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Selection of *Saccharomyces cerevisiae* Starter Strain for Merwah Wine

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Abstract: In order to select *Saccharomyces cerevisiae* starter strains for "Merwah" wine production, three strains (M.6.16, M.10.16, and M.4.17) previously isolated from "Merwah" must and characterized at the lab scale were tested in pilot-scale fermentation in a Lebanese winery during the 2019 vintage. The three inoculated musts were compared to that obtained with a spontaneous fermentation. During the fermentations, must samples were taken to evaluate the dominance of the inoculated strains, and at the end of fermentation, the obtained wines were subjected to chemical and sensorial characterization. Molecular monitoring by interdelta analysis revealed that only M.4.17 was able to complete the fermentation and dominate over the wild yeasts. Based on the analysis of principal technological parameters (i.e., residual sugar, fermentative vigor, sulfur production, and acetic acid) and sensorial analysis of the wines obtained, M.4.17 was selected as an adequate starter for the production of typical "Merwah" wine.

Keywords: indigenous starter; inoculated fermentation; dominant strain; aromatic profile

1. Introduction

In modern technology, winemakers have realized the importance of preserving biodiversity by changing from conventional to organic or biodynamic wine production, which is based on the concepts of sustainable agriculture and preservation of the environment.

Currently, in line with consumer preferences, new trends have emerged in wine fermentation and wine technology. Nowadays, consumers are looking to wine that expresses their terroir, with a good balance in terms of acidity and mouth feels and which can be distinguished from each other [1,2]. Some winemakers argue that authentic expression of terroir and vintage can only be achieved through fermentation with indigenous yeasts. Other winemakers prefer the greater security and controlled variability of specialized strains [3].

If each terroir factor is studied separately, studies remain highly descriptive and fail to explain why wine shows such extraordinary sensory diversity [4].

Unfortunately, in the last few years, climate change phenomena associated to global warming have had both direct and indirect effects on agricultural productivity, which favors a greater use of pesticides that affects the microbial diversity and therefore can have an influence on spontaneous fermentation [5,6].

Therefore, the use of indigenous selected starters represents a useful tool to control alcoholic grape must fermentation, safeguarding the typical sensory characteristics of wine produced from a specific terroir [7].

For this reason, several wineries have developed programs of isolation, selection, and inoculation of starter cultures of *Saccharomyces cerevisiae* for controlled wine fermentations [8]. The selection of indigenous strains with specific phenotypes appears a valuable tool for the differentiation, diversification, and quality improvement of regional wines [9].

One of the major advantages of inoculated fermentation with indigenous yeast lies in the timing and duration of fermentation. Inoculation of fermentation with a starter culture is intended to establish a high population of a selected strain of *S. cerevisiae* from the beginning of fermentation to ensure its dominance [10]. This is one of the most important features determining the starter ability to dominate the process and persist over other yeast strains of the natural microbiota [11]. In addition, inoculated wine fermentation by selected indigenous *S. cerevisiae* strains has been introduced in many wineries for several reasons as it (i) avoids sluggish and stuck fermentations, (ii) provides distinctive characteristics to the wine, (iii) enhances the organoleptic and sensory properties of typical regional wines, and (iv) helps to preserve the native yeast strains that are better adapted to the environment of the viticulture region and to the winemaking process [7,12–14].

The existence of a correlation between the origin of a specific indigenous strain and the characteristics of inoculated wines obtained from strains isolated from different wine regions was reported [7]. In this way, it might be possible to associate specific indigenous strains with a specific region, or with a terroir.

It is well recognized that wines made with indigenous *S. cerevisiae* strains are perceived to be more complex by showing a greater diversity of flavors, where these yeasts produce variable amounts of fermentative by-products, with desirable or undesirable effects on the wine bouquet [15].

Based on relevant features, such as high fermentation vigor, low production of volatile acidity and H_2S, and low residual sugar content at the end of alcoholic fermentation, three indigenous *S. cerevisiae* starter strains, namely M.6.16, M.10.16, and M.4.17, were previously selected from indigenous populations of "Merwah" wine during spontaneous fermentation [16]. These strains were used in this study to inoculate "Merwah" must/wine of the 2019 vintage at the winery Château Bybline (Lebanon). Merwah grape is a light-skinned grape variety that is believed to be native to Lebanon. This variety is usually cultivated in mountain regions at altitudes of 600 m and above and it is characterized by its resistance to frost, and to *Oïdium* and *Mildiou* attack [16,17]. Merwah grapes typically produce rich wines with light citrus and nut flavors. They can at times lack the acidity required to balance their palate weight.

The dominance of the inoculated strains and their influence on the sensorial and aromatic qualities of the wines were evaluated in order to test the suitability of these indigenous strains to be used as starters for "Merwah" wine production at the cellar scale.

2. Materials and Methods

To determine the effects of the previously selected *S. cerevisiae* strains M.6.16, M.10.16, and M.4.17 [16] on wine characteristics, pilot-scale fermentations were carried out on "Merwah" must in the winery during the 2019 vintage (Table 1). The grapes of "Merwah" were harvested from Bekaatet Achout, Mount Lebanon region (1400 m a.s.l.), used for cold maceration (for better extraction of color and flavor from grape skins) at 4 °C for 2 days, and then crushed and rested for 24 h with sulfite (40 mg/L). The temperature of the alcoholic fermentation of "Merwah" grape must (sugar 224.2 g/L, pH 3.45) was stable during all of the pilot fermentation at 19 °C.

The wines obtained using these strains were compared to those fermented spontaneously with wild microflora. At the end of the alcoholic fermentation, the wines were evaluated based on the following parameters: Ethanol, residual sugars, free and total SO_2, total acidity, tartaric, malic, citric, lactic, and acetic acids.

Table 1. *Saccharomyces cerevisiae* strains used to inoculate Merwah must of the 2019 season in pilot fermentation.

Strain Name	Experimental Wine
M.6.16	A *
M.10.16	B *
M.4.17	C *
Control	M **

* Inoculated fermentation; ** uninoculated/spontaneous fermentation.

2.1. Culture Preparation and Inoculation

Prior to inoculation, one colony of each *S. cerevisiae* strain (M.6.16, M.10.16, and M.4.17) was aseptically transferred to separate flasks containing 100 mL of liquid YEPD (1% Yeast Extract, 2% Peptone, 2% Dextrose; Sigma, Darmstadt, Germany), which were incubated in an incubator shaker at 250 rpm (Multi Stack, shaking; LabTech, Sorisole (BG), Italy) at 25 °C.

Approximately 2.5×10^6 cells/mL were inoculated from the YEPD medium (Sigma, Darmstadt, Germany) into 2 L of "Merwah" must of the season 2019 and incubated at 25 °C for 48 h. Each pre-culture was prepared in duplicate and inoculated into 20 L of Merwah must fermented in a glass tank following the diagram reported in Table 1.

2.2. Microbiological Analysis

Eighteen samples taken from the initial, middle, and end of the alcoholic fermentation of the three tested wines, and were inoculated in differential Wallerstein Laboratory Nutrient media (WL nutrient media) (Sigma, Darmstadt, Germany) and incubated at 25 °C for 48 h. Based on the colony color and morphology on WL nutrient media and cell shape and dimension as determined by microscopical observations, three colonies presumably belonging to the *Saccharomyces* genus were isolated from each sample, cultured, and plated on YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% Bacto-agar) (Sigma, Darmstadt, Germany). The selected colonies were purified and maintained in slants of YPD at 4 °C.

2.2.1. DNA Extraction

The yeast colonies isolated as indicated above were cultivated overnight in YPD liquid at 25 ∘C. Then, 1.5 mL of the cell biomass were centrifuged at 13,000× *g* for 5 s, and the supernatants eliminated. The pellets were resuspended in 200 µL of extraction mix: (2% Triton 100×, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl, 1 mM Na2EDTA, phenol: chloroform: isoamyl alcohol (25:24:1)) with 0.3 g 212–300-µm-diameter glass beads (Sigma-Aldrich, St Louis, MO, USA). The samples were vortexed for 2 min and then centrifuged at 13,000× *g* for 5 min. The DNA was precipitated from the supernatants by adding three vol. 100% ethanol and 0.1 vol. 3 M NaOH, with the samples cooled to −80 °C for 20 min. The samples were then centrifuged at 13,000× *g* for 15 min at 5 ∘C. The pellets were washed with 70% ethanol and centrifuged at 13,000× *g* for 15 min, and then vacuum dried. The DNA extracted was dried and suspended in 50 µL of TE buffer (0.1 M Tris, 0.1 M EDTA, pH 8.0) and stored at −20 °C [18]. The efficiency of this DNA extraction procedure, and its purity and concentration, were measured using a spectrophotometer (NanoDrop; BMG Labtech, Offenburg, Germany) [19].

2.2.2. Strain Identification by Interdelta PCR

In order to identify the dominance of the inoculated strains during the fermentation, each colony was subjected to interdelta Polymerase chain reaction (PCR)analysis according to [20]. Genomic DNA (50 ng/µL) was added to the PCR reaction mixtures (25 µL) that contained: Taq buffer (+1.5 mM Mg^{2+}) (1×), 25 mM MgCl2, 0.2 mM primer 1 δ2 (50 -GTGGATTTTTATTCCAAC-30), 0.2 mM primer 2 δ12 (50 -TCAACAATGGAATCCCAAC-30), 0.2 mM dNTP, and 1 U Taq polymerase (Trans Gene Biotech,

Beijing, China). Amplification reactions were performed on a PCR machine (Thermal Cycler T-100; BioRad, Milan, Italy) using the following program: Initial denaturation 95 °C for 4 min, 35 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, and final extension at 72 °C for 10 min. The products of the PCR reactions were analyzed by electrophoresis on 2% agarose gels, submitted to 90 V for 2.5 h, and then stained with SYBR safe and visualized under a UV transilluminator (Chemi Doc XRS imaging system; BioRad, Milan, Italy). All of the visible bands were assigned a number based on their position in relation to the DNA ladder (100pb plus opti-DNA marker G-193 abm, Canada).

2.3. Kinetic of Pilot Fermentations

Fermentations were monitored by measuring the decrease of density by a hydrometer (Brewer's Elite, Valley Cottage, NY, USA) and the residual sugar by UV-visible spectrophotometry, with the dinitrosalicylic acid (DNS) method (expressed as g/L) [21] throughout the pilot fermentations.

2.4. Chemical Analysis

The experimental must/wines were evaluated at the end of alcoholic fermentation by determining several chemical parameters, such as: Ethanol production, total acidity, volatile acidity, pH, free and total SO_2, glycerol, organic acids (tartaric, malic, succinic, and citric acids), and sugars (glucose and fructose) using wine scan (Foss, Hilleroed, Denmark).

2.5. Sensorial Analysis

In order to test the consumer preference and acceptability of the tested wines, a hedonic scaling method [22] was conducted with a panel of 20 persons (10 enologists and wine expert and 10 wine tasters). According to [23], a 5-point hedonic scale was used by giving a score to the studied wines as indicated in Table 2.

Table 2. Five-point hedonic scores given for the studied wines.

Dislike very much	Dislike somewhat	Neither like nor dislike	Like somewhat	Like very much

In addition to the preference test, a qualitative analysis was also conducted to evaluate the studied wines, by giving a score to the relatively set system (Table 3) [24].

Table 3. Principle aspects of the wine tested with a given score scale (from 1 to 5) during the sensorial analysis.

Parameters	1	2	3	4	5
Fines and complexity	Ordinary	Simple	Fine	Elegant	Refined
Aromatic power	very low	Low	Medium	High	Very high
Oxidation odor	very low	Low	Medium	High	Very high
Acidity	very low	Low	Medium	High	Very high
Bitterness	very low	Low	Medium	High	Very high
Balance	very low	Low	Medium	High	Very high

At the end, the quality perception of the three inoculated wines was compared to the spontaneous wine tested.

2.6. Statistical Analysis of Chemical and Sensorial Data

All analytical measurements were performed in duplicate. The values of the parameters are expressed as the mean ± standard deviation.

An analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test were used for mean separation in the chemical analysis, with a significance level of 95% ($p < 0.05$). These statistical analyses, together with principle component analysis (PCA), were conducted using XLSTAT 2019.4.2.

In order to evaluate which category (like or dislike) was "significant" for each wine, a binomial test was applied to the results of the preference test. In this case, the following link: (https://www.socscistatistics.com/tests/binomial/default2.aspx) was used to calculate the *p*-value for the categories of each wine. The data obtained from the sensorial analysis were scored depending on the scale for each parameter and their means were subjected to Radar analysis in order to compare the different tested parameters for each wine. In addition, the results of the sensorial analysis were generated using the ANOVA test (95% confidence interval) through XLSTAT 2019.4.2 to evaluate the significant difference for each parameter in each wine.

Additionally, the correlations between chemical variables and the sensorial parameters were evaluated using the Pearson correlation coefficient through XLSTAT 2019.4.2 [25].

3. Results

3.1. Microbiological Analysis

A dominant yeast colony was observed during the fermentation as early as 3 days. As observed visually on the microscope and on WL medium, the dominant colony in all the samples at all fermentation stages showed a white cream color, similar to that reported for *Saccharomyces cerevisiae* [26].

The interdelta PCR profiles of colonies isolated at the end of the fermentation in each wine were compared to those of the inoculated strains. Only the isolates from wine C presented the same genetic pattern of the inoculated strain (M.4.17) (data not shown).

3.2. Kinetic of Pilot Fermentation

Based on the fermentation results observed in Figures 1 and 2, the wine that fermented spontaneously (M) showed a slower rate of fermentation in the first days respective to wines inoculated with selected *S. cerevisiae* strains. The fermentation lasted between 22 and 24 days, with low residual sugar (<3 g/L) at the end of the alcoholic fermentation in all the tested wines.

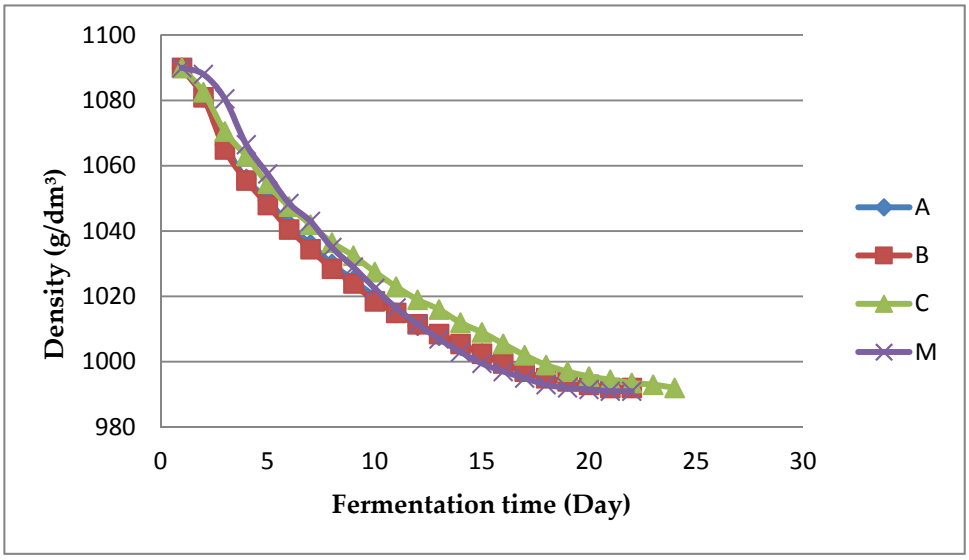

Figure 1. Density evaluation of four experimental Merwah wines during alcoholic fermentation at the winery Château Bybline. A: wine inoculated by the strain M.6.16; B: wine inoculated by the strain M.10.16; C: wine inoculated by the strain M.4.17; M: wine fermented spontaneously (control).

3.3. Technological and Chemical Analysis

The ANOVA analysis showed not-significant differences ($p > 0.05$) in the levels of residual sugars, pH, ethanol, volatile acidity, malic acid, tartaric acid, citric acid, CO_2, glycerol, and free and total sulfur dioxide (SO_2) in the studied wines (A, B, C, and M) (Table S1).

Figure 3 shows the PCA biplot for the first two principal components obtained from 13 technological parameters of the four wines (A, B, C, and M), which explain 74.08% of the total variance. The first component (F1) is positively related to ethanol production, free SO_2, total acidity, volatile acidity, pH, malic acid, reducing sugar, citric acid, and tartaric acid. The second component (F2) is positively related to total SO_2, glucose, fructose, glycerol, and CO_2.

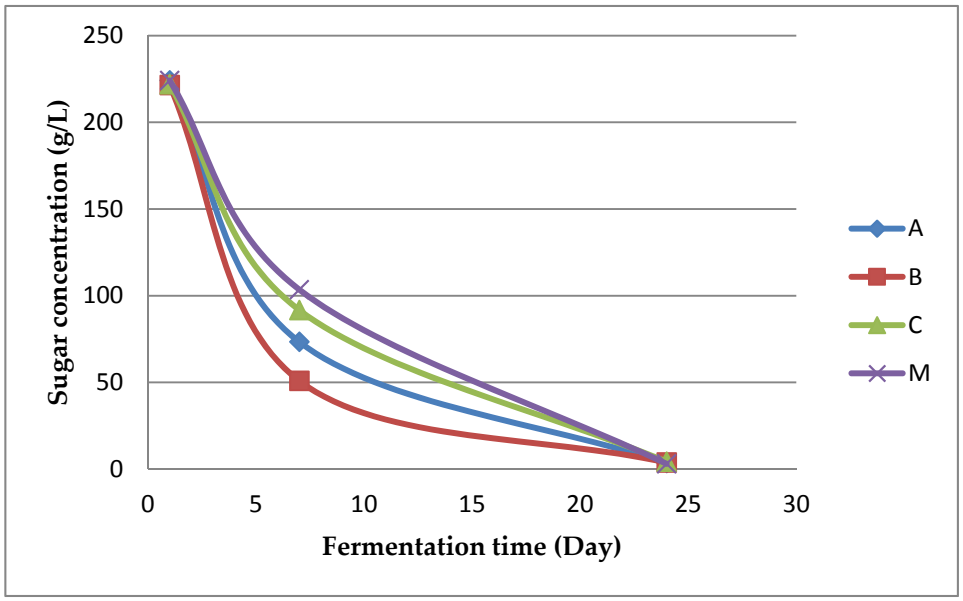

Figure 2. Sugar consumption (g/L) during pilot fermentation in the winery. A: wine inoculated by the strain M.6.16; B: wine inoculated by the strain M.10.16; C: wine inoculated by the strain M.4.17; M: wine fermented spontaneously (control).

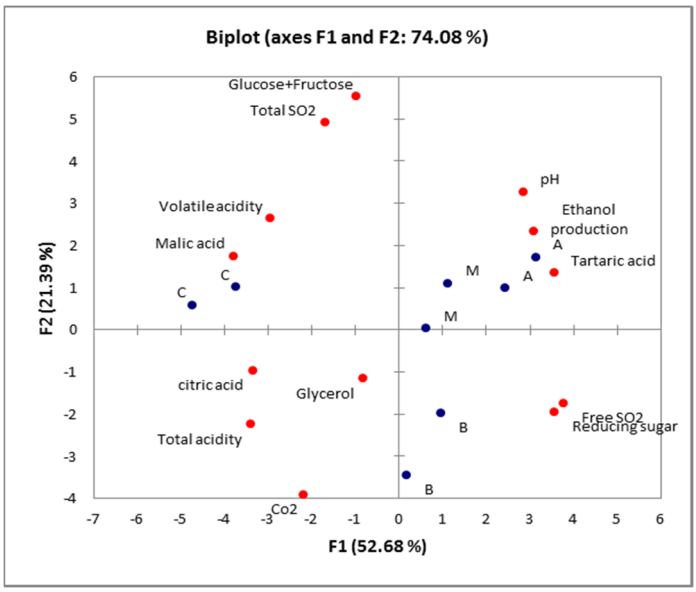

Figure 3. Biplot of two first principal components F1 (52.68%) and F2 (21.39%) obtained from the main technological parameters of three inoculated wines (A, B, and C) and one spontaneous wine (M). The bleu points represent the position of the tested wines in duplicates, with respect to the 13 technological parameters indicated in red points.

The PCA divided the wines into two main groups (Figure 3), where wine C shows more volatile acidity (0.4 g/L) with respect to the others (0.3 g/L). Particularly, in all the tested wines, the levels of acetic acid were below the sensorial threshold (<0.5 g/L), which is an important criterion for the

selection of a wine strain. Figure 3 allows identification of the characteristics of each tested wine in order to choose the most appropriate inoculated strain.

3.4. Sensorial Analysis

A panel of 20 persons (comprising 10 experts and 10 wine consumers with no special training) were used to test the studied wines at the sensorial level. This small panel hedonic method [27] is suitable for generating quality scores for consumer guidance in large-scale wine surveys [28].

A binomial test was applied to the hedonic results (Table 4) to evaluate the significant category for each wine. Wine C was significantly preferred by the judges ($p < 0.05$), while the analysis of the other wines did not reveal any statistical significance.

Table 4. Number of persons/20 that 'liked' and/or 'disliked' the wines with respect to the hedonic scale.

	Categories Respecting to Hedonic Scale				
Wines	**Dislike Very Much**	**Dislike Somewhat**	**Neither Like nor Dislike**	**Like Somewhat**	**Like Very Much**
A	1	4	6	4	5
B	6	5	3	2	4
C	0 *	4	5	3	8 *
M	3	2	5	3	7

* Significant difference in the wine C for both categories "dislike very much" and "like very much" (p-value = 0.0252).

According to the results of the preference test, wine C was "very much liked" by the majority of the judges. Meanwhile, wine B was "very much disliked" by the judges. This suggests a strain effect on the sensorial quality of the wine. In addition, wine M, which was fermented spontaneously, was accepted by the most judges. No significant difference was detected between wine M and C.

In addition to the preference test, a sensorial evaluation was made for each wine considering the following parameters: Finesse and complexity, oxidation odor, aromatic power, acidity, bitterness, and balance in wine, and scored according to the Table 3.

The score given for each parameter in each of the tested wines and the p-values of each parameter were obtained by the ANOVA test. Only the 'aromatic power' demonstrated a significant difference ($p = 0.028$) between the four wines.

Wine C showed a higher aromatic power with a complexity of flavors respective to the other wines (Figure 4). Wine A presented the lowest oxidation odor and bitterness with a moderate aromatic power. In contrast, an oxidation odor was noted for wine M, which fermented spontaneously. High acidity perception was detected in wine B.

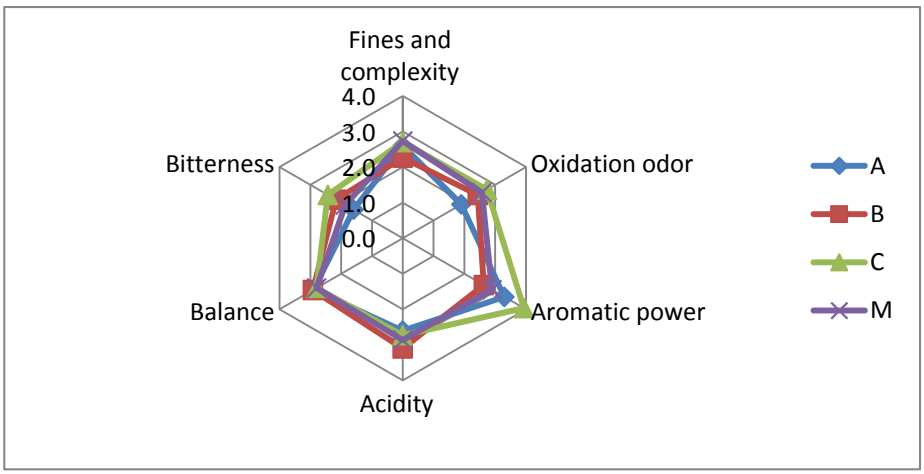

Figure 4. Radar results of the qualitative sensorial parameters for the three inoculated wines (A, B, and C) and the wine that fermented spontaneously (M).

In order to determine the correlation between the sensorial perception and the chemical parameter of the wine perceived by the wine tasters, a Pearson correlation coefficient was calculated for any combination of the chemical compound and sensorial descriptor (Figure 5).

Figure 5. Correlation between the results of the chemical parameters and sensorial perception determined by judges for the tested wines. The color intensity means the grade of correlation (light color: low correlation; intense color: high correlation). The red and blue colors mean positive and negative correlation, respectively.

The aromatic power was found to correlate to the volatile acidity. This can explain the choice of wine C based on the presence of volatile compounds, which increase the aromatic power of the wine. Volatile compounds are important compounds in wine and their composition determines the overall aroma of wine [29]. This confirms the important aspect of aroma in wine quality, and hence, for consumer acceptance [30]. Instead, bitterness was highly correlated with the acids responsible for the fixed acidity of the wines.

4. Discussion

In this study, the fermentative performances of three selected *S. cerevisiae* strains were evaluated during the fermentation of 'Merwah 'must. Chemical, sensorial, and microbiological analysis were carried out in order to choose which strain can be better adapted to the 'Merwah' must environment, revealing interested technological features and producing aromatic compounds.

Certain criteria must be met in order to guarantee the desirable features of the selected yeast strains: Low residual sugar, tolerance to ethanol, high fermentation activity, low volatile acidity, low production of sulfur dioxide, and hydrogen sulfide [7,31]. The technological profile of the inoculated wines (A, B, and C) was compared to the wine that was fermented spontaneously (M).

Based on the results of the ANOVA test for the chemical parameters, the inoculated (A, B, C) and spontaneous (M) wines were not significantly different.

For this reason, sensorial analysis was performed for the four wines tested in duplicate, in order to choose the best fermented "Merwah" wine from an aromatic point of view.

A slightly high total acidity level (>4.45 g/L) in the tested wines was related to the initial acidity concentration in the "Merwah" grape cultivar, in accordance with the observation that grapes from cooler climate wine regions have higher levels of acidity, due to the slower ripening process [32]. Grapes contain several acids in different ratios, including tartaric, acetic, malic, citric, and lactic acids. The predominant acids are tartaric and malic, which together may account for over 90% of the total acidity in the berry, existing at crudely a 1:1 to 1:3 ratio of tartaric to malic acid. At a typical wine pH

(3.4), tartaric acid will be three times as acidic as malic acid [33]. Here, the four wines (A, B, C, and M) were found to contain a concentration of tartaric acid below 3 g/L with a stable pH around 3.

Organic acids in wine play an important role in the taste and quality of wines. Perhaps, wine with an insufficient acidity with a low content of organic acids loses its fullness and roundness of taste and becomes characterless and expressionless [34].

The concentration of volatile acidity in the tested wines was below 0.5 g/L of acetic acid, which is an important criterion for the selection of a wine strain. The maximum acceptable limit for volatile acidity in most wines is 1.2 g/L of acetic acid [35].

Considering the results of the preference test, the wine tasters classified wine C in first place and the wine that fermented spontaneously (M) as second place according to the preference of most of the judges.

Spontaneous fermentations are likely to produce a wider palette of sensory characteristics than those usually found in inoculated fermentations, but whether those characteristics are positive or negative is dictated by factors we do not yet fully understand [36].

It was found that the aromatic wine quality depends on the strict interaction between grapes' must composition and the yeast strains performing the fermentation [7]. These findings confirm that the aromatic quality is related to both the "Merwah" must/wine and the inoculated strain.

Wine C, which was preferred by the wine tasters and judges, was characterized by a high aromatic power related to the volatile compounds. The amount of volatile fermentation products is a key difference between inoculated and uninoculated wines and provides a chemical basis for the 'wild yeast fermentation' character [37].

In this case, the inoculated wine with a yeast strain isolated for its desirable fermenting characteristics might be able to produce pleasant fruity aromas, a high level of alcohol, an attractive mouth texture, and an ability to ferment in low temperatures or high acid [13]. In fact, inoculated wine with a selected indigenous strain from a specific wine region can help the winemaker to avoid the risk of sluggish and stuck fermentation and to increase the stability of the wine quality [10,13].

Furthermore, the results of the molecular analysis showed that the majority of the yeast isolated from wine C corresponded to the inoculated *S. cerevisiae* strain M.4.17. Thus, this strain was characterized by a very high dominance ability with a powerful aromatic quality of wine as demonstrated in a previous study for other strains [7].

Among the studied strains, strain M.4.17 can be selected as a starter to produce wine characterized by interesting oenological and organoleptic features. This strain was chosen by most of the judges for its technological properties and aromatic potential.

The characterization and selection of indigenous *S. cerevisiae* strains adapted to a specific winery has great importance in the biodiversity preservation and exploitation in terms of developing a starter culture collection.

5. Conclusions and Perspectives

The selected wine *S. cerevisiae* strain M.4.17 isolated from "Merwah" must, produced white wine with an equal quality in respect to that obtained from spontaneous fermentation. In addition, this strain showed the ability to also increase the aromatic profile of the wine.

Based on this study, further tests will be carried out to test the drying attitude of the selected strain for its production as dry yeast.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/2/43/s1, Table S1: Chemical profiles of inoculated (A, B, and C) and spontaneously fermented (M) wines at the end of alcoholic fermentation.

Author Contributions: Conceptualization, N.F. and M.B., methodology, N.F., M.B. and G.Z.; software, C.G.; investigation, N.F. and A.B.; resources, N.F., C.G. and M.B., writing—original draft preparation, N.F.; writing—review and editing, C.G., G.Z., A.B. and M.B.; visualization, E.T.; supervision, M.B. All authors have read and approved the final manuscript.

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