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Yeast diversity in the Mediterranean strawberry tree (*Arbutus unedo* L.) fruits' fermentations

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ABSTRACT

In the Mediterranean region the fruits of the strawberry tree (*Arbutus unedo* L.) may be fermented and distilled to produce a traditional beverage very much appreciated in Southern Europe. The aim of the present work was to study the diversity of the yeast population and the killer activity of the isolates identified as *Saccharomyces cerevisiae*, obtained during solid state industrial fermentations of the arbutus berries. The identification of the isolates was performed by the 5.8S rRNA-ITS region restriction analysis and by sequencing the D1/D2 region of the large subunit of the rRNA gene. At the start of the fermentations, various non-*Saccharomyces* species were detected including *Aureobasidium pullulans*, *Dothichiza pithyophila*, *Dioszegia zsoltii*, *Hanseniaspora uvarum* and yeasts belonging to the genera *Metschnikowia*, *Cryptococcus* and *Rhodotorula*. However, as the biological processes progressed the number of different species decreased with *S. cerevisiae* and *Pichia membranaefaciens* becoming dominant at advanced stages of the must fermentation that is characterized by high concentrations of ethanol. Forty three isolates identified as *S. cerevisiae* were tested for killer activity against two sensitive reference strains and *Zygosaccharomyces bailii*. Their killer sensitivity in relation to five killer referenced toxins (K2, K5, K8, K9 and K10) was also studied. Out of the isolates analyzed, 95.3% were sensitive and 4.7% were tolerant against the killer toxins tested. Only three isolates revealed killer activity against one sensitive strain and two of them against the spoiler yeast *Z. bailii*. The microbiota obtained revealed an interesting potential to be used as starter cultures to overcome unpredictable uncontrolled fermentations of the arbutus fruits as well as in other applications of biotechnological interest.

1. Introduction

Arbutus unedo L., strawberry tree, is an evergreen shrub that belongs to the *Ericacae* family growing in the Mediterranean region. It is very important in terms of environmental aspects due to its tolerance to dryness and its ability to regenerate and recolonize forest fires. The fruits are rich in sugars, vitamins, organic acids, phenolic compounds and frequently used to produce jams or jellies, but rarely consumed as fresh fruit (Ayaz, Kucukislamoglu, & Reunanen, 2000; Ruiz-Rodríguez et al., 2011; Soufleros, Mygdalia, & Natskoulis, 2005). The berries are also an interesting source of omega-3 polyunsaturated fatty acids (Oliveira et al., 2011). However, the fruits' main application is the production of a distilled beverage known as "Aguardente de medronho" in Portugal (Cavaco, Longuinho, Quintas, & Carvalho, 2007), "Koumaro" in Greece (Soufleros et al., 2005) and "Corbezzolo" in Italy (Versini et al., 1995). This beverage is a traditional product made in small or industrial scale units during the months of September to January and its production constitutes a agro-sustainable business which makes *A. unedo* an interesting shrub by social and economic reasons (Alarcão-E-Silva, Leitão, Azinheira, & Leitão, 2001; González, Agrasar, Castro, Fernández, & Guerra, 2011; Soufleros et al., 2005). Berries are mixed with small amounts of water in plastic, wood or stainless steel vessels, which are then closed and a solid state fermentation starts depending on the yeasts, present naturally on the fruits and in the environment (Cavaco et al., 2007). The duration of the processes varies between one and three months according to the producers, depending on the amount of sugars present in the fruits, the temperature, the equipment and the species/strains involved. The fermented mass, containing 16–22% ethanol (w/w), is subsequently heated and distilled, to obtain a beverage containing 45–50% ethanol (v/v) and characterized by a specific aroma profile (Cavaco et al., 2007; Galego & Almeida, 2007). However, the quality of the distilled product may be affected by off-flavors that may occur due to uncontrolled fermentations. The nature of yeasts present in a must may contribute to the sensory characteristics of the final product with the synthesis of desirable flavor or off-flavor compounds (Duarte et al., 2010; Loureiro & Malfeito-Ferreira, 2003). Other important characteristic of yeasts involved in the production of food products is the killer phenotype. Killer yeasts

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may offer some advantages over sensitive strains when used in fermentations due to the different interactions among the strains involved that may affect the fermentation processes and the final quality of fermented food and beverages (Ciani & Fatichenti, 2001; Sangorrín et al., 2001; Santos, San Mauro, Bravo, & Marquina, 2009).

In the last few years, an extensive research effort have been made to characterize the yeast microbiota in a vast number of food preparations through techniques based on molecular biology with particular emphasis on PCR amplification of ribosomal RNA genes and spacer regions as internal transcribed spacers (ITS) and non-transcribed spacers (NTS) followed by restriction analysis with restriction enzymes (Baleiras Couto, Reizinho, & Duarte, 2005; Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999; Raspor, Milek, Polanc, Mozina, & Cadez, 2006). The sequence of the large subunit ribosomal DNA (LSUrDNA), especially D1/D2 region, has revealed to be a powerful tool in yeast identification (Kurtzman & Robnett, 1998) becoming more accessible due to new efficient nucleic acids sequencing techniques.

In this context, the aim of this work was to study the diversity of the yeast population and the killer activity of the *S. cerevisiae* isolates obtained during the fermentation period. Up to now, no report was published for strawberry tree fruits fermentation's microbiota. For the evaluation of species diversity, a molecular approach was used involving the restriction fragment length polymorphism (RFLP) of ITS region and sequence analysis of the D1/D2 domains.

The knowledge of yeast diversity could enable the selection of native strains to be used as starter cultures in the production of distilled beverages, ethanol and other applications of biotechnological interest. Using controlled and defined inoculum to initiate the fermentations is certainly a way to obtain more reliable and better quality alcoholic and distilled beverages.

2. Material and methods

2.1. Fermentation conditions

A. unedo fruits were harvested manually when ripe (red surface color) in the Southern region of Portugal, between October and December of 2008, and were transported to the distillery, located in Algarve (Portugal). On arrival, fruits were manually selected to remove the green ones, the peduncles and leaves. Their initial pH value was about 3.3 and °Brix was, on average 15. The fruits (300 kg) were mixed with UV treated water (80 l) in stainless steel containers which were then closed and solid state fermentations were carried out at room temperature. During the mixture with water, the berries may split, facilitating the further hydrolysis of the polysaccharides. The duration of each fermentation process varied as a function of the development of flavor characteristics according to the producer decisions: 135 days for Fermentation A (FA), 150 days for Fermentation B (FB) and 142 days for Fermentation C (FC). Samples of the fermenting mass were collected, under sterile conditions, through taps present in the fermenters and aseptically transferred to sterile 50 ml tubes which were transported, under refrigeration, to the laboratory in Instituto Superior de Engenharia (Universidade do Algarve, Faro, Portugal).

During the fermentation periods, the density (°Brix) and the pH were determined using an Abbe refractometer (ATAGO, Japan) and a pH meter (Crison, Spain), respectively. The concentration of ethanol was determined using the method of headspace-solid-phase microextraction gas chromatography (HS-SPME-GC) with polyacrylate coated fiber. The SPME fiber was exposed for a fixed time (20 min) in the head-space of the vial at laboratory temperature (20 ± 2 °C) with magnetic stirring. The fiber was then withdrawn into the needle and transferred to the injector of the GC (Hewlett Packard 5890 Series II equipped with a FID detector, Avondale, USA). A DB-WAX capillary column (30 m × 0.25 mm I.D. × 0.25 μm) film thickness (J & W Scientific, Folsom, USA) performed with the following oven temperature program: 5 min at 40 °C, 5 °C min^{-1} until 210 °C was used. The quantification was made using standard addition (García & Reichenbächer, 1999). The determinations of pH, °Brix and ethanol were done in triplicate.

2.2. Yeasts growth and isolation

The yeast population was counted twice a week in the first two weeks, once a week in the following two months and twice in the last month of the fermentation. From each sample, 25 ml of fermenting mass was diluted with Ringer solution following the decimal dilution protocol and aliquots were plated, in duplicate by surface spreading on Malt Extract Agar (MEA, Scharlau, Spain) (pH 4.0). After the incubation period at 25 °C during 5 days, colonies were counted and yeast species diversity was studied at four sampling points: on the 4th day (50 isolates), the 11th day (36 isolates), the 32nd day (42 isolates) and on the 108th day (37 isolates) of fermentation. Representative colonies of all morphotypes of yeasts were selected according to their macro morphology and isolated in proportion to their frequencies and subcultured onto YEPD (yeast extract 5 g/l, peptone, 5 g/l, dextrose. 40 g/l agar 20 g/l) or Potato Dextrose Agar (PDA, Scharlau, Spain) for subsequent identification. The isolated strains were preserved at −70 °C using glycerol as cryoprotectant agent (20% v/v).

2.3. Yeast identification

ITS1-5.8rDNA-ITS2 PCR-RFLP: yeast isolates identification was performed by the PCR-RFLP method described by Esteve-Zarzoso et al. (1999). DNAs from liquid cultures were extracted according to Querol, Barrio, and Ramon (1992). PCR reaction mixtures (75 μl) containing 0.5 μM primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and 0.5 μM primer ITS4 (5′-TCCTCCCGCTTATTGATATGC-3′) (White, Bruns, Lee, & Taylor, 1990), 10 μM deoxynucleotides (Promega), 1.5 mM $MgCl_2$ and 1 unit DNA polymerase (Promega) were prepared. Amplifications were performed in a thermocycler (Thermo Electron, USA) in the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturating at 94 °C for 1 min; annealing at 55.5 °C for 2 min; extension at 72 °C for 2 min and a final extension step at 72 °C for 10 min. PCR products (10 μl) were digested without further purification with the restriction endonucleases *Cfo*I, *Hae*III and *Hinf*I (Roche). The PCR products and their restriction fragments were separated on 1% and 3% (w/v) agarose gels, respectively, with 1 × TAE buffer. After electrophoresis, gels were stained with ethidium bromide, visualized under UV light in a G-Box Syngene-Genesis 10 UV Scanner (UK). Fragment sizes were estimated by comparison against a DNA ladder (100 bp BioRad). Yeast isolates were grouped according to their RFLP profiles and compared with the RFLP patterns described by Guillamón, Sabaté, Barrio, Cano, and Querol (1998), Esteve-Zarzoso et al. (1999), Sabate, Cano, Esteve-Zarzoso, and Guillamon (2002) and de Llanos Frutos, Fernandez-Espinar, and Querol (2004).

Sequence analysis of the region D1/D2 of the 26S rRNA gene: Representative strains of each PCR-RFLP profile obtained were treated to perform sequence analysis of the domains D1 and D2 of the 26S rRNA gene. PCR amplification of the referred region in the 26S rRNA gene with the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman & Robnett, 1998) were performed. The amplification reaction and PCR conditions were identical to those described above for ITS 5.8 rRNA region except for the primers used (NL1 and NL4). Amplified products were sequenced by LGC Genomics (Germany) and sequences were compared to those available in GenBank database at the National Center for Biotechnology Information (NCBI) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to be identified by sequence homology with described species.

2.4. Killer behavior

Forty three autochthonous isolates identified as *Saccharomyces cerevisiae* were tested for killer activity (K^+ or K^-) against *Zygosaccharomyces bailii* (ISA 1307). The same isolates were tested for killer sensitivity in relation to five killer reference strains. Killer activity and sensitivity were assayed by the seeded-agar-plate-technique, using YEPD-MB (1% yeast extract, 2% glucose, 2% peptone and 2% agar) containing 0.003% (w/v) methylene blue, buffered at pH 4.0 with 0.5 M phosphate-citrate (Marquina et al., 1992; Sangorrín et al., 2001).

2.4.1. Killer activity

The yeasts *S. cerevisiae* NCYC 1006 and *S. bayanus* PYCC 4456 were used as sensitive references. The killer activity of the isolates obtained during the course of the present work against the spoilage species *Z. bailii* (ISA 1307) was also tested. Each reference species was suspended in sterile water (approximately 10^5 cell/ml) and inoculated as a lawn onto the YEPD-MB medium. These plates were inoculated in concentrated zones (1 cm diameter) with 48 h old cultures of the native isolates to be tested for killing activity. The plates were incubated at 20 °C for 6 days. *S. cerevisiae* isolates were classified as killer (K^+) when they were able to inhibit the growth of the yeasts inoculated on the agar surfaces and the inhibition zone appeared surrounded with a dark blue boundary.

2.4.2. Killer sensitivity

The sensitivity of *S. cerevisiae* isolates were studied in relation to 5 reference killer yeasts, responsible for the production of 5 different types of toxins (K2, K5, K8, K9 and K10), using the method described above. The killer yeasts used were *S. cerevisiae* NCYC 738 (K2 type), *Pichia anomala* NCYC 434 (K5 type), *P. anomala* NCYC 435 (K8 type), *Williopsis saturnus* NCYC 500 (K9 type) and *Kluyveromyces lactis* NCYC 575 (K10 type). The sensitivity of each isolate was identified with the numbers referring the killer type.

3. Results and discussion

The growth of yeasts during the solid-state fermentation of the fruits of the strawberry tree (*A. unedo*) is shown in Fig. 1a. During the first four days, the number of yeasts increased exponentially from 3.7–5.2 log CFU/g to 7–8 log CFU/g of fermenting must, and from then onwards, a slight decrease of those numbers was observed until the end of the fermentation processes (5.9–6.9 log UFC/g). This reduction may be explained by the level of ethanol present at the end of the fermentations (20.7–22.5 g/100 g fermented mass) (Fig. 1b). The concentration of ethanol increased in the three processes achieving about 19.3 g/100 g of fermented mass during the first ten days in Fermentation A and later in the Fermentations B and C. After this sharp increase, the level of the alcohol slightly decreased but increased again stabilizing when values of 20.7–22.5 g/100 g fermented mass were achieved. The stress caused by high levels of ethanol may result in the inhibition of some ethanol sensitive strains and explains the sluggish or stuck fermentations as described by Bisson (1999) in wine production.

The pH value during the fermentation varied from an initial value of 3.3 to a value at the end of the fermentation of 3.5–3.6. The low pH and the high concentration of sugars (°Brix 15) at the beginning of the fermentations favored the multiplication of the yeast biota. According to previously published results, the berries of *A. unedo* are characterized by low pH values and high soluble sugar concentrations reaching more than 20 g/l as described by Cavaco et al. (2007).

A total of 165 yeast isolates were obtained from the three fermentations of the *A. unedo* fruits. The ITS-5.8 rDNA region of the yeast isolates originated PCR products ranging from 390 to 850 pb and their restriction produced 14 different RFLP profiles as shown in Table 1.

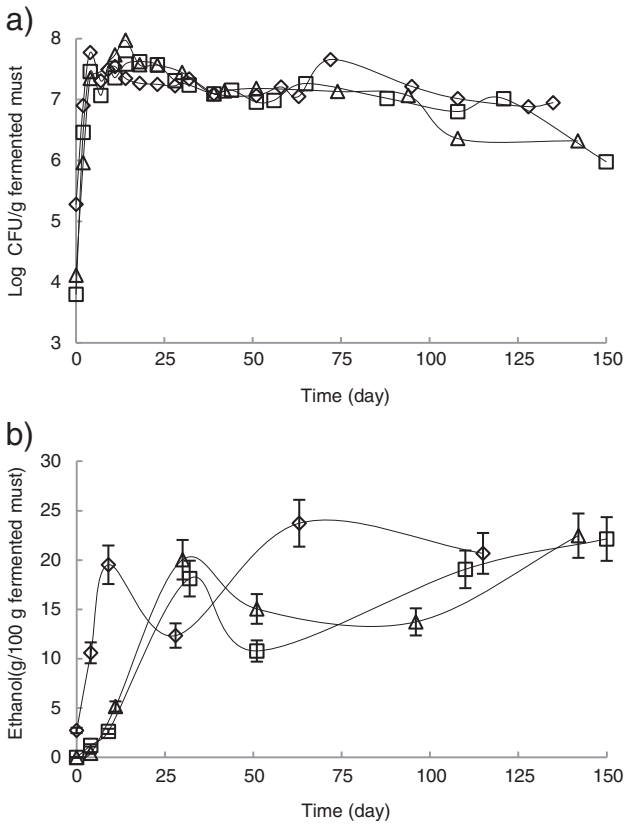

Fig. 1. Yeast population (a) and ethanol evolution (b) during the fermentations of *Arbutus unedo* fruits [fermentations A (◇), B (□) and C (△)].

The number of isolates in each group varied from 1 to 53. Each fragmentation pattern was compared to the yeast ITS-RFLP profiles described previously (de Llanos Frutos et al., 2004; Esteve-Zarzoso et al., 1999; Guillamón et al., 1998; Sabate et al., 2002). The RFLP analysis allowed the identification of the isolates included in the groups II, V, XI, XII, XIII and XIV as *Metschnikowia pulcherrima*, *Pichia membranaefaciens*, *Lachancea thermotolerans* [formerly *Kluyveromyces thermotolerans* (Kurtzman, 2003)], *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *S. cerevisiae*, respectively. The results of BLAST analysis were in full agreement with the ITS–RFLP identifications, yielding sequence similarity values between 98% and 100% (Table 2).

The RFLP Profiles I, IV, VI and VII were not assigned to any of the species associated to already described patterns due to some discrepancies in the restriction banding obtained (de Llanos Frutos et al., 2004; Esteve-Zarzoso et al., 1999; Guillamón et al., 1998; Sabate et al., 2002). As a result, the identification performed was based on the D1/D2 sequence analysis (Table 2). In relation to Profile I, the sequencing analysis displayed high similarity (99%) with *Metschnikowia* aff *fructicola* and low similarity with the type strain of *M. fructicola*. The isolates characterized by profile IV presented 100% similarity with the type strain of *Issatchenkia orientalis*. The two isolates with the Profile VI displayed 100% similarity with the type strain of the basidiomicetous *Dioszegia zsoltii* and the isolates included in Profile VII presented high D1/D2 similarity with a strain of an unknown species of the genus *Cryptococcus*. In relation to Group IX, composed of yeast forming bright orange colonies, it was not possible to perform a satisfactory identification.

The isolates included in RFLP Groups III, VIII and X, were identified as *Candida stellata*, *A. pullulans* and *Rhodotorula mucilaginosa*, respectively However, after analyzing the DNA sequence of the D1/D2 region of the LSU rRNA gene through a BLAST search (Table 2), the yeasts included in Group III were identified as *C. zemplinina*. According to Sipiczki (2004) *C. stellata* and *C. zemplinina* produce similar PCR-RFLP profiles when *Cfo*I, *Hae*III and *Hinf*I are used as restriction enzymes. In the case of

Table 1
RFLP profiles/groups obtained from the restriction of the amplified 5.8S-ITS region of the 165 yeast isolates.

RFLP profile/ group	PCR product (bp)	Restriction fragment (bp)			Number of isolates	Isolation (%)
		Cfo I	*Hae* III	*Hinf* I		
I	390	200 + 100 + 90	290 + 100	390	5	3.0
II	390	200 + 100 + 90	290 + 100	200 + 190	17	10.3
III	475	215 + 110 + 80 + 60	475	235 + 235	4	2.4
IV	480	180 + 170 + 70 + 60	380 + 90	200 + 200 + 90	4	2.4
V	500	175 + 110 + 90 + 75	330 + 90 + 50	275 + 200	25	15.2
VI	550	290 + 260	525	280 + 210	2	1.2
VII	590	250 + 180 + 90	490 + 100	305 + 285	3	1.8
VIII	600	190 + 180 + 100	450 + 150	280 + 180 + 130	9	5.4
IX	600	280 + 220 + 100	600	260 + 150 + 120 + 90	3	1.8
X	640	320 + 240 + 80	430 + 210	340 + 220 + 80	4	2.4
XI	700	310 + 280 + 90	300 + 210 + 95 + 95	340 + 340	11	6.7
XII	775	320 + 310 + 105	775	385 + 200 + 160 + 80	24	14.5
XIII	800	330 + 220 + 150 + 100	800	410 + 380	1	0.6
XIV	850	375 + 325 + 150	320 + 230 + 170 + 125	375 + 365 + 110	53	32.1

Profile VIII, the nine isolates identified as the yeast-like *A. pullulans*, two revealed 98% similarity to *Dothichiza pithyophila* when a BLAST search of GeneBank was done. Finally, regarding Profile X, two of the total isolates were identified as *R. nothofagi*. In the case of yeasts included in Groups III, VIII and X, it would have been useful to introduce more restriction enzymes besides *Cfo*I, *Hae*III and *Hinf*I to increase the discriminatory capacity of the ITS1-5.8S RNA-ITS2 PCR-RFLP method, as suggested in other works (Esteve-Zarzoso et al., 1999; Ferreira et al., 2010). This is especially important in the studying of the ecosystems that have not yet been researched.

Table 2
Results of the comparison of the isolates DNA sequences with those present in GeneBank from NCBI database.

Profile	Fragment sequenced (bp)	GeneBank accession number — species and strain designation	Similarity[a] (%)
I	422	EU373448 *Metschnikowia* aff. *fructicola*	99%
II	432	U45736 *Metschnikowia pulcherrima* strain NRRL Y-7111	428/432 98%
III	436	AY160761 *Candida zemplinina* strain 10-372/CBS 9494	424/424 100%
IV	513	EF550222 *Issatchenkia orientalis* strain NRRL Y-5396	513/513 100%
V	522	EU057561 *Pichia membranaefaciens* strain NRRL Y-2026	522/522 100%
VI	509	AF544246 *Dioszegia zsoltii* var. *yunnanensis* strain AS 2.2091	509/509 100%
VII	553	EU002790 *Cryptococcus* sp. strain CBS 10166	552/553 99%
VIII	530	FJ150916 *Aureobasidium pullulans* var. *pullulans* strain CBS 146.30	530/530 100%
	528	FJ150969 *Dothichiza pithyophila* strain dH 12609	520/528 98%
X	493	AF335986 *Rhodotorula mucilaginosa* strain ATCC 32763	492/493 99.7%
	512	AF189950 *Rhodotorula nothofagi* strain CBS8166	511/512 99%
XI	554	DQ655683 *Lachancea thermotolerans* strain NRRL Y-27911	554/554 100%
XII	546	U84229 *Hanseniaspora uvarum* strain NRRL Y-1614	546/546 100%
XIII	494	U72156 *Torulaspora delbrueckii* strain NRRL Y-866	494/494 100%
XIV	535	AY048154 *Saccharomyces cerevisiae* strain NRRL Y-12632	535/535 100%

Note: All the strains used in the comparisons are type strains with the exception of EU373448 and FJ150969. ATCC — American Type Culture Collection, USA; CBS — Centraalbureau voor Schimmelcultures, Nederland; NRRL — Agricultural Research Culture Collection, USA.

[a] Relation of similarity of number of nucleotides in Domain D1/D2 between isolates and GeneBank accession strains.

As fermentations progressed, a succession of microbial species took place. The different species identified during the fermentation process and their isolation frequencies are presented in Table 3.

The earlier fermentation samples, contained a mixture of species including *A. pullulans* (yeast like), *Cryptococcus* sp., *D. zsoltii*, *D. pithyophila*, *H. uvarum*, *Metschnikowia* spp. and *Rhodotorula* spp. At this early stage, a great diversity of species belonging to the phyla Basidiomycota and Ascomycota was observed. After the first four days, it was no longer possible to isolate basidiomicetous yeasts from the fermenting must, probably due to the decreasing oxygen availability since those microorganisms are aerobes, frequently associated with the phyllosphere and soil (Inácio, Portugal, Spencer-Martins, & Fonseca, 2005). The ascomicetous species *A. pullulans* and *D. pithyophila* present in the initial phases of the fermentation corresponded to 5.4% of the isolates but represent 18% of the isolates obtained in the first days of fermentation. These two species belong to the family *Dothideaceae* and are both associated with plants. *A. pullulans* is a ubiquitous black yeast like species found in habitats with fluctuating water activity such as fruits' surfaces and phyllosphere (Zalar et al., 2008). The isolates belonging to the genus *Metschnikowia* were collected during the early phase of the fermentation but their frequency decreased along the first month. These yeasts represented 13.5% of the total isolates and are characterized by low/medium fermentative metabolisms. *H. uvarum* (14.5% of the total isolates studied) was also isolated during the first month of the fermentative processes. Species of the genera *Metschnikowia* and *Hanseniaspora* are the most common Ascomycota non-*Saccharomyces* found in the first phases of fruits' alcoholic fermentations (Ocón et al., 2010; Sabate et al., 2002; Valles, Bedriñana, Tascón, Simón, & Madrera, 2007). However, in the wine industry, both of them are known to produce ethyl acetate and acetic acid before and during initial fermentations steps leading to wine deterioration (Loureiro & Malfeito-Ferreira, 2003; Romano, Fiore, Paraggio, Caruso, & Capece, 2003).

The yeasts *I. orientalis* (2.4%), *L. thermotolerans* (6.7%) and *C. zemplinina* (2.4%) were also found in the first month and in the beginning of the second month of the three fermentation processes studied and are frequently related to grapes and/or wine production as *T. delbrueckii* that was isolated in a very low frequency (0.6%) (Baleiras Couto et al., 2005; Csoma & Sipiczki, 2008; Ocón et al., 2010). Although in low frequencies, these species may certainly influence the fermentation and the characteristics of the final product, as they tolerate high concentrations of ethanol.

S. cerevisiae was isolated in a low frequency (4%) in the first days of the fermentation confirming the low numbers of this species on fruits surfaces. However, this microorganism took over the processes,

Table 3
Yeast species prevalence (%) isolated from *Arbutus unedo* fruits fermentation.

Species	Fermentation process			
	T_4	T_{11}	T_{32}	T_{108}
A. pullulans	14.0	–	–	–
C. zemplinina	–	2.8	7.1	–
Cryptococcus sp.	6.0	–	–	–
Dio. zsoltii	4.0	–	–	–
Dot. pithyophila	4.0	–	–	–
H. uvarum	28.0	22.2	4.8	–
I. orientalis	2.0	–	7.1	–
L. thermotolerans	–	16.7	11.9	–
Metschnikowia sp.	22.0	16.7	11.9	–
P. membranaefaciens	2.0	–	14.3	48.6
Rhodotorula sp.	8.0	–	–	–
S. cerevisiae	4.0	38.9	42.9	51.4
Unidentified orange colonies	6.0	–	–	–
T. delbrueckii	–	2.8	–	–

–, not detected; T_4: 4 days of fermentation; T_{11}: 11 days of fermentation; T_{32}: 32 days of fermentation; T_{108}: 108 days of fermentation.

due to its efficient fermentation catabolism and ethanol tolerance in the stressful conditions of the fermenting musts during the second and third months, representing 32.1% of the isolates. *P. membranaefaciens* was detected concurrently with *S. cerevisiae*, representing 15.2% of total of the isolates. *P. membranaefaciens* has been associated with biofilm production on the surface of long/old fermentations as described in agave fermentation (Lachance, 1995). This film forming yeast has also been associated with biofilm formation in wine fermentation, where it is considered a spoilage yeast (Loureiro & Malfeito-Ferreira, 2003). In the biological processes described in the present work, this species remained present, forming a thick biofilm at the surface of the fermenters in the three cases studied (which took more than three months) and represented almost half of the yeast biota studied in the final stages of the fermentation.

The microbial succession observed, with the final prevalence of *S. cerevisiae*, is similar to most of the ecological surveys of yeast microbiota in grape and apple musts' fermentations (Sabate et al., 2002; Valles et al., 2007). Additionally, the succession of different yeast species has been reported by several authors on other alcoholic fermentations such as in sugar cane to obtain cachaça (Schwan, Mendonça, da Silva, Rodrigues, & Wheals, 2001) and in agave juice to obtain tequila (Lachance, 1995).

In a first approach to characterize the technological properties of the isolates identified as *S. cerevisiae*, forty three isolates were tested in their ability to kill the reference yeasts *S. cerevisiae* NCYC 1006 and *S. bayanus* PYCC 4456 and the food/beverage spoiler yeast *Z. bailii*. Additionally, the sensitivity of the *S. cerevisiae* isolates, in relation to the well characterized killer toxins K2, K5, K8, K9 and K10, was also studied. From the results obtained, three (F7D56A, F7D51A, F5D51E) of the isolates demonstrated killer activity against one of the sensitive yeast studied (*S. cerevisiae* NCYC1006) and two (F7D56A, F7D51A) revealed killer activity against *Z. bailii*. This is an interesting result as *Z. bailii* is one of the most dangerous spoilage species in the wine industry and soft drinks (Loureiro & Malfeito-Ferreira, 2003) and is highly tolerant to weak organic acids used as preservatives (Quintas, Sotoca, Loureiro-Dias, & Peinado, 2005). In general, *S. cerevisiae* produces active killer toxins, mainly against yeasts of its genus (Ciani & Fatichenti, 2001). However, there are some works describing *S. cerevisiae* active killer toxins against species belonging to other groups such as *Candida* and *Pichia* (Hernández et al., 2008).

With regards to killer sensitivity, almost all wild isolates were sensitive to some of the reference killer strains used in this study, with the exception of two isolates (F7D11C; F5D4F) that were tolerant to all the killer toxins assayed (K2, K5, K8, K9, K10) as summarized in Table 4.

Several different sensitivity patterns can be observed, K2 being the most effective toxin followed by K9, K5, K10 and K8. The *S. cerevisiae* isolates, identified as F7D11C and F5D4F, did not show killer activity but were tolerant to the toxins tested. This is interesting in terms of technological applications because F7D11C and F5D4F isolates may tolerate eventual undesirable contaminant species and strive by competition. These isolates, as well as the ones that revealed killer activity in relation to *Z. bailii* (F7D56A and F7D51A), are considered to be of potential interest for further studies. Some *S. cerevisiae* isolates obtained during the present work have already been tested to produce bioethanol using carob pods as a carbon source (Lima-Costa, Tavares, Rodrigues, Quintas, & Raposo, 2010).

Table 4
Killer behavior of the *S. cerevisiae* isolates in relation to the killer toxins K2, K5, K8, K9, and K10.

No. of Isolates	Isolates identification	Sensitivity pattern
7	F1D44D; F5D39C; F5D9E; F10D11E; F10D14B; F10D23A; F10D30D;	K2 K5 K8 K9 K10
19	F7D39B; F7D121A; F7D44A; F7D110A; F5D51D; F5D135A; F5D18A; F5D58D; F5D51E; F5D28B; F5D4A; F10D11C; F10D11F; F10D14D; F10D18C; F10D23D; F10D37D; F10D76B; F10D9A;	K2 K5 K9 K10
10	F7D90A; F7D44C; F7D18A; F7D28B; F7D56A: F7D56B; F7D51A; F7D150F; F7D90G; F5D23A	K2 K5 K9
2	F7D44E; F7D110D	K2 K9
1	F7D39D	K2 K8
2	F7D18D; F10D37E	K2
2	F7D11C; F5D4F	Resistant to K2 K5 K8 K9 K10

It would be interesting to select a mixture of species isolated from *A. unedo* fruits' fermentation environment, aimed at achieving a rich specific organoleptic profile typical of the region that avoids contaminations, off-flavors and non-permitted compounds in the final product, as described by Duarte et al. (2010) for raspberry wine. It is known that the yeast population is a key parameter in the quality of fermented and distilled beverages. On the other hand, yeast strains adapted to a specific substrate and climate environment give better characterized and certified products.

4. Conclusion

The main application of the fruits of the Mediterranean strawberry tree (*A. unedo*) is in the production of a distilled beverage that constitutes an important social agro-sustainable business permitting an additional income to families. The berries are fermented at room temperature through a solid state process dominated by a rich diversity of autochthonous yeasts. As fermentation progressed, a succession of species occurred culminating with *S. cerevisiae* and *P. membranaefaciens* in a mass with high concentrations of ethanol. The ethanol tolerant and killer resistant *S. cerevisiae* isolates could be tested in a microbial consortium to improve the arbutus berries fermentation and thus contribute to enhancement of the final product's quality. The addition of knowledge to natural sustainable resources is a way of contributing to their preservation and creating financial benefits.

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