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Oenological Capabilities of Yeasts Isolated from High-Sugar Matrices (Manna and Honey) as Potential Starters and Co-Starters for Winemaking

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Abstract: Non-*Saccharomyces* yeasts have recently garnered significant interest in oenology. When co-inoculated with *Saccharomyces cerevisiae*, they contribute to the improvement of wine quality from a sensory point of view. In the present study, a group of yeasts previously isolated from manna and honey by-products were subjected to a genotypic identification. The D1/D2 variable domains of the 26-sRNA gene and the ITS region of the 5.8S gene were sequenced. Additionally, a differentiation of strains was carried out by RAPD-PCR. All strains underwent in vitro screening. Subsequently, a micro-vinification experiment was conducted, focusing on strains with favourable technological characteristics: *Lachancea thermotolerans*, *Starmerella lactis-condensi*, and *Candida oleophila*. These strains were sequentially inoculated alongside a control strain of *Saccharomyces cerevisiae*. Technological screening revealed that some strains exhibited limited H_2S production, ethanol tolerance (up to 8% *v*/*v*), resistance to potassium metabisulphite (200 mg/L), osmotic stress tolerance (up to 320 g/L of glucose), and copper resistance (on average 5 mM). The findings from this study can guide the selection of new starters and co-starters for regional wine production.

Keywords: alcoholic fermentation; non-*Saccharomyces*; oenological selection; *Saccharomyces cerevisiae*; wine aroma; yeasts starter

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1. Introduction

In traditional wine fermentation practices, commercial strains of *Saccharomyces cerevisiae* are used as starters to ensure consistent fermentation and contribute to the production of well-balanced wines. Since the 20th century, *S. cerevisiae* active dry yeast has been widely used for its reliability in achieving fast and predictable fermentations. Nevertheless, there exists a diverse array of both *Saccharomyces* and *non-Saccharomyces* strains that can persist during fermentation [1]. Non-*Saccharomyces* yeasts, while of secondary importance in must fermentation, are sometimes considered spoilage microorganisms [2] due to their limited fermentative capacity and tendency to produce off-flavours such as acetaldehyde, acetic acid, ethyl acetate, and acetoin [3]. Additionally, unwanted volatile phenols, such as those produced by *Brettanomyces* spp. [4], can be associated with these yeasts. Interestingly, strain-dependent studies have revealed that certain non-*Saccharomyces* yeasts exhibit positive

effects. Specifically, they have been contributing to improving wine complexity, texture, and flavour integration in spontaneous fermentations since the 1980s [5].

Non-*Saccharomyces* yeasts play a crucial role in wine production, contributing unique aromatic complexity and mouthfeel. These effects are closely tied to the concept of terroir [6], which resonates with consumer preference for novel wine styles [7]. During the early stages of alcoholic fermentation, non-*Saccharomyces* yeasts, predominantly from genera like *Hanseniaspora*, *Candida*, *Meyerozyma*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces*, and *Metschnikowia*, take the lead. However, as fermentation progresses, they yield the stage to *S. cerevisiae*, which ultimately completes the fermentation process [5,8]. These non-*Saccharomyces* strains often originate from grape berry surfaces, cellar equipment surfaces, or the winery environment. Initially, their demise was attributed to rising ethanol concentrations and the addition of SO_2. However, recent research has revealed a more intricate picture, with strain-specific survival mechanisms. Surprisingly, even in the late stages of fermentation, several non-*Saccharomyces* species persist and thrive at significant levels [5,9–13].

During the pre-fermentative phase, three primary genera dominate: *Hanseniaspora* spp., *Candida* spp., and *Metschnikowia* spp. Among these, *Hanseniaspora uvarum* stands out as a key non-*Saccharomyces* yeast during the initial stages of fermentation. Additionally, *Starmerella bacillaris* is consistently found in grape must across various wine-producing regions and grape varieties, while *Metschnikowia* spp. thrive abundantly in grape must [13]. These non-*Saccharomyces* yeasts can significantly influence wine fermentation. Some impact flavour production directly, while others modulate the growth and metabolism of *S. cerevisiae*. *Metschnikowia pulcherrima* and *S. bacillaris* contribute to the production of 2-phenylethyl alcohol, associated with pleasant flavours at moderate concentrations [14,15]; *Hanseniaspora uvarum*, on the other hand, produces acetate and fruity esters [16,17]. Several non-*Saccharomyces* strains, including *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *M. pulcherrima*, and *Pichia kluyveri*, have recently entered commercial use. These species play specific roles in wine production, aiming to achieve the following objectives: (i) enhancing varietal aromas [18]; (ii) regulating acidity characteristics [19]; (iii) improving colour extraction and mouthfeel characteristics [20]; (iv) reducing ethanol content [2]; (v) in the context of sparkling wines, playing a role in improving effervescence [21]. Despite their valuable contributions, non-*Saccharomyces* yeasts generally exhibit lower fermentation performance and do not numerically dominate the entire fermentation process due to their limited tolerance to ethanol and SO_2 [22,23].

During the initial stages of fermentation, yeasts play a relevant role by significantly impacting the metabolic processes, leading to noticeable changes in the volatile characteristics of wine. This makes them suitable candidates for co-inoculation or sequential inoculation alongside *S. cerevisiae* [1,24]. Numerous studies indicate that matrices with high sugar content harbour both *Saccharomyces* and non-*Saccharomyces* yeasts, which have potential applications in oenology and the production of fermented beverages. For instance, Matraxia et al. [17] explored the use of *H. uvarum*, isolated from honey by-products (honeycombs and capping waxes) during beer fermentation, in co-inoculation with *S. cerevisiae*. Alfonzo et al. [25] successfully employed *S. cerevisiae* strains isolated from honey in winemaking, revealing significant differences compared to *S. cerevisiae* strains isolated from grapes.

Microbial communities specific to a particular food matrix significantly contribute to its composition and properties for food-related purposes. Guarcello et al. [26] conducted a study analyzing the microbial ecology of Sicilian manna ash (the phloem sap, a cerulean liquid that, on contact with air, quickly thickens and forms a light crystalline whitish layer that represents manna). Their goal was to gain insight into the hygienic quality, shelf- life, and potential applications of this traditional food. The study characterised the microorganisms associated with different products obtained during manna processing. Additionally, Gaglio et al. [27] investigated the microbial biodiversity of honey by-products used in the

production of "Spiritu re fascitrari". They discovered a niche rich in *Saccharomyces* and non-*Saccharomyces* yeasts.

Based on the above considerations, the aims of the present study are as follows: (i) identify a group of yeasts isolated from manna and honey; (ii) assess oenology through specific resistance, osmotolerance, and enzymatic activity tests; (iii) determine the fermentation performance of the best strains (such as starter or co-starter) by micro-fermentation.

2. Materials and Methods

2.1. Isolate Origins, DNA Extraction, and RFLP Analysis

The yeasts studied here in this research work belong to the collection of the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Palermo, Italy); they were isolated from manna and honey by-products (Table S1). Specifically, 27 isolates have already been characterised genotypically at the species level [26], while 38 isolates have been identified using molecular techniques. DNA was extracted using the Quick-DNA Microprep Kit (Zymo Research, Orange, CA, USA) according to the instructions of the manufacturer. For initial discrimination, 38 yeast isolates were analysed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8 S rRNA gene. DNA amplification was performed with the ITS1/ITS4 primer pair in accordance with Esteve-Zarzoso et al. [28]. The resulting amplicons were then digested with *Cfo*I, *Hae*III, and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. ITS amplicons and the corresponding restriction fragments were analysed on an agarose gel using 1.5% and 3% (*w*/*v*) agarose in a 1 × TBE (89 mM Tris-borate, 2 mM EDTA, pH 8) buffer, stained with SYBR safe DNA gel stain (Invitrogen, Waltham, MA, USA), visualised by UV transillumination, and captured on the Gel Doc 1000 video gel documentation system (Bio-Rad, Richmond, CA, USA). The standard DNA ladders used were 1 kb Plus and 50 pb (Invitrogen, Waltham, MA, USA).

2.2. Strain Typing and Species Identification

The intraspecific characterisation of the isolates belonging to the *S. cerevisiae* strains was carried out by Interdelta analysis with primers delta 12 and delta 21 [29]. The intraspecific characterisation of the isolates belonging to the non-*Saccharomyces* strains was carried out using different RAPD-PCR assays with primers M13 [30] and XD5 [31]. PCR products were analysed and visualised as described by Settanni et al. [32]. At least one strain per RAPD group was further processed by 26S rRNA gene D1/D2 region sequencing [27]. The data were compared with the sequence published in the GenBank database by means of the BLAST alignment tool http://blast.ncbi.nlm.nih.gov/ (accessed on 22 January 2024).

2.3. Technological Screening

All strains were tested for technological characteristics, H_2S production, osmotolerance, and resistance to ethanol, potassium metabisulphite, and copper. In addition, growth tests on lysine were conducted. The ability to produce H_2S was tested using a qualitative method performed on Bismuth Sulphite Glucose Glycerin Yeast extract (BiGGY) agar (Oxoid, Milan, Italy) [33]. Hydrogen sulphide was estimated by colony blackening after 3 days of incubation at 28 °C. A four-level scale was used: −, no growth; +, growth and low H_2S production; P, growth and medium H_2S production; PP, growth and high H_2S production. Only strains with low H_2S production were subjected to additional tests. The resistance tests were performed in a modified YPD medium containing different doses of each stress agent and according to the selection criteria for non-*Saccharomyces* yeasts described by Mestre Furlani et al. [34]. Accordingly, the following concentrations were used 4, 8, or 12% (*v*/*v*) of ethanol; 220, 270, or 320 g/L of glucose to test osmotolerance; 150 or 200 mg/L of sulphur dioxide (SO_2) by addition of potassium metabisulphite ($K_2S_2O_5$); and 2.5, 5, or 10 mM of copper, supplied as copper sulphate.

2.4. Growth Kinetics on a Single Source of Sugar

The strains were also evaluated for their ability to grow in the presence of single-sugar matrices using the procedure described by Kurtzman et al. [35] with the following modifications: the tests were performed in rimless tubes (16 × 180 mm), each containing 10 mL medium broth (yeast extract, 3 g/L; triptone, 5 g/L; glucose or fructose, 200 g/L) and inoculated with the pure strain cultures as reported by Hall et al. [36].

Growth of pure strain cultures in synthetic media was assessed by measuring optical density (OD) at 600 nm in a 96-well microtitre plate. Measurement was performed every 24 h for 4 days using ScanReady microplate photometer P-800 (Life Real Biotechnology Co., Ltd., Hangzhou, China). The temperature of the incubation was set at 25 °C. A blank measurement was subtracted from each OD reading. All analyses were performed in triplicate. Total growth of the strains was calculated as the integrated area underlying the curve up to 4 days as described by Hall et al. [36].

2.5. Fermentation of Grape Must

The strains with low H_2S production, high resistance to ethanol and potassium metabisulphite, and the ability to grow rapidly on glucose and fructose substrates were evaluated for their ability to ferment a grape must.

The grapes cv. Traminer were harvested during the 2023 vintage. All the micro-vinifications were carried out in the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Palermo, Italy). The grapes were harvested, destemmed, and crushed by hand. The must obtained was divided into 21 batches (1 L each) and pasteurised at 72 °C for 15 s. The yeasts were inoculated in liquid concentrated form (approximately 6.0 Log CFU/g) the TR1 to TR3 trials were inoculated with different strains of non-*Saccharomyces*, each belonging to the species *Lachancea thermotolerans* MN400, *Starmerella lactis-condensi* MN412, and *Candida oleophila* YS209. While experiment TR4 was inoculated with a strain of *S. cerevisiae* MN113 from manna, TRC was inoculated with a commercial strain of *S. cerevisiae* EC1118 (Lallemand Inc., Montreal, QC, Canada). The experimental design is shown in Figure 1.

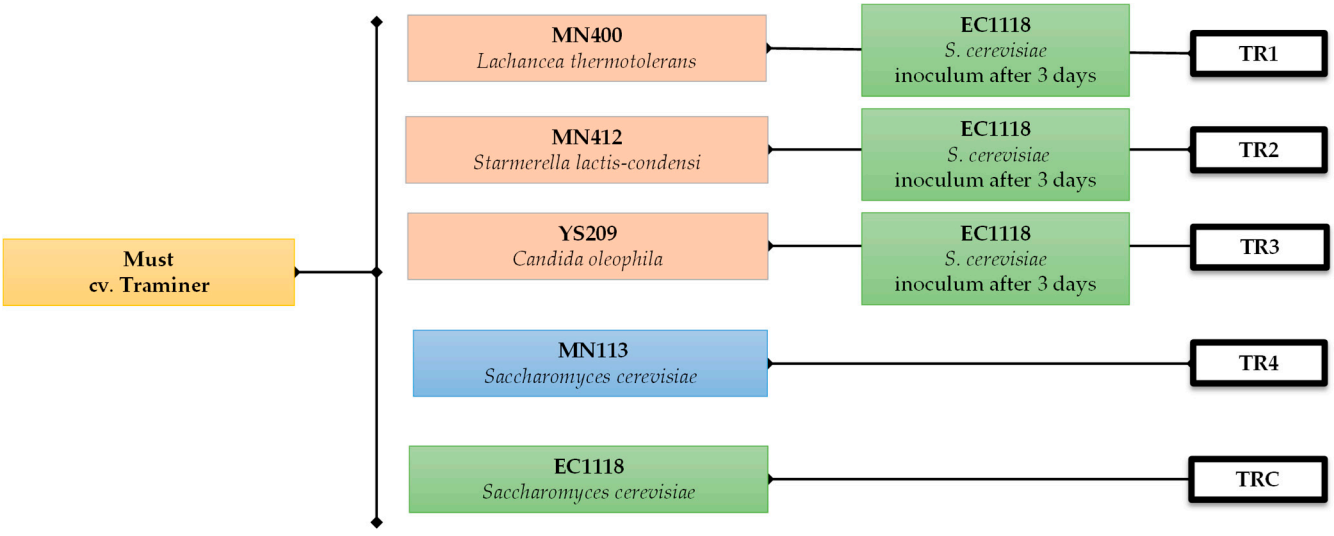

Figure 1. Experimental plan of micro-vinification.

After 3 days, each experiment from TR1 to TR3 was inoculated with *S. cerevisiae* EC1118. The alcoholic fermentation of all experiments was carried out at 20 °C for 30 days.

At the end of the alcoholic fermentation, potassium metabisulphite (8 g/hL) was added to all experiments. Samples were collected at different stages of vinification: at the time of non-*Saccharomyces* strain inoculation (0 days), after the inoculation of *S. cerevisiae* (3 days of alcoholic fermentation), after 8 days of fermentation, and at the end of alco-

holic fermentation. The collected samples were immediately analysed. All analyses were performed in triplicate. To remove CO_2, the flasks were sealed with a Müller valve [37], and the weight loss was monitored until it fell below 0.01 g per day, indicating the end of fermentation.

2.6. Microbiological and Oenological Parameters

All samples collected during alcoholic fermentation were analysed for yeast populations. Musts samples were diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy) and analysed in triplicate for presumptive *Saccharomyces* spp. yeasts on Wallerstein Laboratory (WL) nutrient agar [38], and non-*Saccharomyces* were counted on lysine agar [39]. All media and supplements were purchased from Oxoid (Thermofisher, Milan, Italy).

The wines obtained were analysed by means of WineScan (FOSS, Hillerød, Denmark) to determine volatile acidity (VA), reducing sugars, ethanol, glycerol, malic acid, and lactic acid. The instrument was calibrated according to the EEC 2676 standard procedure [40]. pH was determined according to the OIV-MA-AS313-15 method [41] and total acidity (TA) according to the method described in OIV-MA-AS313-01 [42]. All chemical analyses were performed in triplicate.

2.7. Statistical Analysis

The ANOVA test was employed to ascertain the statistical significance between the microbial loads (presumed *Saccharomyces* and non-*Saccharomyces*) and the chemical parameters observed during the winemaking process (residual sugar, ethanol, glycerol, malic acid, lactic acid and volatile acidity and total acidity). The post-hoc Tukey method was used for pairwise comparison of all data. Statistical significance was set at $p < 0.05$ [43].

An exploratory multivariate approach using Agglomerative Hierarchical Clustering (AHC) was used to investigate the relationships between the data obtained at the end of the alcoholic fermentation (ethanol, residual sugar, glycerol, malic acid, lactic acid, pH, total acidity, and volatile acidity) from the different treatments [25]. The software used for agglomerative statistical data processing was XLStat ver. 2019.2.2 (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. Isolation, Identification, and Strain Typing of Yeasts

Out of a total of 65 yeast isolates, 38 were subjected to genotypic characterisation. The restriction analysis of the ITS1-5.8S-ITS2 region led to the separation of these isolates into five distinct profiles (Table 1).

Table 1. Molecular identification of yeast species isolated from manna and honey samples.

Strain Origin	Number of Isolates	Size Amplicons 5.8S-ITS (bp)	Size of Restriction Fragment (bp) [a]			Number of Strains [b]	Species	Range Size of the PCR Products (bp)	Acc. No. (Range % Similarity)
			*Cfo*I	*Hae*III	*Hinf*I				
Manna	4 + 1 [c]	700	330 + 210	450 + 200 + 80	390 + 320	4 + 1 [c]	*Citeromyces matritensis*	543–565	PP695356-59 (99.26–100)
Manna	29 + 19 [c]	680	320 + 275	300 + 210 + 80	345	3 + 4 [c]	*Lachancea thermotolerans*	551–581	PP695351-53 (99.82–100)
Honey	2 + 1 [c]	600	310 + 260	400 + 125 + 80	320	1 + 1 [c]	*Meyerozyma guillermondii*	563–568	PP695355 (99.64)
Honey	1	400	180 + 175	280 + 190	220	1	*Starmerella magnoliae*	441	PP695354 (100)
Manna	1 + 1 [c]	850	370 + 330	310 + 240 + 175 + 130	370 + 360 + 120	1[c]	*Saccharomyces cerevisiae*	Guarcello et al. [23]	Guarcello et al. [23]

[a] Values refer to the number of base pairs per fragment. [b] Strain typing was performed with RAPD-PCR for non-*Saccharomyces* and by Interdelta analysis for *Saccharomyces* yeast. [c] Yeast isolates previously identified by Guarcello et al. [26].

Preliminary species identification was performed by comparing the restriction profiles with those reported in the literature [28,30,40]. Specifically, the isolates were identified as *Citeromyces matritensis*, *L. thermotolerans*, *Meyerozyma guillermondii*, *Starmerella magnoliae*, and *S. cerevisiae*. To further confirm the species identification, genotypic analysis involved pairwise alignment of D1/D2 sequences (Table 1). Strain typing allowed the 65 isolates to be grouped into 21 strains, distributed across eight yeast species (Table 2). Among these, *L. thermotolerans* had the highest number of strains (n = 7), while *S. magnoliae*, *C. oleophila*, and *S. cerevisiae* were each represented by a single strain.

Table 2. Technological screening of 21 yeast strains.

Strain	H_2S [α]	Ethanol Resistance [β]			MBSK Resistance [γ]		Osmotic Resistance [δ]			Copper Resistance [ε]		
		4%	8%	12%	150 mg/L	200 mg/L	220 g/L	270 g/L	320 g/L	2.5 mM	5 mM	10 mM
MN114	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MN117	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS209	−	+	−	−	+	+	+/−	+/−	+/−	+	+	+
MN85	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MNF138	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MNF289	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MNF308	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS82	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MN28	−	+	+	−	+	+	+	+	+	+	+	+/−
MN93	−	+	+	−	+	+	+	+	+	+	+/−	−
MN136	−	+	+	−	+	+	+	+	+	+	+	+/−
MN400	−	+	+	−	+	+	+	+	+	+	+	+/−
MNF104	−	+	+	−	+	+	+	+	+	+	+	+/−
MNF105	−	+	+	−	+	+	+	+	+	+	+	+/−
YS1	−	+	+	−	+	+	+	+	+	+	+	+/−
YS246	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS300	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MN113	+	+	+	+	+	+	+	+	+	+	+	+/−
MN412	−	+	+	−	+	+	+	+	+	+	+/−	−
MN417	−	+	+	−	+	+	+	+	+	+	+/−	+/−
YS292	−	+	−	−	+	+	+/−	+/−	+/−	+	+	+

[α] H_2S Production: −, no growth; +, growth and low H_2S production; P, growth and medium H_2S production; PP, growth and high H_2S production; [β] growth on YPD supplied with different ethanol percentages (4, 8, and 12% (*v*/*v*)); [γ] growth on YPD supplied with different concentrations of potassium metabisulphite (150 and 200 mg/L); [δ] growth on YPD supplied with different glucose concentrations (220, 270, and 320 g/L); [ε] growth on YPD supplied with different copper concentrations (2.5, 5, and 10 mM). Abbreviations: n.d., not determined.

3.2. Technological Characteristics of Yeast Strains

Table 2 reports the results of technological screening.

Several differences were observed between different yeast species. All strains underwent assessment for their ability to produce H_2S [44]. However, only strains from the species *C. matritensis*, *C. aaseri*, and *M. guillermondii* showed high H_2S production and were therefore excluded from subsequent resistance tests. In ethanol resistance tests, strains belonging to the species *L. thermotolerans* and *S. lactis-condensi* demonstrated resistance to 8% (*v*/*v*) ethanol. Meanwhile, strains from the species *S. magnoliae* and *C. oleophila* exhibited resistance to 4% (*v*/*v*) ethanol, whereas *S. cerevisiae* displayed resistance to 12% (*v*/*v*) ethanol. Moreover, all strains showed growth in the presence of 200 mg/L of potassium metabisulphite. Regarding copper resistance, there was significant variability among strains of the species *L. thermotolerans*. However, only strains from the species *S. magnoliae* and *C. oleophila* resisted the highest copper concentrations (10 mM). Based on the results of previous technological tests, all 12 strains were selected for further investigation of their growth kinetics on fructose and glucose media (Figure 2).

During fructose fermentation, the highest OD value on the 4th day of fructose fermentation was observed for *S. cerevisiae* MN113, reaching 1.15. Among non-*Saccharomyces*,

strain MN400 *L. thermotolerans* showed the best growth, with an OD value of 0.92 after 4 days of glucose fermentation. Notably, during fructose fermentation, *S. lactis-condensi* MN412 showed OD values (0.90) higher than the control strain *S. cerevisiae* EC1118 (0.79) after 4 days of incubation. This characteristic suggests potential fructophilic activity of strain MN412. The complete growth values are summarised in Table 3.

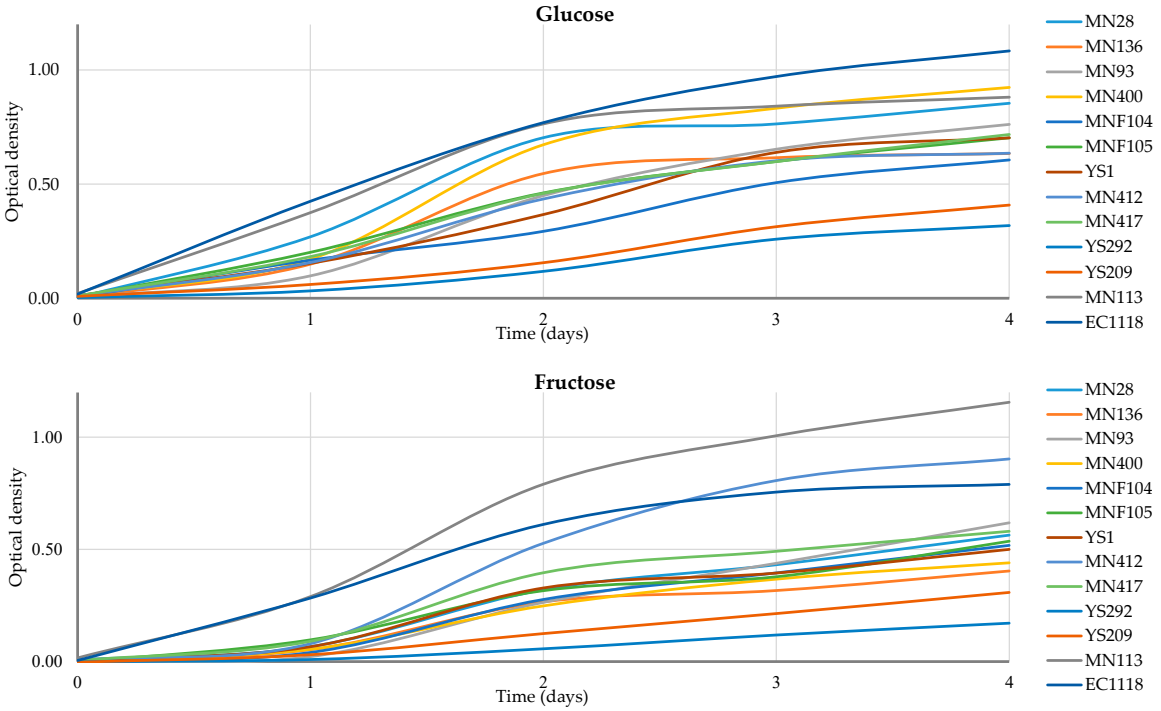

Figure 2. Growth of different strains in a single-sugar matrix of glucose or fructose. The growth was measured using OD values at 600 nm in triplicate. Values of standard deviation ranged between 0 and 0.16 but are not shown for a better graphical visualisation of the figures.

Table 3. Total growth of the strains on a synthetic medium containing exclusively glucose or fructose as a sugar source. Total growth was calculated as the integral area under the curve determined after 4 days of incubation.

Strain	Glucose	Fructose
non-*Saccharomyces* spp.		
MN28	2.11	1.10
MN136	1.63	0.84
MN93	1.58	1.03
MN400	2.14	0.89
MNF104	1.27	0.97
MNF105	1.61	1.06
YS1	1.51	1.04
MN412	1.51	1.87
MN417	1.60	1.27
YS292	0.57	0.27
YS209	0.73	0.52
Saccharomyces spp.		
MN113	2.42	2.66
EC1118 (Control)	2.71	2.04

In terms of overall growth on glucose, among the non-*Saccharomyces* strains, *L. thermotolerans* MN400 showed the highest value (2.14). On the other hand, for fructose growth, *S. lactis-condensi* MN412 achieved the highest value (1.87). Additionally, when it comes

to fructose, *S. cerevisiae* MN113, isolated from manna, displayed greater growth than the EC1118 strain (control).

3.3. Micro-Fermentation

For the TR1 trial, *L. thermotolerans* MN400 was chosen due to its robust copper resistance and optimal growth dynamics on both glucose and fructose. In the TR2 experiment, *S. lactis-condensi* MN412 served as a co-starter, exhibiting vigorous growth, specifically on fructose. In the TR4 fermentation experiment, *S. cerevisiae* MN113 acted as the primary starter. Throughout the micro-fermentations, daily weight loss (CO_2 emitted) was monitored over a period of 30 days, spanning the completion of alcoholic fermentation. The results from the fermentation kinetics (Figure 3) demonstrated that 3 days after inoculation the non-*Saccharomyces* species with the most substantial weight loss were *S. lactis-condensi* (TR2) and the *L. thermotolerans* strain (TR1), while among *Saccharomyces*, the commercial strain EC1118 (TRC) and manna-isolated MN113 strain (TR4) showed the highest fermentation rates. Other strains exhibited minimal fermentation activity.

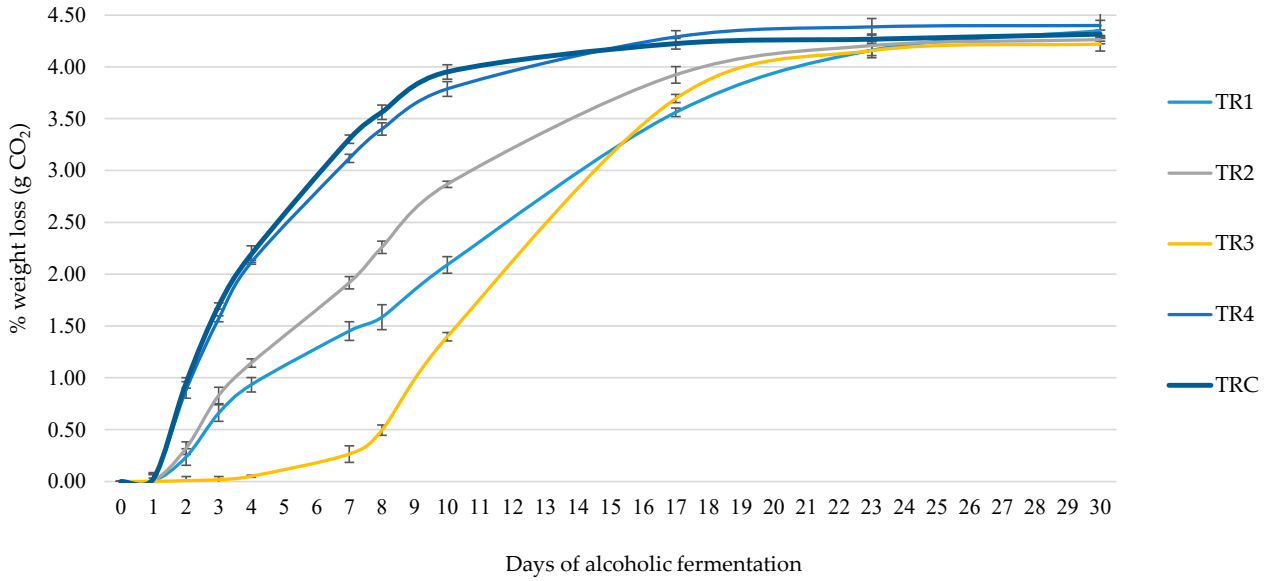

Figure 3. Weight loss during micro-vinification.

3.4. Microbiological Counts

Table 4 presents the microbial yeast counts during fermentation.

Table 4. Monitoring of yeast populations during experimental micro-fermentation.

Samples	Microbial Loads (Log CFU/mL)					
	TR1	**TR2**	**TR3**	**TR4**	**TRC**	**S.S.**
Saccharomyces spp.						
T0	n.d.	n.d.	n.d.	6.49 ± 0.15 [a]	6.79 ± 0.23 [a]	n.s.
T3 [α]	6.48 ± 0.08 [b]	6.53 ± 0.12 [b]	6.57 ± 0.07 [b]	7.44 ± 0.24 [a]	7.36 ± 0.17 [a]	***
T8	7.18 ± 0.20 [a]	7.13 ± 0.11 [a]	7.26 ± 0.37 [a]	7.35 ± 0.22 [a]	7.26 ± 0.24 [a]	n.s.
End of AF	6.67 ± 0.33 [a]	6.36 ± 0.15 [a]	6.32 ± 0.16 [a]	6.40 ± 0.16 [a]	6.23 ± 0.21 [a]	n.s.
Non-*Saccharomyces* spp.						
T0	5.94 ± 0.27 [a]	6.05 ± 0.11 [a]	6.21 ± 0.10 [a]	n.d.	n.d.	n.s.
T3	7.20 ± 0.32 [a]	7.23 ± 0.10 [a]	7.11 ± 0.13 [a]	n.d.	n.d.	n.s.
T8	6.65 ± 0.28 [a]	6.48 ± 0.22 [a]	<2.00	n.d.	n.d.	n.s.
End of AF	4.25 ± 0.20 [a]	4.30 ± 0.14 [a]	<2.00	n.d.	n.d.	n.s.

[α] inoculum of *S. cerevisiae* EC1118 in trials TR1, TR2, and TR3. Results indicate average values ± standard deviation of three plate counts. Abbreviations: T0, must after yeast inoculum; T3, 3 days of alcoholic fermentation; T8, 8 days of alcoholic fermentation; AF, alcoholic fermentation. S.S., statistical significance; n.d., not determined. Data in the same line followed by the same letter are not significantly different according to Tukey's test. *p* value: ***, $p < 0.001$; n.s., not significant [45].

Microbiological monitoring involved presumptive counts of both *Saccharomyces* and non-*Saccharomyces* at various stages: T0 (inoculation of the starter/co-starter), day 3 (inoculation of *S. cerevisiae* in TR1, TR2, and TR3 trials), day 8, and at the end of alcoholic fermentation. The non-*Saccharomyces* strains were initially inoculated within the range of 5.9 to 6.2 log (CFU/mL), whereas *Saccharomyces* strains (MN113 and EC1118) were inoculated at a density around 6.5–6.8 log (CFU/mL). By day 3 of alcoholic fermentation across all treatments, yeast populations exhibited growth, reaching values of 6.5 and 7.4 Log CFU/mL. On the third day, *Saccharomyces* was inoculated at approximately 6.5 Log CFU/mL for each of the experiments (TR1, TR2, and TR3). After 8 days of alcoholic fermentation, TR1 and TR2 trials showed non-*Saccharomyces* counts approximately 0.5 logarithmic cycle higher than presumptive *Saccharomyces*. In TR3, the non-*Saccharomyces* counts fell below the detection limit due to their low resistance to ethanol. This trend aligns with findings by Binati et al. [2] who studied three different non-*Saccharomyces* species combined with *S. cerevisiae*. At the end of alcoholic fermentation, TR1 and TR2 exhibited non-*Saccharomyces* counts of 4.2–4.3 Log CFU/mL, while *Saccharomyces* counts were approximately 6.4–6.7 Log CFU/mL. Several authors agree that co-inoculating *S. cerevisiae* and non-*Saccharomyces* yeast species can lead to the demise or reduced variability of non-*Saccharomyces* once *S. cerevisiae* dominates the fermentation and becomes stress-resistant to inhibitory ethanol.

Furthermore, the secretion of inhibitory substances has been identified as a potential cause of inhibition in non-*Saccharomyces* yeasts [46]. Therefore, researchers recommend a sequential inoculation approach (non-*Saccharomyces* followed by *S. cerevisiae*) over a mixed culture. This technique allows for greater expression of non-*Saccharomyces* yeast metabolism [47]. In trials where single-culture MN113 *S. cerevisiae* (isolated from manna) was used (TR4), similar trends were observed compared to control trials (TRC) inoculated with grape yeasts. Alfonzo et al. [25] used *S. cerevisiae* isolated from honey by-products in wine production and found its microbiological behaviour to be comparable to that of *S. cerevisiae* isolated from grapes.

3.5. Physico-Chemical Analysis

The influence of manna yeasts (both *Saccharomyces* and non-*Saccharomyces*) on the chemical composition of wines was evaluated by quantifying key analytical components at the end of alcoholic fermentation. The summarised results of these chemical analyses are presented in Table 5.

Table 5. Chemical parameters determined during the micro-vinification process.

Parameters	Musts	Micro-Vinification					
		End of Alcoholic Fermentation					
		TR1	TR2	TR3	TR4	TRC	S.S.
Residual sugars $^{\alpha}$	221.83 ± 2.26	0.24 ± 0.05 ^{a}	0.15 ± 0.04 ^{a}	0.26 ± 0.09 ^{a}	0.28 ± 0.05 ^{a}	0.24 ± 0.04 ^{a}	n.s.
Ethanol $^{\beta}$	n.d.	11.34 ± 0.05 ^{b}	11.36 ± 0.05 ^{ab}	11.35 ± 0.05 ^{ab}	11.47 ± 0.05 ^{ab}	11.50 ± 0.05 ^{a}	*
Malic acid $^{\alpha}$	1.71 ± 0.15	1.47 ± 0.04 ^{b}	1.57 ± 0.06 ^{ab}	1.59 ± 0.04 ^{ab}	1.66 ± 0.07 ^{ab}	1.66 ± 0.06 ^{a}	*
Lactic acid $^{\alpha}$	n.d.	0.69 ± 0.04 ^{a}	0.02 ± 0.04 ^{b}	0.03 ± 0.02 ^{b}	0.06 ± 0.02 ^{b}	0.03 ± 0.01 ^{b}	*
Glycerol $^{\alpha}$	n.d.	7.40 ± 0.15 ^{a}	6.20 ± 0.17 ^{b}	5.30 ± 0.11 ^{c}	4.80 ± 0.10 ^{d}	5.10 ± 0.14 ^{cd}	***
pH	3.63 ± 0.01	3.67 ± 0.02 ^{a}	3.68 ± 0.02 ^{a}	3.69 ± 0.02 ^{a}	3.69 ± 0.02 ^{a}	3.68 ± 0.01 ^{a}	n.s.
VA $^{\alpha}$	n.d.	0.36 ± 0.02 ^{ab}	0.18 ± 0.02 ^{c}	0.28 ± 0.03 ^{b}	0.30 ± 0.04 ^{b}	0.38 ± 0.02 ^{a}	***
TA $^{\alpha}$	5.10 ± 0.02	5.55 ± 0.05 ^{a}	4.96 ± 0.10 ^{b}	4.98 ± 0.06 ^{b}	5.04 ± 0.12 ^{b}	5.04 ± 0.07 ^{b}	***

Results indicate mean value ± standard deviation of three determinations from three replicates. $^{\alpha}$, expressed in g/L; $^{\beta}$, expressed in % (*v*/*v*). Abbreviations: VA, volatile acidity (acetic acid g/L); TA, total titratable acidity (tartaric acid g/L); n.d., not detected; S.S., statistical significance. Data within a line followed by the same letter are not significantly different according to Tukey's test. *p* value: *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant.

In terms of glycerol content, the highest values were observed in trials inoculated with *L. thermotolerans* (7.4 g/L, TR1), followed by TR2 (6.20 g/L), TR3 (5.30 g/L), and TRC (5.10 g/L). Lastly, the trial involving the use of manna-isolated strain MN113 showed the lowest value (4.80 g/L). This trend aligns with findings reported by Hranilovic et al. [48]. Volatile acidity showed the highest value in the TRC trial (0.38 g/L acetic acid), while the lowest value was recorded in the TR2 trial (0.18 g/L acetic acid). Across the other treatments, VA values remained below 0.80 g/L, which is the threshold beyond which wine quality is compromised [49].

At the conclusion of alcoholic fermentation, many of the obtained wines displayed low residual sugar content (<0.5 g/L), a common characteristic of dry wines [50]. This confirms the successful completion of fermentation by the yeasts. The ethanol content in trials T2, T3, and T4 was comparable to the TRC control trial. However, trial T1 showed lower ethanol values than TRC. Additionally, an AHC analysis was conducted on the primary chemical data from the wines to better visualize the technological variability introduced by the strains used (Figure 4).

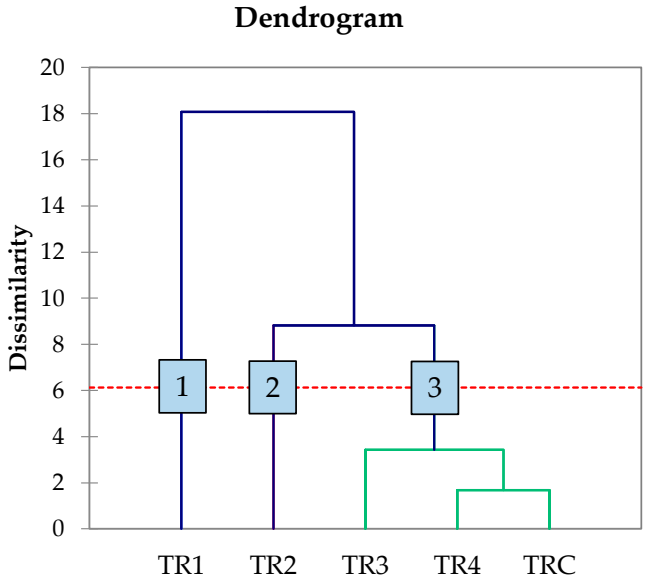

Figure 4. Dendrogram generated by AHC analysis of principal component analysis of the main oenological parameters at the end of alcoholic fermentation. Numbers indicate the different clusters emerged throught AHC analysis. The red dotted line represents the level of significance.

Through data processing, the five treatments were categorised into three distinct groups. Group 1 was represented by trial TR1, which differed from the others due to variations in glycerol and TA content. Group 2, associated with trial TR2, exhibited distinct volatile acidity compared to the remaining trials. Group 3 consisted of trials TR3, TR4, and TRC. In terms of chemical parameters, strains *L. thermotolerans* MN400 and *S. lactis-condensi* MN412 yielded different wine profiles. Interestingly, *S. cerevisiae* strain MN113 showed a fermentation performance similar to that of the commercial strain, suggesting its potential as a starter for winemaking. However, a comprehensive evaluation of the aromatic and sensory impact of these strains on wines produced at both medium and large scales remains necessary.

4. Conclusions

In this research, both culture-dependent and molecular techniques were used to explore the diversity of yeasts in high-sugar matrices such as manna and honey. The primary focus was on yeasts, aiming to investigate their potential application in oenology. The study revealed a rich variety of non-*Saccharomyces* and *Saccharomyces* yeasts present in manna and honey by-products. In order to ascertain their suitability for use as starters or co-starters in winemaking, an extensive technological characterisation was conducted on the pasteurised grape juice. The objective of this characterisation was to facilitate more precise control over the yeast population's development. In particular, strains with limited H_2S production and enhanced tolerance to ethanol, osmotic stress, and copper were carefully selected.

Given that the characteristics analysed are influenced by the yeast species and strain, the results underscore the importance of characterising a diverse range of isolates. These selected starters and co-starters can be employed in monoculture or mixed fermentations, ultimately enhancing wine quality and imparting distinct characteristics to the final product. However, additional research is necessary to assess how these chosen strains impact the volatile organic components when subjected to sequential inoculation with *S. cerevisiae*. The use of pasteurised must allowed an initial screening of strains of oenological interest. Further studies will be carried out in real fermentations to verify the suitability of the selected strains.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages10030048/s1, Table S1: List of yeast isolates from manna and honey subjected to strain typing.

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