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Selection of Three Indigenous Lebanese Yeast *Saccharomyces cerevisiae* with Physiological Traits from Grape Varieties in Western Semi-Desert and Pedoclimatic Conditions in the Bekaa Valley

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Abstract: Wine production depends on the fermentation process performed by yeasts, especially (but not solely) strains of the species *Saccharomyces cerevisiae*, which is a technique that has been practiced from the Middle Ages till modern days. Selecting indigenous starters offers a beneficial technique to manage alcoholic grape juice fermentation, conserving the particular sensory qualities of wine produced from specific regions. This paper investigated yeast biodiversity of four grape varieties (Carignan, Syrah, Grenache, and Aswad Karesh) grown in the pedoclimatic western semi-desert Bekaa Valley. Further research identified, characterized, and selected strains with the most industrial wine interest and economic value to Lebanon. By using molecular methods and by the ITS PCR analysis, the isolates belonging to the *Saccharomyces* and *non-Saccharomyces* genus were identified. These isolates taken from four varieties were further characterized by amplification with Interdelta and δ12/δ21 primer pairs, permitting the identification of 96 *S. cerevisiae* strains. Forty-five genomically homogenous groups were classified through the comparison between their mtDNA RFLP patterns. Based on physiological characterization analysis (H_2S and SO_2 production, killer phenotype, sugar consumption, malic and acetic acid, etc.), three strains (NL28629, NL28649, and NL28652) showed interesting features, where they were also vigorously fermented in a synthetic medium. These strains can be used as a convenient starter for typical wine production. In particular, Carignan and Syrah had the highest percentage of strains with the most desirable physiological parameters.

Keywords: *Saccharomyces cerevisiae*; molecular identification; physiological characterization; inoculated fermentation; PCR; yeast selection; Lebanese vineyard

Citation: El Dana, F.; Hayar, S.; Colosio, M.-C. Selection of Three Indigenous Lebanese Yeast *Saccharomyces cerevisiae* with Physiological Traits from Grape Varieties in Western Semi-Desert and Pedoclimatic Conditions in the Bekaa Valley. *Fermentation* **2021**, *7*, 280. https://doi.org/10.3390/fermentation7040280

Academic Editor: Antonio Morata

Received: 7 October 2021
Accepted: 18 November 2021
Published: 26 November 2021

1. Introduction

The transformation of grape juice into wine is basically a fermentation process historically carried out by indigenous yeasts and considered to be the most economically significant of all biotechnologies [1]. The wine yeast plays a vital role in catalyzing a quick, complete, and effective conversion of sugar in grape, especially glucose and fructose into ethanol, carbon dioxide, heat as a byproduct, but, most importantly, metabolites without the development of off-flavor [1–4].

Unfortunately, the climate change associated with global warming has directly and indirectly effected agricultural output over the last few years. This has promoted a more extensive usage of pesticides, disrupting microbial diversity and adversely affecting spontaneous fermentation [5–9].

Therefore, selecting suitable indigenous yeast strains is essential to optimize alcohol yield, must fermentation, and preserve wine sensory and quality from a specific terroir [10]. For this reason, some wineries and laboratories have created new techniques for isolation, identification, characterization, and selection of starter cultures of *Saccharomyces cerevisiae* for controlled wine fermentation [11–13]. The selection of indigenous strains with unique phenotypes presents a powerful instrument for regional wines' distinctiveness, diversity, and quality development [14].

The yeast species that dominates in the formation of wine worldwide is *Saccharomyces cerevisiae*. Selecting a particular strain of this species for fermentation can substantially impact various beverages' flavor and aroma characteristics [15,16]. These yeast species live in a wide variety of ecological niches. Still, the most prevalent habitat is the fruit surface of mainly grapes and berries. They become actively involved in the decomposition of ripe fruit as they assist in the fermentation process [17,18]. Indeed, the indigenous yeasts' effectiveness on the surface of these undamaged grapes relies on their diverse physiological characteristics such as killer phenotype, sugar availability, total acidity, pH, etc. [19]. In this natural habitat, *S. cerevisiae* can successfully carry out their metabolism and their fermentation process as they have the essential nutrients and substrates. Yeasts are not very demanding in comparison with other microorganisms such as lactic acid bacteria. However, their growth is promoted by providing essential substances such as fermentable sugars, vitamins, amino acids, oxygen, and minerals [20].

The biodiversity of wine-producing *Saccharomyces cerevisiae* and *non-Saccharomyces cerevisiae* has been investigated at the molecular level in order to determine their diverse microflora characteristics [21,22].

As the significance of *S. cerevisiae* in wine-making has long been acknowledged, the use of commercial strains of yeast cultures in fermentation becomes one of the most prevalent techniques to assure a consistent product and to prevent wine spoiling. However, this approach might establish a progressive substitution of local microflora and a corresponding diminution or lack of several beneficial and distinctive organoleptic properties of natural or spontaneous alcoholic fermentation. Thus, understanding the evolution of the yeast microflora during natural alcoholic fermentation will help in the selection of the most representative indigenous strains in order to improve wine quality [23,24].

Since the beginning of recorded history through the Middle Ages, the Lebanese monasteries have perfected wine production and maintained this art of their know-how through local practices of the indigenous grape varieties' orchard. Thus, it is essential to study the deep-rootedness of its yeast strain biodiversity [25]. Château Kefraya is located on the foothills of Mount Barouk, 10^3 m above the Mediterranean Sea, in the Bekaa Valley. This terroir is mainly composed of sandy, clay-chalk, gravelly, and clay-limestone soil.

In this study, we investigated: (i) the biodiversity of indigenous yeast microflora at the species level hosted by 12 distinct grape varieties remotely grown in vineyards residing in the Bekaa–Château Kefraya domain (Lebanon), (ii) the molecular identity of genomically different yeast strains, (iii) the physiological characteristics of selected strains such as H_2S and SO_2 production, killer phenotype, sugar consumption, acids present, etc. (iv) the differences among the 4 varieties regarding the most desirable hosted strains, (v) and the best grape varieties that must be included at higher percentage during wine preparation.

2. Materials and Methods

2.1. The Grape Trees Origin

Twelve Lebanese grape varieties (1—Carignan, 2—Syrah, 3—Grenache, 4—Aswad Karesh, 5—Obeidy, 6—Merwah, 7—Ahmar Mawardi, 8—Assali El Arous, 9—Asmi Black, 10—Zeini, 11—Zeini Abiad, 12—Asmi Red) samples were harvested in 2019 from vineyards residing in Château Kefraya (300 hectares), a village in the Western Bekaa District in the Republic of Lebanon. The first vineyards were established in 1951. In 1979, Château Kefraya began making its own wine with its own grapes grown in its vineyard and vinified in its cellar.

2.2. Sampling Protocol

Samples were collected in sterile plastic bottles according to the Institut Français de la Vigne et du Vin (IFV) protocol. All the grapes found in this vineyard were grown in the same region, but each variety has been bred independently and far away from each other. Using 12 different sterile gloves, each grape sample was hand-squeezed separately in sterile bottles and then transferred into a 1 L flask through a coarse-mesh sieve to discard skins and seeds. Each flask was capped by sterile hydrophobic cotton to avoid external microbial contamination. It was permitted for fermentation to continue spontaneously for 14 days at 18–22 °C (fermentation progress measured by mass loss). To track the different molecular and physiological traits, each strain was serially coded with the NL (National Lebanese) strains prefix, (e.g., NL28571).

2.3. Reagents and Microorganisms

The different media culture Yeast Extract Peptone Dextrose (YPD/YEPD) were purchased from Merck KGaA, Darmstadt, Germany. Bismuth-containing indicator medium (BiGGY agar) was purchased from Oxoid, Ltd., Basing Stoke, Hampshire, UK. The two commercial reference strains used for this study are X16 and VL1 (LAFFORT l'oenologie par nature/ZYMAFLORE®). Petri dishes were obtained from PHOENIX Biomedical, Mississauga, Canada.

In order to simulate the composition of grape standard juice, a synthetic media (SM) was used with the following composition (expressed per 100 mL for each strain tested): (12 g glucose, 12 g fructose, 0.4 g L-(+)- Tartaric acid, 0.03 g citric acid monohydrate, 0.5 g (L-) malic acid, 0.2 g monobasic potassium phosphate, 0.02 g magnesium sulfate, 0.03 g ammonium sulphite, 0.03 g Meso-Inositol, 0.24 g CaCO3, 0.01 g anaerobic factors, 0.05 g turbicel, 0.6 mL amino acid, 0.1 mL vitamins, 0.02 mL 10% SO_2 solution, 0.1 mL oligo elements of the following composition (expressed per mg per liter): 750 KH_2PO_4, 500 KH_2SO_4, 250 $MgSO_4.7H_2O$, 155 $CaC1_2.2H_2O$, 200 NaC1, 4 $MnSO_4.H_2O$, 4 $ZnSO_4$, 1 $CuSO_4.5H_2O$, 1 KI, 0.4 $CoC1_2.6H_2O$, 1 H_3BO_3, 1 $(NH_4)6Mo_7O_{24}$. All minerals and oligoelements were purchased from (Sigma-Aldrich, Schnelldorf, Germany).

2.4. Identification and Molecular Characterization of Yeast Strains

2.4.1. Culture Preparation and Inoculation

During alcoholic fermentation, the indigenous yeasts were isolated from each grape juice sample. Each grape juice was diluted and spread on CHROMagarTM (22.0 g/L Chromogenic mix L; 10.2 g/L Chloramphenicol; 10.2 g/L Peptone; 15.0 g/L Agar) using easy Spiral dilute (Interscience, France) for cultivation [26]. Thirty yeast colonies derived from the isolation of grape juice were inoculated for 3 days on solid YPD medium at 20 °C, where they were chosen according to color, surface feature, and shape. Solid YPD plates were used to preserve these yeast cultures for 3 days at 18 °C.

Each strain was inoculated in synthetic media at a concentration of 10^6 cells/mL, from pre-culture grown for 2 days in grape must. All of the trails were performed in duplicate.

After inoculation, one colony of each *S. cerevisiae* strain was transferred to distinct centrifuged tube (NEST Biotechnology, Wuxi, Jiangsu, China), each containing 5 mL of liquid YEPD (10 g yeast extract, 10 g pancreatic peptone, 20 g glucose, 0.1 g chloramphenicol, 15 g agar) [27].

2.4.2. DNA Extraction

The isolated yeast colonies were placed in an Eppendorf tube (NEST Biotechnology, China) that contained 660 µL of 50TE+SDS (TE: Tris-EDTA (Eurobio Scientific, Les Ulis Cedex, France), SDS: sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO USA) to allow cell lysis. After shaking well, these tubes were incubated for 30 min at 65 °C in the stove (Heraeus). For the precipitation of rubbish compounds, approximately 340 µL of 5 M potassium acetate KAc (Sigma-Aldrich, USA) were added and refrigerated for 30 min.

Centrifugation for 10 min at 13,000 rpm took place after getting the small tubes out from the fridge [28,29].

After the centrifuge, 750 µL of the supernatant were removed and placed in new tubes (contain the DNA). Then, 750 µL of isopropanol were added to each tube and left at room temperature for about 10 min after being mixed well. A second centrifuge for 10 min at 13,000 rpm was also done to get DNA as a pellet at the end [28]. The isopropanol was removed from each tube and the latter was left to dry for 20 min. The DNA pellets were left to dry also for 20 min after rinsing them with cold 95% ethanol (to get rid of the isopropanol). The pellets were suspended by adding 250 µL of TE buffer and left to dry, then stored at −20 °C [28–30].

2.4.3. Yeast Identification at Species Level by ITS PCR

From a total of 1321 strains, a total genomic DNA was prepared from each strain according to the methods used by IFV to distinguish *S. cerevisiae* from the non-*S. cerevisiae*. Primer pairs 18S and 28S were purchased from Sigma and used to amplify ITS region (ITS1 and ITS2) and the 5·8S rRNA gene; this was performed in an internal transcribed spacer (ITS) PCR [16–31]. Internal transcribed spacer PCR enables the modification according to conditions described by [29–32] as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles consisting of 30 s at 95 °C for denaturation, 1 min for primer annealing at 46 °C and 1 min for primer extension at 72 °C, followed by a final extension at 72 °C for 5 min. The restriction endonucleases HinfI and HaeIII were used to digest the amplified DNAs. On agarose gel and using an electrophoresis (Avantor®, VWR®), the amplified products were separated when exposed to 400 V for 45 min in 1xTBE buffer (Eurobio Scientific, Les Ulis Cedex, France) [33].

2.4.4. Strain Identification by Interdelta PCR

Using Interdelta PCR (Thermo Fisher Scientific, Singapore), a TY1 retrotransposon region was amplified with δ12/δ21 primer pairs in order to identify 96 strains (taken from the first 4 grape varieties) within the same yeast species [28,32,34]. The amplification of the δ region was done by placing the PCR tubes (SSI Bio, California, CA, USA) in a SimpliAmp thermal cycler (Thermo Fisher Scientific, Singapore) for initial denaturation at 95 °C for 5 min. After the latter denaturation, the reaction mixture was cycled 40 times as follows: 30 s denaturation, 1 min primer annealing, and 1 min primer extension at 95 °C, 46 °C, and 72 °C, respectively. At the end, a final extension for 5 min at 72 °C was performed [12,16,22,29].

Restriction fragments were separated on agarose gel (Sigma-Aldrich, Chemie GmbH, Munich, Schnelldorf, Germany) composed of 1.5 g mixture of 50% standard agarose +50% resophor agarose, 100 mL of TBE EDTA 1X, and 5% Midorigreen (NIPPON Genetics EUROPE, Japan). Gels were stained with 100 bp DNA Ladder inside an electrophoresis to allow the DNA migration, then these DNA bands were visualized under E-box-VX2/20MX UV light (VILBER, Marne-la-Vallée Cedex 3, France) and compared to DNA length standards to select gnomically different strains [35].

2.5. Physiological Characterization of Identified Strains

The *Saccharomyces cerevisiae* strains identified through molecular sequence analysis were further tested for their physiological characteristics. For further characterization, the different genomically selected strains were incubated in Liquid YPD and left for 2–3 days at 20 °C.

2.5.1. H_2S Production Test

To assess hydrogen sulphide production, 10 µL of each strain from the previously prepared YPD liquid were inoculated on the BIGGY medium and kept for 2–3 days at 24 °C, respectively. Visual scale was used as a function of the increasing level of H_2S produced [10,29,36,37].

2.5.2. Assay of Killer/Sensitive Phenotype

For the killer/sensitive phenotype test, two layers of PYG, composed of malt extract broth (2%) and agar (2%), were prepared inside petri dishes along with methylene blue (0.0003%) (Sigma-Aldrich) and buffered at pH 4.5 with 4 mol/L hydrochloric acid. Then, 100 µL of each reference strain were incorporated into the medium, and the strains were inoculated on the plates as spots, which were incubated for 3 days at 23 °C. *S. cerevisiae* IOC Harmonie and *S. cerevisiae* IOC 18-2007 were used as sensitive and killer reference strains, respectively [10,38,39].

2.5.3. Initiation of Fermentation and Sugar Consumption

Yeast strains were inoculated in bottles each containing 100 mL of SM with 24 g initial sugar concentration (50% of glucose and fructose) at 20 °C over 37 days [19]. The weight loss was obtained by the selected Lebanese strains and compared with the reference strains: VL1 and X16. Alcoholic fermentation was determined to be completed when the weight was steady. The ability of the strains to utilize glucose and fructose as a carbon source, and reduce their concentrations below 3 g/L, was determined by spectrophotometry Y-15 (FoodQuality, BioSystems, Barcelona, Spain) connected to Y15-Raccouri Microsoft using a specific enzymatic test kit (FoodQuality, BioSystems, Barcelona, Spain) as soon as the weight loss by percentage by each strain exceeded 6%.

2.5.4. Sulfur Dioxide, Malic Acid, and Volatility Production

The production of SO_2, volatility, and malic acid by each strain were determined also by spectrophotometrically using the Y-15 Biosystem after the weight reached a steady state in each of their SM [16].

2.5.5. Total Acidity and pH

The capability of selected yeast strains to produce an acceptable amount of total acidity (11.0–14.0% vol/vol) and stay within the permitted range of pH (3–6) was tested by infrared (FT-IR) analyzer and potentiometric titration apparatus, respectively, using the facilities of subcontracted Céania Analysis laboratories [40].

2.6. Strain and Grape Variety Selection

To select the best strain of *Saccharomyces cerevisiae*, the physiological traits were compared within the isolates. The selection of preferable variety was based on the percentage of effective strains hosted within the 4 varieties.

3. Results

3.1. Isolation and Molecular Characterization of Yeast Strains

3.1.1. Identification of Yeast Taxonomy at the Species Level

Our data permitted the classification and identification of isolates belonging to the *Saccharomyces* and *non-Saccharomyces* genus (Tables 1 and 2).

Table 1. Number of yeast species isolated from 12 grape varieties after analyzing yeast biodiversity of year 2019 samples at the genus and species level.

Yeast	Diversity Counts
Saccharomyces cerevisiae	690
non-Saccharomyces cerevisiae	631
Unknown	46

Table 2. Distribution number of the various strains found under the species level of 631 *non-Saccharomyces cerevisiae*.

Non-Saccharomyces cerevisiae	**Counts (631)**
Hanseniaspora uvarum	315
Metschinikovia pulcherrima	179
Candida	48
Kluyveromyces/Zygosaccharomyces	31
Metschinikovia pulcherrima/Candida melibiosa	29
Torulaspora delbrueckii	14
Pichia nakasei/Candida soli/Pichia occidentalis	6
Trigonopsis californica	4
Pichia SP	3
Candida stellata	1
Candida zemetschinikovia pulcherrimalinina	1

As shown in Table 1, 690 strains belonged to *Saccharomyces cerevisiae*, 631 to *non-Saccharomyces cerevisiae*, and 46 unknown strains were identified within grape varieties harvested in 2019.

Table 2 depicts the non-Saccharomyces strains: Kluyveromyces/Zygosaccharomyces, Metschinikovia pulcherrima/Candida melibiosa, and Pichia nakasei/Candida Soli/Pichia occidentalis. The further distinction among the species mentioned above was not within the scope of our study. However, the three genera Hanseniaspora uvarum, Metschinikovia pulcherrima, and Candida seemed to be the most dominant non-Saccharomyces cerevisiae (Table 2). Similar results were achieved by [41]. Indeed, non-Saccharomyces yeasts belonging to the genera Candida and Hanseniaspora have gained more interest due to their outstanding ability to be used in the production of good fermented wines [41].

3.1.2. Genomically Different Strain Identification

The PCR amplification showed that all the isolates exhibiting PCR-RFLP patterns with size 370/360/110 bp for Hinf I and 320/230/170/120 bp for Hae III corresponded to *S. cerevisiae*. Hence, 96 S. cerevisiae strains were identified and confirmed by ITS PCR analysis in the 4 tested grape juice varieties. Only 45 strains were found to be genomically different among the 96 S. cerevisiae community through Delta PCR analysis (Figure 1). The 4 varieties Carignan, Syrah, Grenache, and Aswad Karesh hosted 22, 14, 7, and 2 strains out of 45, respectively.

3.2. Physiological Characterization of Selected Strains

3.2.1. Fermentation Initiation

As shown in Table 3 (column V), out of the 45 selected strains, only 24 could initiate fermentation quickly (above 0.10%) on day 2 of the experiment at 22 °C. Among these strains, Carignan, Syrah, Grenache, and Aswad Karesh hosted 10, 9, 4, and 1, respectively. Concerning the sugar consumption, and according to the important feature mentioned by [42], it seems that the NL28629 (0.18%), NL28649 (0.19%), and NL28651 (0.22%) provoke the best percentage CO_2 loss compared to the commercial strain X16 (0.08%) Table 3 (column V).

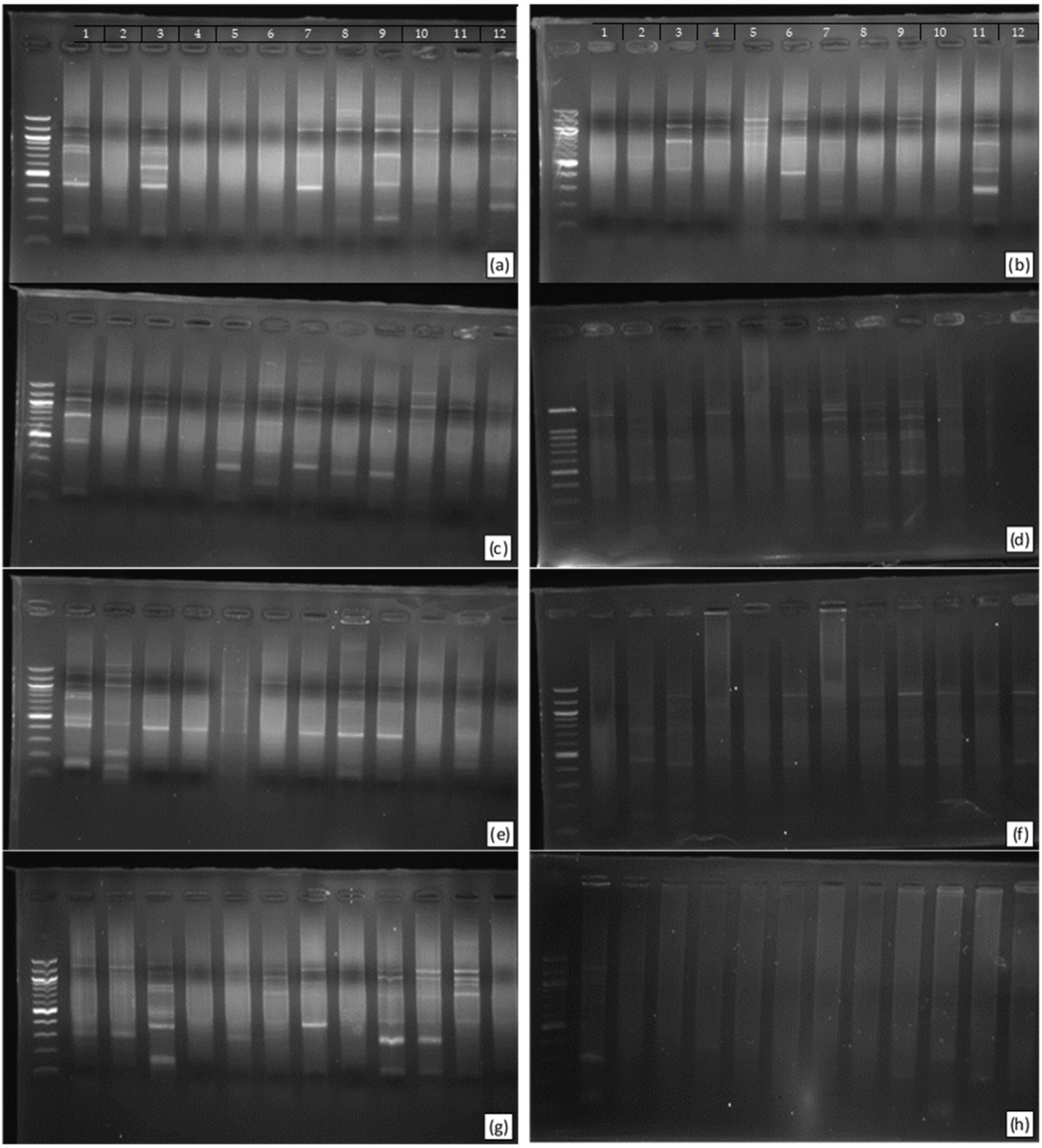

Figure 1. Interdelta electrophoretic patterns of 96 different Saccharomyces cerevisiae strains taken from Château Kefraya. The figure is divided into 8 pictures (**a**–**h**) each one contains 12 lanes representing the different yeast strains.

Table 3. Physiological features of 45 selected *Saccharomyces cerevisiae* strains isolated from four grape varieties compared with commercial wine yeasts X16 and VL.

		H_2S Production		Killer Phenotype		Weight Loss		Sugar Residue	Total SO_2	Acetic Acid	Malic Acid
Grape Varieties	**Strains**	**Color Intensity**	**Sulphite Reductase Activity**	**Killer**	**Sensitive**	**Day 2 (%)**	**Day 37 (%)**	**(g/L)**	**(mg/L)**	**(g/L)**	**(g/L)**
Carignan	NL28571	4	High	K−	R+	0.03	4.39	Out High	37	0.58	4.21
	NL28572	3	Medium	K−	R+	0.12	4.74	Out High	57	0.56	3.97
	NL28578	4	High	K−	R+	0.09	4.60	Out High	44	0.53	4.23
	NL28584	4	High	K−	R+	0.17	6.32	3.06	35	0.71	3.91
	NL28586	4	High	K−	R+	0.11	4.24	1.23	49	0.11	2.68
	NL28587	4	High	K−	R+	0.13	4.59	Out High	41	0.48	4.19
	NL28590	3	Medium	K−	R+	0.05	4.83	9.26	43	0.54	3.91
	NL28594	2	Low	K+	R+	0.12	4.97	7.87	50	0.64	3.99
	NL28597	5	High	K−	R+	0.16	5.02	Out High	43	0.36	4.14
	NL28600	4	High	K−	R+	0.00	4.47	Out High	38	0.62	4.18
	NL28601	4	High	K−	R+	0.14	4.78	2.08	54	0.41	4.2
	NL28602	4	High	K−	R+	0.07	4.73	10.27	48	0.49	4.1
	NL28603	4	High	K−	R+	0.09	4.61	9.65	42	0.54	4.23
	NL28609	2	Low	K−	R+	0.04	4.72	2.69	44	0.76	3.8
	NL28610	2	Low	K−	R−	0.09	4.70	10.45	26	0.5	4.34
	NL28612	4	High	K−	R+	0.13	5.15	2.32	62	0.57	3.93
	NL28613	5	High	K−	R+	0.01	4.92	2.29	41	0.61	4.2
	NL28614	5	High	K−	R+	0.01	4.63	6.96	41	0.73	4.16
	NL28616	4	High	K−	R+	0.05	6.59	Out High	10	1.07	4.02
	NL28619	4	High	K−	R+	0.18	6.66	2.33	40	0.70	3.84
	NL28627	5	High	K−	R+	0.05	7.17	0.4	22	0.73	3.39
	NL28628	5	High	K−	R+	0.11	6.96	0.17	20	0.78	3.85
	Columns	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**
Syrah	NL28629	3	Medium	K−	R+	0.18	6.95	0.19	27	0.54	3.93
	NL28634	4	High	K−	R+	0.17	5.90	0.22	29	0.5	3.99
	NL28640	5	High	K−	R+	0.08	6.44	0.52	24	0.7	4.20
	NL28643	5	High	K−	R+	0.14	6.73	0.92	20	0.78	3.98
	NL28644	4	High	K−	R+	0.16	10.78	0.33	31	0.75	3.90
	NL28649	3	Medium	K−	R+	0.19	6.68	0.75	36	0.58	3.83
	NL28651	4	High	K−	R+	0.22	6.45	0.29	27	0.74	3.98
	NL28652	3	Medium	K−	R+	0.14	6.69	0.73	21	0.86	3.82
	NL28653	5	High	K−	R+	0.06	5.41	0.5	26	1.05	4.22
	NL28654	5	High	K−	R+	0.05	6.33	0.18	34	0.41	4.13
	NL28656	5	High	K−	R+	0.08	6.37	1.03	27	0.87	3.94
	NL28657	5	High	K−	R+	0.05	6.87	0.48	24	0.77	3.91
	NL28661	4	High	K−	R+	0.12	6.69	0.75	40	0.58	3.97
	NL28666	4	High	K−	R+	0.20	6.50	0.26	35	0.58	4.00

Table 3. *Cont.*

		H_2S Production		Killer Phenotype		Weight Loss		Sugar Residue	Total SO_2	Acetic Acid	Malic Acid
Grape Varieties	**Strains**	**Color Intensity**	**Sulphite Reductase Activity**	**Killer**	**Sensitive**	**Day 2 (%)**	**Day 37 (%)**	**(g/L)**	**(mg/L)**	**(g/L)**	**(g/L)**
Grenache	NL28668	4	High	K−	R+	0.16	6.10	1.22	33	0.73	4.23
	NL28669	4	High	K−	R+	0.14	4.77	11	43	0.49	4.26
	NL28674	4	High	K−	R+	0.06	8.47	Out High	36	0.51	4.18
	NL28680	5	High	K−	R+	0.08	4.53	2.28	42	0.59	4.32
	NL28693	4	High	K−	R−	0.07	4.54	Out High	35	0.68	4.12
	NL28694	4	High	K−	R+	0.12	4.57	Out High	43	0.44	4.39
	NL28699	5	High	K−	R+	0.12	8.46	11.15	33	0.52	4.27
Aswad	NL28702	5	High	K−	R+	0.11	4.49	Out High	31	0.77	4.51
Karesh	NL28703	4	High	K−	R+	0.08	4.75	9.78	35	0.59	4.20
French yeasts	X16	3	Medium	K+	R+	0.08	5.27	1.32	50	0.89	3.97
	VL1	2	Low	K−	R+	0.42	5.61	0.28	37	0.44	4.09
	Columns	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**

3.2.2. Sugar Consumption

Regarding the most crucial trait, which is sugar residues, and comparing to the reference strains X16 and VL1, only 25 out of 45 Lebanese strains were capable of consuming sugar and maintaining residues below 3 g/L [42]. Only 3 varieties, Carignan, Syrah, and Grenache, hosted 9, 14, and 2 of these 25 strains, respectively. The levels of sugar residues were near zero for the mostly selected strains (Table 3, column VII).

3.2.3. H_2S and SO_2 Production

Only 8 of the 45 representative *S. cerevisiae* strains showed good sulphite reductase activity when transferred to BiGGY medium, indicating that 8 strains (NL28594, NL28609, NL28610, NL28572, NL28590, NL28629, NL28649, and NL28652) produced low-to-medium hydrogen sulphide (H_2S) (Table 3, columns I and II).

As for the sulfur dioxide production and as assessed by spectrophotometry, only 27 indigenous *S. cerevisiae* strains were characterized by a low yield of SO_2 (below 40 mg/L), ranging from 10 to 38 mg/L, whereas the amount of SO_2 in the fermented synthetic media by the commercial strains ranged from 37 to 50 mg/L for VL1 and X16, respectively (Table 3, column VIII). This makes most of the indigenous strains have the ability to produce the needed SO_2 level to prevent browning and keeps the wine fresher. Yet the level of SO_2 produced by these strains will not allow the production of diacetyl, which can negatively affect wine quality and human health risk.

3.2.4. L-Malic and Acetic Acids

With regard to L-malic acid production, all strains showed the same production level (ranged between 3.9 and 4.5 g/L) compared with commercial strains VL1 and X16 (Table 3, column X). In addition, 62% of the selected strains presented a production level of volatile acid (acetic acid) lower than 0.62 g/L, compared to the commercial strains 0.44; 0.89 g/L for VL1 and X16, respectively, which is believed to be an acceptable level (Table 3, column IX) [43].

3.2.5. Killer/Sensitive Phenotype

Only one representative *S. cerevisiae* strain (NL28594) showed good killing ability (K+) (Table 3, column III). Regarding the sensitive phenotype (R-), 2 strains, which are NL28610 and NL28693, were killed and thus sensitive (Table 3, column IV). Carignan happens to host 1 killer strain and 1 sensitive one among the abovementioned strains, whereas the 3rd sensitive strain belonged to Grenache.

3.2.6. pH and Total Acidity

All strains attained a pH value similar to that of the two commercial strains between 3.08 and 3.16.

All the NL strains showed acidity values similar to the commercial strains between 11.53 and 14.37% vol/vol.

According to their physiological and genetic features, *Saccharomyces* yeasts were able to control wine acidity, either by raising the wine acidic level (biological acidification) or by lowering it (biological deacidification) [44].

3.3. Selection of Indigenous Yeast Strains

Three strains, NL28629, NL28649, and NL28652, appeared to be prevalent over the remaining 42 NL strains. The first trait, sulphite reductase activity of these 3 NL strains, was similar to that of the commercial strain X16 (Table 4); hence, highlighting the importance of selecting them to be used subsequently for further physiological testing (H_2S production is the first test to be done). Indeed, these yeasts were neutral; neither killer, nor sensitive like VL1 (Table 4), making their implementation and competition in wine media more dominant. On the other hand, in line with the Mylona et al. allegation, the three selected strains were able to initiate fermentation quickly, thus guaranteeing effective establishment of the fermentation process [45].

Table 4. Represents the physiological characteristics of the 3 indigenous Lebanese *S. cerevisiae* versus the reference commercial strains.

Parameters	Yeast Strains				
	NL28629	**NL28649**	**NL28652**	**X16**	**VL1**
H_2S production	Medium	Medium	Medium	Medium	Low
Killer/Sensitive phenotype	Neutral	Neutral	Neutral	Killer	Neutral
Sugar consumption (g/L)	0.19	0.75	0.73	1.32	0.28
SO_2 production (mg/L)	27	36	21	50	37
Acetic acid (g/L)	0.54	0.58	0.86	3.97	4.09
L-Malic acid (g/L)	3.93	3.83	3.82	3.97	4.04
Acidity	11.54	12.25	12.54	14.09	14.35
pH	3.09	3.10	3.12	3.15	3.13

Otherwise, their ability in sugar consumption was very high as the sugar residues tested by spectrophotometry was 0.19 for NL28629 strains comparing to its counterpart's strains, 0.75 and 0.73 g/L for NL28649 and NL28652, respectively (Table 4).

The findings shown in Table 3 indicate that 29% of the 45 indigenous wine yeasts taken from Kefraya domain were low SO_2 producers. In the same way, as it is obvious in Table 4, the commercial strains X16 and VL1 produced high levels of SO_2 compared to the 3 NL strains where the level remained less than 36 mg/L. Therefore, these strains are unlikely to allow a delay in the malolactic fermentation as mentioned by [46].

The performance of the 3 selected strains, NL28629, NL28649, and NL28652, also appeared in the acetic acid level produced, 0.54, 0.58, and 0.86, respectively (Table 4), and remained not only below the legalization limit (1.2) [47], but also below the preferable level (0.7 g/L) for NL28629 and NL28649 determined by [48].

As for malic acid, spectrophotometry measurements indicated that the 3 NL strains along with the reference commercial strains had similar levels of production (3.8–4.0 g/L). These levels may allow the diversification in the final taste of the Lebanese wine as mentioned by [49].

Thus, all tested yeast strains provide an acidic medium, between 11.54 and 12.54% vol/vol. According to [50,51], these levels are required for well-processed fermentation. Consequently, this parameter gives more value to the selected strains, specifically that the pH value is similar to the commercial strains and within the range between 3.09 and 3.11.

3.4. Selection of Grape Variety

The comparison of the results from the 4 grape varieties allowed the determination of the percentage distribution of the yeast with the most desirable physiological traits (Table 5).

Table 5. Distribution by percentage of the yeasts with the most desirable physiological traits within the 4 grape varieties.

Varieties	Percentage of Strains						
	Hydrogen Sulphide	Killer/Sensitive Phenotype		Initiation of Fermentation	Sugar Residue	Total SO_2	Acetic Acid
		Killer	Sensitive				
Carignan	22.7	4.5	9	45.4	40.9	31.1	68.1
Nb:22	*(5)*	*(1)*	*(2)*	*(10)*	*(9)*	*(7)*	*(16)*
Syrah	21.4	-	7.1	71.4	100	28.8	42.8
Nb:14	*(5)*		*(1)*	*(10)*	*(14)*	*(4)*	*(6)*
Grenache	-	-	-	57.1	28.5	57.1	85.7
Nb:7				*(4)*	*(2)*	*(4)*	*(6)*
Aswad Karesh	-	-	-	50	-	100	50
Nb:2				*(1)*		*(2)*	*(1)*

(*Nb*): The numbers in italic represent the effective number of strains within each variety. (-): no yeasts with such characteristics present.

The selection was done according to specific physiological priorities: sugar residue, hydrogen sulphide, and killer/sensitive phenotype. Regarding sugar residue, all strains hosted by Syrah (100%) have the ability to reduce sugar below 3 g/L, followed by Carignan (40.9%) (Table 5). Along the same line, these two varieties hosted approximately the same percentage of strains that produce an acceptable amount of H_2S, 22.7 and 22.1%, respectively. However, the Carignan was the only variety that appeared to host one killer strain. For the sensitive phenotype, Syrah and Carignan host 1 and 2 sensitive strains, respectively, they can remain preferable to be selected when compared to counterpart varieties. Since the three previous selected strains, NL28629, NL28649, and NL28652, are hosted by Syrah, this makes it a very interesting variety. Wineries may consider using Syrah more often and in higher percentage.

4. Conclusions

There is a prevalent assumption that spontaneous alcoholic fermentation, generated by indigenous microflora specifically yeast, is determined by each specific vineyard, and it provides a particular characteristic and quality of each wine. The last stage of natural wine fermentation is generally dominated by alcohol-tolerant *S. cerevisiae* strains. About the vineyard niche habitats, the *S. cerevisiae* component and other specialized yeasts are recognized as autochthonous and their engagement in natural fermentation permits the development of wines with distinct traits in each micro-climatic location. However, the amount and the occurrence of these yeasts fluctuate depending on the location, climatic change and type of grape.

To our knowledge, this study is distinguished among other scientific papers for highlighting the biodiversity of the Lebanese *S. cerevisiae* hosted by grape varieties grown

in the pedoclimatic western semi-desert Bekaa Valley vineyards. Twelve grape varieties from the Château Kefraya domain were harvested and studied in the IFV facilities.

The total biomass obtained through ITS PCR molecular identification showed that 690 of the yeasts belonged to *Saccharomyces cerevisiae* and 631 to *non-Saccharomyces cerevisiae*. The genera *Hanseniaspora Uvarum*, *Metschinikovia Pulcherrima*, and *Candida* are the most dominant *non-Saccharomyces cerevisiae*. The delta sequences amplification combined with mtDNA analysis using delta PCR, permitting the differentiation of 45 genomically different *S. cerevisiae* strains: among them 22, Carignan; 14, Syrah; 7, Grenache; and 2, Aswad Karesh.

The physiological characterization allowed the selection of 3 strains, NL28629, NL28649, and NL28652, due to their traits and features compared to the commercial X16 and VL1 strains. These 3 strains were of a medium reductase activity, neither were killed by or killed other strains, and could initiate fermentation quickly. Moreover, they had high sugar consumption ability, producing acetic acids, and SO_2 levels below 1, 0.7 (g/L), and 40 (mg/L), respectively. As for malic acid, pH, and total acidity, the 3 strains were within the acceptable range.

Furthermore, when comparing the four varieties used in this study, Carignan and Syrah seemed to host the highest percentage of strains with the most desirable physiological traits, and they appeared to be the preferred varieties to be used in a higher amount during wine making.

In short, these promising results must encourage the Lebanese authorities, and particularly the Association of Lebanese Industrialists, to exploit our findings by increasing investment for research on indigenous yeasts, and encouraging them to produce these strains on a commercial scale via collaboration contracts with industrial companies.

Author Contributions: Conceptualization, investigation, resources, project administration, funding acquisition, writing-review and editing, S.H.; Methodology, formal analysis, data curation, writing original draft preparation, writing-review and editing, F.E.D.; Methodology, formal analysis, resources and supervision, M.-C.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Lebanese Ministry of Industry and the Association of Lebanese Industrialists, grant number 221527. All testing laboratories were performed gratefully in the Institut Français de la Vigne et du Vin (IFV).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The members of the research team deeply thank all those who have contributed to the success of this project: Kefraya domain (Edward Kosremelli and Fabrice Guiberteau), the Lebanese Ministry of Industry, the Association of Lebanese Industrialists, the Institut Français de la Vigne et du Vin, and the Doctoral School of Science and Technology, Research Platform for Environmental Sciences, (EDST-PRASE), Lebanese University.

Conflicts of Interest: The authors declare no conflict of interest.

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