indicated that the diversity of yeast in the red pitaya increased and then decreased. However, the opposite was true for the white pitaya during spontaneous fermentation. In general, the yeast species in red pitaya were more diversified than in white pitaya.

Yeast species composition during spontaneous fermentation of red and white pitaya

A Venn diagram was used to visualise the common and different genera and species in the red and white pitaya at different stages during spontaneous fermentation. As shown in Figure 1, there were significant differences between red and white pitaya in terms of the total number, as well as common and unique yeast genera and species. A total of 49 genera and 69 species were identified in the five spontaneous fermentation samples for the red pitaya (Figures 1C and 1D), and the numbers of common genera and species for the five stages were 15 and 17, respectively. The unique genera and species were 12 and 18 in F1, 0 and 1 in F5, 1 and 1 in F7, and 1 and 2 in F15. A total of 32 genera and 37 species were identified in the white pitaya (Figures 1E and 1F), and two genera and two species were found in the five stages. The numbers of unique genera and species were 12 and 15 in F1, 1 and 2 in F3, 1 and 2 in F7, and 3 and 3 in F15. However, unique species and genus were absent in the red pitaya for F3, and in the white pitaya for F5.

The number of yeast species decreased in the red and white pitaya as fermentation progressed. On the first day of spontaneous fermentation, the number of yeast species was the highest, but the lowest on the third day. During the middle and late stages of fermentation, the number of species increased, and then decreased. Overall, there were similarities in the number of yeast genera and species variations between the red and white pitaya throughout the five fermentation stages. However, the red pitaya exhibited a significantly higher number of yeast species during spontaneous fermentation.

The compositions of the yeast species during spontaneous pitaya fermentation are shown in Figure 2 which indicates that yeast species richness in the red pitaya was significantly higher than that in white pitaya. At the species level, F1 in red pitaya was dominated by unclassified_g_*Vishniacozyma* (29.69%), followed by *Hannaella siamensis* (17.98%), *Filobasidium* sp. (15.61%), unclassified_o_*Saccharomycetales* (9.70%), and *Gibberella intricans* (4.74%). F3 was dominated by unclassified_o_*Saccharomycetales* (85.07%), followed by *Clavispora opuntiae* (7.09%), unclassified_g_*Hanseniaspora* (3.17%), *Pichia barkeri* (2.01%), and *Wickerhamomyces anomalus* (1.32%). In addition, F5 was dominated by unclassified_o_*Saccharomycetales* (79.87%), followed by *Clavispora opuntiae* (13.69%) and *Wickerhamomyces anomalus* (3.05%), and F7 contained unclassified_o_*Saccharomycetales* (62.16%), followed by *Clavispora opuntiae* (11.68%), unclassified_f_*Dipodascaceae* (11.07%), *Dipodascaceae* sp. (8.61%), and *Wickerhamomyces anomalus* (4.41%). F15 contained unclassified_f_*Dipodascaceae* (32.47%), *Dipodascaceae*_sp. (24.98%), *Clavispora opuntiae* (22.30%), unclassified_o_*Saccharomycetales* (14.53%), and *Pichia barkeri* (4.65%). At the species level, F1 for white pitaya was dominated by *Clavispora opuntiae* (99.78%), while *Wickerhamomyces anomalus* and unclassified_o_*Saccharomycetales* accounted for only 0.02 and 0.01% of the total amount, respectively. F3 contained *Clavispora opuntiae* (99.98%), followed by *Wickerhamomyces anomalus* (0.01%) and other yeast species (0.01%). F5 contained *Clavispora opuntiae* (79.84%), as well as *Wickerhamomyces anomalus* (19.16%) and *Candida quercitrusa* (0.97%). F7 was dominated by *Clavispora opuntiae* (70.60%), followed by *Wickerhamomyces anomalus* (25.50%) and *Candida quercitrusa* (3.85%), while F15 was dominated by unclassified_o_*Saccharomycetales* (56.30%), followed by *Wickerhamomyces anomalus* (36.40%), *Candida quercitrusa* (4.08%), and *Clavispora opuntiae* (2.53%).

A comparative analysis was performed on the presence and abundance of dominant yeast species during the spontaneous fermentation of red and white pitaya. The dominant yeast species in red pitaya was unclassified_o_*Saccharomycetales* (Figure 2A), and its abundance changed considerably as fermentation progressed. This species accounted for only 9.70% of the amount of yeast during the initial stage of fermentation (F1). However, its abundance increased to 85.07, 79.87, and 62.16% during the early and middle stages of fermentation (F3, F5, and F7), and then decreased to 14.53% during the later stage of fermentation. The abundance of *Clavispora opuntiae* decreased, and then increased during spontaneous fermentation of red pitaya from 7.09, 13.69, and 11.68% for stages F3, F5, and F7, respectively, and to 22.30% in stage F15. As shown in Figure 2B, *Clavispora opuntiae* was the dominant yeast species in white pitaya which accounted for 99.78, 99.98, 79.84, and 70.60% of the species in F1, F3, F5, and F7, respectively. After 15 d of spontaneous fermentation, the yeast community composition in white pitaya varied significantly, and the dominant *Clavispora opuntiae* species was replaced by unclassified_o_*Saccharomycetales*. The abundance of *Clavispora opuntiae* decreased sharply to 2.53%, while unclassified_o_*Saccharomycetales* increased from 0.01% in F1 to 56.30% in F15.

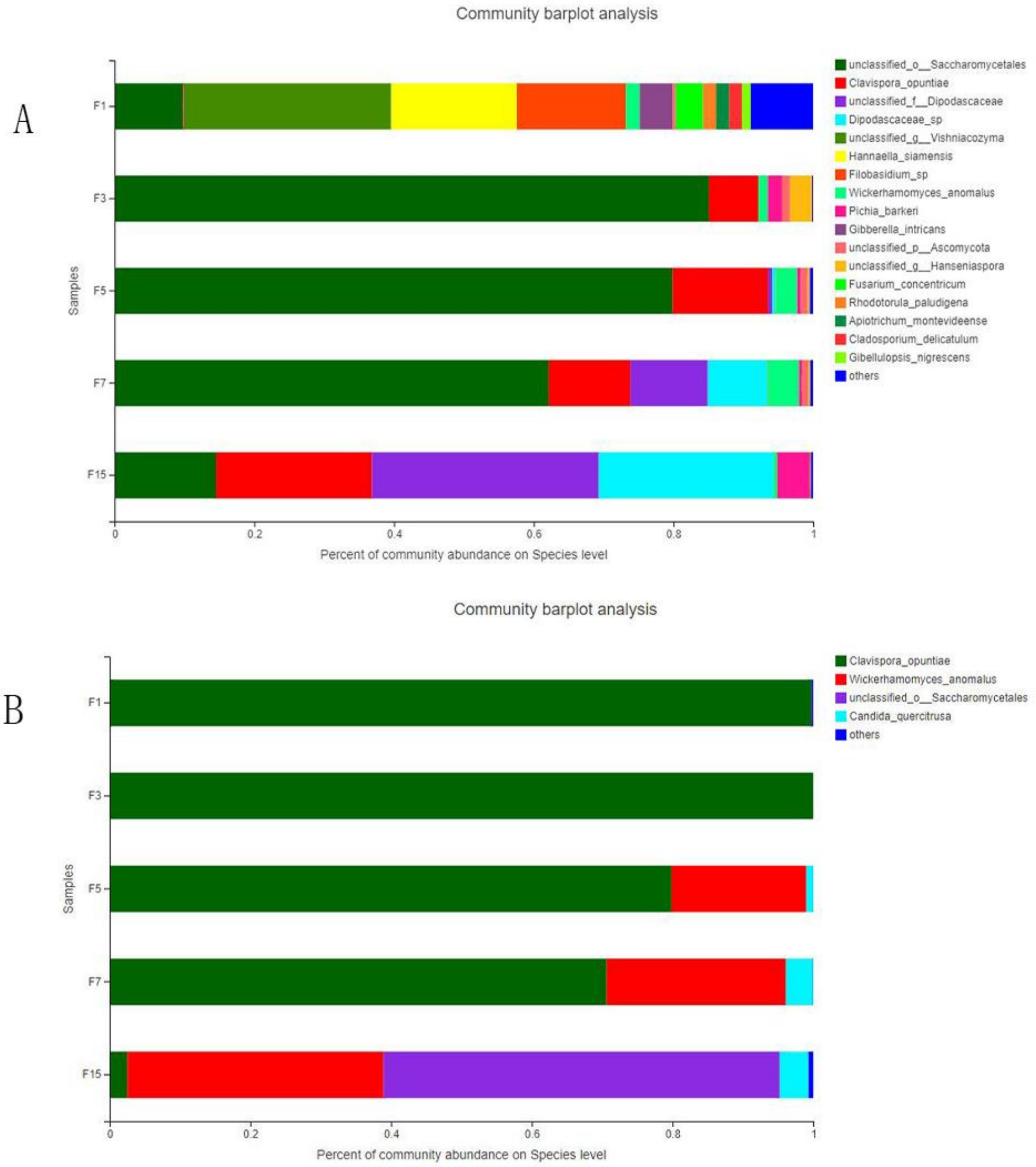

Figure 2. Barplot of yeast community composition at different spontaneous fermentation stages of **(A)** red (*Hylocereus polyrhizus*) and **(B)** white (*Hylocereus undatus*) pitaya.

Analysis of differences among yeast communities during spontaneous red and white pitaya fermentation

Red pitaya

One-way ANOVA was used to analyse the differences in yeast species composition during the five stages of spontaneous red pitaya fermentation. Results showed highly significant differences in unclassified_o_*Saccharomycetales* ($p \leq 0.01$) species, and significant differences in unclassified_p_*Ascomycota* ($p \leq 0.05$) species throughout the five stages (Figure 3A).

White pitaya

One-way ANOVA showed highly significant differences ($p \leq 0.001$) in *Clavispora opuntiae* between the five stages of spontaneous fermentation of white pitaya (Figure 3B).

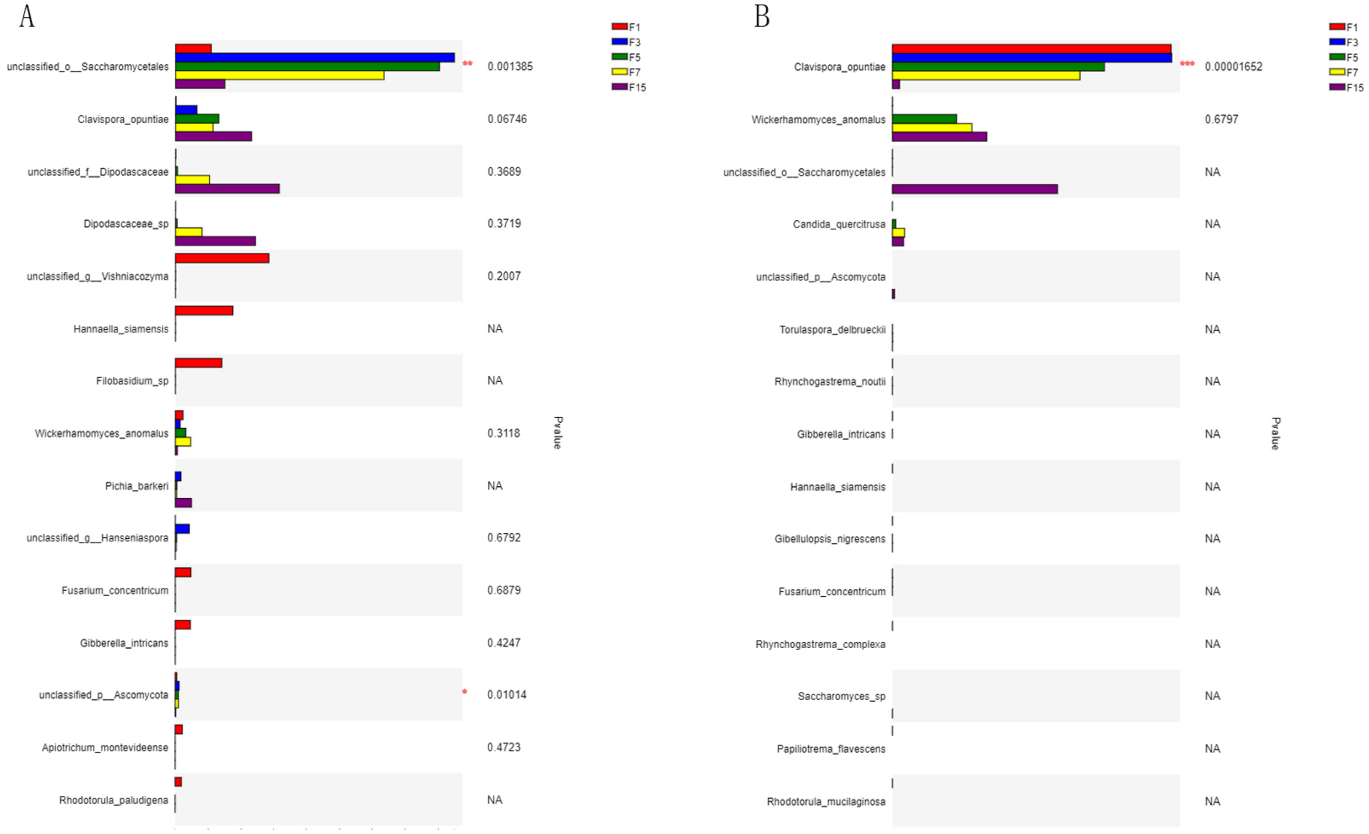

Figure 3. Difference analysis for the *Saccharomyces* yeast community of **(A)** red (*Hylocereus polyrhizus*) and **(B)** white (*Hylocereus undatus*) pitaya during different spontaneous fermentation stages at the species level. *$p \leq 0.01$; **$p \leq 0.001$; and ***$p \leq 0.0001$.

Analysis of yeast community evolution during spontaneous fermentation of red and white pitaya

Red pitaya

The phylogenetic relationships of the predominant yeast species in red pitaya during the five stages of spontaneous fermentation were analysed using FastTree. A phylogenetic tree was constructed using the maximum likelihood (ML) method which showed that unclassified_*Dipodascaceae* was closest to *Dipodascaceae* sp., and unclassified_*Hanseniaspora* was close to *Pichia barkeri* and *Clavispora opuntiae*, but far away from unclassified_*Saccharomycetales* (Figure 4A).

White pitaya

The phylogenetic relationships of the predominant yeast species in white pitaya in the five fermentation stages were analysed using FastTree. A phylogenetic tree by the ML method showed that *Pichia barkeri* was closest to *Wickerhamomyces anomalus*, *Candida quercitrusa* was close to *Debaryomyces nepalensis* and unclassified_*Saccharomycetales*, but far from *Clavispora opuntiae* (Figure 4B).

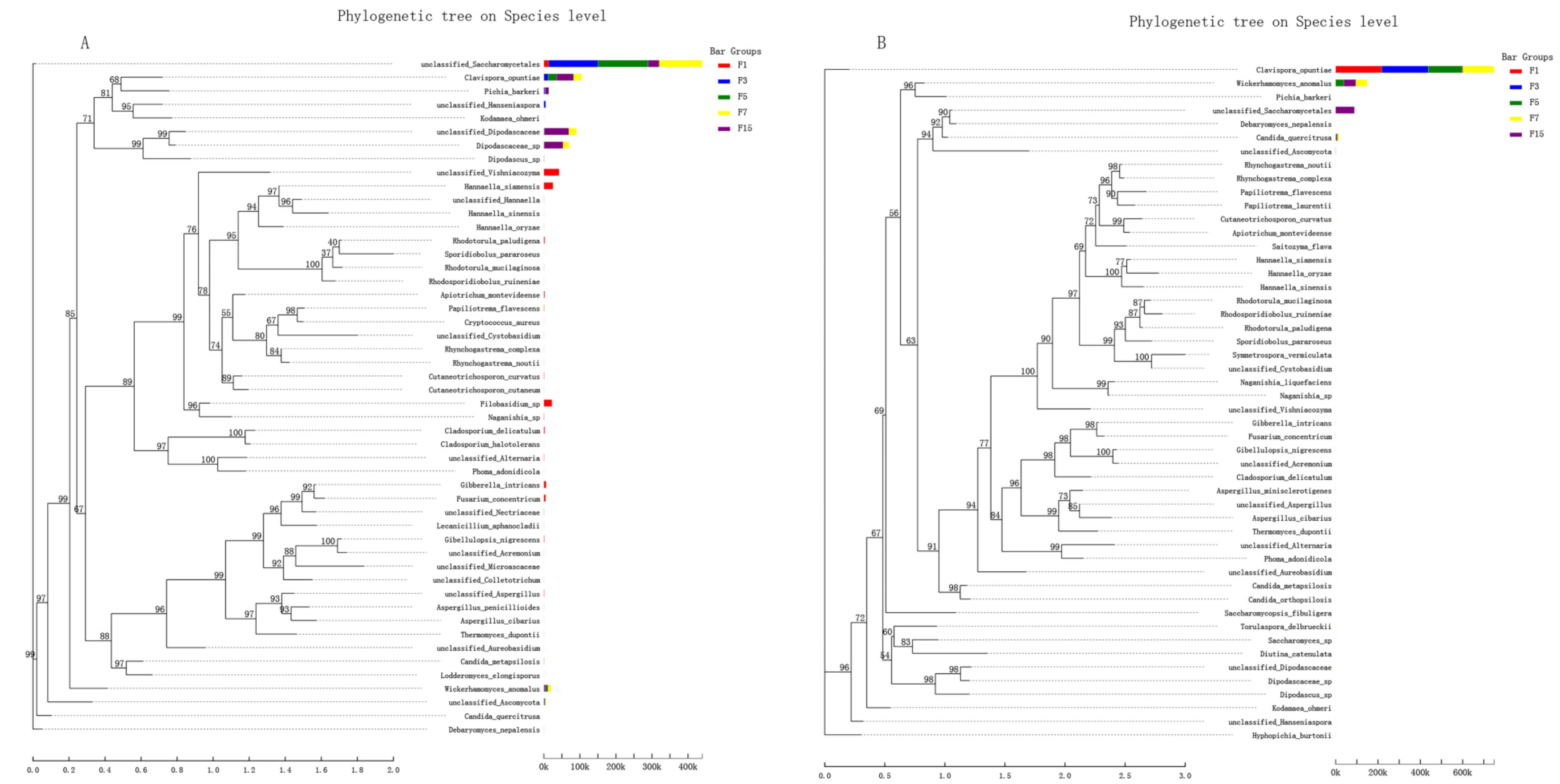

Figure 4. Phylogenetic analysis for the *Saccharomyces* yeast community of **(A)** red (*Hylocereus polyrhizus*) and **(B)** white (*Hylocereus undatus*) pitaya during different spontaneous fermentation stages (F1, F3, F5, F7, and F15) at the species level.

Conclusion

High-throughput sequencing is an important tool for analysing the diversity of brewing microorganisms because it is simple, fast, accurate, and offers high throughput. As a result, it has been frequently used to analyse microorganisms (Reuter *et al*., 2015). The high-throughput sequencing results showed abundant yeast resources in the red and white pitaya, with 69 species and 49 genera of yeast in the red pitaya, and 37 species and 32 genera of yeast in the white pitaya, thus suggesting great potential for using pitaya yeast resources (Fan *et al*., 2021).

In the present work, we compared and analysed the alpha diversity and species composition of yeast during different stages of spontaneous red and white pitaya fermentation. The diversity index showed that yeast diversity was significantly higher in red pitaya as compared to white pitaya. The physicochemical parameters such as sugar content of the two types of pitaya suggested that the higher yeast diversity might have been related to the higher sugar content in red pitaya, which is consistent with the research results of other researchers (Li *et al*., 2015; Zhou *et al*., 2018). Li *et al*. (2015) showed that soluble solid content was significantly higher in red pitaya as compared to white pitaya, and Zhou *et al*. (2018) also found significantly higher total sugar and titratable acid content in red rather than white pitaya. The Venn diagram showed that there were large differences in the total number of yeast species, common species, and endemic species between the two types of pitaya. Additionally, the number of yeast species in red and white pitaya increased, and then decreased during different stages of spontaneous fermentation, which could have been attributed to the decreased ethanol tolerance of some of the yeast species.

A comparative analysis revealed unclassified_o_*Saccharomycetales* and *Clavispora opuntiae* were the dominant yeast species in red and white pitaya during spontaneous fermentation. *Clavispora opuntiae* can be used for ethanol production (Nigam *et al*., 2015). In addition, unclassified_o_*Saccharomycetales* were present throughout the red pitaya fermentation process and dominated during the early and middle stages of fermentation (F3, F5, and F7), thus suggesting good tolerance to the ethanol or brewing environment. Additional studies may also consider isolating and culturing this yeast species to determine its fermentation performance, as the isolation of excellent brewing yeast species can better serve the entire pitaya wine industry.

Clavispora opuntiae was highly dominant during the early and middle stages of white pitaya fermentation. However, its abundance decreased gradually with the progression of fermentation and sharply decreased to 2.53% during the late stages of fermentation. Conversely, the abundance of unclassified_o_*Saccharomycetales* increased dramatically during the late stages of fermentation. Interestingly, these two dominant yeast species exhibited opposite trends during spontaneous red pitaya fermentation. The abundance of unclassified_o_*Saccharomycetales* also decreased while the abundance of *Clavispora opuntiae* increased with prolonged fermentation. This implied a competitive relationship between these two species during red and white pitaya fermentation, and could have been attributed to the varietal differences and sequencing analysis results. However, more specific reasons will be studied in the future.

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