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Unusual Non-*Saccharomyces* Yeasts Isolated from Unripened Grapes without Antifungal Treatments

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Abstract: There a lot of studies including the use of non-*Saccharomyces* yeasts in the process of wine fermentation. The attention is focused on the first steps of fermentation. However, the processes and changes that the non-*Saccharomyces* yeast populations may have suffered during the different stages of grape berry ripening, caused by several environmental factors, including antifungal treatments, have not been considered in depth. In our study, we have monitored the population dynamics of non-*Saccharomyces* yeasts during the ripening process, both with biochemical identification systems (API 20C AUX and API ID 32C), molecular techniques (RFLP-PCR) and enzymatic analyses. Some unusual non-*Saccharomyces* yeasts have been identified (*Metschnikowia pulcherrima*, *Aureobasidium pullulans*, *Cryptococcus sp*. and Rhodotorula mucilaginosa). These yeasts could be affected by antifungal treatments used in wineries, and this fact could explain the novelty involved in their isolation and identification. These yeasts can be a novel source for novel biotechnological uses to be explored in future work.

Keywords: yeast; non-*Saccharomyces*; grape berry; population dynamics; ripening; wine

1. Introduction

Grape berries microbiota refers to all species of filamentous fungus, yeast and bacteria that have been found in grape berries, in vineyard soil or in wine. Regarding yeasts, these microorganisms belong to Ascomycetes, Basidiomycetes and Deuteromycetes classes [1]. Yeasts that are found in grape berries can be classified into two groups: (i) *Saccharomyces*, those which are responsible for sugar fermentation in grape berries, and (ii) non-*Saccharomyces* or wild yeast. Some species that belong to the last group can participate in the beginning of the fermentation process [2]. The non-*Saccharomyces* species present in the grape juice and, in the first stages of fermentation, are divided into three groups: yeasts that are mostly aerobic (*Pichia* spp., *Rhodotorula* spp., or *Cryptococcus albidus*), yeasts with low fermentation ability (*Kloeckera apiculata*, *K. apis* and *K. javanica*) and yeasts that display fermentative metabolism (*Metschnikowia pulcherrima*, *Kluyveromyces marxiamus* and *Zygosaccharomyces bailii*) [3,4]. Nevertheless, non-*Saccharomyces* yeasts can also be detected before the fermentation process, i.e., during ripening and harvest processes. The development of these yeasts has been observed not only on the surface of grape berries and their must but also in the environment where the wine production takes place and in the wine cellar equipment. Therefore, these places can be considered specialized niches for non-*Saccharomyces* yeasts [5,6].

The importance of non-*Saccharomyces* yeasts is their contribution to different wine features, flavor and bouquet principally. This contribution depends on metabolites concentration, that is affected directly by yeast activity. Initially it was believed that all non-*Saccharomyces* species died after the beginning of alcoholic fermentation but further studies do not support this statement [7–11]. Even so,

environmental factors, such as oxygen concentration, play an important role in the survival and growth of the different species [10,12,13].

According to Fleet [10], yeasts influence beverage aroma by different mechanisms; of these, *de novo* biosynthesis of aroma compounds is probably the most important [14]. The variety of odor compounds produced by non-*Saccharomyces* yeasts is already known. The contribution of non-*Saccharomyces* yeasts to flavor quality can take various forms. Production of glycerol by *Candida stellata* and esters by *C. pulcherrima* has been reported [11]. Other non-*Saccharomyces* yeasts are also widely recognized for producing glucosidase enzymes, which, by hydrolyzing such bonds, can release volatile compounds linked to sugars, giving greater complexity to the aromatic profile [1,15].

Furthermore, metabolic interactions have been noticed between *S. cerevisae* and non-*Saccharomyces* wine species during the fermentation process. There are evidences that the interaction between some yeast species, when they develop together during fermentation, produces hardly predictable metabolites, which could alter the wine composition in chemical and aromatic ways [16,17]. In addition, these synergic metabolic interactions between different yeast species could be used to conceive new technology in the fermentation field [13].

Viticulture represents an important agricultural practice in many countries and the long-term use of organic and inorganic pesticides in vineyards has resulted in increased concentrations of these pollutants in soils and other environmental compartments [18]. Contamination with metals and organic pollutants, together with erosion and tillage, reduces the quality of the soils and poses important environmental and toxicological threats. Vineyard soils are usually highly degraded soils in terms of biochemical properties [19] and are thus more susceptible to contamination. Nevertheless, few studies have been developed regarding the impact of these products on the yeast population diversity [20].

The aim of this work was to study the origin of non-*Saccharomyces* yeasts along the grape ripening process in vineyards untreated with antifungal products, isolating, identifying and characterizing different microorganisms detected.

2. Materials and Methods

2.1. Samples

Samples were collected in a winery without antifungal treatments located in Iniesta, Cuenca, Spain (39°26′ N, 1°48′ W), during a period between 25 July and 1 September (2017). The samples were taken in different stages of the ripening process and were stored in sterile bags, keeping them at −20 °C until its analysis.

2.2. Yeasts Isolation

Samples were homogenized by using a mortar and then left for 24 h at 4 °C. Decimal dilutions were made in saline solution, and 100 µL for each dilution were inoculated in Malt Agar plates (malt extract, 20 g/L; glucose, 20 g/L; agar, 20 g/L; mycological peptone, 1 g/L). All reagents were purchased form Pronadisa (Madrid, Spain). Lastly, plates were incubated at 28 °C for 24–48 h and viable cells were determined. A total of 105 colonies were selected and cultured in Malt Agar plates in order to obtain pure cultures. The colonies that grew were observed under the optic microscope in order to establish their cellular morphology.

2.3. Physiological Characterization of Isolates

The yeast identification systems API 20C AUX and API ID 32C (BioMerieux, Craponne, France) were used to assess the strain-specific pattern of carbon compound assimilation and other phenotypic assays. Strips were inoculated and inspected for growth daily, according to the manufacturer's instructions.

2.4. Molecular Identification Using rDNA Sequence

Chromosomal DNA was isolated by using Ultraclean Microbial DNA isolation Kit (MoBio, Carlsbad, CA, USA). Then, it was subjected to PCR amplification using the primers ITS1/ITS4 and NL1/NL4, as previously described [21,22].

The amplified DNAs (15 µg) were digested with the restriction endonucleases: *Cfo*I, *Hae*III and *Hinf*I (Takara, Shiga, Japan). The PCR product and the restriction fragments were separated on 2.5% (w/v) agarose gels. Molecular weight was estimated by comparison against 100 bp and 20 bp DNA ladders (Takara, Shiga, Japan). PCR and RFLP fragment lengths were used for identification of yeasts using www.yeast-id.com (Universitat de València and CSIC, Paterna, Spain). Moreover, yeast strain adscription was verified by PCR amplification for the D1/D2 domain of 26S rDNA of sample strains using the primers NL1 and NL4 [21]. The PCR product was purified using UltraClean PCR Clean Up kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. Direct sequencing of the purified PCR products was performed by ABI Prism BigDye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, TX, USA). The sequences were aligned, by using the BLAST program, with complete or nearly complete 26S rDNA gene sequences retrieved from the EMBL nucleotide sequence data libraries [21].

2.5. Enzymatic Characterization

2.5.1. Protease Activity

Exocellular protease production was determined by spreading yeast colonies onto YPD agar plates containing 20 g/L casein. Plates were incubated at 28 °C for 7 days. A clear zone around the colony is indicative of protease activity.

2.5.2. Beta-Glucosidase Activity

The assay was carried out in a medium containing 1 g/L yeast extract, 1 g/L peptone, 2 g/L glucose, 0.01 g/L ferric ammonium citrate, 0.3 g/L esculin and 20 g/L agar. Plates were incubated with 24-h yeast cultures at 28 °C for 2 days. The presence of the enzymatic activity was visualized as a dark halo surrounding yeast growth.

2.5.3. Pectinase Activity

The assays were carried out in the following medium: 1 g/L yeast extract, 1 g/L ammonium sulphate, 6 g/L $NaHPO_4$, 3 g/L, KH_2PO_4, 5 g/L apple pectin. Yeast cultures 48-h old were spread on the medium and incubated at 28 °C for 5 days and then revealed by the addition of a solution of 1 g/L hexadecyltrimethylammonium bromide. Pectinase activity was evidenced by the presence of a clear halo around the colonies.

2.5.4. Xylanase Activity

The assays were carried out on yeast extract agar containing 5 g/L beechwood xylan, 5 g/L peptone and 5 g/L NaCl. Yeast cultures48-h old were spread on the medium and incubated at 28 °C for 7 days. Xylanase activity was evidenced by the presence of a clear halo around the colonies.

2.5.5. Lipase Activity

Yeast isolates were used to determine lipase activity on rhodamine olive-oil agar media [21]. After 48 h of incubation at 28 °C in the media, colonies were investigated. For detection of lipase activity, they were irradiated with UV light at 350 nm; lipase activity was detected by an orange fluorescent halo around colonies.

All the biochemical activities previously described were assayed in triplicate. Reagents were purchased from Pronadisa (Madrid, Spain), Sigma-Aldrich (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany).

3. Result

3.1. Morphological Groups

Attending to their morphology, a total of seven groups of yeasts were obtained (Figure 1).

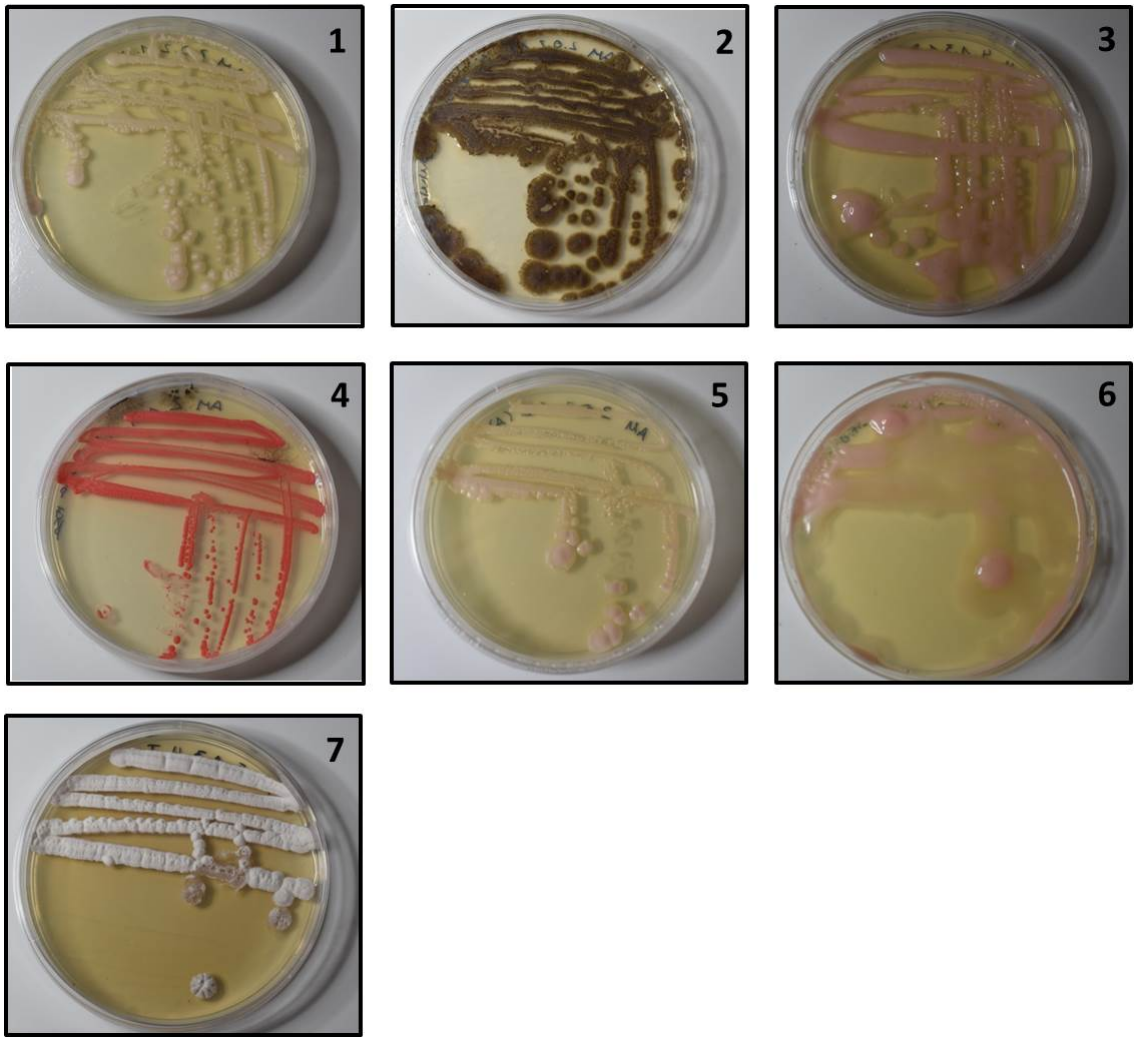

Figure 1. Morphology of isolated yeasts (seven groups).

3.2. Population Dynamics

The number of yeasts on the grape surface raises from 3.33×10^1 cfu/mL of a single species at the beginning to 1.67×10^3 cfu/mL at the end of the ripening process (Table 1).

Table 1. Count of different yeast populations (cfu/mL).

Group	Time (days)				
	0	**14**	**23**	**31**	**38**
1	3.33×10^1 (10.6) *	3.67×10^1 (15.4)	3.33×10^1 (14.3)	1.66×10^2 (10.5)	3.80×10^1 (11.6)
2	nd	4.66×10^2 (12.8)	2.30×10^2 (16.8)	9.66×10^2 (13.6)	6.66×10^1 (16.2)
3	nd	1.00×10^2 (9.6)	nd	nd	3.00×10^1 (14.9)
4	nd	6.67×10^0 (11.2)	nd	nd	nd
5	nd	nd	2.33×10^1 (13.2)	nd	nd
6	nd	nd	2.33×10^1 (10.7)	3.33×10^1 (10.5)	nd
7	nd	nd	nd	nd	1.54×10^3 (9.8)
Total	3.33×10^1	5.19×10^2	3.10×10^2	1.17×10^3	1.67×10^3

* Values in brackets represent standard deviation (n = 3).

After the incubation period, seven different morphological groups of yeasts were observed on Malt Agar plates. At the beginning of the study (day 0), 100% of the grown colonies belong to the morphological group 1, but after two weeks (14th day) morphological types 2, 3 and 4 appear, becoming the fourth the main (89.78%). After 23 days since the beginning of the study, the second morphological group is still the prevailing (74.19%), but two new morphological groups have shown up, 5 and 6, and 3 and 4 do not grow anymore. At the 31st day, the second morphological group is still being the main (82.56%) and the morphological group 5 does not grow. At the end of the study (38th day) there is a new morphological group, the 7, which is predominant (72.64%) at the end of the study.

3.3. Physiological Characterization

At a second stage, a physiological characterization of the strains was carried out by using two complementary commercial strip kits (API 20 C AUX, Table 2) and (API 32C, Table 3).

Table 2. API C AUX assays for different groups of isolates.

Group	Carbohydrate Assayed																			
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1	-	+	-	-	-	+	+	+	-	+	+	-	+	-	-	-	+	-	-	-
2	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
3	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
4	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+
5	-	+	+	-	-	+	+	+	+	+	+	-	+	-	+	-	+	+	-	+
6	-	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+
7	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+

(-): No assimilation; (+): Assimilation; 0: Control; 1: D-Glucose; 2: Methyl-D-Glucopyranoside; 3: Glycerol; 4: N-Acetyl-Glucosamine; 5: calcium 2-Keto-Gluconate; 6: D-Cellobiose; 7: L-Arabinose; 8: D-Maltose; 9: D-Xylose; 10: D-Sucrose; 11: Adonitol; 12: D-Trehalose; 13: Xylitol; 14: D-Melezitose; 15: D-Galactose; 16: D-Raffinose; 17: Inositol; 18: D-Lactose; 19: D-Sorbitol.

Table 3. API 32C assays for different groups of isolates.

Group		Carbohydrate Assayed															
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**A**	**B**	**C**	**D**	**E**	**F**
1	***1***	-	-	+	+	-	+	+	+	+	+	-	-	+	-	-	-
	0	+	+	-	-	+	+	-	-	+	+	-	-	+	-	-	+
2	***1***	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+	-
	0	+	+	-	-	+	+	+	+	-	+	-	-	+	+	-	+
3	***1***	-	-	+	-	-	+	+	+	+	+	+	+	+	-	-	-
	0	+	+	-	-	-	+	-	-	+	+	+	-	+	-	-	+
4	***1***	-	-	+	+	-	+	+	+	-	+	-	-	+	-	+	-
	0	+	-	+	+	-	+	+	+	-	+	-	-	+	-	-	-
5	***1***	-	-	+	-	-	+	+	+	-	+	+	-	+	-	-	-
	0	-	+	-	-	-	+	-	-	+	-	-	-	+	-	-	+
6	***1***	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-
	0	+	+	+	+	+	+	-	-	+	+	-	-	+	-	-	+
7	***1***	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-
	0	+	-	+	+	+	+	+	+	-	+	+	-	+	-	-	-

1.0 D-Galactose; *1*.1 Actidione; *1*.2 D-Saccharose; *1*.3 N-Acetyl-glucosamine; *1*.4 Lactic acid; *1*.5 L-Arabinose; *1*.6 D-Cellobiose; *1*.7 D-Raffinose; *1*.8 D-Maltose; *1*.9 D-Trehalose; *1*.A Potassium 2-Ketogluconate; *1*.B Methyl-*α*-D-glucopyranoside; *1*.C D-Mannitol; *1*.D D-Lactose (bovine origin); *1*.E Inositol; *1*.F No substrate; *0*.0 D-Sorbitol; *0*.1 D-Xylose; *0*.2 D-Ribose; *0*.3 Glycerol; *0*.4 L-Rhamnose; *0*.5 Palatinose; *0*.6 Erythritol; *0*.7 D-Melibiose; *0*.8 Sodium glucuronate; *0*.9 D-Melezitose; *0*.A Potassium gluconate; *0*.B Levulinic acid; *0*.C D-Glucose; *0*.D L-Sorbose; *0*.E Glucosamine; *0*.F Esculin+ferric citrate.

Regarding API 20C AUX strip, results were introduced into the Apiweb identification system and, according this software, group 1 yeasts were identified as *Metschnikowia pulcherrima*, group 2 as *Aureobasidium pullulans*, grups 3, 5 and 6 as *Cryptococcus sp*. and group 4 as *Rhodotorula mucilaginosa*. Apiweb system was no able to identify isolate of group 7. API Zym strip has not been designed for identification purposes (no option has been included in Apiweb software). The only object of this strip is to propose an enzymatic profile of the microorganism assayed; small differences among isolates were observed.

3.4. Molecular Identification

For further characterization, the PCR product of the ITS of 31 isolates was examined by electrophoresis. RFLP of these amplicons produced a characteristic band profile for each strain (Table 4) that was used to make a comparison to data recorded in the Yeast-ID database at the CECT web server, but no match was recorded. Subsequently, genomic DNA of all isolates were subjected to PCR amplification of the D1/D2 region and were identified by comparing sequences using the NCBI blast program. According to the criteria for discrimination of yeast species using D1/D2 rDNA sequencing, a match greater than 99% is required to assess that an isolate of microorganism is a member of a species (Table 4).

Table 4. Restriction analysis of the 5.8S-ITS PCR products and sequence information for the D1/D2 domains of 26S rDNA gene of groups of isolates.

Group	5.8S-ITS-RFLP Analysis (bp)					Closest Relative Species	Matching Nucleotides (%) [b]
	PCR [a]	*Hha*I	*Hae*III	*Hin*dIII	*Hin*fI		
1	630	340 + 280 + 190	500	600	260 + 250 + 150	*Metschnikowia pulcherrima*	99.5
2	500	160+ 100	150 + 110	500	280 + 180 + 140	*Aureobasidium pullulans*	99.8
3	600	300 + 260	470	650	320 + 250	*Crytococcus uzbekistanensis*	99.5
4	740	320 + 270 +210	390 + 270 + 210	580	220 + 170	*Rhodotorula mucilaginosa*	99.9
5	700	300 + 270	700	700	310 + 240	*Crytococcus adeliensis*	99.5
6	600	340 + 280	280 + 200	300 + 260	240	*Crytococcus sp.* CF-285748	99.7
7	630	630	270	630	270 + 150	*Quambalaria cyanescens*	99.5

[a] PCR amplified rDNA size. [b] Sequence identity in the D1/D2 region of isolates of the 26S ribosomal gene and closest relative species in the NCBI GenBank database.

3.5. Enzymatic Activities

The results of the tests conducted in culture mediums for qualitative detection of the different enzymatic activities are shown in Table 5 where it is observed that the great majority of the identified species present β-glucosidase and protease activities are detected in almost all the yeasts studied, while only isolates from *Quambalaria cyanescens* present lipase activity. Furthermore, no microorganism presents xylanase nor pectinase activity. These results do not agree with the review published by Esteve-Zarzoso et al. [23] but are consistent with experimental data reported by Charoenchai et al. [24]. Nevertheless, small differences among isolates were observed, so that these results can not be used to establish physiological differences.

Table 5. Enzymatic characterization of the isolates.

Microorganism	Enzymatic Activities				
	β-glucosidase	**Protease**	**Lipase**	**Xylanase**	**Pectinase**
Crytococcus adeliensis	+	+	-	-	-
Cryptococcus sp. CF-285748	+	+	-	-	-
Metschnikowia pulcherrima	+	+	-	-	-
Aureobasidium pullulans	+	+	-	-	-
Cryptococcus uzbekistanensis	-	+	-	-	-
Rhodotorula mucilaginosa	+	+	-	-	-
Quambalaria cyanescens	+	+	+	-	-

4. Discussion

Comparing these results with those obtained in the same vineyard in previous years, some qualitative and quantitative differences can be observed [15]. In samples from untreated vineyards, yeast population increased during the ripening process, being at its maximum in the final stage. At the beginning of this process, the predominant specie is *Metschnikowia pulcherrima*. However, while the ripening process progresses, the population of this specie decreases, being replaced by *Aureobasidium pullulans*, *Cryptococcus uzbekistanensis* and *Rhodotorula mucilaginosa*, although the numbers of these yeasts are lower than *M. pulcherrima* population at the beginning of the process. *R. mucilagionsa* is a pigmented single-cell yeast that belongs to Basidiomycota division. Its different color, red-orange colonies, are the result of pigments that they produce in which the function is to block certain wavelengths that may cause a harmful effect [21]. On the other hand, *C. uzbekistanensis* is not a capsulated round yeast and was first identified by Maksimova and Chernov [25]. These species are also replaced, with *M. pulcherrima* being the only one that presents during all of the ripening process. Thereby, in the middle of the process, the species *Cryptococcus adeliensis* and *Cryptococcus* sp. CF-285748 appear. Although *Cryptococcus* genus is mentioned as one of the typical one pertaining to non-*Saccharomyces* yeasts and, therefore, present in the surface of grapes [26,27], these two species are not typical ones, but both have been isolated in other studies [28–30]. There are not many references regarding the isolation and characterization of *Cryptococcus* sp. CF-285748; however, *C. adeliensis* is described by Scorzetti et al. [31] as a species from the *Cryptococcus* genus. This yeast is not present during fermentation but can use a wide variety of carbon sources and produces starch during its growth. Furthermore, the low presence of this species during the ripening process can be due to the fact that during the incubation of the replicas the incubation temperature was 28 °C, being 25 °C its optimal growth temperature; the growth of this yeast is weak at 30 °C [31]. Finally, in the last phase of ripening, a new species appears, *Quambalaria cyanescens*, of which the population number is well above the rest of isolated microorganisms. This microorganism has been found in a wide range of ecological niches, being symbiont in species of *Corymbia* and *Eucalyptus* [32]. Hence, although this last species is well described [32], there are not studies regarding its presence in the must nor in the grape surface. In fact, one of the objectives of this study was to confirm previous results obtained in our laboratory. On the other hand, the fact that this yeast only appears at the end of the ripening process

may be due to the application of antifungal treatments during most parts of the period in which the samples were collected, except in the last week, during which there was no treatment applied. This hypothesis seems to fit with certain studies about antifungal treatments that produced a decrease in the yeast population [20,33,34]. Some of the microorganisms described in this work are not the common type of yeast founded on grape surface. This fact may be caused by several factors. First, there is a limited number of works studying the yeast-like microbiota present during the ripening of grapes and its dynamics during this process [35]. On the other hand, the origin of the grapes should be considered, which could explain one part of the dissimilarity in terms of diversity of non-*Saccharomyces* yeasts isolated in the different studies. Regarding the population dynamics, *A. pullulans* is one of the main yeasts isolated in unripe grapes, although its population decreases along the ripening process, being undetectable when grapes are harvested [36]. This result agrees with our data, as *A. pullulans* appears at the beginning of the ripening process, but it has not been isolated in the later phases of the process. It is also reported the presence of *R. mucilaginosa* and *Cryptococcus* sp. during the midpoint of the ripening process, although the abundance of *R. mucilaginosa* in our study is smaller than the one observed in other studies [36].

If only glycosides with the most flavorant aglycons were considered, the most abundant in grape juice are apiosylglycosides (more than 50%), followed by rutinosides (6% to 13%), and lastly, glucosides (4 to 9%). All glycosides are not existing in all cultivars and their amounts also differ according to the original grape. The glycoside flavor potential remains rather constant during fermentation and in drinks as well. This fact opened a new field of rigorous investigation on the chemistry of glycoconjugated flavor compounds to exploit this significant flavor source [37]. Terpene glycosides can be enzymatically hydrolyzed to enrich wine flavor by release of free aromatic complexes from natural glycoside precursors. This procedure is carried out with several enzymes, which act consecutively according to two phases: firstly, α-L-rhamnosidase, α-L-arabinosidase or β-D-apiosidase make the cleavage of the terminal sugar and rhamnose, arabinose or apiose and the corresponding β-D-glucosides are released; then, the deliverance of monoterpenol takes place after action of a β-D-glucosidase [37]. The sensorial features of the wines produced with Muscat grapes are connected to the level of terpene alcohols, so an improvement of such a level, as a result of hydrolytic processes conducted by non-*Saccharomyces* yeasts is expected. Isolates from *Hanseniaspora uvarum* and *H. vineae* have been proved to be candidates to be used in vinification procedures to improve wine olfactive properties [38]. Optimal conditions to induce β-glucosidase activity have also been determined [38]. On the other hand, a total of 17 *Pichia* and *Wickerhamomyces* isolates belonging to the species *P. fermentans*, *P. membranifaciens* and *W. anomalus* have been tested for exocellular β-glucosidase production. W. anomalus and *P. membranifaciens* were obtained from enological ecosystems in Utiel-Requena Spanish region and characterized by physiological and molecular techniques (PCR-RFLP and sequencing). They were proved to be the most interesting species to be used as a source of enzymes because they show tolerance to high levels of ethanol and glucose, making them of great interest to be used in vinification procedures [39]. Interest in the health benefits of red wine has augmented over the last several years due to the occurrence of resveratrol, which can be found mainly as the glucoside form. Yeasts-endowed β-glucosidase activity improves free-resveratrol concentration in wine. After screening 308 non-*Saccharomyces* yeast strains for β-glucosidase, Gaensly et al. [40] found 14 yeasts, which increased the resveratrol concentration up to 102% without any noteworthy difference, and nine of these yeast strains also created high ethanol contents. Four autochthonous *H. uvarum* β-glucosidase-producer strains displayed adequate oenological characteristics and hydrolyzed resveratrol-glucosides during the alcoholic fermentation of *V. labrusca* grape must. Extracellular proteolytic activity of yeasts may increase the nitrogen sources for the grown of microorganisms during alcoholic fermentation [41]. Initial low content in nitrogen sources may lead to stop fermentations. On the other hand, yeasts can produce esters, higher alcohols and volatile fatty acids, compounds contributing to the fermentation bouquet of beverages, as primary metabolites from sugar and amino acid metabolism [42]. Proteolytic activity of some *H. guilliermondii*

strains have been studied as a biotechnological solution to reduce turbidity due to proteins in fermented beverages [43]. Our findings confirm that some *Hanseniaspora* strains can synthesize proteolytic enzymes [44], but protease activity in *Pichia* and *Wickerhamomyces* isolates was too low [39], according to results obtained by other authors [43]. Usually described non-*Saccharomyces* yeasts are an interesting source of enzymes to be used in wine-making. Isolation of non-usual yeasts in the surface of unripened grapes opens a new door to obtain new enzymes with new potential to be used in biotechnological processes. Further studies should be developed to characterize these "new" enzymes and determine their potential use in enology.

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Abbreviations

The following abbreviations are used in this manuscript:

PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism

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