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Impact of Non-*Saccharomyces* Yeast Fermentation in Madeira Wine Chemical Composition

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Abstract: Madeira wine is produced via spontaneous alcoholic fermentation arrested by ethanol addition. The increasing demand of the wine market has led to the need to standardize the winemaking process. This study focuses on identifying the microbiota of indigenous yeasts present during Madeira wine fermentation and then evaluates the impact of selected indigenous non-*Saccharomyces* as pure starter culture (*Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans*, and *Pichia kluyveri*) in the chemical and phenolic characterization of Madeira wine production. Results showed that the polyphenol content of the wines was influenced by yeast species, with higher levels found in wines produced by *Pichia* spp. (ranging from 356.85 to 367.68 mg GAE/L in total polyphenols and 50.52 to 51.50 mg/L in total individual polyphenols through HPLC methods). Antioxidant potential was higher in wines produced with *Hanseniaspora uvarum* (133.60 mg Trolox/L) and *Starmerella bacillaris* (137.61 mg Trolox/L). Additionally, *Starmerella bacillaris* stands out due to its sugar consumption during fermentation (the totality of fructose and 43% of glucose) and 15.80 g/L of total organic acids compared to 9.23 g/L (on average) for the other yeasts. This knowledge can be advantageous to standardizing the winemaking process and increasing the bioactive compounds, resulting in the production of high-quality wines.

Keywords: non-*Saccharomyces*; Madeira wine; polyphenols; antioxidant potential; indigenous yeasts

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1. Introduction

Wine spontaneous fermentation is a complex biochemical process that mainly involves interaction between yeasts and bacteria. Although *Saccharomyces cerevisiae* is the primary yeast involved in alcoholic fermentation, non-*Saccharomyces* yeasts species have been increasingly used to ensure the wine's final quality [1,2].

The microbiology of wine, as well as the indigenous yeasts, has been extensively studied. Non-*Saccharomyces* species originate mainly from the vineyard environment, being present in the soil and on the surface of the grapes. Non-*Saccharomyces* species and their concentration are conditioned by factors such as grape variety, geographical and climatic conditions, and viticulture practices [3]. The yeasts found in the vineyard are divided into three categories according to their fermentation performance [4]. The most common oxidative yeasts initially isolated in grapes belong to the first category—*Cryptococcus*, *Rhodotorula*, and *Aerobasidium* genera. Their concentration decreases in the middle and late

grape ripening stages due to the competition for nutrients [5]. In the initial stage of the spontaneous fermentation, the semi-fermentative non-*Saccharomyces* yeast genera, such as *Hanseniaspora*, *Candida*, *Pichia*, and *Metschnikowia*, are dominant [6,7]. As fermentation continues and the level of ethanol increases, these species are replaced by highly fermentative yeasts, such as *Saccharomyces*, *Lachancea*, and *Torulaspora* [4]. Another origin of indigenous yeasts that affects the wine's flavor and its final quality is the microbial communities inhabiting the winery environment, such as the floor, air, and equipment. Even though non-*Saccharomyces* yeasts represent the major winery inhabitants, namely from the genera *Hanseniaspora*, *Candida*, *Pichia*, *Aureobasidium*, *Metschnikowia*, *Cryptococcus*, *Torulaspora*, and *Williopsis*, many studies have found *S. cerevisiae* in wineries [3,8,9]. Additionally, several studies show that both yeasts species (*Saccharomyces* and non-*Saccharomyces*) tend to persist in the wineries over consecutive vintages and, for that reason, these resident microbial communities have an important role during both the fermentation process and in the wine's final quality [10,11]. Besides influencing the wine physicochemical parameters and its volatile composition, non-*Saccharomyces* species have been reported to influence the non-volatile composition, namely the final concentration of acids in wines, depending on the species and strains involved [12,13] in addition to the polyphenolic composition [14]. Polyphenols contribute to the wine's sensory properties, such as color, bitterness, and astringency, and are involved in protein interactions, oxidation reactions, and other wine aging processes. Additionally, they are known for having significant health benefits due to their pharmacological properties. Their concentrations in wines are influenced by several factors, such as technological practices, climate, location, ripening stage, and the strain of the yeast present during the vinification process. Indeed, some non-*Saccharomyces* yeasts are described to enhance the wine's polyphenol composition and concentrations due to the metabolic activities of yeast or by the enzymatic reactions during the fermentation process. The β-glycosidase is the main responsible for hydrolyzed the glycoside bonds and release the phenols aglycones [15–17]. Additionally, non-*Saccharomyces* present a significant interest in the oenological properties since these yeasts, when used in pure or mixed culture with *S. cerevisiae*, can modulate the wine's acidity [18].

Madeira wine (17–22% ABV) is a worldwide recognized wine with a significant impact on the Madeira Island economy. This fortified wine is characterized by its complex flavor and typicity, resulting from the chemical reactions between alcohols, acids, sugars and polyphenols during its peculiar winemaking and ageing processes. These chemical reactions contribute to the transformation of novel compounds and can promote changes in the final wine's qualitative and quantitative volatile and non-volatile composition [19]. Madeira wine is produced by spontaneous alcoholic fermentation by indigenous yeasts, which is then interrupted by the addition of natural grape spirit. Madeira wine can be aged by two distinct processes: *canteiro* or/and *estufagem*. In the first one, the wine is exclusively aged in oak casks in the lofts of wine cellars (where temperatures can range between about 15 and 30 °C) for at least 3 years before being commercialized. In the *estufagem* process, the wine is submitted to thermal processing for 3 months at about 45 °C, usually followed by an aging period in oak casks. Tinta Negra *Vitis vinifera* L. grape variety represents about 80% of Madeira wine's total production. These wines can be produced in different styles, namely sweet (total of sugars >96.1 g/L), medium sweet (80.4–96.1 g/L), medium dry (64.8–80.4 g/L), and dry (49.1–64.8 g/L) [20].

Currently, Madeira winemakers aim to improve fermentation process standardization, upgrading the quality of certain wines. In order to respond to this challenge, there is an increasing interest in identifying and studying the indigenous yeast populations, in particular the non-*Saccharomyces* species, since these can positively influence wine chemical parameters—such as acidity, polyphenols, aroma, color—and food safety [21]. Therefore, this work aimed to isolate, identify, and characterize the non-*Saccharomyces* native yeast population in musts of different wineries and vineyard locations of Tinta Negra variety and then study the impact of the inoculation of five selected indigenous

non-*Saccharomyces* yeasts, as pure starter culture, in the wine's chemical composition, particularly the phenolic compounds.

2. Materials and Methods

2.1. Spontaneous Fermentations for Yeast Isolation

2.1.1. Winery Samples

The spontaneous alcoholic fermentation of winery samples was performed by sampling grape musts from Tinta Negra red grape variety (*Vitis vinifera* L.) collected from three different local wineries (Winery 1, Winery 2, and Winery 3). The fermentation was performed under controlled temperature (20 ± 3 °C), with the addition of potassium metabisulfite (60 mg/L) to the grape juice, and without adding commercial yeast. Fermentation was carried out without maceration and was stopped by the addition of neutral grape spirit (95% (*v*/*v*) ethanol), raising the natural alcohol content up to about 17% (*v*/*v*) when the total sugar content reached about 50 g/L. In each winery, three samples were collected from three different fermentations tanks into two fermentation steps: must from the beginning of fermentation (M0), with a density of around 1075 g/L, and must before fortification (MBF), with a final density of around 1002 g/L.

2.1.2. Vineyard Samples

The spontaneous alcoholic fermentation of Tinta Negra grapes collected from two different geographical locations on Madeira Island: Câmara de Lobos (L1, L2, and L3) and São Vicente (L4, L5, and L6) were also performed. Grapes were destemmed and crushed by hand. Microvinifications were carried out at laboratory scale (in duplicate) in 3 L amber glass flasks under controlled temperature (20 ± 3 °C), and 60 mg/L of potassium metabisulfite was added without maceration. Alcoholic fermentation followed the same procedure as previously described for the winery samples. For each location, two samples were collected in the following fermentation steps: must from the beginning of fermentation (M0) and must before fortification (MBF). M0 samples were taken from the grape juice after the manual crushing and before the addition of potassium metabisulfite and MBF samples from the must after the fermentation process.

2.2. Yeast Counting and Isolation

The indigenous yeast strains used in the current study were isolated from the samples taken from the trials performed in the wineries and vineyards at the different fermentation steps (M0 and MBF). Juice samples were serially diluted (10^{-1} to 10^{-3}) in peptone water (Merck, Darmstadt, Germany), and 100 µL were surface plated onto different culture media (in duplicate). The yeasts isolates were obtained using general glucose–yeast–peptone (GYP) medium composed of 20 g/L of glucose (Sharlab, Barcelona, Spain), 5 g/L of peptone, 20 g/L of nutrient agar, and 5 g/L of yeast extract (Himedia, Einhausen, Germany) with 1 mL of Biphenil solution (0.075 g/mL) and 1 mL of chloramphenicol (0.01 g/mL) from Fisher Scientific (Lisbon, Portugal). The incubation was performed at 25 °C for 5 days. For the isolation of non-*Saccharomyces*, an identical GYP medium was prepared with the addition of a cycloheximide solution (10 µg/L) (Merck, Darmstadt, Germany). The *Dekkera/Brettanomyces* isolates were obtained following the procedures described by Rodrigues et al. (2001) [22], using the selective media DBDM with samples being incubated at 25 °C for 12 days. *Zygosaccharomyces* species were isolated in a ZDM medium, with samples being incubated at 28 °C for 48 h, according to the Schuller et al. (2000) [23] studies. The total yeast counting was obtained by recording the number of colony-forming unit (CFU) counts. Different colony morphologies were registered, and two to five representative isolates were selected and purified on GYP plates.

The fermentative yeast species were selected using the urease and fermentation tests (Glucose). For the urease test, 1 L of Christensen's medium was prepared with 1 g of peptone (Himedia, Einhausen, Germany), 1 g of glucose (Himedia, Einhausen, Germany), 5 g of NaCl, 2 g of KH_2PO_4, and 0.012 g of red phenol from Merck (Darmstadt, Germany)

and pH-adjusted to 6.8. Solutions of 20% urea (Panreac, Barcelona, Spain) were prepared before being added (0.5 mL) to tubes previously loaded with 4.5 mL of Christensen's medium. The colonies were inoculated into the tubes and then incubated for 3 days at 25 °C. The development of a yellow color was considered as positive result. The fermentation test was prepared with 1 L of distilled water, 10 g/L of yeast extract (PVL, Lisbon, Portugal), and 20 g/L of glucose monohydrated (Merck, Darmstadt, Germany). Ten milliliters of this solution were placed into Durham tubes and sterilized at 121 °C for 15 min. The colonies were inoculated for 2 to 4 days at 25 °C. The gas formation was considered a positive result. Fermentative yeast species had a positive response for both urease and fermentation tests.

2.2.1. Yeast Identification

The isolates were collected from fresh yeast colonies, and then DNA extraction was performed by thermal shock (95 °C for 15 min, −80 °C for 15 min, and then 95 °C for 15 min). The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was carried out using an ITS1 primer (50-TCCGTAGGTGAACCTGCGG-30) and an ITS4 primer (50-TCCTCCGCTTATTAGATATGC-30) from STABVida (Lisbon, Portugal) and the digestive enzyme NZYTaq II DNA polymerase from NZYTech (Lisbon, Portugal) was used. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. Agarose at 1.5% in 1 × TAE buffer (Cleaver Scientific, Warwick, United Kingdom) was used to separate DNA products and their restriction fragments. The PCR-amplified fragments were sequenced by STABVida (Lisbon, Portugal), and then a blast analysis was performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, accessed on 1 November 2022). ITS1 and ITS4 sequences were considered for accurate results, and the identification was considered valid when at least 98% of correspondence was revealed.

2.2.2. Yeast Inoculation and Wine Production

Five non-*Saccharomyces* yeasts strains were selected to perform the current study: *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans*, and *Pichia kluyveri*. All fermentations used Tinta Negra grapes. The inoculation was performed at laboratorial scale and the procedure was based on Benito el al. (2017) [24]. The initial must, which had a density of 1075 g/L (at 20 °C), was pasteurized at 105 °C for 5 min. After cooling, yeasts were individually inoculated using the GYP medium, containing 10^9 CFU/mL (measured by the Thoma cell counting chamber method), at 25 °C for 24 h, and then 3 mL of the solution was added to 3 L of must. The fermentations were performed in duplicate. The alcoholic fermentation was conducted under a controlled temperature (20 ± 3 °C), and the density was measured using a pycnometer. After fermentation, the samples were fortified by adding natural grape spirit (95% (v/v) of ethanol) raising the alcohol content up to 17% (v/v) ethanol. After vinification, all wines were kept at 45 °C for 120 days (*estufagem* aging simulation). Samples were collected, in duplicate, at different stages of the winemaking and aging: M0 (initial must), MAI (must after 24 h of inoculation), MBF (must before fortification), WAF (wine after fortification), and WAE (wine after *estufagem*).

*2.3. Characterization of the Selected Non-*Saccharomyces *Species*

2.3.1. Ethanol, Reducing Sugars, and Organic Acids

The concentration levels of ethanol, fructose, glucose, glycerol, and organic acids were quantified based on the procedure proposed by Miranda et al. (2017) [20]. The analyses were carried out on a Waters Alliance high-performance liquid chromatographer (HPLC-DAD-RID) from Waters Corporation (Santa Clara, CA, USA) equipped with an auto-injector (Waters 2695 separation module), a photodiode array detector (Waters 2996), a refractive index detector, and the Empower Pro software from Waters Corporation for data handling. The chromatographic separation was performed using a Hi-Plex H column (300 × 7.7 mm,

and 8 µm particle size) from Agilent (Santa Clara, CA, USA) with an isocratic elution, using an aqueous solution of sulphuric acid (0.0025 M), at a flow rate of 0.6 mL/min. The column temperature was set to 65 °C and the injection volume was 10 µL. Samples were previously filtered through 0.20 µm PP Chromafil Xtra syringe filters (from Macherey-Nagel, Düren, Germany). The concentrations of six organic acids were determined based on their standard curves between the following ranges: tartaric, malic, and acetic acids, 100–5000 mg/L; lactic acid, 50–5000 mg/L; citric and succinic acids, 20–1000 mg/L; and formic acid, 100–1000 mg/L. All samples were analyzed in triplicate.

2.3.2. Polyphenolic Composition

Samples were assayed for the total polyphenolic composition (TP) using the Folin–Ciocalteu method, based on Pereira et al. (2013) [25]. Briefly, 100 µL of samples/standards were added to the following reagents: 5 mL of distilled water, 0.5 mL of Folin–Ciocalteu reagent (Fluka Biochemika AG, Buchs, Switzerland), and 2 mL of a 20% (w/v) aqueous solution of Na_2CO_3 (Panreac Química S.A., Barcelona, Spain). Then, the volume was adjusted to 10 mL and the solutions mixed. The absorbance was measured at 750 nm after a 30 min reaction period using an ultraviolet–visible (UV–Vis) spectrophotometer, model UV-2600 from Shimadzu (Kyoto, Japan), equipped with UVProbe 2.42 software. TP was determined based on the following standard curve of gallic acid (Fluka Biochemika AG, Buchs, Switzerland) in the range 25–750 mg/L: A_{750} = 0.0011 GAE (mg/L) + 0.0213 (R^2 = 0.998). Samples were analyzed in triplicate.

The identification and quantification of individual polyphenols were based on Pereira et al. (2013) [25]. Briefly, 20 µL of each sample was directly injected into an HPLC-DAD from Waters Corporation (Santa Clara, CA, USA) equipped with an auto-injector (Waters 2695 separation module), photodiode array detector (Waters 2996), and the Empower Pro Software from Waters Corporation. The polyphenolic compounds were separated in an Atlantis T3 column (250 × 4.6 mm, i.d.; 5 µm, from Waters, Milford, MA, USA) using three mobile phases for the chromatographic separation: A (10 mM of phosphate buffer adjusted to pH 2.7 with phosphoric acid), B (acetonitrile), and C (methanol). The column temperature and the flow rate were set to 30 °C and 1.0 mL/min, respectively. The gradient program varied from 100% aqueous mobile phase (Phase A) to 60% organic phase (Phase B) in 58 min and then 12 min of re-equilibration. All standards and wine samples were injected in triplicate. The analytes were identified based on their retention time and UV–Vis spectra (between 200–780 nm) and by spiking samples with a mixture of pure standards. All standards and samples were previous filtered through 0.20 µm PP Chromafil Xtra syringe filters. The quantitative determination was performed according to the external standard calibration method. Wavelengths used for quantification were 210 nm (flavan-3-ols, hydroxybenzoic acids, and hydroxybenzaldehydes), 315 nm (*trans*-resveratrol and hydroxycinnamic acids), and 360 nm (flavonoids and ellagic acid). Anthocyanins were not evaluated in the current study.

2.3.3. Antioxidant Potential

The antioxidant potential was evaluated by the ability of wines to scavenge DPPH free radicals, and the procedure was adapted from Pereira et al. (2013) [25]. Firstly, the calibration curve was defined using Trolox solutions (25–1250 mg/L). A 60 µM DPPH solution in methanol was prepared daily. A volume of 22 µL of standard solution/sample was added to 3.0 mL of DPPH solution, and the absorbance was determined at 515 nm after 20 min, every 30 s. Methanol was used as a blank, and samples were analyzed in triplicate. The antioxidant potential was calculated based on the inhibition percentage (%I), with the following equation: $\%I = [(A_{515}\ (0\ min) - A_{515}\ (20\ min))/A_{515}\ (0\ min)] \times 100$, where the A_{515} (0 min) was the absorbance measured at the beginning of the reaction and A_{515} (20 min) the absorbance after 20 min.

2.4. Data Processing

All results were presented as mean ± standard deviation (SD), and the significant differences were evaluated by the analysis of variance (One-way ANOVA, Holm–Sidak method) using the statistical software SigmaPlot, version 12.0.

3. Results and Discussion

3.1. Identification of Non-Saccharomyces Derived from Wineries and Vineyards

Madeira wine fermentative yeasts of three different wineries were identified during the fermentation process (M0 and MBF stages), as well as those of two main vineyard regions of the Madeira wine appellation. A total of 287 isolates were identified from the spontaneous fermentation of Tinta Negra wines (154 isolates in the samples collected from wineries and 133 in the samples collected from different vineyard locations). Table 1 reports the distribution of the 11 yeasts identified in the studied wineries: *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia kluyveri*, *Pichia fermentans*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida apicola*, *Cystobasidium minutum*, *Pichia terricola*, *Cystobasidium slooffiae*, and *Wicheramomyces anolalus*. In turn, six yeasts species were identified in the samples derived from the different vineyards: *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia kluyveri*, *Pichia fermentans*, *Saccharomyces cerevisiae*, and *Hanseniaspora opuntiae*. *Dekkera/Brettanomyces* and *Zygosaccharomyces* species were not detected in all samples.

Table 1. Distribution (%) of the yeasts identified during the fermentation (M0 and MBF stages) of the must samples collected at the different winery (W) and vineyard locations (L).

	Wineries			**Vineyard Locations**					
Yeast Strain	**W 1**	**W 2**	**W 3**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**
M0 stage									
Pichia terricola	18								
Starmerella bacillaris	32	43	9	7			5	6	
Pichia kluyveri	12			2		13			
Candida apicola	14	19							
Cystobasidium minutum	3								
Hanseniaspora uvarum	21	32	83	91	98	86	95	94	100
Pichia fermentans		6			2	1			
Cystobasidium slooffiae			8						
Total Ascomycetes	79	100	92	100	100	100	100	100	100
MBF Stage									
Pichia fermentans	3				1				
Saccharomyces cerevisiae	94	68	85	72		5	10	3	11
Wicheramomyces anomalus	3								
Hanseniaspora uvarum		31	15	28	99	95	90	38	89
Torulaspora delbrueckii		1							
Hanseniospora opuntiae								59	

Wineries: W1, W2 and W3. Locations: South Locations (Câmara de Lobos—L1, L2 and L3); North Locations (São Vicente—L4, L5 and L6). Stages from grape collection: grape juice (M0) and must before fortification (MBF).

The yeast microbiota varied between wineries, demonstrating that each winery has its own native yeast culture (M0 stage). Most of the yeasts found in Madeira wine wineries belong to the *Ascomycota* phylum, ranging between 79% and 100% in the M0 stage and 100% in the MBF stage. The yeasts belonging to *Basidiomycota* phylum were only *C. minutum* and *C. slooffiae*. Similar to other studies [1,8], the proportion of non-*Saccharomyces* yeasts in wine cellars at the initial stage (M0) was higher compared to *Saccharomyces*. At this stage, *S. bacillaris* and *H. uvarum* species were present in all wineries, representing about 73% of the total yeasts. Both yeasts are recognized for persisting in cellar environments and are capable of reimplantation during the next vintage, becoming an integral part of the winery

yeast resident microbiota [10]. As expected, *S. cerevisiae* took over the process at the end of the fermentation (MBF stage), representing about 82% of the total yeast in all wineries. Also, at the MBF stage, *H. uvarum* was detected in Wineries 2 and 3 (15%), followed by other non-*Saccharomyces* species, such as *P. fermentans*, *W. anomalus*, and *T. delbrueckii*. The diversity and distribution of yeasts in wineries can vary depending on the winery and antiseptic conditions [8,10]. *H. uvarum* was the predominant non-*Saccharomyces* species isolated from the grape musts collected in the main Madeira wine vineyards (M0 stage), in agreement with previous studies performed in other vineyards involved in the production of fortified wines [13] and others [26–28]. Although grape samples belong to different vineyard locations, the yeast microbiota found at the M0 stage were practically similar in all locations. At this stage, other yeast species such as *S. bacillaris*, *P. kluyveri*, and *P. fermentans* were also present in lower concentrations. These yeasts were previously identified in grape vineyards in several studies [12,29,30]. *S. cerevisiae* emerged in the MBF stage in five vineyards. Even though *S. cerevisiae* is extremely rare on grapes or vineyards [9,31,32], this yeast can be found during the spontaneous alcoholic fermentation in sterilized vessels [33] or even on damaged berries [34].

3.2. Analytical Characterization

The selection and utilization of indigenous cultures is a topic of interest in regions with enological tradition, such as in the case of Madeira. In this sense, five non-*Saccharomyces* yeasts were selected and individually inoculated in the grape must for evaluating their role in the chemical composition to produce Madeira wines, namely: *H. uvarum*, *S. bacillaris*, *P. terricola*, *P. fermentans*, and *P. kluyveri*. The fermentation process was monitored through the control of the must density and the ethanol produced (Figure 1).

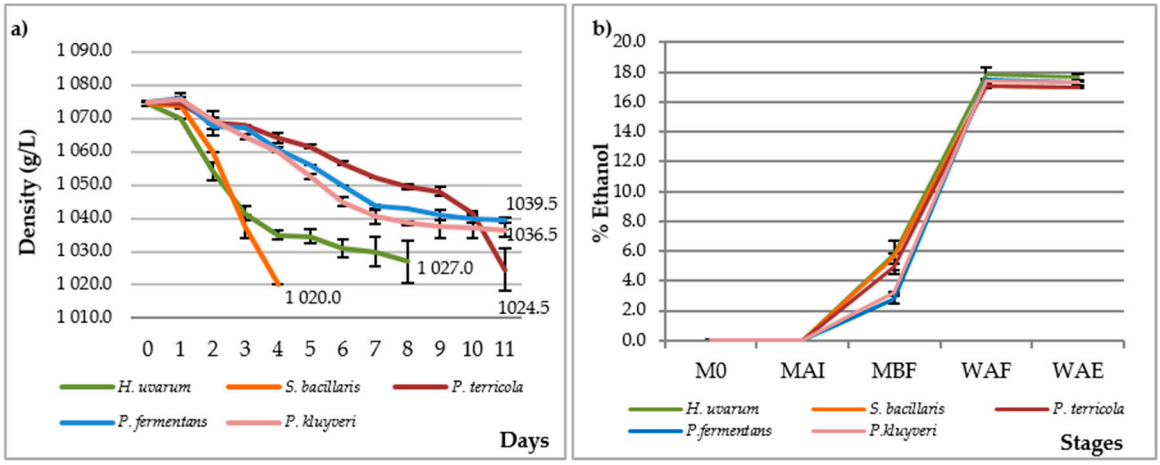

Figure 1. Density depletion (**a**) and the ethanol formation (**b**) during the Madeira winemaking using different selected non-*Saccharomyces* yeasts (*H. uvarum*, *S. bacillaris*, *P. terricola*, *P. fermentans*, and *P. kluyveri*) as starter cultures.

The fermentative performance varied according to the yeast species. Figure 1 depicts that *S. bacillaris* showed higher fermentative capacity, reaching a density of 1020 g/L within 4 days, within the range allowed for dry wines. Indeed, most strains of this non-*Saccharomyces* species are tolerant to relatively high levels of ethanol and can persist up to the middle–end phase of the fermentation [35]. *H. uvarum* and *P. terricola* also showed fermentation capacity to produce this wine style, reaching densities of 1027 g/L and 1024.5 g/L within 8 and 11 days, respectively. In contrast, *P. fermentans* and *P. kluyveri* species presented low fermentative capacity (densities reached 1039.5 g/L and 1036.5 g/L, respectively)—characteristic of *Pichia* species [36]—and, therefore, can only be considered for the sweet wines production. In line with these findings, ethanol production before fortification (Figure 1b) in the inoculates of *H. uvarum* (5.8%), *S. bacillaris* (5.6%), and *P.*

terricola (5.0%) was also higher than that found in *P. fermentans* (2.7%) and *P. kluyveri* (3.2%). Some studies previously demonstrated that ethanol production was 4% in wines produced by *H. uvarum*, 10% for *S. bacillaris*, and up to 7% for those produced by *Pichia* species [13,36,37].

Individual sugars (fructose and glucose) and glycerol were also quantified to evaluate the impact of the non-*Saccharomyces* species during the vinification and aging processes (*estufagem*) of Madeira wine production (Figure 2).

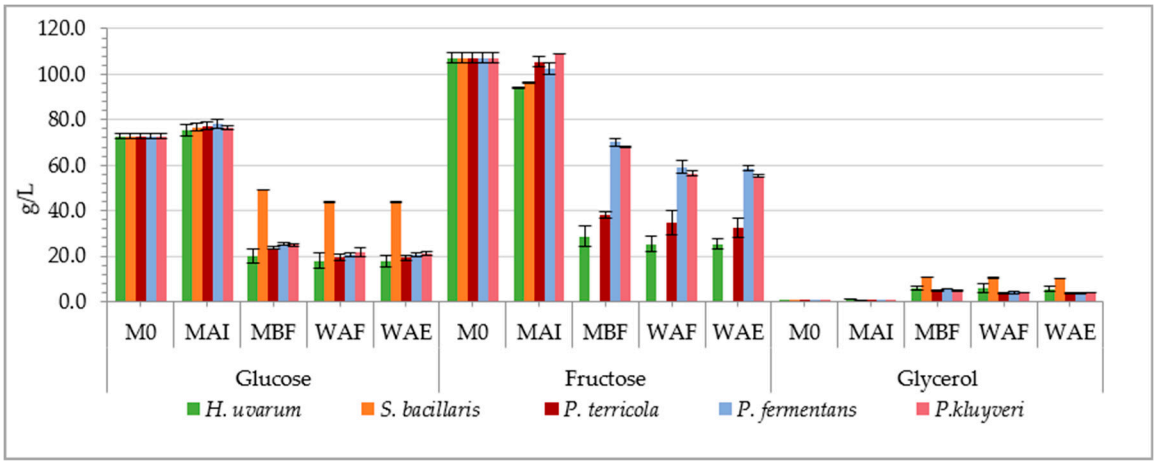

Figure 2. Glucose, fructose, and glycerol amounts during Madeira winemaking using different selected non-*Saccharomyces* yeasts (*H. uvarum*, *S. bacillaris*, *P. terricola*, *P. fermentans*, and *P. kluyveri*) as starter cultures.

H. uvarum showed similar preference for both individual sugars (glucose consumed 76% and fructose consumed about 77%). In accordance with findings by Lemos Junior et al. (2019) [38], fructose was completely consumed in the presence of *S. bacillaris*, while only about 43% of glucose was consumed. On the other hand, as reported previously [36], *Pichia* species revealed a preference for glucose (on average 74%). Regarding Pereira et al. (2017) studies, the presence of fructose and its degradation mechanisms during the *estufagem* process greatly contributes for the development of typical features of Madeira wines, such as color and aroma [39]. The increase in glycerol concentration during fermentation was observed in all non-*Saccharomyces* species. However, *S. bacillaris* stood out, reaching a concentration of 10.3 g/L of glycerol. This yeast tends to improve the wine softness and body through the increase of glycerol (up to 14 g/L), as previously reported by Lemos Junior et al. (2019) [38].

The main individual organic acids found in Madeira wine samples in the WAE stages are described below in Figure 3.

In accordance to a previous study Pereira et al. (2010) [40], tartaric, malic, and lactic acids were the main organic acids found in the current Madeira wine samples. Tartaric acid ranged from 2.88 to 5.07 g/L, malic acid from 0.97 to 2.27 g/L, and lactic acid from 0.31 to 2.92 g/L. *S. bacillaris* stood out from the other non-*Saccharomyces* species, showing higher levels of tartaric, malic, lactic, and citric acids. The total concentration of organic acids for wines produced by *S. bacillaris* and the other yeasts was 15.80 g/L and 9.23 g/L (on average), respectively. This non-*Saccharomyces* species acts as a natural acidification agent, increasing the total acidity of wines by reducing the pH [35]. In this sense, the inoculation of musts with *S. bacillaris* could promote a natural acidity correction in Madeira wines with low acidity. Regarding the main acid involved in the volatile acidity of wines, acetic acid concentration varied between 1.49 g/L (*S. bacillaris*) and 3.04 g/L (*P. kluyveri*). Despite high levels of this compound being able to contribute negatively to the wine's aroma with vinegar notes, Miranda et al. (2017) [20] reported that the odor rejection thresholds for acetic acid in Madeira wines can range between 1.96 and 5.72 g/L depending on the evaluation

panel (regular or non-regular wine consumers) and the age and sweetness degree of the wine. Other study Pereira et al. (2010) [40] showed that the concentration of this organic acid ranged from 0.67 to 2.21 g/L in Madeira wine samples. Even though some studies report no significant differences in acetic acid production on fermentations performed by *P. kluyveri*, the concentration of acetic acid produced may depend on the strain involved [36].

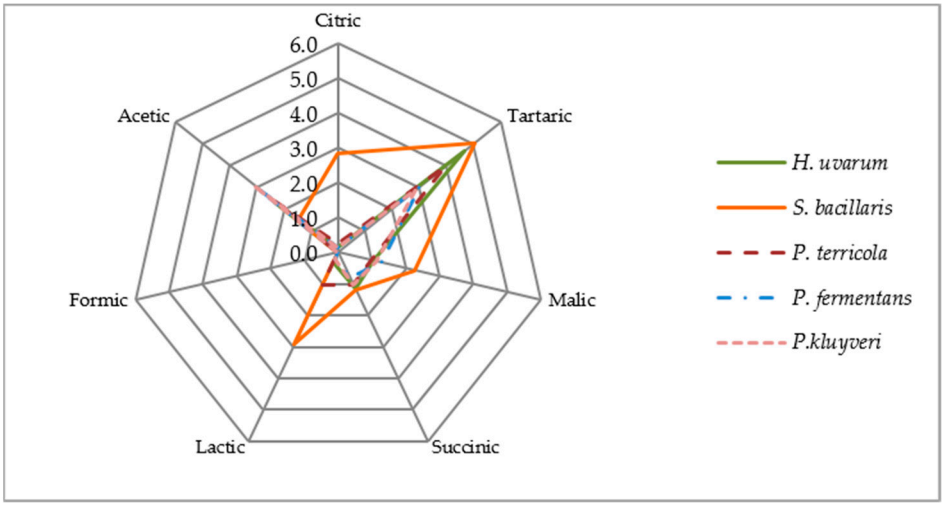

Figure 3. Concentration of individual organic acids (g/L) in Madeira wines produced using different selected non-*Saccharomyces* yeasts (*H. uvarum*, *S. bacillaris*, *P. terricola*, *P. fermentans*, and *P. kluyveri*) as starter cultures at the WAE stage.

3.3. Antioxidant Potential and Total Polyphenols

Table 2 summarizes the TP and antioxidant potential (DPPH) results found during the winemaking process of the Madeira wines produced from the different non-*Saccharomyces* yeasts.

Table 2. Antioxidant potential (DPPH) and total polyphenols (TP) during the Madeira winemaking using different selected non-*Saccharomyces* yeasts (*H. uvarum*, *S. bacillaris*, *P. terricola*, *P. fermentans*, and *P. kluyveri*) as starter cultures.

	H. uvarum	*S. bacillaris*	*P. terricola*	*P. fermentans*	*P. kluyveri*
TP (mg GAE/L)					
M0	398.52 ± 0.00 a	398.52 ± 0.00 a	398.52 ± 0.00 a	398.52 ± 0.00 a	398.52 ± 0.00 a
MAI	255.94 ± 3.74 a	248.44 ± 6.82 ab	269.27 ± 8.31 c	252.30 ± 0.00 ab	217.00 ± 3.65 d
MBF	174.35 ± 0.00 a	215.48 ± 2.65 b	186.09 ± 0.00 c	177.98 ± 0.00 d	186.17 ± 0.00 c
WAF	201.92 ± 0.00 a	185.94 ± 0.00 b	212.15 ± 0.00 c	201.09 ± 0.00 d	205.94 ± 0.00 e
WAE	294.95 ± 2.04 a	263.74 ± 3.93 b	356.85 ± 1.70 c	361.85 ± 3.65 cd	367.68 ± 8.10 d
DPPH (mg Trolox/L)					
M0	209.91 ± 0.00 a	209.91 ± 0.00 a	209.91 ± 0.00 a	209.91 ± 0.00 a	209.91 ± 0.00 a
MAI	159.18 ± 0.00 a	145.21 ± 0.00 b	161.48 ± 0.00 c	159.87 ± 0.00 d	167.56 ± 0.00 e
MBF	141.37 ± 0.00 a	147.28 ± 0.00 b	136.03 ± 0.00 c	136.77 ± 0.00 d	131.60 ± 0.00 e
WAF	126.15 ± 0.00 a	111.58 ± 0.00 b	121.46 ± 0.00 c	124.98 ± 0.00 d	134.15 ± 0.00 e
WAE	133.60 ± 0.00 a	137.61 ± 0.00 b	120.95 ± 0.00 c	129.96 ± 0.00 d	125.27 ± 0.00 e

Different letters in the same row denote statistically significant differences at ($p < 0.05$). M0 (grape juice), MAI (must after 24 h of inoculation), MBF (must before fortification), WAF (wine after fortification and WAE (wine after *estufagem*).

One can observe that all fermentations (from M0 to MBF) promoted a decrease in TP regardless of the non-*Saccharomyces* yeasts strain used, between 45% and 56% when wines were inoculated with *S. bacillaris* and *H. uvarum*, respectively. This decrease can be due to physical processes, mostly involving the reversible interaction between anthocyanins and the yeast walls by absorption [40,41]. On the other hand, the *estufagem* process (WAF to WAE stage) promoted an increase on TP, greater in the wines that underwent fermentation with the *Pichia* species (between 68% using *P. terricola* and 79% using *P. fermentans*).

Similarly, the antioxidant potential decreased between 29.8–37.3% during the fermentation process of all wine samples (from M0 to MBF). *S. bacillaris* promoted the highest antioxidant potential in the final wines of 137.61 mg Trolox/L. On the other hand, *Pichia* spp. revealed the lowest levels (120.95 to 125.27 mg Trolox/L). The final results were lower than those found in Madeira wines produced by spontaneous fermentation (305.52 mg Trolox/L for sweet wines and 409.66 mg Trolox/L for dry wines after the *estufagem* process) [25]. However, in the present study the vinification underwent without grape skins, contributing to lowering the initial antioxidant potential levels.

3.4. Polyphenolic Composition

In total, 24 individual polyphenols were identified and quantified in the wines produced by different non-*Saccharomyces* species (Table 3), including non-flavonoids (7 hydroxybenzoic acids, 8 hydroxycinnamic acids, and 1 stilbene) and flavonoids (4 flavonols and 4 flavan-3-ols).

Table 3. Individual polyphenols (mg/L) in Madeira wine samples produced by different non-*Saccharomyces* species in the initial must (M0 stage) and in the final wine samples (WAE stage).

	M0 Stage	**WAE Stage**				
		H. uvarum	***S. bacillaris***	***P. terricola***	***P. fermentans***	***P. kluyveri***
Non-flavonoids						
Hydroxybenzoics						
Gallic	0.46 ± 0.04 a	0.622 ± 0.004 b	1.2 ± 0.1 c	1.502 ± 0.001 d	1.21 ± 0.02 c	0.83 ± 0.04 e
Protocatechuic acid	1.09 ± 0.05 a	1.95 ± 0.03 b	2.10 ± 0.03 b	1.9 ± 0.2 b	1.90 ± 0.08 b	2.025 ± 0.004 b
Syringaldehyde	n.q.	1.5 ± 0.1 a	1.09 ± 0.04 b	0.40 ± 0.01 c	0.680 ± 0.002 d	1.288 ± 0.002 e
Syringic	n.q.	4.5 ± 0.24 a	4.19 ± 0.01 b	4.7 ± 0.12 c	5.19 ± 0.01 d	5.89 ± 0.07 e
Vanillic acid	n.q.	1.92 ± 0.04 a	2.4 ± 0.2 b	3.2 ± 0.2 c	2.7 ± 0.1 d	3.29 ± 0.06 c
p-Hydroxybenzoic acid	n.q.	1.18 ± 0.01 a	0.436 ± 0.001 b	0.97 ± 0.09 c	2.5 ± 0.3 d	2.2 ± 0.1 e
Ellagic	n.d.	0.99 ± 0.09 a	1.07 ± 0.08 b	0.88 ± 0.03 c	0.88 ± 0.02 c	n.q.
Total	1.55	12.63	12.42	13.49	15.11	15.53
Hydroxycinnamates						
Caffeic acid	n.q.	1.08 ± 0.08 a	1.28 ± 0.08 b	1.54 ± 0.01 c	0.89 ± 0.06 d	1.05 ± 0.07 a
trans-Caftaric acid	4.89 ± 0.04 a	3.551 ± 0.003 b	3.7 ± 0.1 c	3.69 ± 0.09 c	3.26 ± 0.07 d	3.14 ± 0.06 e
Ferrulic acid	0.78 ± 0.06 a	0.98 ± 0.08 b	1.26 ± 0.01 c	0.86 ± 0.04 a	0.85 ± 0.04 a	0.805 ± 0.001 a
Sinapic acid	n.d.	0.23 ± 0.01 a	0.36 ± 0.01 b	0.24 ± 0.02 a	0.26 ± 0.02 c	n.q.
p-Coumaric acid	n.q.	n.q.	0.41 ± 0.01 a	0.639 ± 0.002 b	0.40 ± 0.04 a	0.410 ± 0.003 a
cis-Coutaric	0.13 ± 0.01 a	0.142 ± 0.001 b	0.139 ± 0.003 b	0.15 ± 0.01 c	0.114 ± 0.001 d	0.135 ± 0.001 b
trans-Coutaric	1.66 ± 0.01 a	2.05 ± 0.08 b	2.22 ± 0.04 c	2.01 ± 0.06 b	2.01 ± 0.02 b	2.12 ± 0.00 d
trans-Fertaric	0.43 ± 0.02 a	0.44 ± 0.04 a	0.468 ± 0.001 b	0.228 ± 0.001 c	0.377 ± 0.001 d	0.390 ± 0.003 d
Total	7.89	8.47	9.82	9.36	8.16	8.05
Stilbene						
trans-Resveratrol	n.q.	0.341 ± 0.005 a	0.265 ± 0.002 b	0.29 ± 0.01 c	0.344 ± 0.003 a	0.36 ± 0.02 d
Flavonoids						
Flavan-3-ols						
(+)-Catechin	1.16 ± 0.07 a	14.3 ± 0.8 b	10.8 ± 0.4 c	19.7 ± 0.6 d	17 ± 2 e	17 ± 2 e
(−)-Epicatechin	6.8 ± 0.2 a	1.5 ± 0.1 b	1.36 ± 0.08 b	1.28 ± 0.01 b	1.6 ± 0.1 b	1.76 ± 0.06 b
(−)-Epigallocatechin	0.15 ± 0.01 a	2.1 ± 0.1 a	1.36 ± 0.06 a	2.37 ± 0.05 a	1.15 ± 0.05 a	1.6 ± 0.1 a
(−)-Epigallocatechin gallate	1.68 ± 0.04 a	1.85 ± 0.05 a	2.0 ± 0.1 a	1.40 ± 0.01 a	1.70 ± 0.02 a	1.45 ± 0.02 a
Total	9.83	19.82	15.54	24.75	21.88	22.07
Flavonols						
Kaempferol	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Myricetin	n.q.	0.30 ± 0.03 a	0.32 ± 0.02 a	0.24 ± 0.01 b	0.29 ± 0.01 c	n.q.
Quercetin	n.d.	1.01 ± 0.03 a	0.57 ± 0.01 b	0.24 ± 0.01 c	1.21 ± 0.02 d	1.45 ± 0.02 e
Rutin	n.d.	0.66 ± 0.05 a	0.65 ± 0.01 a	0.50 ± 0.01 b	0.54 ± 0.01 c	0.506 ± 0.003 b
Total	–	4.98	4.59	3.61	5.03	4.99
Total polyphenols	19.27	46.24	42.64	51.50	50.52	51.00

n.q.—not quantified, below LOQ; n.d.—not detected, below LOD; different letters in the same row denote statistically significant differences at ($p < 0.050$) according to Holm–Sidak test.

According to Table 3, the concentration of total individual polyphenols increased from the M0 to WAE stage in all samples produced by non-*Saccharomyces* species, from 19.27 mg/L up to 51.50 g/L (*P. terricola*). The proportion of non-flavonoids (53.39%) was similar to flavonoids (54.76%). Although the total individual polyphenol contents were similar for all non-*Saccharomyces*, *Pichia* species produced a slightly higher concentration. (+)-Catechin was the most abundant compound found in all final wines, varying from 10.79 mg/L (*S. bacillaris*) to 19.71 mg/L (*P. terricola*). This polyphenol showed the highest concentration in Madeira wine samples previously studied (337 µg/mL) [42]. Catechin can affect the quality of wines, conferring flavor (bitterness) and oxidation resistance [43]. Regarding the hydroxybenzoic acids, syringic and vanillic acids were the ones that stood out, mainly in wines produced by *P. kluyveri* (5.89 mg/L and 3.29 mg/L, respectively). These

compounds showed similar concentrations to those previously described inGonçalves et al. (2013) [44] for commercial table wines, namely 0.2 to 1.0 g/L for vanillic acid and 2 to 4 mg/L for syringic acid. The most abundant hydroxycinnamic acid was trans-caftaric acid, showing higher levels in wines produced by *S. bacillaris* (3.68 mg/L) and *P. terricola* (3.69 mg/L). The heating process promoted a decrease in this compound of at least 28%, which is in agreement with the findings by Pereira et al. (2010) [40]. Trans-resveratrol concentration increased from the M0 to the WAE stage, varying according to the yeast species used from 0.27 mg/L (*S. bacillaris*) to 0.36 mg/L (*P. kluyveri*). This bioactive compound is usually found in wine at concentrations ranging from undetectable to 14.3 mg/L depending on the type of the wine [45,46]. The levels of resveratrol tend to be higher when grapes are exposed to biotic or abiotic stress or by the yeasts-endowed *β-glucosidase* activity [14,46]. Gaensly et al. (2015) [46] demonstrated that four strains of *H. uvarum* increased free resveratrol after alcoholic fermentation of *V. labrusca* without modifying its composition or sensorial properties. Flavonols have an important role in the color and sensory perception of wines. According to Table 3, quercetin was the flavonol with the highest concentration, ranging from 0.24 mg/L in wines inoculated with *P. terricola* to 1.45 mg/L in wines produced by *P. kluyveri*. Although skin maceration was not carried out in this study, some wine samples showed higher levels of quercetin when compared to other Madeira wine studies (0.65 mg/L) [40]. Quercetin levels varied between wine samples (WAE) according to the yeast species used, revealing that yeast species can influence its concentration in wines during the fermentation process, consistent with results from other studies [47].

Although Madeira wines are produced by indigenous fermentations, it is essential to the standardization of the vinification process. Therefore, the use of indigenous yeast inoculation in the production of these fortified wines, according to the type of wines, can lead to enhancement of the wine's quality, producing more uniform wines. The results revealed that despite wines inoculated with *S. bacillaris* revealing high fermentative capacity, acidity, and antioxidant potential levels, *Pichia* spp. showed higher polyphenol amounts. Thus, complementary studies with the inoculation of a mixture culture of non-*Saccharomyces* yeasts as started culture in the production of Madeira wines are fundamental to enhancing their quality.

4. Conclusions

The current study showed the diversity of non-*Saccharomyces* indigenous yeasts involved in the production of Madeira wines, with *H. uvarum*, *S. bacillaris*, *P. terricola*, *P. fermentans*, and *P. kluyveri* being the most representative species. *H. uvarum* and *S. bacillaris* represented about 73% of the total yeast in grape juices. Only *H. uvarum*, *S. bacillaris*, and *P. terricola* showed fermentation capacity to produce dry Madeira wines. The different non-*Saccharomyces* species promoted great variability on the wine characteristics. Wines produced with *S. bacillaris* evidenced higher acidity (15.80 g/L of total organic acids) when compared to the ones produced with other non-*Saccharomyces* yeasts. The contents of polyphenols in wines were influenced by the yeast species and were higher in the *Pichia* inoculates, reaching 51.50 mg/L of the total polyphenols in wines produced with *P. terricola*. The antioxidant potential was higher in musts inoculates with *H. uvarum* and *S. bacillaris*. Even though the evaluated non-*Saccharomyces* species showed promising results to be used as starter cultures in the production of Madeira wines, additional information is needed to evaluate the sensorial properties of these wines.

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