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Molecular Identification and VOMs Characterization of *Saccharomyces cerevisiae* Strains Isolated from Madeira Region Winery Environments

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Abstract: The quality and typical characteristic of wines depends, among other factors, on the volatile organic metabolites (VOMs) that are biosynthesized by yeasts, mainly *Saccharomyces cerevisiae* species. The yeast strain influences the diversity and proportions of the VOMs produced during the fermentation process, as the genetic predisposition of the strains is a by-product of selective adaptation to the ecosystem. The present work reports the characterization of *S. cerevisiae* strains isolated from grape must, used in the Demarcated Region of Madeira (DRM) for winemaking. Yeast species were identified by amplification and by restriction fragment length polymorphism (RFLP) analysis of the region 5.8S-internal transcribed spacers (PCR-RFLP of 5.8S-ITS) of ribosomal DNA (rDNA). The strains identification was performed by analyzing the RFLP pattern of mitochondrial DNA (RFLP-mtDNA). The representative strains were selected for the characterization of the volatile profile through headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS) analysis. A total of 77 VOMs were identified. Higher alcohols, esters, and fatty acids were the major chemical families representing 63%, 16%, and 9%, respectively, in strain A and 54%, 23%, and 15% in strain B. The results indicate the influence of the strain metabolism in the production of VOMs, many of which probably participate in the aroma of the corresponding wines.

Keywords: Demarcated Region of Madeira; *Saccharomyces cerevisiae*; strain; molecular identification; volatile organic metabolites

1. Introduction

The quality and typicity of wines result from the conjugation of several natural and human factors [1,2]. In the Demarcated Region of Madeira (DRM), specific agro-edaphoclimatic conditions such as insular Atlantic territory, volcanic origin, combined with selective noble grapes and specific agronomic techniques adapted to the area and orography of the island, in close relationship with traditional and sui generis vinification process, confer to the wines produced in the DRM unique organoleptic properties, among which aroma is an important parameter that defines its quality and typicity [3].

The contribution of *S. cerevisiae* to the aroma of the wine is well known. During alcoholic fermentation, as a secondary metabolomic pathway, *S. cerevisiae* biosynthesizes semi-volatile and non-volatile organic metabolites, especially a large number of different volatile organic metabolites

(VOMs), belonging to different chemical families such as esters, alcohols, fatty acids, aldehydes, ketones, and miscellaneous compounds. These metabolites are associated with a wide variety of aromatic descriptors that are likely to participate in the aroma of the wine. The diversity and proportions of VOMs are conditioned by different factors including the yeast strain that drives the fermentative process [3–5], due to a genetic predisposition as a result of selective adaptation to certain ecosystems [6].

The yeast population (*S. cerevisiae*) naturally present in the must grapes come from resident microbiota of wine cellar and mainly from endemic microbiota that naturally lives in pruine (cerous grape skin) whose diversity and proportions depend directly of agro-edaphoclimatic conditions of the vineyards [7,8].

Considering that the DRM represents one of the oldest Demarcated Regions of Portugal that secularly produces the famous Madeira Wine (MW) fundamental in the local economy [3].

In this context, the present work focuses on the isolation, molecular identification, and characterization of VOMs from *S. cerevisiae* strains from the DRM, resulting in the first type of study in the region, with the aim of increasing knowledge and conservation of an important part of this vitiviniculture heritage.

2. Materials and Methods

2.1. Materials and Reagents

All the culture mediums were of microbiology use. Yeast peptone dextrose (YPD) agar (yeast extract, 1% *w/v*, peptone, 1% *w/v*, glucose, 2% *w/v*, agar, 1% *w/v*) and YPD broth (yeast extract, 1% *w/v*, peptone, 1% *w/v*, glucose, 2% *w/v*) were purchase from Sigma-Aldrich (St. Louis, MO, USA). Potato dextrose agar (PDA; potato infusion, 20% *w/v*, glucose, 2% *w/v*, agar, 2% *w/v*)) and nutrient agar (NA; peptone, 0.5% *w/v*, beef extract/yeast extract, 0.3% *w/v*, sodium chloride, 0.5% *w/v*, agar, 1.5% *w/v*) were purchase from Merck (Darmstadt, Germany). All the culture mediums were prepared according to manufactured instructions. Glycerol was obtained from Fischer Scientific (Loughborough, UK).

Molecular biology grade and reagents grade were used for species and strains identification. Lithium acetate (LiOAc), potassium acetate (KOAc), tris-hydrochloride (Tris-HCl), D-sorbitol and lyticase enzyme were purchase from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) and tris-EDTA were purchase from Merck (Germany). Sodium dodecyl sulphate (SDS), PCR buffer, deoxynucleotides (dNTPs), DyNAzyme II DNA Polymerase, *Hae*III (*BsuR*I) enzyme, *Hinf*I enzyme, R buffer, GeneRuler 1 kb and 100 bp DNA Ladder were purchase from Thermo Scientific (Waltham, MA, USA). ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTTATTGATATGC-3′) primers were obtained from Eurogentec (Belgium) and RNase was obtained from Macherey Nagel Bioanalysis (Germany). Agarose electrophoresis grade was obtained from Fischer Scientific (Loughborough, UK).

Analytical grade reagents were used for VOMs characterization. Sodium chloride (NaCl) was purchase from Panreac (Spain) and 3-octanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). SPME fiber coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) StableFex was supplied by Supelco (Palo Alto, CA, USA). GC carrier gas, helium of purity 5.5 was purchase from Air Liquide (Portugal). Ultra-pure water was obtained from a Milli-Q® system (Millipore, Bedford, MA, USA) and mQ was purchase from Braun (Germany).

2.2. Sampling

The sampling was performed during 2015 campaign (15 September to 05 October). A total of 10 grape musts were selected and processed from authorized/recommended grapevine varieties for the production of MW, namely: Sercial, Verdelho, Malvasia de São Jorge, Malvasia Cândida, Bastardo, Terrantez, Boal (white grapes) and Tinta Negra (red grapes) from vineyards located in traditional zones of the DRM (Figure 1). The grapes were harvested manually, complying with the quality and

oenological parameters stipulated in the statute of DRM and internal winery processes. The grape musts were obtained through pneumatic press and immediately collected aseptically in sterilized flask.

Figure 1. Sampling zones (varieties): 1—Seixal (Sercial); 2—São Vicente (Tinta Negra); 3—São Vicente (Verdelho); 4—São Jorge (Malvasia de São Jorge); 5—São Jorge (Bastardo); 6—Prazeres (Verdelho); 7—Calheta (Terrantez); 8—Campanário (Boal); 9—Fajã dos Padres (Malvasia Cândida); 10—Jardim da Serra (Sercial) (Madeira Island map adapted from Google Maps. Imagens ©2020 TerraMetrics).

2.3. Microfermentation and S. cerevisiae Isolation

Ten different grape musts (500 mL) were submitted to spontaneous microfermentation according to Schuller et al. [9], with some modification, namely, room temperature (27.9 °C; ±1) and, stirring or orbital shaker (160 rpm). The microfermentations progress was monitored daily through the determination of must mass loss until weight stabilization. Serial dilutions (10^{-2} to 10^{-8}) were spread on YPD plates. After incubation (48 h, 30 °C), 10 isolates were selected based on colonies morphology (corresponding to *S. cerevisiae*) and purified. The pure isolates (100) were stored in YPD broth supplemented with glycerol (30%, *v/v*) at −42 °C until analysis.

2.4. Molecular Identification

2.4.1. DNA Isolation

For the yeast species identification, DNA was isolated and purified according to Lõoke et al. [10], with slight modifications. YPD plates were divided in six sections. Isolates (each pure culture) were spread on each section and incubated (48 h, 30 °C). Lithium acetate-sodium dodecyl sulphate (LiOAc-SDS) solution was used for yeast cell lysis. Stock solution of LiOAc was prepared (200 mM), autoclaved and 1% of SDS was added to this solution. A pure culture was picked from the YPD section plate, suspended in 100 µL of the solution described previously, and incubated at 70 °C, for 20 min. Then, DNA was precipitated adding 300 µL of ethanol (96%), mixed briefly by vortex and then centrifuged at ≈15,000× *g* (approximately) for 3 min. Supernatant was removed and pellet was washed with 500 µL of ethanol (70%). Finally, the dried DNA was suspended in 50 µL of ultrapure autoclaved water. DNA concentration was determined using the NanoDrop 2000c Spectrophotometer (Thermo Scientific™).

For strain identification, DNA was extracted by enzymatic method according to Querol et al. [11]. A pure culture was picked from YPD section plate, suspended in 1 mL of YPD broth, strongly vortexed and incubated overnight at 30 °C. The cells were washed with ultrapure water (Millipore) autoclaved and collected by centrifugation (≈15,000× *g*, 3 min). The pellet was resuspended in 100 µL of sorbitol (1 M), EDTA (100 mM, pH 7.5) and 4 µL of Lyticase enzyme (2.5 mg/mL). The tubes were incubated at 37 °C, 60 min, centrifugated (10,000× *g*, 1 min) and then, 50 µL of tris-HCl (50 mM), EDTA (20 mM, pH 7.4) and 3 µL of SDS (10%, *w/v*) solution were added. The tubes were incubated again at 65 °C, 30 min, immediately, 40 µL of KOAc (5 M) was added, homogenized by inversion, and placed in ice bath (30 min). Finally, the tubes were centrifugated at 4 °C, ≈22,000× *g*, during 15 min. The supernatant

was transferred to a fresh tube, mixed with isopropanol and incubate at room temperature (5 min) and centrifugated (≈9500× *g*, 10 min). The supernatant was discarded, and DNA was washed with ethanol (70%), dried, and dissolved in TE buffer. DNA concentration was determined using the NanoDrop 2000c Spectrophotometer and diluted in TE approximately to 55–60 µg/µL.

2.4.2. PCR-RFLP/5,8S-ITS of rDNA Analysis

ITS1-5,8S-ITS2 fragment was amplified in a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA) using ITS1 and ITS4 primers as described by White et al. [12]. The amplification reactions were performed in 25 µL containing 18.25 µL of mQ; 2.5 µL (1X) of PCR buffer; 2 µL (50 µM) of dNTPs; 0.5 µL (0.5 µM) of each primers; 0.25 µL (0.5 U) of DyNAzyme II DNA Polymerase and 1 µL of DNA.

PCR amplification was carried out according to Esteve-Zarzoso et al. [13]: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primers annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min, followed by cooled at 4 °C. The fragments were digested with the restriction endonucleases *Hae*III (*BsuR*I) and *Hinf*I as recommended by the manufacturer. The PCR products and their restriction fragments were analysed by agarose gel electrophoresis (1.4 and 3% *w/v* respectively). Gels were stained with ethidium bromide and observed under UV light. Digital images were acquired using a Kodak 290C camera.

2.4.3. RFLP—mtDNA Analysis

The digestion was performed according to Shuller et al. [14]. The mix reaction contained 17 µL of DNA (extracted by enzymatic method), 0.5 µL (10 U/µL) of restriction endonucleases *Hinf*I, 2 µL (10X) of R buffer and 0.5 µL (10 mg/mL) of RNase was incubated overnight at 37 °C. The restrictions fragments were separated on agarose gel electrophoresis (1.5% *w/v*) and a 1-kb DNA ladder was used as a molecular size marker. Gels were stained with ethidium bromide and observed under UV light. Digital images were acquired using a Kodak 290C camera.

2.5. VOMs Characterisation

2.5.1. Inoculated Microfermentations

Two dominant *S. cerevisiae* strains were selected for the VOMs characterization. Commercial strain QA23 (Lavin) was selected as positive control. 10^5 and 10^6 cells/mL were inoculated in commercial rectified concentrated grape must clarified (MC), diluted in mQ to 12.5% of probable alcohol. Before the assay, the MC was subject to microbiological control on YPD, PDA and NA plates. Inoculated microfermentations were carried out in homologues conditions (28 °C, 160 rpm). The evolution of the microfermentations was monitored by weight loss until stabilization. At the end of microfermentations, the wines were clarified and refrigerated at 4 °C and analyzed for no more than 12 h.

2.5.2. HS-SPME Conditions

The extractions conditions, namely, sample volume, sample temperature, ionic strength, fibre type and contact time between fibre and headspace were carried out according to Perestrelo et al. [15] with some modifications. 3-octanol (1:1, *v/v*) was selected as internal standard, 10 mL for sample volume, 20% NaCl and 10 µL of internal standard. The VOMs extraction was performed with a DVB/CAR/PDMS StableFex fiber in headspace mode at 40 °C during 40 min under continuous stirring (9000 rpm).

2.5.3. GC-MS Conditions

The VOMs separation was performed in an Agilent Technologies 6890N (Palo Alto, CA, USA) gas chromatography, equipped with a fused silica capillary column BP20 (SGE, Dortmund, Germany) of 60 m × 0.25 mm I.D. × 0.25 µm film thickness. Helium at a flow of 1 mL/min was used as carrier gas.

The temperature gradient used was: 45 °C during 1 min, increasing 2 °C/min until 100 °C maintaining this temperature for 3 min, following an increase of 5 °C/min to 130 °C and remain for 5 min, rising again 20 °C/min until 220 °C for 15 min. The total run time was 72 min [15].

The VOMs detection was performed using an Agilent Technologies 5975N quadrupole mass selective detector operated in electronic impact ionization (EI) mode at 70 eV and mass spectra was recorded in the range of 30–300 *m/z*. The ions source, transfer line, and quadrupole temperatures were 220, 250, and 150 °C, respectively.

The VOMs identification was performed by comparison of mass spectra with NIST 05 Al library. The VOMs with percent of similarity >75% were considered for the present analysis. Additionally, some VOMs were identified and confirmed by comparison of Kovats retention index (KI) value with the reported in the literature for similar chromatographic columns. The KI was calculated using the Equation (1), where "*n*" is the number of carbon atoms present in the alkane with *i* carbon atoms (*i*), *N*, the number of carbon atoms relative to alkane *i* + 1, *t'r (n)* the retention time adjusted for minor alkane, and *t'r (N)* the retention time adjusted for major alkane. The retention times used in the equation correspond to the times obtained by direct injection of the C8–C20 *n*-alkane series.

$$\mathrm{KI} = 100\left[n + (N-n)\frac{logt'_r(d) - logt'_r(n)}{logt'_r(N) - logt'_r(n)}\right] \quad (1)$$

3. Results

3.1. Spontaneous Microfermentation and Yeast Isolation

All the samples fermented spontaneously. The microfermentations had a loss of mass between 31.25 and 80.50 g/L and a duration between six and 12 days. Three types of fermentative profiles were obtained as shown in Figure 2.

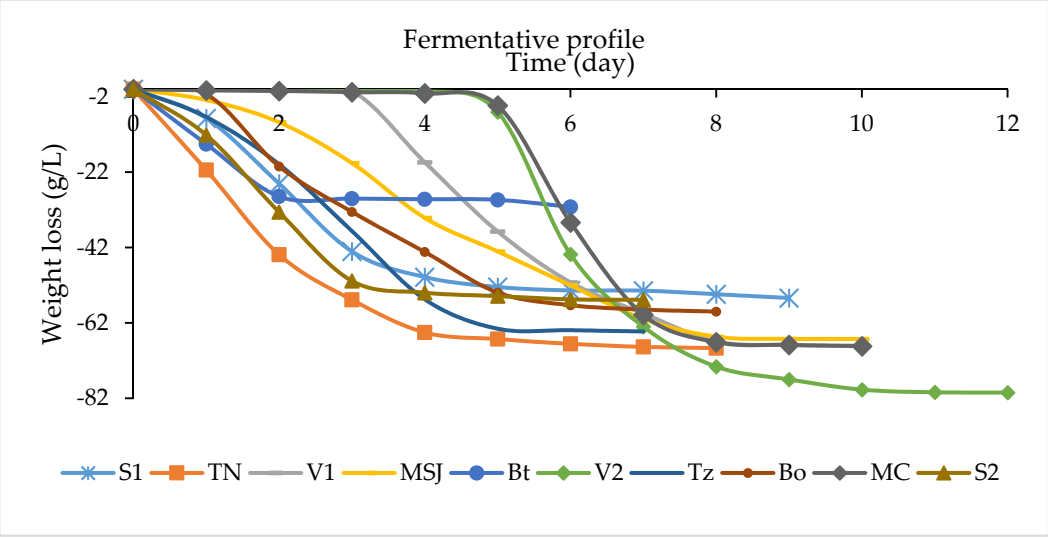

Figure 2. Fermentative profile **S1**—Sercial 1 (Seixal); **TN**—Tinta Negra; **V1**—Verdelho 1 (São Vicente); **MSJ**—Malvasia de São Jorge; **Bt**—Bastardo; **V2**—Verdelho 2 (Prazeres); **Tz**—Terrantez; **Bo**—Boal; **MC**—Malvasia Cândida; **S2**—Sercial 2 (Jardim da Serra) (average standard deviation for each point is lower than 12%).

In the first type, the fermentations started in the 3rd or 4th day followed by a significant weight loss, this profile was observed in Malvasia Cândida, Verdelho 1 (Prazeres) and Verdelho 2 (São Vicente) varieties. The second type showed regular loss weight during all fermentation process. This pattern

was observed in Malvasia de São Jorge variety. In the third type, the fermentations started quickly with significant weight loss until stabilization. This profile was observed in the remaining samples (Figure 2).

After microfermentations, the number of viable yeasts per milliliter was estimated for each wine (Table 1).

Table 1. Number of colonies obtained for each microfermented wine.

N°	Grape	Zone (*)	Id.	CFU/mL
1	Sercial	Seixal	S1	4.20×10^6
2	Tinta Negra	São Vicente	TN	1.20×10^7
3	Verdelho	São Vicente	V1	5.30×10^5
4	Malvasia SJ	São Jorge	MSJ	1.55×10^7
5	Bastardo	São Jorge	Bt	1.26×10^9
6	Verdelho	Prazeres	V2	9.70×10^8
7	Terrantez	Calheta	Tz	1.37×10^8
8	Boal	Campanário	Bo	4.70×10^2
9	Malvasia Cândida	Fajã dos Padres	MC	7.70×10^5
10	Sercial	Jardim da Serra	S2	4.70×10^6

(*)—Vineyard locations; Id.—identification of sample, CFU/mL—Colony-forming-unit per millilitre.

The lowest concentration of yeasts per mililitre was obtained for Boal variety (470 CFU/mL), followed by Verdelho 1 (530,000 CFU/mL) and Malvasia Cândida (770,000 CFU/mL). In the remaining wines, yeasts were quantified in an order of magnitude in the millions of CFU/mL. Sercial 1, Sercial 2, Tinta Negra and Malvasia de São Jorge were quantified in 4.2, 4.7, 12, and 15.5 million CFU/mL, respectively. However, the highest concentration of yeasts was found in the Bastardo (1260 million CFU/mL) followed by Terrantez and Verdelho 2 in which 137 and 970 million CFU/mL were quantified.

3.2. Molecular Identification

From 10 microfermentations, 100 isolates whose macromorphological colony characteristics corresponding to the description of *S. cerevisiae* were tested. The DNA concentration obtained was between 700–100 ng/µL and an average degree of purity of 1.9 (d_s 0.05) for the genus and species identification. The DNA concentration for the strain identification was diluted until 5500–6000 ng/µL and an average degree of purity of 2.0 (d_s 0.10). DNA concentration and purity were measured by NanoDrop 2000c Spectrophotometer.

Regarding genus identification by 5.8S-ITS of rDNA amplification, the amplicon obtained in the isolate analysis corresponded to a fragment between 850–880 pb, after visualization in agarose gel (Figure 3).

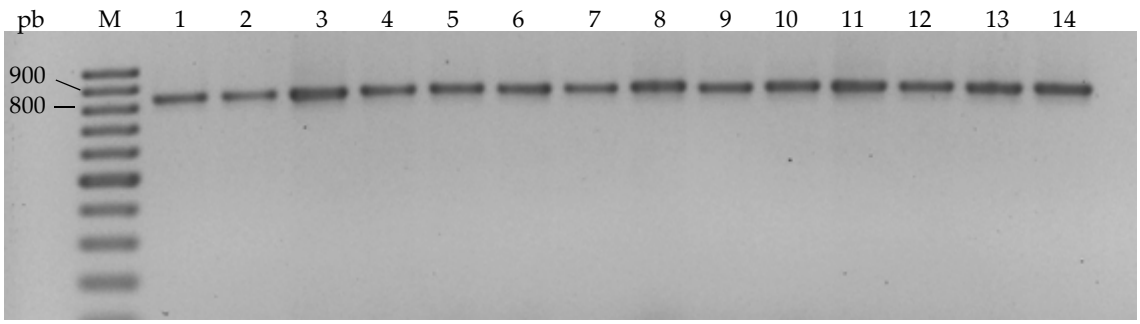

Figure 3. Amplification of ITS-5.8S region of rDNA; 1–M: 100 bp GeneRuler DNA Leader; 1–14: characteristic fragment obtained from isolated analysed; agarose gel (1.4%).

Regarding species identification, the amplicon digested with restriction endonucleases resulted in restriction pattern with fragments with approximately 320, 230, 180, and 150 pb with HaeIII enzyme, and 365 and 155 pb with the HinfI enzyme (data no presented).

From 100 isolates of *S. cerevisiae*, 8 RFLP-mtDNA patters were identified (assigned as A to O), each pattern was considered as a strain. Pattern B occurred in 81% of the isolates and pattern A was identified in 11% (Figure 4). The pattern C and L were identified in 2% while other four patterns occurred only in 1% of the isolates. We also identified the presence of two or three isolates in most microfermentations: strains B and O in Malvasia (São Jorge), B and A in Tinta Negra (São Vicente), and B and C in Verdelho (Prazeres), all with 9:1 proportion, B and A in Sercial 2 (Jardim de Serra) with 6:4 proportion, and B with K in Malvasia Cândida (Fajã dos Padres) with 6:4 proportion. In two microfermentations, three strains were identified: B, A and L in Sercial 1 (Seixal) with 7:2:1 proportion and B, A and M in Verdelho (São Vicente) with 6:3:1 proportion, respectively. In Boal and Bastardo varieties, strain B was identified, and on Terrantez B, A, L, and N strains were identified in 7:1:1:1 proportion, respectively.

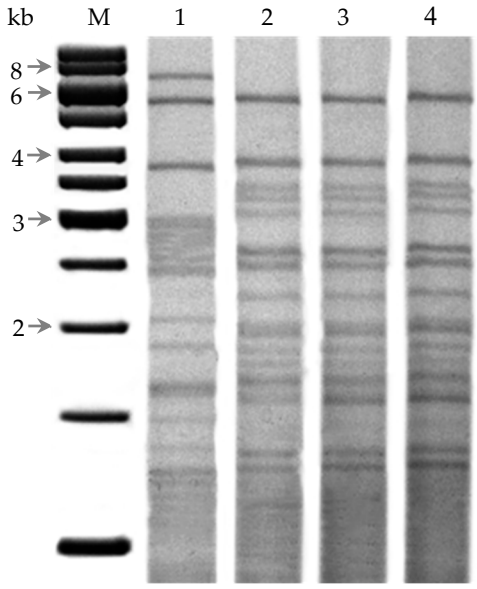

Figure 4. RFLP-mtDNA restriction patters which *Hinf*I enzyme. Line 1—M: 1 kb GeneRuler DNA Leader; 1—strain A, 2–4—strain B; agarose gel (3%).

The dominant strains were A and B. Strain A was identified at Sercial 1, Tinta Negra, Verdelho 1, Terrantez and Sercial 2 grape musts microfermentations. Strain B was identified in all grape must microfermentations.

3.3. VOMs Characterization

Inoculated Microfermentations

Supplementary Figure S1 shows the overlap of the chromatograms obtained for A and B strains. For better visualization of the VOMs biosynthesized by each strain, the MC chromatogram was included. The chromatogram of strain B is more complex with a greater number and more intense peaks, followed by the control whose chromatogram (data no shown) presents the same number of peaks than B, although somewhat less intense. Finally, the chromatogram of strain A showed the least number of peaks and the lowest intensity.

Regarding to total GC peak areas, the results expressed in arbitrary units (a.u.) were 2.28×10^8 for MC; 8.53×10^8 for strain A; 1.72×10^9 for strain B and 1.23×10^9 for the control strain. Figure 5 shows the total areas of VOMs identified from each strain and positive (C) and negatives (MC) controls.

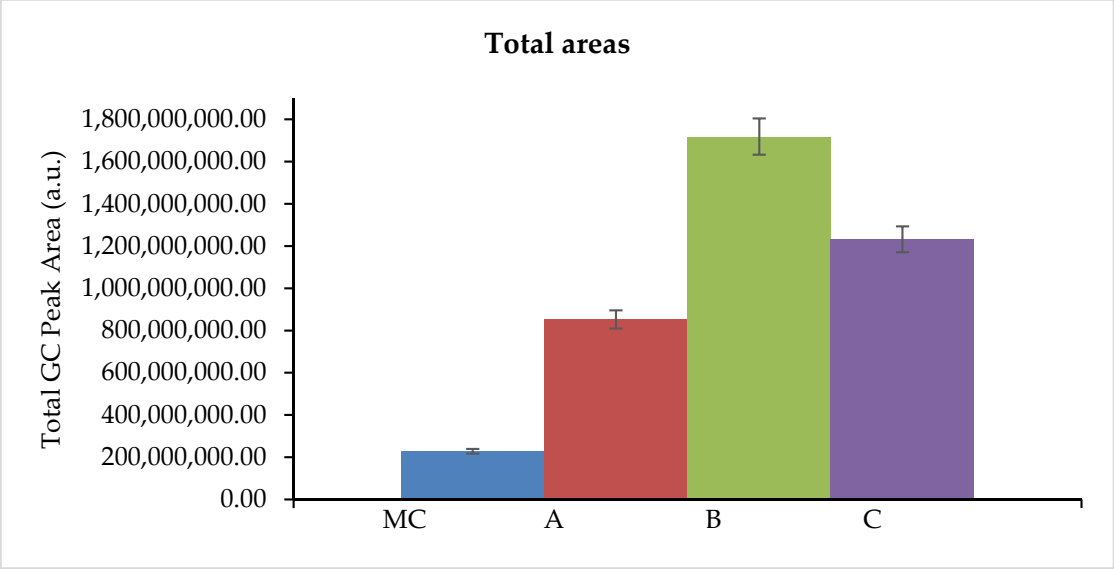

Figure 5. Total areas obtained by HS-SPME/GC-MS analysis, MC—Commercial rectified concentrated grape must (clarified and diluted until 12.5% of probable alcohol), A—Strain A; B—Strain B; C—Control strain C (strains identified by RFLP-mtDNA).

As expected, the volatile fraction increased after fermentation. The A, B and C registered an increase of 374%, 540%, and 754% respectively, in comparison to the volatile fraction of MC. The highest production of secondary metabolites was obtained by strain B whose volatile fraction exceeded about 100% the volatile fraction of A and approximately 27% of the control strain C.

The VOMs identified were grouped by chemical families as follows: esters, high alcohols, carbonyl compounds, fatty acids, terpenes and miscellaneous. Table 2 shows the contribution of each chemical family to the total volatile profile and the number of compounds identified.

Table 2. Percentage (%) of VOMs for each chemical family to the total volatile profile (in brackets the standard deviation).

	Total Volatile Fraction (%)					
Strain/Substrate	**Esters**	**High Alcohols**	**Carbonyl Compounds**	**Fatty Acids**	**Terpenes**	**Miscellaneous**
MC	4.10 (8)	1.11 (2)	89.07 (11)	0.78 (1)	3.02 (6)	1.92 (4)
A	15.85 (15)	63.18 (10)	4.80 (12)	9.24 (5)	0.77 (6)	6.10 (4)
B	23.13 (16)	54.22 (12)	3.84 (12)	15.03 (7)	1.24 (6)	2.87 (7)
C	23.77 (17)	54.92 (11)	2.49 (13)	10.48 (6)	0.95 (7)	7.38 (6)

Before fermentation, MC volatile profile showed that carbonyl compounds are the major chemical family, representing 89%, followed by esters (4.10%) and terpenes that represent approximately 3%. Miscellaneous and higher alcohols represent 1.92 and 1.11%, respectively. In a lesser extent, fatty acids (0.78%). The most abundant volatile compound is 3-octanone (74.76%), followed by cyclohexane representing 9.82% of the total volatile fraction. Between 1% and 2% are benzaldehyde, 1,1-diethoxy-ethane, and 4-ethylresorcinol (Table 3). The remaining compounds represent less than 1% each.

Table 3. VOMs identified by HS-SPME/GC-MS analysis, obtained from wines inoculated with pure strains (RFLP-mtDNA), indicating the contribution of each compound in the chemical family and in the total volatile fraction of the wine, as well as the olfactory descriptor.

N°	RT (min)	KI	Compound Families/VOMs	Id	Total Volatile Fraction (%)								Aromatic Descriptors
					MC		A		B		C		
			ESTERS		**PRF**	**PRT**	**PRF**	**PRT**	**PRF**	**PRT**	**PRF**	**PRT**	
1	6.793	1113	Ethyl acetate	MS	25.589	0.983	8.591	1.362	6.881	1.592	5.716	1.359	Fruity, buttery [16]
2	10.913	1216	Ethyl butanoate	MS	10.295	0.395	1.008	0.160	0.563	0.130	0.677	0.161	Apple [17]
3	14.627	1290	Isoamly acetate	MS	2.958	0.114	9.035	1.432	3.458	0.800	4.663	1.108	Fresh, banana, pear [16]
4	20.831	1396	Ethyl hexanoate	MS	9.468	0.364	14.743	2.337	7.640	1.768	10.070	2.393	Fruity, strawberry, anise [16]
5	23.413	1442	Ethyl 5-hexanoate	MS	-	-	0.610	0.097	0.286	0.066	0.163	0.039	-
6	26.982	1500	Ethyl heptanoate	MS	-	-	0.259	0.041	0.228	0.053	0.101	0.024	Fruity [17]
7	33.725	1612	Ethyl octanoate	MS	24.993	0.960	33.605	5.328	43.386	10.037	45.557	10.828	Floral, menthol, anise [16]
8	36.369	1659	Ethyl 7-Octanoate	MS	-	-	1.282	0.203	0.885	0.205	0.566	0.135	-
9	38.914	1702	Ethyl nonoate	MS	-	-	-	-	0.261	0.060	0.261	0.062	-
10	39.661	1717	Ethyl-3-(methylsulfanyl) propanoate	MS	-	-	-	-	0.112	0.026	-	-	Onion, sulfurous, pineapple [18]
11	44.266	1800	Ethyl decanoate	MS	10.757	0.413	6.030	0.956	15.957	3.691	13.388	3.182	Fruit, grape [16]
12	45.212	1819	3-Methylbutyl octanoate	MS	-	-	-	-	-	-	0.077	0.018	Sweet fruit [18]
13	45.421	1823	Diethyl Butanedioate	MS	14.708	0.565	3.719	0.590	3.284	0.760	3.478	0.827	Wine, fruit [18]
14	46.057	1835	Ethyl hexadecanoate	MS	-	-	-	-	-	-	0.193	0.046	-
15	46.300	1840	Ethyl decanoate	MS	-	-	3.942	0.625	6.223	1.440	2.212	0.526	Fruit, fat
16	49.130	1892	Ethyl benzenoacetoate	MS	-	-	0.415	0.066	0.312	0.072	0.311	0.074	Sweet, cinnamon, waxy [16]
17	49.947	1908	2-Phenylethyl acetate	MS	-	-	14.633	2.320	8.957	2.072	9.217	2.191	Rose, honey, tobacco
18	50.945	1927	Ethyl dodecanoate	MS	1.234	0.047	2.129	0.338	1.567	0.362	3.349	0.796	Leaf, mango [16]
			ALCOHOLS										
19	10.809	1214	1-Propanol	MS	-	-	0.692	0.426	0.541	0.280	0.729	0.416	Fruit, plastic, penetrating [16]
20	13.204	1264	2-Methyl-1-propanol	MS	-	-	8.398	5.176	8.147	4.222	4.772	2.724	Leek, glue, liquor [16]
21	15.257	1301	1-Butanol	MS	-	-	-	-	0.107	0.056	0.087	0.050	Medicines, fruit [17]
22	19.131	1370	3-Methyl-1-butanol	MS	-	-	71.005	43.761	65.329	33.854	65.293	37.280	Balsamic, astringent [16]
23	29.132	1537	3-Ethoxy-1-propanol	MS	-	-	0.061	0.038	0.042	0.022	0.293	0.168	Fruit [18]
24	36.525	1662	2-Ethylhexanol	MS	74.718	0.792	0.096	0.059	0.087	0.045	-	-	Soft, oily, floral [16]
25	42.597	1771	2-(2-Ethoxyethanol)	MS	-	-	0.104	0.064	0.062	0.032	0.082	0.047	slightly ethereal [18]
26	44.909	1813	1-Nonanol	MS	-	-	-	-	0.072	0.038	0.054	0.031	Fresh, clean [18]
27	46.874	1851	Methionol	MS	-	-	0.214	0.132	0.444	0.230	0.378	0.216	Sweet, potato [16]
28	47.211	1857	2-Undecanol	MS	-	-	0.023	0.014	0.042	0.022	0.127	0.072	Fresh, fat [18]
29	52.219	1952	2-Phenylethanol	MS	25.282	0.268	19.406	11.960	25.128	13.022	28.185	16.093	Honey, floral, roses [17]
			CARBONYL COMPOUNDS										
30	4.900	1071	Acetaldehyde	MS	0.358	0.285	12.413	0.524	8.499	0.287	27.120	0.581	Fresh, fruity [16]
31	5.766	1089	Butanal	MS	-	-	0.517	0.022	0.547	0.018	0.532	0.011	Chocolate [18]
32	7.409	1131	3-Methylbutanal	MS	0.631	0.502	-	-	-	-	0.847	0.018	Caramel, cream, pineapple [16]
33	15.013	1296	4-Methyl-3-penten-2-one	MS	0.559	0.444	-	-	-	-	-	-	Flowers [17]
34	17.676	1346	2-Heptanone	MS	0.212	0.168	-	-	-	-	-	-	-

Table 3. *Cont.*

N°	RT (min)	KI	Compound Families/VOMs	Id	Total Volatile Fraction (%)								Aromatic Descriptors
					MC		A		B		C		
			ESTERS		**PRF**	**PRT**	**PRF**	**PRT**	**PRF**	**PRT**	**PRF**	**PRT**	
35	19.107	1369	4-Methyl-2-heptanone	MS	0.256	0.204	-	-	-	-	-	-	Almond, toasted sugar [16]
36	21.278	1403	4,6-Dimethyl-2-heptanone	MS	0.798	0.635	-	-	-	-	-	-	-
37	22.007	1417	3-Octanone	MS	94.423	75.091	74.797	3.158	80.984	2.732	54.653	1.171	Mushrooms, hebaceous [17]
38	23.546	1444	3-Hydroxy-2-butanone	MS	-	-	1.261	0.053	1.410	0.048	6.140	0.132	Fat, milk [18]
39	24.029	1452	Octanal	MS	0.185	0.147	-	-	-	-	-	-	-
40	30.304	1557	2-Nonanone	MS	-	-	0.610	0.026	0.686	0.023	2.952	0.063	Fruit, fresh [18]
41	36.990	1670	Decanal	MS	0.850	0.676	1.656	0.070	1.455	0.049	2.417	0.052	Apple, rose, honey [16]
42	37.719	1682	Benzaldehyde	MS	1.555	1.236	4.330	0.183	4.831	0.163	1.152	0.025	-
43	41.930	1759	2-Undecanona	MS	-	-	-	-	-	-	4.188	0.090	Fruit, fat [18]
44	49.827	1905	3,5-Dimethylbenzaldehyde	MS	-	-	2.312	0.098	1.587	0.054	-	-	-
45	50.858	1926	2,4,6-Trimethyl acetophenone	MS	0.173	0.138	2.106	0.089	-	-	-	-	-
			ACIDS										
46	34.506	1626	Acetic acid	MS	-	-	63.237	5.846	53.430	8.030	54.558	5.718	Sour [17]
47	40.355	1730	2-Methyl propanoic acid	MS	-	-	5.992	0.554	4.228	0.635	2.730	0.286	Sour cheese [18]
48	43.524	1788	Butanoic acid	MS	-	-	-	-	0.342	0.051	0.618	0.065	Acetic, cheese [18]
49	50.847	1925	Hexanoic acid	MS	100.000	0.654	2.340	0.216	12.798	1.923	5.384	0.564	Sweet [17]
50	55.032	2104	Octanoic acid	MS	-	-	18.874	1.745	13.519	2.032	19.896	2.085	Sweet, cheese [17]
51	58.788	2182	Decanoic acid	MS	-	-	9.555	0.883	13.033	1.959	16.813	1.762	Rancidity, fat [17]
52	67.582	2362	Hexadecanoic acid	MS	-	-	-	-	2.650	0.398	-	-	Oily [17]
			TERPENES										
53	14.213	1283	Geranyl oxide	MS	5.657	0.164	-	-	-	-	-	-	-
54	18.931	1366	D-Limonene	MS	0.642	0.019	-	-	-	-	-	-	Citrus, lemon [17]
55	39.068	1705	Linalool	MS	2.456	0.071	10.124	0.078	8.629	0.107	3.534	0.034	Citrus, floral
56	40.259	1728	4-Ethylresorcinol	MS	49.511	1.435	-	-	-	-	5.176	0.049	-
57	48.052	1873	Dehydro-air-ionene	MS	26.521	0.769	-	-	-	-	-	-	Liqueur [18]
58	48.391	1879	Nerol	MS	-	-	4.481	0.035	6.769	0.084	3.669	0.035	Floral, sweet [18]
59	48.606	1883	R-Citronelol	MS	-	-	16.249	0.125	8.396	0.104	45.597	0.434	-
60	50.717	1923	Cis-Geraniol	MS	-	-	15.477	0.119	16.274	0.201	4.880	0.046	Floral, sweet [18]
61	51.015	1929	Geranyl acetone	MS	15.213	0.441	-	-	-	-	-	-	-
62	54.716	1998	E-Nerolidol	MS	-	-	20.593	0.159	13.315	0.165	14.828	0.141	Citrus, wood [18]
63	60.577	2220	Farnesol	MS	-	-	33.076	0.255	46.617	0.577	22.315	0.213	Fresh, sweet [17]
			MISCELLANEOUS										
64	4.623	1065	Etoxyethane (isomer I)	MS	-	-	0.703	0.056	-	-	0.630	0.052	-
65	4.828	1069	Etoxyethene (isomer II)	MS	-	-	1.634	0.129	1.343	0.069	2.394	0.198	-
66	5.100	1075	Cyclohexane	MS	82.049	9.861	5.055	0.400	6.183	0.316	1.715	0.142	Fruit, crema [17]
67	5.897	1091	Ethyl hydrogenoxalate	MS	-	-	0.920	0.073	0.283	0.014	-	-	-
68	6.914	1117	1.1, Diethoxy-ethane	MS	14.321	1.721	64.828	5.125	41.600	2.128	76.575	6.331	Ether, nuts
69	11.057	1220	Toluene	MS	-	-	-	-	1.390	0.071	0.654	0.054	Ink [17]
70	14.045	1280	1-(1-Ethoxyethoxy)-pentane	MS	-	-	6.979	0.552	3.458	0.177	10.157	0.840	-

Table 3. *Cont.*

N°	RT (min)	KI	Compound Families/VOMs	Id	Total Volatile Fraction (%)								Aromatic Descriptors
					MC		A		B		C		
			ESTERS		PRF	PRT	PRF	PRT	PRF	PRT	PRF	PRT	
71	33.334	1604	1,3-Bis (1,1-dimethylethyl)-benzene	MS	-	-	-	-	1.194	0.061	0.436	0.036	-
72	43.005	1778	Butyrolactone-dihydro-2 (3H)-furanone	MS	-	-	-	-	0.394	0.020	0.368	0.030	-
73	43.613	1789	Hexadecane	MS	-	-	-	-	0.743	0.038	-	-	-
74	45.113	1817	2,6-Dimethyl, 2,6-octadiene	MS	-	-	-	-	-	-	1.000	0.083	-
75	48.548	1882	Cyclodecane	MS	1.908	0.229	-	-	0.664	0.034	-	-	Sweet, fresh [16]
76	56.808	2142	2,6-Diisopropylnaphthalene	MS	1.722	0.207	-	-	-	-	-	-	-
77	59.492	2197	2,4-bis(dimethylethylphenol)	MS	-	-	19.881	1.572	42.748	2.187	6.071	0.502	Phenolic [18]

Tr—Retention time (min), KI—Kovats index, Id—compound identification method, MS—Mass spectrometry, MC—rectified concentrated must diluted to 12% (*v/v* of probable alcohol), A—Strain A, B—Strain B, C—Control strain, PRF—Percentage of the compound relative to the family; PRT—Percentage of the compound in relation to the total profile.

After fermentations, between 77% and 79% of the identified VOMs belong to higher alcohols and esters. Higher alcohols are found in a greater percentage, especially in strain A, representing 63.20%, while in B and C they are found in a similar proportion (54% approximately). In relation to esters, strains B and C have the highest proportion (about 23%), while A has about 16%.

Minor chemical families represent between 20 and 23% of the identified VOMs. Of this fraction, fatty acids are the most abundant VOMs, representing approximately 15.0, 10.5 and 9.2 in strains B, C and A, respectively. The miscellaneous compounds represent 7.4% and 6.1% in C and A respectively, and in strain B they represent only 2.9%. The carbonyl compounds showed more heterogeneous results, representing 4.8%, 3.8%, and 2.5% in A, B, and C respectively. Terpenes are the minor chemical family with the lowest percentage (<1.2%) in all samples.

Regarding the number of VOMs, were identified 78, from which 22 were carbonyl compounds, 18 esters, 12 higher alcohols, 11 terpenes, seven fatty acids, and eight miscellaneous. In the MC, 33 VOMs were identified and, in each wine fermented by pure strain, a total of 53 VOMs were identified in the A, and 60 in the B and C, respectively.

It is verified that the studied volatile profile is composed of three majority VOMs, representing approximately 57% of the total volatile fraction in B and 61% in A and C. The chemical family with the greatest contribution is the higher alcohols, representing 55.7% of the total volatile fraction of A, approximately 47% of B and 50.8% of control. Moreover, 3-methyl-1-butanol was the major compound, mainly in A (44%) and in a smaller percentage in B and C (between 34% and 36%). The second major VOM was 2-phenylethanol (flowers odour), which represents about 12%, 13%, and 15% of the total volatile fraction of A, B, and C, respectively. The third major identified volatile was ethyl octanoate, contributing approximately 10–11% to the total volatile profile of B and C. However, in A it is present in a smaller percentage (5.3%). The fourth most abundant VOM was acetic acid, contributing 8% of the total volatile fraction of B and approximately 5.8% of A and C (Table 3).

4. Discussion

Several demarcated regions of the world present in Portugal, Spain, and Italy [9,19,20], and emerging wine regions in Chile and Australia [21], characterized molecular and technologically endemic strains of *S. cerevisiae*. However, this is the first study carried out at the DRM.

Through 5.8S-ITS fragment analysis, we verified that all isolates belonged to the species *S. cerevisiae*, since an amplified and specific restriction fragments were obtained for this species [13,22–26].

Since the must grapes were fermented under similar conditions, it appears that the different fermentative profiles may be due to the chemical composition of the must [11] and/or the association of strains during fermentation. The fermentation process is completed by one to three strains as reported by Versavaud et al. [27] and with some results obtained by Schuller et al. [9]. The concentration of viable cells/mL of waste varies in all varieties studied, probably agro-edafoclimatic factors may be directly related to this CFU difference. Fermentation can be affected not only by the substrate, in this case by the grape variety, but also by the flora endemic on the grape surface and symbiosis, variety/ecosystem.

4.1. Intraspecific Variability

The success of spontaneous microfermentation (100%) in this study was the same reported by Cappello et al. [22] and Blanco et al. [28] while this value was much lower (60%) in the work of Schuller et al. [9]. On the other hand, they reported a higher rate of intraspecific variability (18.3%) compared to the present study (8%) and to those reported by Cappello et al. [22] and Blanco et al. [28] (2.3% and 4.3%, respectively).

These differences may be due to the sampling methodology used in the different studies. It appears that *S. cerevisiae* from cellar environments have a very high rate of microfermentation but a lower rate of intraspecific variability. These results possibly suggest that some (endemic) strains may be more susceptible to enological treatment, making a first selection in an important technological parameter.

The RFLP-mtDNA restriction patterns obtained in the present work do not coincide with the RFLP-mtDNA patters in the consulted bibliography, until the date of completion of this work.

4.2. Screening of Volatile Profile

In total, 46 VOMs were biosynthesized by yeasts, mainly higher alcohols and esters, and to a lesser extent, fatty acids, miscellaneous, and some carbonyl compounds. In addition, 8 esters present in the wort in a low percentage, were obtained in wines in higher percentage, so it is inferred that the surplus was the product of biosynthesis by the respective yeast. Also, three VOMs are possibly used as precursors in different metabolic pathways, since they were in a high percentage in the wort, decreasing drastically after fermentation as is the case of 3-octanone. In the particular case of terpenes, it appears that they were possibly subject to modifications, probably metabolic, since they are present in the must and absent in the wine.

4.2.1. Higher Alcohols

After fermentation, the volatile fraction corresponding to the higher alcohols is predominantly the most representative [5,29]. This result agrees with those obtained in the present study (Table 3). Higher alcohols are biosynthesized by yeasts during alcoholic fermentation, in two ways, namely via the amino acid catabolism or Ehrlich mechanism [4,7,30], and through the anabolic route from α-keto acids [4,7].

It was found that the production of higher alcohols, both in quantitative terms and in diversity of VOMs, it is strongly conditioned by the *S. cerevisiae* strain that carried out the fermentation process, since, on identical substrates and similar fermentation conditions, different profiles were obtained, both in the contribution of alcohols to the total volatile fraction (about 54% in B and C, and 64% in A), and in the number of higher alcohols synthesized by each strain (10, 12, and 11 in A, B, and C respectively (Table 2)), and also in the individual proportion of each alcohol within the chemical family itself, and in the total volatile profile (Table 3).

Generally, 3-methyl-1-butanol, the main aliphatic alcohol, represents between 40–72% of the volatile fraction of higher alcohols. In this study, it represented 62–70% of this fraction, concluding that the results obtained is in accordance with the percentage range described in the literature [4,29]. Moreover, 3-Methyl-1-butanol mimics the smell of bitter, balsamic, and astringent and is produced from leucine catabolism [18,30].

MW in the manufacturing process (younger non-marketable), was also found as the majority VOC, extracted and identified using the same technique used in the present study [31].

The second VOM in percentage is 2-phenylethanol (Table 3). This phenolic alcohol is biosynthesized from phenylalanine of great oenological interest since it is associated with aromatic descriptors that imitate the smell of roses [4,31] evolving into honey, contributing to the bouquet of some aged wines [4].

Regarding the screening of the volatile profile from VM of the Malvasia variety [32] it was verified through the respective chromatogram, that the peak referring to 3-methyl-1-butanol and 2-methyl-1-butanol is the seventh most intense peak in wines aged for 20 years (1988), and the fifth most intense peak in wines aged for two years (2006). Additionally, it is still the second most intense peak belonging to the chemical family of higher alcohols. In the same study, 2-phenylethanol is the third most intense peak in wines aged for 20 years, and the most intense peak in wines aged for two years, being the most representative of higher alcohols. In this sense, it was inferred that its biosynthesis possibly occurs from the initial stage of alcoholic fermentation, since VM Malvasia belongs to the type of sweet wine [32]. The literature also reports that the characteristic heating process in VM does not substantially modify the percentage of this chemical family [33].

Higher alcohols are also important in esterification reactions with carboxylic acids, precursors responsible for the formation of odorant esters, with a positive impact on the wine's aroma [4,30], especially in the bouquet.

4.2.2. Esters

A chemical family with positive aromatic descriptors, which mimic aroma of fruits and flowers, sometimes found in small percentages in the grape. Its abundance in wine results mainly from the secondary metabolism of *S. cerevisiae* during alcoholic fermentation [4].

In the present study, fatty acid ethyl esters are the most common. Ethyl octanoate, which gives a fruity aroma and anise [16], is the major ester in the three strains analyzed, representing approximately 10% of the total volatile fraction in wines produced by strains B and C. However, in wine produced with A, it represents just over 5% confirming the influence of the strain on the biosynthesis of this VOM of oenological interest. Ethyl decanoate obtained the second highest percentage in B and C (about 3.2%), however, in A the value was <1%. Ethyl hexanoate, on the other hand, represented about 2.3% in A and C, reducing its synthesis in B.

Ethyl acetate, isoamyl acetate and 2-phenylethyl acetate were the only esters identified, belonging to the group of higher alcohol acetates, being strain C with the highest percentages of these volatiles (5.1%). These results can be explained by the enzymatic esterification reaction between acetic acid and the corresponding higher alcohol, as well as the influence of the strain on the biosynthesis of these compounds [5].

4.2.3. Fatty Acids

In general, strain A has the lowest percentage of fatty acids and, in opposite, strain B showed the highest percentage and the largest number of these VOMs (Table 3).

Acetic acid is the largest volatile fatty acid in wines, representing about 90% of this chemical family [4]. However, in the present study only about 54% of the fatty acid fraction was identified in strains B and C. The same was verified by Jiang et al. [29] which obtained between 45% and 60% of this compound, differing slightly with the results obtained in A (63%). Mauriello et al. [5], analyzed 36 strains of *S. cerevisiae*, in which acetic acid represented between 10% and 100% of the chemical family of fatty acids, and in other cases was not identified, with a relationship between the geographical area from which they were isolated and the production of acetic acid. The contribution of acetic acid to the total volatile profile is approximately 5.8% in A and C, whereas in B, a percentage of about 8% was registered.

In cellar environments, the conditions that provide the greatest biosynthesis of this compound are reported in wines whose musts have been clarified and fermented under anaerobic conditions [4]. According to Gayon et al. [7] and Garden-Cedal et al. [34], *S. cerevisiae* biosynthesizes low percentages of acetic acid in musts produced from grapes in good phytosanitary conditions and moderate levels of initial sugar (<13% *v/v* of probable alcohol), depending on the strain. The octanoic acid is the second fatty acid in percentage, registered in the three strains studied, whereas, the decanoic acid was biosynthesized in a greater percentage in the strains B and C. Regarding hexanoic acid, it was biosynthesized to a greater extent by strain B.

4.2.4. Carbonyl Compounds

A chemical family composed mainly of aldehydes and ketones, in which 3-octanone is the major carbonyl compound in MC and the most representative volatile organic compound identified in the wines under study. 3-Octanone mimics the aroma of mushrooms and herbaceous aromas [17].

Probably, the presence of 3-octanone in wines is due to the substrate and not to biochemical or chemical reactions.

Considering the carbonylated compounds product of the metabolism of *S. cerevisiae*, they are found in a very low percentage, representing about 1.7%, 1.0%, and 1.2% of the total volatile fraction of A, B, and C, respectively, with acetaldehyde being the major carbonyl compound, followed by benzaldehyde.

4.2.5. Terpenes

This chemical family constitute the varietal or primary aroma, associated with positive aromatic descriptors, mainly floral aromas [35].

Except for linalool identified only in strain C, it is observed that the terpenes identified in the MC were not found in wines. In this sense, it is concluded that these VOMs underwent rearrangements, resulting in a product of chemical reactions due to fermentation conditions (pH, ethanol content, etc.) [35]. On the other hand, evidence shows the biotransformation and biosynthesis of specific terpenes by *S. cerevisiae* under winemaking conditions [36–38].

5. Conclusions

The strains of *S. cerevisiae* play a fundamental role in the quality and typicality of wines produced in Demarcated Regions, through biosynthesis of VOMs with an impact on wine aroma, whose diversity and proportion depend closely on the yeast strain that drives the fermentative process.

Strains residing in wine-growing environments (grapes and wineries) have genetic variability adapted to the specificities of the ecosystem, which are reflected in the final product. Thus, in order to produce high quality wines, highlighting the typical characteristics, several studies have been carried out in the molecular identification and VOMs characterization of indigenous strains of *S. cerevisiae* from several demarcated regions. To date, to the best of our knowledge, no scientific study has been reported on this subject in DRM.

In this first approach, most of the VOMs (extracted and identified through HS-SPME/GC-MS) produced during alcoholic fermentation, persist in aged MWs. It is verified the importance of the identification and selection of the strain, considering its relevant impact during alcoholic fermentation and in the following vinification processes.

Eight strains of *S. cerevisiae* were identified by analyzing 100 RFLP-mtDNA restriction patterns. Comparing with those reported in scientific studies, it is possible to infer that the strains identified in the present study are probably indigenous from DRM.

The two predominant strains (B and A) were selected to characterize the volatile profile. The microfermentation tests inoculated with the pure strain showed that each of the strain of *S. cerevisiae* influenced (biosynthetized) differently the volatile profile with possible impact on the wine aroma. Higher alcohols, esters, and fatty acids were the dominant chemical families, and can be used as a differentiating parameter of the strain. Therefore, VOMs biosynthesized during fermentation can be called "the raw material of the bouquet" and "the fingerprint of wine".

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/9/1058/s1, Figure S1: GC-MS overlay chromatograms from strain.

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