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Fermentative and Enological Features of *Saccharomyces cerevisiae* Populations Generated Through Adaptive Laboratory Evolution

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Abstract: Adaptive laboratory evolution (ALE) is a non-GMO technique utilized for the amelioration of wine yeast strains. Employing two-step ALE strategies, we recently acquired six evolved *Saccharomyces cerevisiae* populations with improved fermentative abilities compared to their parental strains in synthetic broths. Herein, we evaluated the qualities of the abovementioned evolved populations under real winemaking conditions, using the grape musts Assyrtiko and Roditis. The ethanol-tolerant populations evolved solely with glucose delayed to complete the fermentation due to slow fructose assimilation, albeit showing improved ethanol yields, compared to their parental strains. The volatile compounds of the evolved populations were significantly different from those of parental strains. Statistically significant differences were observed in the organoleptic profiles between the evolved populations' and parental strains' wines. Notably, wine from one evolved population (BLR200) was rated higher in overall aroma and quality. This study supports the magnitude of ALE strategies for the generation of novel wine yeasts.

Keywords: *Saccharomyces cerevisiae*; evolved populations; winemaking; alcoholic fermentation; volatile compounds

Citation: Mavrommati, M.; Christofi, S.; Kallithraka, S.; Papanikolaou, S.; Aggelis, G. Fermentative and Enological Features of *Saccharomyces cerevisiae* Populations Generated Through Adaptive Laboratory Evolution. *Beverages* **2024**, *10*, 102. https://doi.org/10.3390/beverages10040102

Academic Editor: Agustín Aranda

Received: 15 September 2024
Revised: 10 October 2024
Accepted: 16 October 2024
Published: 22 October 2024

1. Introduction

Yeasts play a major role in the formulation of the final wine aroma. The so-called "yeast bouquet" comprises ethyl esters, acetate esters, fusel alcohols, carbonyls, and volatile fatty acids. Wine yeast strains can be classified as fruity–floral, neutral, or cheesy–rancid–spirituous, depending upon the volatile compounds they are capable of producing through their metabolism [1]. The contribution of *Saccharomyces cerevisiae* metabolism in the final wine aroma has been extensively studied. Esters, which confer fresh and fruity aromas to the wine, derive from the reaction of acetyl-CoA either with ethanol (for ethyl esters production) or with fusel alcohols (for acetate esters production) [2,3]. Fusel alcohols are produced through a metabolic pathway known as the "Ehrlich pathway", utilizing several amino acids as precursors [4,5]. They generally should not be exceeding the concentration of 300 mg/L in the wine, since they produce a strong smell that resembles solvent, whereas in smaller concentrations, they contribute to the wine's fruity character [6]. Acetaldehyde, a metabolic intermediate of alcoholic fermentation, offers apple-like, citrus-like, and nutty aromas to the produced wine [7]. Acetic acid is an undesirable volatile compound of yeast metabolism, produced through a bypass metabolic pathway of alcoholic fermentation,

where pyruvic acid is converted into acetyl-CoA. By law, its concentration in wine should not exceed 1.0–1.5 g/L [7,8]. Finally, hydrogen sulfide (H_2S), emitting a distinctive odor of rotten egg, is formulated from the abundant inorganic sulfur compounds of grape must, through the sulfate reduction sequence (SRS) pathway [7,9]. It has been proven that there are metabolic diversities even between different strains of the same species, which crucially affect the final sensory profile of the produced wine, even when fermenting the same grape must [10].

Over the years, there has been a constant demand in the wine market for new yeast strains with improved characteristics. Methods based on genetic engineering have been extensively and successfully used for this purpose [11,12]. Notwithstanding, the utilization of genetically modified yeast strains in winemaking tends to be avoided, owing to consumer distrust and to the strict regulation for the use of GMOs for food applications in many countries [13]. One efficient non-GMO strategy employed for wine yeast refinement is Adaptive Laboratory Evolution (ALE) [14], an experimental technique in which the principles of natural evolution are implemented on the laboratory bench, aiming at the better fitting of one specific cell population in a specific environment with the desired conditions [15–18]. *S. cerevisiae* populations which combine ethanol tolerance with improved fructose assimilation can result in being particularly useful in winemaking. It has been proven that ethanol concentrations above 15% *v*/*v* affect *S. cerevisiae* cells' physiology by increasing the concentration of unsaturated fatty acids (mainly oleic and palmitoleic acid) of their membrane and decreasing its saturated fatty acids [19–25]. In practice, high ethanol concentrations in the fermentation broth might provoke a decreased fermentation rate and reduced ethanol production [26]. In addition to this, *S. cerevisiae* is a glucophilic yeast, meaning that it prefers glucose to other sugars, such as fructose [27,28]. Consequentially, in a typical grape must fermentation, glucose is assimilated faster than fructose, leading to a discrepancy between these two sugars in the fermentation kinetics [29], a situation often causing stuck or sluggish fermentations in winemaking [30].

Recently, Mavrommati et al. [31,32], employing two different two-step ALE strategies, generated six different evolved populations, derived from two different parental *S. cerevisiae* strains, named BLR and CFB [33]. In accordance with the initial ALE strategy [31], the process took place in two distinct steps. First, during the evolution step, yeast growth occurred in a medium containing 20 g/L of glucose. Subsequently, in the selection step, yeast cells were subjected to an aqueous solution containing 18–25% *v*/*v* ethanol for 1–3 h of incubation. Only the cells demonstrating the capacity to withstand such elevated ethanol concentrations were able to survive this stringent selection process. Subsequently, the surviving cells from the aqueous ethanolic solution were transferred to a new medium with 20 g/L glucose, and the process was repeated in a loop. Two evolved populations derived from the parental strain *S. cerevisiae* CFB after 100 and 150 generations of evolution, respectively (named here CFB100 and CFB150), and two evolved populations derived from the parental strain *S. cerevisiae* BLR after 100 and 200 generations of evolution, respectively (named here BLR100 and BLR200), were proven ethanol tolerant, exhibiting higher fermentation rates than their parental strains in synthetic broth with 200 g/L glucose [31]. In the second ALE strategy [32], the first (evolution) step was realized in a medium with 20 g/L fructose, and the second (selection) step in aqueous solution with 18% *v*/*v* ethanol for 3 h of incubation, repeated in a loop, as stated previously. One evolved population derived from the parental strain *S. cerevisiae* CFB after 100 generations of evolution (named here CFB100Fr) and one evolved population derived from the parental strain *S. cerevisiae* BLR after 100 generations of evolution (named here BLR100Fr) presented diverse fermentative abilities in synthetic broth with 100 g/L glucose and 100 g/L fructose (grape must assimilating) [32].

The aim of this study is to investigate the fermentative and enological capabilities of previously evolved populations [31,32], in comparison to their parental strains. This investigation involves tailored enological fermentations using grape musts obtained from indigenous Greek grape varieties. The primary focus is on analyzing the volatile com-

pounds present in the resulting wines, with a specific emphasis on conducting sensory evaluations of wines. Through this study, the researchers aim to identify and compare statistically significant differences in the volatile compounds of wines originating from the same must but produced from both parental strains and evolved populations. Furthermore, the research seeks to elucidate variations among the differently evolved populations. These findings are anticipated to provide evidence regarding the effectiveness of the ALE technique in cultivating evolved populations with improved technological capabilities.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

The strains *Saccharomyces cerevisiae* ho_SB (CFB) and *S. cerevisiae* DBVPG1973 (BLR) [33] were used, as well as the evolved populations CFB100, CFB150, BLR100, BLR200 [31], CFB100Fr, and BLR100Fr [32]. They were kept at −80 °C in 30% *v*/*v* glycerol and grown out of the frozen stock in 250 mL Erlenemeyer flasks containing 100 mL yeast extract–peptone–dextrose (YPD) medium (2% *w*/*v* glucose, 1% *w*/*v* peptone, and 1% *w*/*v* yeast extract) incubated in an incubator shaker (ZHICHENG ZHWY 211C, Shanghai, China) at 28 °C, agitated at 180 rpm for 48 h. The media were sterilized at 121 °C for 20 min.

2.2. Fermentations on Grape Must

The fermentation performances of the parental strains and the evolved populations were tested under different wine fermentation conditions. Assyrtiko and Roditis grapes (indigenous Greek grape varieties) were mechanically crushed and pressed with the utilization of a small-scale crusher commonly used in experimental wineries, specially designed for precise control over the crushing process, ensuring consistency and quality in small-batch wine production. The must was left for 12 h for clarification at 4 °C. After clarification, the total sulfur dioxide (SO_2) of the must was adjusted at 10 mg/L with the addition of the appropriate quantity of metabisulphite, and filtration with 0.45 μm filters took place in order to eliminate indigenous yeast cells. The initial pH, titratable acidity, sulfites, and sugar content (expressed as Brix) [34] of the grape musts used in the present study are presented in Supplementary Materials Table S1. Four different musts were used, namely Assyrtiko I, Assyrtiko II, Roditis I, and Roditis II, due to their different chemical composition. Prior fermentation, the sugar content of grape must was adjusted to 21.5 Brix via the addition of equimolar concentrations of glucose and fructose. Nitrogen enrichment of must was accomplished with the addition of 20g/hL diammonium phosphate (DAP). Yeasts were inoculated at 10^7 cfu/mL to initiate alcoholic fermentations carried out in 2 L vessels, at 20 °C, in duplicate. A spontaneous fermentation (without yeast inoculation) was also realized for each must. The fermentation progress was monitored by measuring the must density daily, with parallel determination of reducing sugars content (glucose and fructose) by HPLC, as described by Terpou et al. [35]. At the end of fermentation, the wine was racked with a consequent addition of 40 mg/L SO_2.

2.3. Analytical Methods

2.3.1. Sugars and Fermentation Products' Determination

Glucose, fructose, ethanol, and glycerol were determined using High-Performance Liquid Chromatography (HPLC, Waters 600E) equipped with an Aminex HPX-87H (300 mm × 7.8 mm, Bio Rad CA) column, coupled to a differential refractometer (RI Waters 410) with the following operating conditions: sample volume, 20 μL; mobile phase, 10 mM H_2SO_4; flow rate, 0.5 mL/min; and column temperature, 65 °C. Samples were centrifuged at 9000 rpm for 10 min at 4 °C (Hettich Universal 320-R, Germany), and the supernatant was filtered through a 0.2 μM membrane filter before the injection to the chromatograph. The retention times for each substance were the following: glucose, 12.3 min; fructose, 13.6 min; glycerol, 17.5 min; and ethanol, 26.9 min. Several chemical analyses were applied in both must and wine. Classical analytical parameters (total SO_2 content, pH, and titratable acidity) were determined according to the OIV (2020) methods [34].

2.3.2. Analysis of Volatile Compounds

Wine volatile compounds were extracted using a mixture of pentane and diethyl ether, according to the method described in [36], as proposed in earlier studies [37,38], using 3-octanol (2500 ppm) as an internal standard. Determination of the individual volatile compounds was performed with Gas Chromatography coupled with Mass Spectrometry (GC-MS), using an Agilent 6890 series GC System (Wilmington, DE, USA), as described previously [39].

2.3.3. Sensory Analysis

A sensory evaluation of the wines derived by fermentation of Assyrtiko II must was realized by 8 trained panelists. The samples were labeled with three-digit numbers and presented in a randomized order to each panelist. They were assessed in duplicate regarding their odor (white flowers, tropical fruits, citrus fruits, stone fruits, botanicals, and oxidation) and flavor (sourness and mouthfeel as the perception of the body of wine) attributes. An overall quality assessment was also conducted. The intensity of the sensory attributes examined was rated on a 5-point scale (0, null; 5, very strong).

2.3.4. Statistical Analysis

Analyses of volatile compounds were run in duplicate. Data were subjected to one-way analysis of variance (ANOVA), with STATISTICA V.7 Software (Statsoft® InC., Tulsa, OK, USA). Mean values were compared using Tukey's HSD test when the samples were significantly different ($p < 0.05$).

3. Results and Discussion

3.1. Vinification with CFB100 and BLR100 Populations

CFB100 and BLR100 are ethanol-tolerant populations derived from the strains *S. cerevisiae* CFB and *S. cerevisiae* BLR, respectively, after 100 generations of evolution in a two-step ALE experiment (i.e., growth in synthetic glucose medium followed by exposure to high ethanol concentration solution) [31]. In the present work, we evaluated their performances compared to their parental strains under real vinification conditions in Assyrtiko I, Roditis I, and Roditis II grape musts.

3.1.1. Fermentative Behaviors of CFB100 and BLR100 Populations on Different Grape Musts

The glucose and fructose uptake rate, and ethanol and glycerol production of the populations CFB100, BLR100, and their parental strains, as well as the spontaneous fermentation in Assyrtiko I grape must, are shown in Figure 1. It is evident that the parental strains displayed better fermentative abilities than the evolved populations. The CFB strain consumed all the available sugars 48 h earlier than the CFB100 population (Figure 1a–c). Nevertheless, CFB100 consumed glucose 22 h earlier than CFB, ultimately completing the fermentation in 380 h owing to the remaining fructose in the broth (Table 1). The ethanol yield ($Y_{P/S}$) of CFB was slightly lower than that of CFB100, i.e., 0.45 g/g and 0.47 g/g, respectively, whilst maximum ethanol concentrations were 106.4 g/L and 103.4 g/L and maximum glycerol concentrations were 7.9 g/L and 5.5 g/L, respectively (Table 1). The BLR100 population realized a sluggish fermentation, of 738 h, whereas the BLR strain brought the must to dryness 428 h earlier (Figure 1d–f). Apparently, the sluggish fermentation of BLR100 was a result of the slow assimilation of the must's fructose, since the whole glucose had already been consumed at 402 h (Table 1). Ethanol yield ($Y_{P/S}$) had the value of 0.45 g/g, in both cases, whilst maximum ethanol concentrations were 107.0 g/L and 95.1 g/L and maximum glycerol concentrations 6.6 g/L and 5.7 g/L, respectively. Spontaneous fermentation also was sluggish, lasting for 690 h (Figure 1g). However, it was completed 48 h earlier than the fermentation of BLR100, although glucose was consumed in 402 h in both cases. Furthermore, the ethanol yield was particularly low (0.32 g/L), with

a maximum ethanol concentration of 79.6 g/L and a maximum glycerol concentration of 5.2 g/L (Table 1).

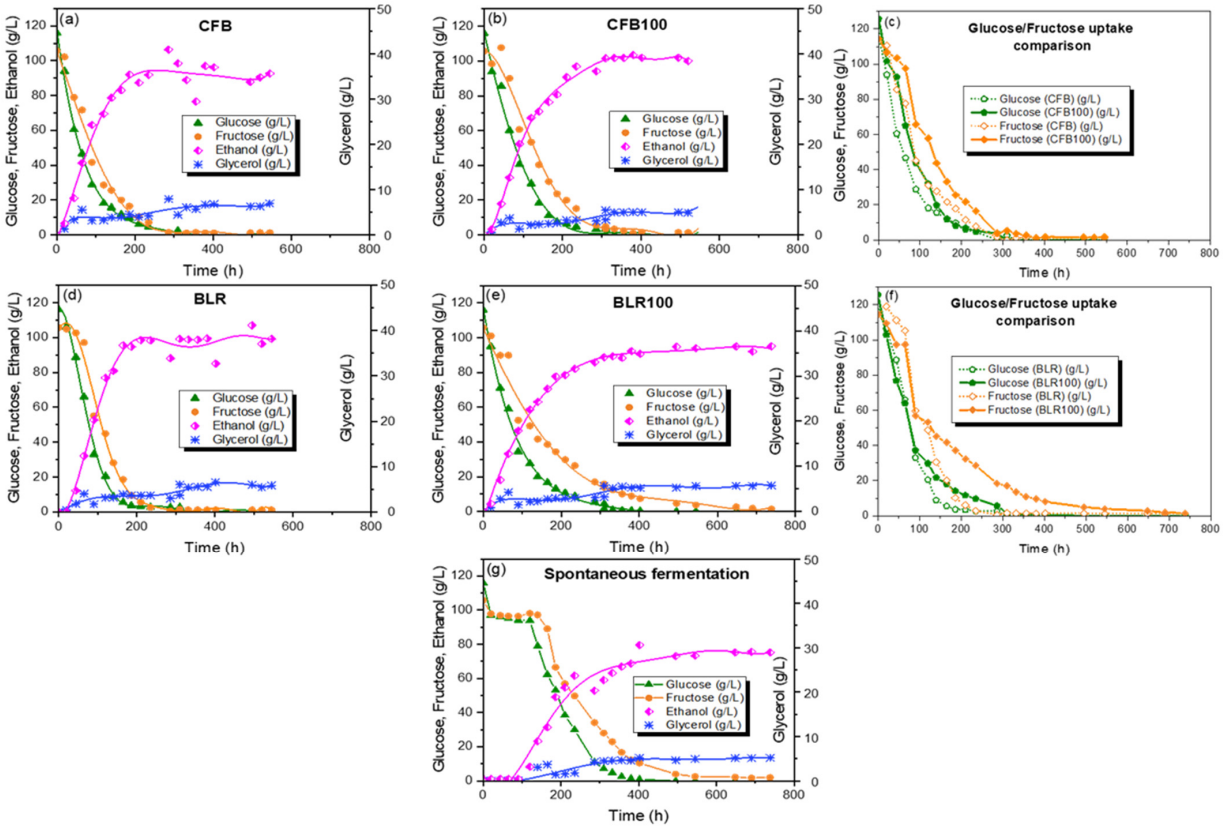

Figure 1. Glucose and fructose consumption (g/L) and ethanol and glycerol production (g/L) of parental strains *S. cerevisiae* CFB (**a**) and *S. cerevisiae* BLR (**d**) and evolved populations CFB100 (**b**) and BLR100 (**e**) in Assyrtiko I grape must. Comparisons of glucose and fructose uptake rates between parental strains and evolved populations are also presented (**c**,**f**). Spontaneous fermentation is also shown (**g**).

The glucose and fructose uptake rate and the ethanol and glycerol production of the populations CFB100 and BLR100 and their parental strains, as well as the spontaneous fermentation in Roditis I grape must, are shown in Figure 2. The CFB strain terminated the fermentation 72 h earlier than the CFB100 population (Figure 2a–c). In both of these fermentations, glucose was consumed in 280 h, and CFB consumed fructose 98 h earlier than glucose (Table 2), which is generally an unusual pattern. The ethanol yield ($Y_{P/S}$) of CFB100 was higher than CFB (0.48 g/g and 0.45 g/g, respectively), one difference reflected in the maximum ethanol concentrations, which were 113.7 g/L in CFB100 and 102.8 g/L in CFB (Table 2). The BLR100 population delayed completing the fermentation 259 h compared to BLR strain, having consumed the must's glucose 214 h earlier than fructose, whereas BLR consumed fructose 25 h earlier than glucose (Figure 2d–f and Table 2). The ethanol yield ($Y_{P/S}$) of BLR100 was rather high (0.49 g/g), compared to 0.47 g/g of BLR, and the maximum ethanol concentrations were 116.8 g/L and 108.9 g/L, respectively (Table 2). The spontaneous fermentation lasted for 352 h (Figure 2g), which was the exact same duration as the fermentation of CFB100, albeit 142 h quicker than the fermentation of BLR100. It is noteworthy that in all three of these fermentations, glucose was consumed in 280 h. The ethanol yield ($Y_{P/S}$) of the spontaneous fermentation was relatively low, presenting the value of 0.34 g/g, with a maximum ethanol concentration of 83.3 g/L (Table 2). Maximum glycerol concentrations were similar in all fermentations, ranging between 5.3 and 5.9 g/L (Table 2).

Table 1. Fermentative abilities of *S. cerevisiae* parental strains and evolved populations CFB100 and BLR100 during vinification in Assyrtiko I grape must. Spontaneous fermentation is also shown.

	CFB	CFB100	BLR	BLR100	Spontaneous Fermentation
$Y_{P/S}$ (g of ethanol/g of glucose consumed)	0.45 ± 0.01	0.47 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	0.32 ± 0.00
Fermentation time (h)	332	380	310	738	690
Glucose consumption time (h)	332	310	310	402	402
Fructose consumption time (h)	332	380	310	738	690
Maximum ethanol concentration (g/L)	106.4 ± 5.6	103.4 ± 1.0	107.0 ± 5.3	95.1 ± 0.1	79.6 ± 2.8
Residual glucose in maximum ethanol concentration (g/L)	2.4 ± 1.7	0	0	0	0.9 ± 0.6
Residual fructose in maximum ethanol concentration (g/L)	1.4 ± 1.2	1.0 ± 0.5	1.2 ± 0.0	1.4 ± 0.4	10.4 ± 1.4
Maximum glycerol concentration (g/L)	7.9 ± 0.7	5.5 ± 0.3	6.6 ± 0.4	5.7 ± 0.1	5.2 ± 0.0

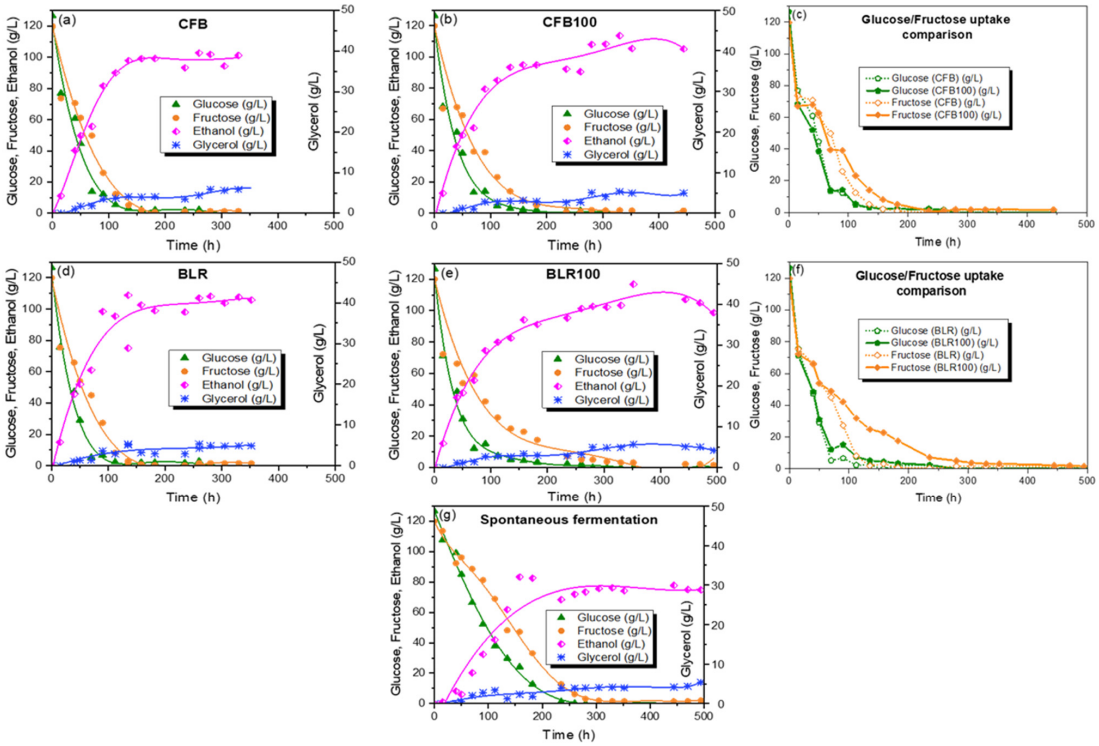

Figure 2. Glucose and fructose consumption (g/L) and ethanol and glycerol production (g/L) of parental strains *S. cerevisiae* CFB (**a**) and *S. cerevisiae* BLR (**d**) and evolved populations CFB100 (**b**) and BLR100 (**e**) in Roditis I grape must. Comparisons of glucose and fructose uptake rates between parental strains and evolved populations are also presented (**c**,**f**). Spontaneous fermentation is also shown (**g**).

Table 2. Fermentative abilities of *S. cerevisiae* parental strains and evolved populations CFB100 and BLR100 during vinification in Roditis I grape must. Spontaneous fermentation is also shown.

	CFB	CFB100	BLR	BLR100	Spontaneous Fermentation
$Y_{P/S}$ (g of ethanol/g of glucose consumed)	0.45 ± 0.00	0.48 ± 0.01	0.47 ± 0.00	0.49 ± 0.00	0.34 ± 0.01
Fermentation time (h)	280	352	260	494	352
Glucose consumption time (h)	280	280	260	80	280
Fructose consumption time (h)	182	352	235	494	352
Maximum ethanol concentration (g/L)	102.8 ± 0.6	113.7 ± 3.8	108.9 ± 0.5	116.8 ± 6.9	83.3 ± 0.6
Residual glucose in maximum ethanol concentration (g/L)	2.3 ± 0.2	0	2.3 ± 0.1	0	24.1 ± 4.0
Residual fructose in maximum ethanol concentration (g/L)	1.2 ± 0.3	1.9 ± 0.1	1.9 ± 0.8	3.2 ± 0.0	47.2 ± 0.7
Maximum glycerol concentration (g/L)	5.9 ± 0.0	5.3 ± 0.2	5.3 ± 0.0	5.7 ± 0.4	5.4 ± 0.7

The data concerning the glucose and fructose uptake rate and the ethanol and glycerol production of the populations CFB100 and BLR100 and their parental strains, as well as the spontaneous fermentation in Roditis II grape must, are provided in Supplementary Materials Figure S1. The CFB strain and CFB100 population exhibited similar fermentative behavior, assimilating all available sugars in 260 h and 280 h, respectively (Supplementary Figure S1a–c). Additionally, contrary to the fermentations in Assyrtiko I and Roditis I, CFB100 consumed both must's glucose and fructose simultaneously in 280 h, whereas CFB consumed glucose 78 h earlier than fructose (Table 3). The ethanol yield ($Y_{P/S}$) was low in both cases, presenting the value of 0.34 g/g, accompanied by low maximum ethanol concentrations, i.e., 86.7 g/L in CFB and 91.3 g/L in CFB100. Maximum glycerol concentrations were 7.1 g/L in CFB and 6.6 g/L in CFB100 (Table 3). The pattern of the BLR100 population terminating the fermentation significantly later than BLR strain was repeated, with a gap of 215 h (Supplementary Figure S1d–f). BLR assimilated both glucose and fructose in 235 h, whereas BLR100 assimilated fructose 190 h later than glucose (Table 3). The ethanol yield ($Y_{P/S}$) remained low, i.e., 0.35 g/g for BLR and 0.37 g/g for BLR100, with maximum ethanol concentrations of 95.3 g/L and 97.1 g/L and maximum glycerol concentrations of 6.4 g/L and 6.9 g/L, respectively (Table 3). The spontaneous fermentation was completed in 352 h, i.e., 98 h earlier than the fermentation of BLR100 (Supplementary Figure S1g), with an ethanol yield ($Y_{P/S}$) of 0.37 g/g, maximum ethanol concentration of 93.9 g/L, and maximum glycerol concentration of 5.3 g/L (Table 3).

Evidently, in all three must fermentations, the parental strains completed the fermentation distinctively more rapidly than the evolved populations, with the BLR100 population exhibiting a sluggish fermentation in all cases. This was mainly attributed to the fact that the time needed for fructose assimilation in evolved populations significantly exceeded the time required for glucose assimilation, with the exception of the CFB100 population in Roditis II must. This time divergence could be interpreted by the fact that the foresaid populations originate from an ALE experiment where glucose was used as the sole carbon source [31], which could possibly have attuned them to better glucose assimilation, while

deteriorating their ability for fructose assimilation. On the other hand, the ethanol yield ($Y_{P/S}$) was increased by 2–3 units in all the fermentations of the evolved populations compared to the parental strains, indicating their improved ethanol tolerance.

Table 3. Fermentative abilities of *S. cerevisiae* parental strains and evolved populations CFB100 and BLR100 during vinification in Roditis II grape must. Spontaneous fermentation is also shown.

	CFB	CFB100	BLR	BLR100	Spontaneous Fermentation
$Y_{P/S}$ (g of ethanol/g of glucose consumed)	0.34 ± 0.00	0.34 ± 0.00	0.35 ± 0.01	0.37 ± 0.01	0.37 ± 0.01
Fermentation time (h)	260	280	235	450	352
Glucose consumption time (h)	182	280	235	260	280
Fructose consumption time (h)	260	280	235	450	352
Maximum ethanol concentration (g/L)	86.7 ± 1.5	91.3 ± 0.4	95.3 ± 2.2	97.1 ± 0.5	93.9 ± 0.1
Residual glucose in maximum ethanol concentration (g/L)	1.2 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.6 ± 0.0	1.5 ± 0.1
Residual fructose in maximum ethanol concentration (g/L)	12.0 ± 0.6	10.5 ± 4.0	6.5 ± 3.5	0.6 ± 0.4	1.1 ± 0.5
Maximum glycerol concentration (g/L)	7.1 ± 0.1	6.6 ± 0.4	6.4 ± 0.0	6.9 ± 0.1	5.3 ± 0.0

3.1.2. Chemical Properties and Volatile Compounds of Assyrtiko I and Roditis Wines

Data concerning pH and titratable acidity (TA) of Assyrtiko I wines of the present study are provided in Supplementary Material Table S2. The wine derived from CFB100 was the more acidic (TA = 7.2, pH = 3.0) wine, while the rest of the wines presented similar acidity values (TA = 6.8, pH = 3.0). The data concerning the concentration of the volatile contents of Assyrtiko I wines are provided in Supplementary Materials Figure S2. Statistically important differences were observed in three alcohols, six esters, and three acids. CFB100 wine exhibited higher amounts of 1-hexanol, decanoic acid ethyl ester, and octanoic acid compared to CFB wine (where decanoic acid was not detected). Conversely, CFB wine displayed lower concentrations of isoamyl acetate, octanoic acid ethyl ester, and ethyl hydrogen succinate (with no detectable amounts), as well as hexadecanoic acid and octadecanoic acid, in comparison to CFB100 wine. BLR100 wine similarly exhibited a higher concentration of 1-hexanol compared to the wine produced by its parental strain (BLR). However, it demonstrated lower levels of isoamyl alcohol and tyrosol, along with decreased amounts of isoamyl acetate, decanoic acid ethyl ester, acetic acid 2-phenylethylester, and octanoic acid ethyl ester (with no detectable amounts). Additionally, BLR100 wine displayed reduced quantities of hexadecenoic acid, octadecanoic acid, and octanoic acid (with no detectable amounts) compared to BLR wine. It is worth mentioning that the esters such as amyl acetate, coumaric acid ethyl ester, and ethyl-9-decenoate, as well as several acids, such as hexanoic and decanoic acids, were only detected in the wines derived from the parental strains CFB and BLR.

Data concerning the pH and titratable acidity (TA) values of Roditis I wines in the current study are provided in Supplementary Materials Table S3. In more detail, the wine originating from BLR100 exhibited the highest acidity, with a TA of 6.3 and a pH of 3.5, while

the wine derived from BLR displayed lower acidity levels, with a TA of 5.4 and a pH of 3.5. The data concerning the concentration of volatile content in Roditis I wines is presented in Supplementary Materials Figure S3. Significant differences were observed in one alcohol (1-hexanol), six esters, and two acids. In comparison to CFB wine, CFB100 wine displayed higher amounts of 1-hexanol, isoamyl acetate, and ethyl dodecanoate. Conversely, CFB wine exhibited higher quantities of acetic acid 2-phenylethylester, ethyl hexadecanoate, ethyl octadecenoate, and ethyl hydrogen succinate (with undetectable levels in CFB100 wine), as well as the acids hexadecenoic acid and octadecanoic acid, in contrast to CFB100 wine. On the other hand, BLR100 wine demonstrated higher concentrations of the esters ethyl hydrogen succinate and ethyl octadecenoate compared to BLR wine, which had no detectable ethyl octadecenoate. BLR100 wine also exhibited lower levels of 1-hexanol, the esters acetic acid 2-phenylethylester and ethyl hexadecanoate, and the acids hexadecenoic acid and octadecanoic acid, in comparison to BLR wine.

The fact that some populations, i.e., CFB100 and BLR100, produced low amounts of certain acetate esters, such as isoamyl acetate and acetic acid 2-phenylethylester in Assyrtiko I wines and acetic acid 2-phenylethylester in Roditis I wines, compared to their parental strains' wines, is of great interest. Both BLR100 and CFB100 populations have been proven to be ethanol tolerant [31], suggesting that, most likely, these populations are able to synthesize increased amounts of unsaturated fatty acids to enrich cell membranes with these for ethanol protection [19–25]. Unsaturated fatty acids are known as negative regulators for acetate esters' production by repressing the *ATF1* gene expression [40,41]. Lower amounts of the acetate esters isoamyl acetate and acetic acid 2-phenylethylester have been observed in wines derived from synthetic must supplemented with oleic acid [42]. Lower amounts of isoamyl acetate have also been detected in wines derived from Cabernet Sauvignon must, where a mixture of unsaturated fatty acids was added [43]. In both cases, these lower contents were attributed to the repression of *ATF1* gene expression in *S. cerevisiae*. Nevertheless, acetate esters are highly desirable in wine, owing to their significant contribution to its fruity character [7]. Increased concentrations of isoamyl acetate, acetic acid 2-phenylethylester, and ethyl caproate were achieved in wines from Colombard grape juice, with overexpression of the *ATF1* gene in a commercial *S. cerevisiae* strain [44].

The statistically important differences detected between the volatile compounds of the Assyrtiko I and Roditis I wines derived from the parental strains and the evolved populations indicate the presence of biochemical disparities between them. These differences are in agreement with a previous study [45], where the wines produced from evolved strains obtained through an ALE experiment targeting enhanced ethanol tolerance, exhibited varying concentrations of volatile compounds, compared to wines derived from the parental strains. This observation was made following fermentations conducted on red Grenache and Chardonnay grape musts. Significant alterations in important aroma compounds were also detected between plum wines derived from a parental *S. cerevisiae* strain and three evolved mutants obtained through an experiment which combined ALE with atmospheric and room-temperature plasma (ARTP) and high-throughput screening (HTS), aiming at enhanced tolerance to low-pH environments [46].

Data concerning the pH and titratable acidity of Roditis II wines of the present study are provided in Supplementary Materials Table S4. More acidic was the wine derived from BLR100 (TA = 6.5, pH = 3.2), whereas the wine derived from BLR was the least acidic (5.7, pH = 3.3).

3.2. Vinification with CFB150, BLR200, CFB100Fr, and BLR100Fr Populations

CFB150 and BLR200 are ethanol-tolerant populations derived from the strains *S. cerevisiae* CFB and *S. cerevisiae* BLR, after 150 and 200 generations of evolution, respectively, from the already mentioned two-step ALE experiment [31]. CFB100Fr and BLR100Fr are populations of 100 generations derived from a two-step ALE experiment (alternating synthetic fructose medium with high-ethanol-concentration solution), which aimed at enhanced ethanol tolerance, combined with improved assimilation of fructose [32]. CFB100Fr

presented exceptionally improved fermentation ability compared to its parental strain, CFB, in a fermentation of a synthetic broth with 100 g/L glucose and 100 g/L fructose [32]. In the present study, we assessed the performances of these four evolved populations compared to their parental strains under real vinification conditions in Assyrtiko II grape must.

3.2.1. Fermentative Behaviors of CFB150, BLR200, CFB100Fr, and BLR100Fr Populations in Assyrtiko II Grape Must

The data concerning the glucose and fructose uptake rate and the ethanol and glycerol production of the populations CFB150 and CFB100Fr and their parental strain, *S. cerevisiae* CFB, as well as the spontaneous fermentation in Assyrtiko II grape must, are provided in Supplementary Materials Figure S4. The CFB strain and CFB150 population both completed the fermentation in a short time, i.e., in 140 h, with a concurrent consumption of the must's glucose and fructose (Supplementary Figure S4a–d). Their ethanol yields, maximum ethanol, and glycerol concentrations were also similar (Table 4). On the other hand, the evolved population CFB100Fr presented an entirely different behavior, finalizing the fermentation in 236 h (Supplementary Figure S4c) and assimilating the must's glucose 71 h earlier than fructose (Table 4). Its ethanol yield, maximum ethanol concentration, and maximum glycerol concentration were 0.35 g/g, 97.3 g/L, and 4.2 g/L, respectively (Table 4). It is worth mentioning that the CFB strain and CFB150 and CFB100Fr populations behaved differently in fermentations of synthetic must with 100 g/L glucose and 100 g/L fructose [32]. More precisely, CFB realized a stuck fermentation, and CFB150 completed the fermentation in 495 h, whereas CFB100Fr finalized the fermentation as quickly as in 170 h [32]. These different behaviors are in accordance with a previous study, where an evolved isolate from an experiment with random mutagenesis combined with directed evolution, aiming at the generation of *S. cerevisiae* strains with improved fructophilicity, presented improved fermentative abilities in defined medium with 115 g/L glucose and 115 g/L fructose, but not in Semillon grape juice [47]. These distinctions are consistent with expectations, as must represents a natural grape juice with a multifaceted chemical composition. In addition to fermentable sugars, such as glucose and fructose, must encompasses non-fermentable carbohydrates, pectic substances, organic acids, nitrogenous compounds, aroma compounds, tannins, pigments, vitamins, enzymes, and phenolic compounds [48]. This variety of components collectively exerts a profound influence on yeast physiology throughout the fermentation process. In contrast, the synthetic must was prepared exclusively to simulate the composition of natural must in terms of glucose and fructose content. In addition, it has been observed that a synthetic must which contained the majority of natural must's substances was metabolized differently than natural grape must by the yeast cells [49]. The remarkable fermentative performance observed in both the CFB strain and CFB150 population during vinification, contrasted with their challenges in assimilating synthetic glucose and fructose broth, may be ascribed to the presence of unsaturated fatty acids and sterols inherent in the natural composition of must. These components are known to enhance the integrity of yeast cell membranes [50,51]. In contrast, the CFB100Fr population completed wine fermentation 66 h after the completion of synthetic broth fermentation. This delay might be attributed to the adverse effects of vinification's stressful conditions, including high osmotic pressure, the presence of organic acids and phenolic compounds in the must, and the addition of sulfur compounds [52].

The data concerning the glucose and fructose uptake rate and the ethanol and glycerol production of the populations BLR200 and BLR100Fr and their parental strain, *S. cerevisiae* BLR, as well as the spontaneous fermentation in Assyrtiko II grape must, are provided in Supplementary Materials Figure S5. The BLR strain completed fermentation earlier than the evolved populations accomplishing this in 165 h (Supplementary Figure S5d). Specifically, glucose consumption preceded fructose consumption by 25 h (Supplementary Figure S5a). BLR100Fr completed the fermentation in 236 h, while the whole must's glucose had already been consumed in 186 h (Supplementary Figure S5c). Finally, BLR200 completed the fermentation in 380 h, having consumed the must's glucose 144 h earlier than

fructose (Supplementary Figure S5b). It is noteworthy that the disparity in time between glucose and fructose consumption was diminished in BLR100Fr (evolved on fructose) compared to BLR200 (evolved on glucose) (50 h difference and 144 h difference, respectively) (Supplementary Figure S5d). The ethanol yields were 0.38 g/g for BLR, 0.36 g/g for BLR100Fr, and 0.37 g/g for BLR200 (Table 4). The values of the maximum ethanol concentrations were 103.4 g/L for BLR, 98.7 g/L for BLR100Fr, and 106.2 g/L for BLR200, and the maximum glycerol concentrations had the values of 5.2 g/L, 4.1 g/L, and 4.6 g/L, respectively (Table 4). The BLR strain and the evolved populations behaved differently in fermentations of synthetic must with 100 g/L glucose and 100 g/L fructose [32]. In particular, the BLR strain assimilated both sugars in 354 h, BLR200 in 312 h, and BLR100Fr in 436 h [32]; thus, the BLR strain and BLR100Fr population showed a better fermentative ability in natural grape must, whereas the BLR200 population fermented the synthetic broth more rapidly.

Table 4. Fermentative abilities of *S. cerevisiae* parental strains and their evolved populations, CFB150, CFB100Fr, BLR200, and BLR100Fr, during vinification in Assyrtiko II grape must. Spontaneous fermentation is also shown.

	CFB	CFB150	CFB100Fr	BLR	BLR200	BLR100Fr	Spontaneous Fermentation
$Y_{P/S}$ (g of ethanol/g of glucose consumed)	0.37 ± 0.01	0.36 ± 0.00	0.35 ± 0.01	0.38 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.43 ± 0.00
Fermentation time (h)	140	140	236	165	380	236	455
Glucose consumption time (h)	140	140	165	140	236	186	332
Fructose consumption time (h)	140	140	236	165	380	236	455
Maximum ethanol concentration (g/L)	101.9 ± 1.0	103.0 ± 0.1	97.3 ± 7.6	102.1 ± 11.8	106.2 ± 7.6	98.7 ± 2.9	123.6 ± 5.6
Residual glucose in maximum ethanol concentration (g/L)	1.2 ± 1.3	0.5 ± 0.4	0	0.8 ± 0.1	0.2 ± 0.3	0.4 ± 0.6	0
Residual fructose in maximum ethanol concentration (g/L)	2.1 ± 4.2	0	9.7 ± 0.5	0	0.2 ± 0.3	0	4.5 ± 0.7
Maximum glycerol concentration (g/L)	5.8 ± 0.1	5.9 ± 0.0	4.2 ± 0.7	5.2 ± 1.3	4.6 ± 0.5	4.1 ± 0.3	3.5 ± 0.4

The spontaneous fermentation, as illustrated in Supplementary Figures S4e and S5e, exhibited a prolonged duration of 455 h in comparison to the other fermentation processes. Despite its sluggishness, it yielded a higher ethanol yield of 0.43 g/g, reaching a maximum ethanol concentration of 123.6 g/L. Additionally, it displayed a notably lower maximum glycerol concentration of 3.5 g/L, as shown in Table 4.

3.2.2. Chemical Composition of Assyrtiko II Wines

Data concerning the pH and titratable acidity of Assyrtiko II wines of the present study are provided in Supplementary Materials Table S5. Higher acidity was observed in the wine from BLR200 (TA = 5.7, pH = 3.7), while the wine derived from BLR presented lower acidity values (TA = 4.3, pH = 3.7). The concentrations of the volatile content of Assyrtiko II wines are shown in Figure 3. Significant differences were observed in seven alcohols, five esters, and five acids among the analyzed samples. CFB wine exhibited

elevated concentrations of phenyl-2-ethanol, 3-methylthio-1-propanol, isoamyl acetate, and phenylethyl acetate, while displaying reduced contents of hexanoic acid ethyl ester, octanoic acid ethyl ester, hexanoic acid, octanoic acid, and decanoic acid compared to wines derived from evolved populations CFB150 and CFB100Fr. Isobutanol, 1-hexanol, butyrolactone, and isobutyric acid concentrations were similar in wines from the CFB strain and CFB150 population. CFB150 wine contained higher concentrations of 2,3-butanediol, isoamyl acetate, hexanoic acid ethyl ester, phenylethyl acetate, isobutyric acid, butyric acid, hexanoic acid, and decanoic acid compared to CFB100Fr wine, whereas the latter exhibited elevated levels of isobutanol, isoamyl alcohol, 1-hexanol, butyrolactone, phenyl-2-ethanol, 3-methylthio-1-propanol, and ethyl-L-lactate compared to CFB150 wine. Regarding BLR, BLR200, and BLR100Fr wines, isoamyl alcohol, butyric acid, and hexanoic acid concentrations were similar among them. The wine derived from the BLR strain exhibited higher concentrations of isobutanol, 2,3-butanediol, 3-methylthio-1-propanol, isoamyl acetate, hexanoic acid ethyl ester, phenylethyl acetate, and butyric acid, while displaying lower concentrations of phenyl-2-ethanol and decanoic acid compared to wines from the evolved populations BLR200 and BLR100Fr. BLR200 wine contained higher levels of phenyl-2-ethanol, 3-methylthio-1-propanol, isoamyl acetate, and octanoic acid ethyl ester, as well as decanoic acid, compared to BLR100Fr wine. Conversely, BLR100Fr wine exhibited elevated concentrations of 1-hexanol, 2,3-butanediol, butyrolactone, hexanoic acid ethyl ester, ethyl L-lactate, and octanoic acid in comparison to BLR200 wine (Figure 3). It is worth noting that wines from all four evolved populations demonstrated decreased levels of the acetate esters isoamyl acetate and phenylethyl acetate compared to wines from the parental strains. These populations underwent evolution under selective pressure from high ethanol concentrations [31,32], potentially leading to enhanced cell membranes with increased quantities of unsaturated fatty acids [19–25]. As previously noted, unsaturated fatty acids have been shown to downregulate the expression of the *ATF1* gene, which plays a pivotal role in the production of acetate esters [40,41]. Reduced levels of acetate esters, such as isoamyl acetate and phenylethyl acetate, have been observed in wines derived from synthetic must supplemented with oleic acid and fermented by *S. cerevisiae* [42].

The significant differences detected in the volatile compounds between wines from parental strains and evolved populations indicate biochemical distinctions between them, as well as among populations generated through various ALE experiments [31,32]. This underscores the effectiveness of the ALE technique in generating novel yeast strains tailored for winemaking purposes.

3.2.3. Sensory Analysis of Assyrtiko II Wines

The results of the sensory evaluation of the Assyrtiko II wines derived from the populations CFB150, BLR200, CFB100Fr, and BLR100Fr and their parental strains, as well as from the spontaneous fermentation in Assyrtiko II grape must, are shown in Figure 4.

Significant differences in color intensity were observed among the various wines, with the wine resulting from spontaneous fermentation exhibiting a closer proximity to a green tone, while wines derived from the BLR strain and BLR100Fr population tended toward a brown tone. The wine from the CFB150 population displayed a deeper brown hue compared to wines from the CFB strain and the CFB100Fr population. Conversely, the wine from the BLR200 population exhibited a greener hue in comparison to wines from the BLR strain and BLR100Fr population.

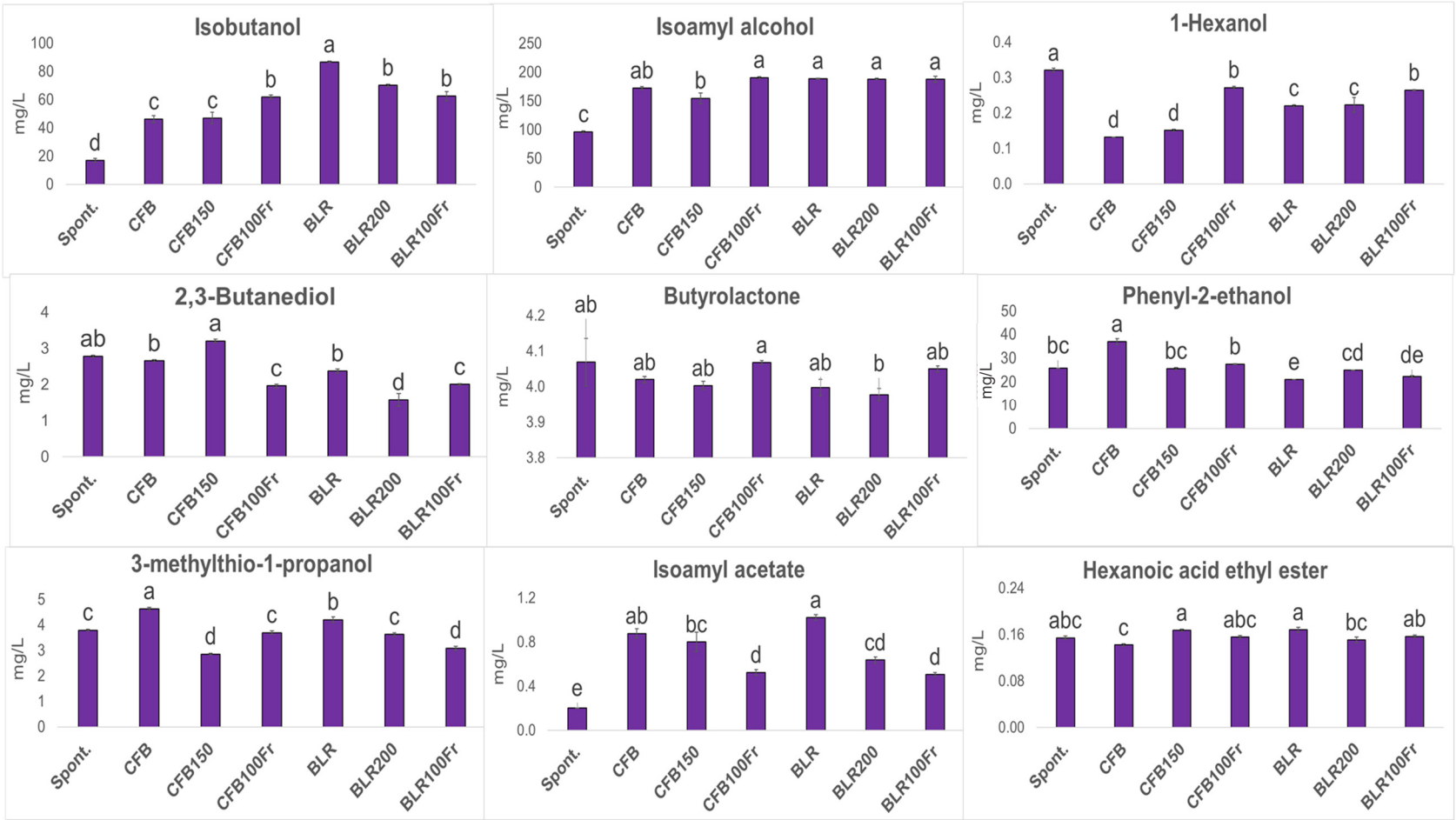

Figure 3. *Cont.*

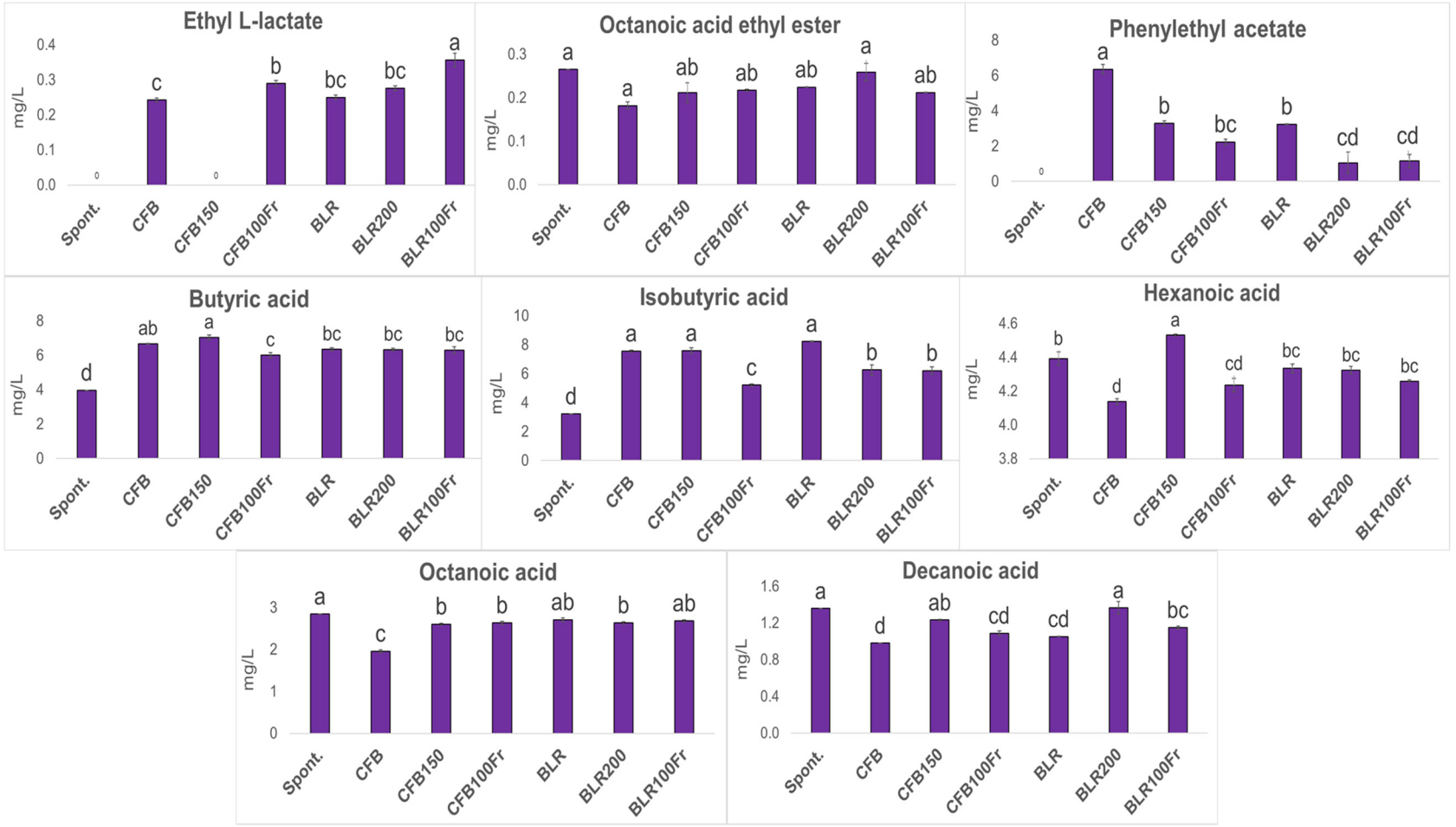

Figure 3. Percentage content of volatile compounds in Assyrtiko II wines of the present study. Different letters in each column evince significant statistical differences ($p < 0.05$) between different volatile compounds.

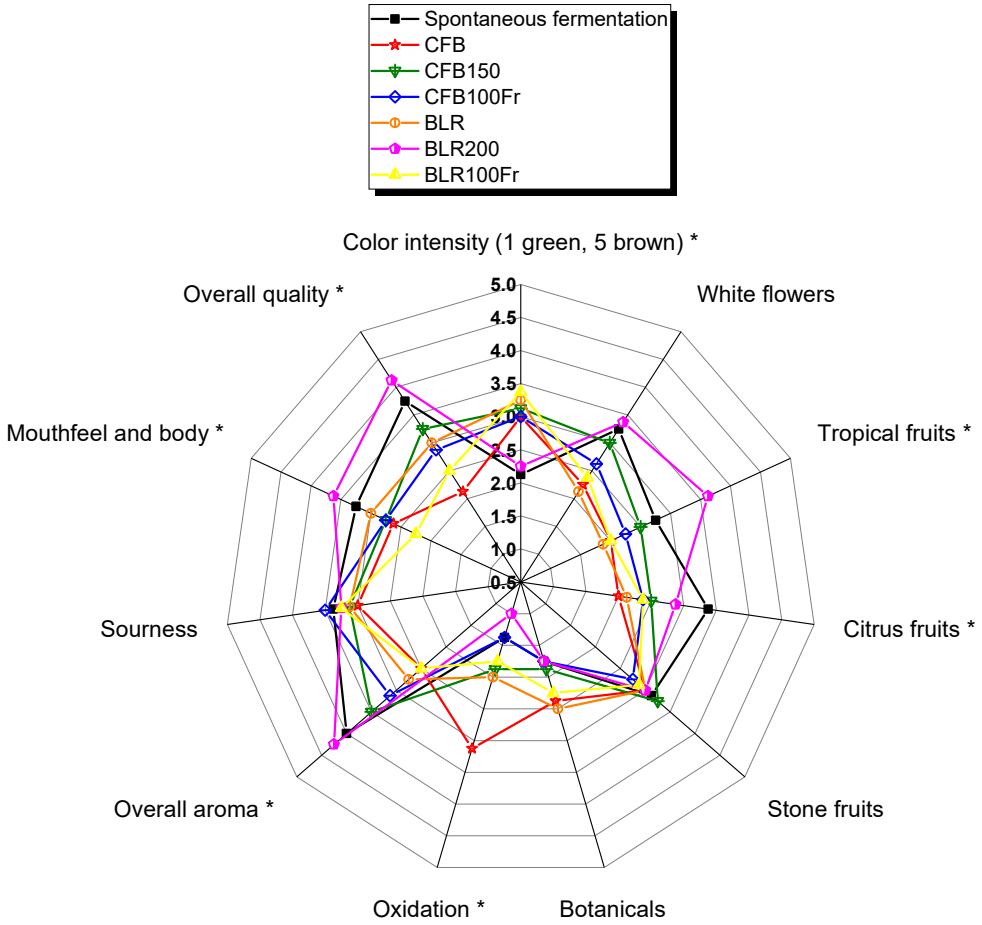

Figure 4. Organoleptic profile of wines produced by parental strains and evolved population from Assyrtiko II grape must. * Samples differ significantly ($p < 0.05$).

In assessing the olfactory characteristics of the wines, significant differences were observed in the perception of tropical fruits and citrus fruits, as well as in oxidation nuances. The wine derived from the BLR200 population exhibited the most pronounced tropical fruit aroma, followed by the wine resulting from spontaneous fermentation. This observation is substantiated by the elevated concentrations of octanoic acid ethyl ester in these wines, known for imparting aromas of pineapple and fresh flowers [53]. Conversely, wines from the CFB strain displayed significantly lower intensity in tropical fruit aroma compared to those from the evolved populations derived from it (CFB150 and CFB100Fr). The spontaneous-fermentation wine exhibited a more pronounced citrus aroma, potentially attributed to its higher concentration of several acids, such as hexanoic, octanoic, and decanoic acid, despite their typical characterization as cheese-like odors in the literature [54]. In contrast, wines derived from the CFB strain displayed significantly lower citrus aroma scores compared to other wines, including those from the evolved populations, CFB150 and CFB100Fr. Notably, the BLR strain exhibited similar citrus aroma scores to wines from its two evolved populations (BLR200 and BLR100Fr). Furthermore, the wine derived from the CFB strain indicated significantly higher oxidation levels compared to other wines. Significant differences were also observed in the overall aroma scores among the wines. The wine from the BLR200 population scored higher than other wines, including those from the BLR strain and BLR100Fr population. This could be attributed to its elevated content of esters with pleasant aromas, such as hexanoic acid ethyl ester (strawberry aroma), ethyl L-lactate (raspberry aroma), and octanoic acid ethyl ester (pineapple and pear aroma), as well as its lower content of 3-methylthio-1-propanol, known for its unpleasant cauliflower aroma [6,53,55]. Additionally, the overproduction of esters has been observed in

an evolved strain with enhanced consumption capacity for gluconate, obtained through an ALE strategy aiming to increase the flux of *S. cerevisiae* cells toward the pentose phosphate pathway [56,57]. Moreover, wines derived from the CFB150 and CFB100Fr populations scored significantly higher in overall aroma compared to the wine derived from the CFB strain.

Regarding the wines' flavor, significant differences were observed in mouthfeel and body, while all samples exhibited similar sourness. The wine derived from the BLR200 population scored significantly higher than other wines in terms of mouthfeel and body, including even those from the BLR strain and BLR100Fr population. This superior score is corroborated by its elevated concentration of butyric acid, known for imparting aromas of butter and cheese [58,59]. In contrast, wines from the CFB strain, CFB150, and CFB100Fr populations exhibited similar characteristics in this regard.

Finally, the wines exhibited significant variations in overall quality. The wine derived from the BLR200 population received higher scores than other wines, possibly attributable to its superior performance in overall aroma and its minimal oxidation levels. Additionally, its color intensity, leaning closer to "green" than "brown," may have contributed to its higher quality assessment. Following closely in quality, as determined by the panelists, was the wine resulting from spontaneous fermentation, followed by the wine derived from the CFB150 population. Wines from the CFB100Fr population and BLR strain were rated with similar, average quality, followed by the wine from the BLR100Fr population. The wine from the CFB strain received the lowest quality score, probably due to its elevated concentration of 3-methylthio-1-propanol, characterized by a cauliflower aroma, and its higher oxidation levels, despite its richness in phenyl-2-ethanol and phenylethyl acetate, offering aromas of roses, and isoamyl acetate, providing a banana aroma [54,55].

4. Conclusions

In this study, six distinct evolved *S. cerevisiae* populations, generated through two previous ALE experiments [31,32], were evaluated in comparison with their respective parental strains. Tailored wine fermentations were performed utilizing Assyrtiko and Roditis grape musts, originating from indigenous Greek grape varieties. Across all experimental conditions, the parental strains, CFB and BLR, demonstrated superior fermentative capacities compared to the evolved populations. The evolved populations CFB100 and BLR100 exhibited a slower assimilation rate of fructose from the grape musts compared to glucose, potentially attributed to their adaptation to glucose as the primary carbon source during the evolutionary process. Discrepancies were noted between the wine fermentations conducted in this study and those in synthetic grape musts previously examined. The Gas Chromatography–Mass Spectrometry (GC-MS) analysis revealed statistically significant variations in the volatile-compound profiles between wines derived from the parental strains and those from evolved populations, as well as among wines from populations generated through different ALE experiments. Sensory assessments of Assyrtiko II wines are in agreement with the results obtained from the GC-MS analysis. The outcomes of this investigation highlight the efficacy of ALE as a potent technique in the winemaking industry for the development of improved, non-genetically modified (non-GMO) wine yeasts, thus emphasizing its potential for advancing oenological practices.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/beverages10040102/s1, Table S1: Properties of grape musts utilized in the present study; Table S2: pH and titratable acidity of Assyrtiko I wines of the present study; Table S3: pH and titratable acidity of Roditis I wines of the present study; Table S4: pH and titratable acidity of Roditis II wines of the present study; Table S5: pH and titratable acidity of Assyrtiko II wines of the present study; Figure S1: Glucose and fructose consumption (g/L) and ethanol and glycerol production (g/L) of parental strains *S. cerevisiae* CFB (a) and *S. cerevisiae* BLR (d) and evolved populations CFB100 (b) and BLR100 (e) in Roditis II grape must. Comparisons of glucose and fructose uptake rates between parental strains and evolved populations are also presented (c,f). Spontaneous fermentation is also shown (g); Figure S2: Percentage content of volatile compounds in

Assyrtiko I wines of the present study. Different letters in each column evince significant statistical differences ($p < 0.05$) between different volatile compounds; Figure S3: Percentage content of volatile compounds in Roditis I wines of the present study. Different letters in each column evince significant statistical differences ($p < 0.05$) between different volatile compounds; Figure S4: Glucose and fructose consumption (g/L) and ethanol and glycerol production (g/L) of parental strain *S. cerevisiae* CFB (a) and evolved populations CFB150 (b) and CFB100Fr (c) in Assyrtiko II grape must. Comparison of glucose and fructose uptake rates between parental strains and evolved populations is also presented (d). Spontaneous fermentation is also shown (e); Figure S5: Glucose and fructose consumption (g/L) and ethanol and glycerol production (g/L) of parental strain *S. cerevisiae* BLR (a) and evolved populations BLR200 (b) and BLR100Fr (c) in Assyrtiko II grape must. Comparison of glucose and fructose uptake rates between parental strains and evolved populations is also presented (d). Spontaneous fermentation is also shown (e).

Author Contributions: Conceptualization, M.M. and G.A.; methodology, M.M., S.C. and S.K.; software, M.M. and S.C.; validation, S.K., S.P. and G.A.; formal analysis, M.M.; investigation, M.M.; resources, S.K., S.P. and G.A.; data curation, M.M.; writing—original draft preparation, M.M.; writing—review and editing, S.K., S.P., G.A. and M.M.; visualization, M.M., S.K. and G.A.; supervision, S.K., S.P. and G.A.; project administration, M.M., S.C. and S.K.; funding acquisition, S.K. and S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project entitled "Exploitation of new natural microbial flora from Greek origin amenable for the production of high-quality wines" (Acronym: Oenovation; Code: T1EΔK-04747; Action "Investigate—Create—Innovate" Intervention II; Call: "COMPETITIVENESS, ENTREPRENEURSHIP AND INNOVATION" (ΕΥΔ ΕΠΑνΕΚ).

Data Availability Statement: Dataset available upon request from the authors.

Acknowledgments: The authors would like to thank Evangelia A. Tsapou (University of West Attica) for the implementation of GC-MS analyses.

Conflicts of Interest: The authors declare that there are not any known conflicts of interest.

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