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YEAST dynamics during the natural fermentation process of table olives (*Negrinha de Freixo* cv.)

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ABSTRACT

Yeast population and dynamics associated to spontaneous fermentation of green table olives *Negrinha de Freixo* cv. were evaluated. Olives and brine samples were taken at different fermentation times, and yeast were enumerated by standard plate count and identified by sequencing of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). *Saccharomyces cerevisiae* was the most frequent, followed by *Candida tropicalis*, *Pichia membranifaciens* and *Candida boidini*, representing together 94.8% of the total isolates. *Galactomyces reessii* was also identified for the first time in table olives. The highest species diversity was found between 44 and 54 days of fermentation, both in brine and olive pulp. Furthermore, high similarity was observed between brine and olive pulp microbiotas. In conclusion, these results give valuable information to table olive industrials in order to achieve more knowledge on the fermentation process of this important Protected Designation of Origin product.

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1. Introduction

Table olives are one of the most important fermented vegetables in Portugal. Trás-os-Montes is the main producing Portuguese region, being recognized the Protected Denomination Origin (PDO) of table olive *Negrinha de Freixo* cultivar [Commission Regulation (EC) No 1107/96]. In its preparation, fruits are hand-harvested, graded and further treated in one of the following types: green olives in brine, oxidized black olives, and ripe olives in brine. All are natural fermented olives, resulting fermentation from the competitive activities between autochthonous microbiota and contaminating microorganisms (Heperkan, 2013), which diversity and abundance depends of several factors, such as the cultivar, type of olive processing and applied conditions (Corsetti et al., 2012). Yeasts are one of the most important groups of microorganisms in fermented olive production. They play a key role in the organoleptic characteristics of the end product, and in the promotion of lactic acid bacteria (Arroyo-López et al., 2008). However, an excessive growth of fermentative yeasts species can also cause several problems such as spoilage of olives, gas pocket formation and clouding of the brines (Tofalo et al., 2012).

So far, the production of table olives *Negrinha de Freixo* PDO has been conducted in an empirical way without knowledge about the microbiota and the chemical properties occurring over the fermentation process. The aim of this study was to evaluate changes in yeast population, both in brines and olives, over the spontaneous fermentation of green table olives *Negrinha de Freixo* cv. in brine. The chemical properties (pH and titratable acidity) of brines were also evaluated at the same time, and further correlated with yeasts population. The acquired knowledge will be useful to improve the fermentation process, but also to obtain a more stable, safer and healthier product for consumers. This information could also be transferred to the industrial sector.

2. Material and methods

2.1. Sampling

Olives *Negrinha de Freixo* cv. from the crop year 2012 were kindly provided by the producers association "Adega Cooperativa de Freixo de Espada à Cinta" (Freixo de Espada à Cinta, NE Portugal). Fruits were harvested, transported in 20 kg plastic containers to the factory, sorted and washed. Afterward, natural fermentation took place in fermenters of 14,500 L of capacity with 9500 kg of olives covered with brine (NaCl 7%, w/v, pH 4, regulated with lactic acid addition) at room temperature.

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Samples (olives and brine) were collected over the fermentation process (0-raw material, 4, 17, 27, 32, 44, 54 and 149 days) from three different vats and immediately transported to the laboratory at 4 °C.

2.2. Chemical parameters

pH and titratable acidity were evaluated in brine samples over the fermentation. pH was measured by a digital pHmeter (Hanna HI 99163). Titratable acidity was determined by titration up to pH 8.2 with 0.1 N NaOH and expressed as g of lactic acid/100 mL brine. All measures were performed in triplicate.

2.3. Yeast isolation and identification

Aliquots of 25 mL of brine and 25 g of olive pulp were diluted with 225 mL of sterile peptone water (0.1%, w/v) and plated in triplicate on potato dextrose agar (PDA, Liofilchem, Italy) supplemented with chloramphenicol (1 mg/mL, Sigma–Aldrich, Milan, Italy). After incubation at 25 °C for 4 days, the colonies were counted and the means and standard deviations of three replicates were calculated. Results were expressed as log CFU/g or mL.

Pure culture of yeast isolates growing on PDA medium at 25 ± 2 °C for 24–48-h were firstly grouped according to their morphological similarity. One representative strain of each morphotype was selected and molecularly identified. Isolation of genomic DNA was performed by transferring a loopful of yeast cells, removed from a 24 to 48 h-old growing pure culture on PDA medium, into a microtube containing 500 μL of Lysis buffer (200 mM Tris–HCl pH 8.0, 250 mM NaCl, 25 mM EDTA pH 8.0 and 0.5% SDS) and 0.5 g of sterile glass spheres (0.4–0.6 mm of diameter, Sartorius). The tubes were vortexed for 5 min to disrupt cells. After addition of 250 μL of cold 3M NaOAc pH 5.5, the mixture was gently homogenized by inversion and incubated for 20 min at −20 °C. Following centrifugation at 10,500 rpm (4 °C) for 10 min, the supernatant was collected to another microtube and one volume of isopropanol (−20 °C) was added. This mixture was slowly homogenized and incubated overnight at −20 °C. The DNA precipitate was collected by centrifugation at 10,500 rpm (4 °C) for 10 min and the pellet washed with cold 70% ethanol. The DNA pellet was resuspended in 40 μL of ultra pure water and stored at −20 °C until use. Molecular identification of yeast strains was based on the sequencing of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. The ITS region (ITS1, 5.8S, ITS2) was amplified using ITS1 and ITS4 primers (White et al., 1990), in a PCR protocol formerly described by Oliveira et al. (2012). Amplified products were purified using JETQUICK PCR Product Purification Spin Kit (Genomed), following the manufacturer's instructions, and further sequenced using the STABVida services (Oeiras, Portugal). The obtained DNA sequences were analyzed with DNASTAR v.2.58 software, and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm.

2.4. Data analysis

The species richness, *Simpson* (1/D, Simpson's reciprocal index) and *Shannon–Wiener* (*H*) diversity indexes, as well as the Chao & Lee 1 and first-order Jackknife species richness estimators (Magurran, 2004), was estimated using the software *Species Diversity and Richness*, v.4.0 (Seaby and Henderson, 2006). The proportion of isolates from each yeast species in relation to the total number of yeast isolates was considered as the frequency of occurrence (FO, %) of a certain taxa. The number of yeast species shared between olive pulp and brine over the fermentation was estimated using Sørensen, Jaccard and Bray–Curtis similarity indexes (Magurran, 2004). These measures were computed in the *Community Analysis Package*, v.4 (Henderson and Seaby, 2007). Correlations between chemical parameters (pH and titratable acidity) and both yeast diversity and abundance (total and individual species) found in brine were measured by the Spearman coefficient, determined by SPSS v.20 software.

Statistical analysis of the chemical parameters and microbial counts was carried out by one-way analysis of variance, and means were compared by Tukey's test (at $p < 0.05$) using SPSS v.20 software. Yeast counts data was first transformed to $\log_{10}$ before statistical analysis.

3. Results and discussion

3.1. Chemical changes during fermentation

The titratable acidity (lactic acid percentage) increased significantly after 54 days of fermentation (0.96 g/100 mL brine) in relation to the beginning (Fig. 1), and afterward remained constant until the end of the fermentation (0.90 g/100 mL). This increment could be related to the presence of organic acids such as acetic, malic and lactic acids, which are reported to be common in fermentations of green and black olives (Chorianopoulos et al., 2005; Panagou et al., 2008; Tassou et al., 2002). These compounds are produced by microorganisms through sugars consumption (e.g., glucose, fructose and sucrose) present in olives (Tofalo et al., 2012). As expected, the pH values decreased during the fermentation, reaching 3.46 at the end of the process. This value is satisfactory to protect table olives against deterioration processes and inhibit the growth of pathogenic microorganisms during storage (Tofalo et al., 2012).

3.2. Yeast dynamics during fermentation

The yeast dynamics in pulp and brine along the fermentation process are represented in Fig. 2. Before immersion in brine the counts of the indigenous yeast in olive pulp were 3.59 log CFU/g. Along fermentation yeast counts varied between 5.1–6.5 log CFU/g and 5.0–6.3 log CFU/mL in the olive pulp and brine, respectively. Similar patterns were obtained during natural fermentations of Italian (Aponte et al., 2010; Cocolin et al., 2013) and Spanish (Bautista-Gallego et al., 2011; Hurtado et al., 2008) table olive cultivars.

The isolation of yeast from raw, brined olive pulp and fermenting brine allowed the identification of 11 taxa (Table S1, Supplementary data). The greatest number of species belonged to

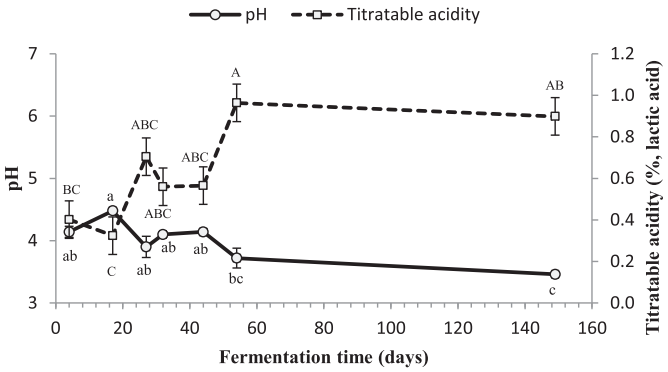

Fig. 1. pH and titratable acidity values along the fermentation. Each value is expressed as mean ± SE ($n = 3$). Bars with different lowercase (pH) and uppercase (titratable acidity) letters indicate significant differences ($p < 0.05$).

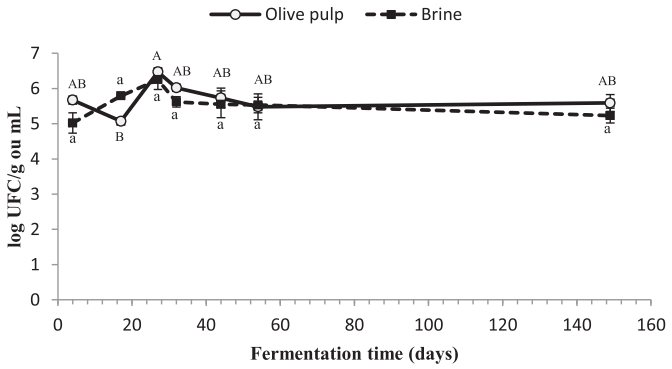

Fig. 2. Yeasts growth in olive pulp and brine along fermentation. Each value is expressed as mean ± SE ($n = 3$). Bars with different lowercase (brine) and uppercase (olive pulp) letters indicate significant differences ($p < 0.05$).

the phyla Ascomycota and genera *Candida*, *Pichia*, *Debaryomyces*, *Saccharomyces* and *Galactomyces*. The remaining species belonged to the phyla Basidiomycota and genus *Rhodotorula*. In what concerns abundance, a total of 2622 isolates (1492 in pulp and 1130 in brine, Table 1), were obtained, being the greatest percentage from Ascomycota (98.8%) and only 1.2% from Basidiomycota. Among the species identified *Saccharomyces cerevisiae*, *Candida tropicalis*, *Pichia membranifaciens* and *Candida boidinii* were the most frequently isolated representing together 94.8% of the total isolates. Those species (Bautista-Gallego et al., 2011) and others from the same genera (Heperkan, 2013), were also reported to be the most frequently isolated in table olive fermentations.

As fermentation began, changes on yeast population were noticed on olive pulp and on the fermenting brine (Table 1). Overall, it was found more yeast species diversity in brine (7 species) than in olive pulp (5 species). This pattern was generally observed throughout the fermentation process, being the highest species diversity found between 44 and 54 days of fermentation, both in brine and olive pulp. At the end of the process (149 days), the diversity decreased. All the diversity indexes and species richness estimators corroborated these results (Table 1).

Yeast community on the olive pulp before brining (0 days) was composed by 7 species, being *S. cerevisiae*, *Pichia guilliermondii*, *Rhodotorula graminis* and *C. tropicalis* the most frequently isolated (Fig. 3). During the first 4 days the yeasts species most abundant in brine was *C. boidinii* (63% of the total isolates) and in olive pulp was *P. membranifaciens* (56% of the total isolates) (Fig. 3). Although found in less number, the yeasts *C. tropicalis* and *S. cerevisiae* were also present in olive pulp; whereas in brine was additionally verified the presence of *Pichia manshurica*. The presence of *C. boidinii* in brines was previously described in the early (Alves et al., 2012) and later phase (Bautista-Gallego et al., 2011) of the fermentation of various table olives types, as well as in all fermentation phases of natural black olives (Nisiotou et al., 2010). This specie showed to exhibit lipase activity (Bautista-Gallego et al., 2011), contributing to improve the flavor of olives through the formation of esters from free fatty acids of the fruit (Hernández et al., 2007; Rodríguez-Gómez et al., 2010). It was also shown to have capacity to colonize and adhere to the olive skin, thereby proving to be a possible starter culture (Arroyo-López et al., 2012). To our knowledge, the presence of *P. membranifaciens* on olives through the fermentation has never been reported before. However, this specie has been frequently detected on brines at the later phase (Lucena-Padrós et al., 2014) or throughout (Bautista-Gallego et al., 2011) the Spanish-style green olive fermentation process. Although *P. manshurica* was only detected in brine at the beginning of the fermentation (first 4 days), several authors have yet reported its presence in brines throughout (Abriouel et al., 2011) and at later stages (Lucena-Padrós et al., 2014; Nisiotou et al., 2010) of the fermentation of several table olive types.

After 17 days until the end of fermentation, the yeast community changed, being *S. cerevisiae* and *C. tropicalis* the main species present on both olive pulp and brine (Fig. 3). *Saccharomyces cerevisae* is reported to be well adapted to the olive fermentation environment (e.g., low pH and oxygen availability, and high salt concentration) (Arroyo-López et al., 2006; Heperkan, 2013), which could explain its high presence in our study. These features makes *S. cerevisiae* as one of the most relevant yeast species to be used as starter (Heperkan, 2013).

Comparison of yeast community composition found on raw material, olive pulp and brine over the fermentation, could partly explain their source. The presence of *S. cerevisiae*, *C. tropicalis* and *Debaryomyces hansenii* on raw olive pulp and their further detection over fermentation, suggested that the origin of these species was probably the olive fruits. The exclusively occurrence of *Candida norvegica*, *R. graminis*, *P. guilliermondii* and *R. glutinis* on raw material, suggested that they are not involved in the fermentation process. The environmental conditions occurring over the fermentation were probably unfavorable for these species, limiting their growth. By contrast, *P. membranifaciens*, *C. boidinii*, *P. manshurica* and *Galactomyces reessii* were uniquely detected along fermentation suggesting that they may have originated from the fermentation vessels and other devices in contact with the olives and brine. To our knowledge, the presence of *G. reessii* has not been yet associated to table olive fermentation.

Overall, Jaccard, Sørensen and Bray–Curtis indexes show high similarity between brine and olive pulp microbiotas (Table 2). This similarity changed over the fermentation, becoming higher from the initial to 44 days of fermentation. After that, the similarity was maintained until the end of the fermentation. These results highlight that yeast species richness, composition and abundance on olive pulp and brine were less similar in the first stage of the fermentation (up to 44 days) and more similar after that period. The high similarity found after 44 days was mainly due to the fact that both olive pulp and brine were dominated by two shared species, *S. cerevisiae* and *C. tropicalis*.

Spearman correlation tests demonstrate significant negative correlations between pH and abundance (-0.32, $p < 0.05$) and richness (-0.43, $p < 0.001$) of yeasts in brine. Among the yeasts isolated in brine, only *C. boidinii* (-0.25, $p < 0.05$), *C. tropicalis*

Table 1
Yeast species diversity and abundance in olive pulp and brine over fermentation.

Indexes	Fermentation time (days)							
	4	17	27	32	44	54	149	Total
Olive pulp								
Total yeast isolates	127	Ne[a]	45	261	132	369	411	1492
Species number	3	Ne	2	4	4	4	3	5
Shannon–Wiener	0.9	Ne	0.7	1.0	1.0	1.3	0.7	1.2
Simpson's reciprocal index	2.3	Ne	2.0	2.2	2.4	3.4	1.6	2.6
Chao & Lee 1	3.0	Ne	2.0	4.0	4.0	4.0	3.0	5.0
Jackknife 1	3.0	Ne	2.0	4.0	4.0	4.0	3.0	5.0
Brine								
Total yeast isolates	51	108	Ne	83	82	577	229	1130
Species number	4	2	Ne	3	5	6	4	7
Shannon–Wiener	1.0	0.7	Ne	1.0	1.5	1.2	0.9	1.3
Simpson's reciprocal index	2.2	2.0	Ne	2.7	3.9	2.8	2.1	2.9
Chao & Lee 1	4.1	2.0	Ne	3.0	5.0	7.3	4.0	7.0
Jackknife 1	4.0	2.0	Ne	3.0	5.0	6.0	4.0	7.0

[a] Ne – Not evaluated.

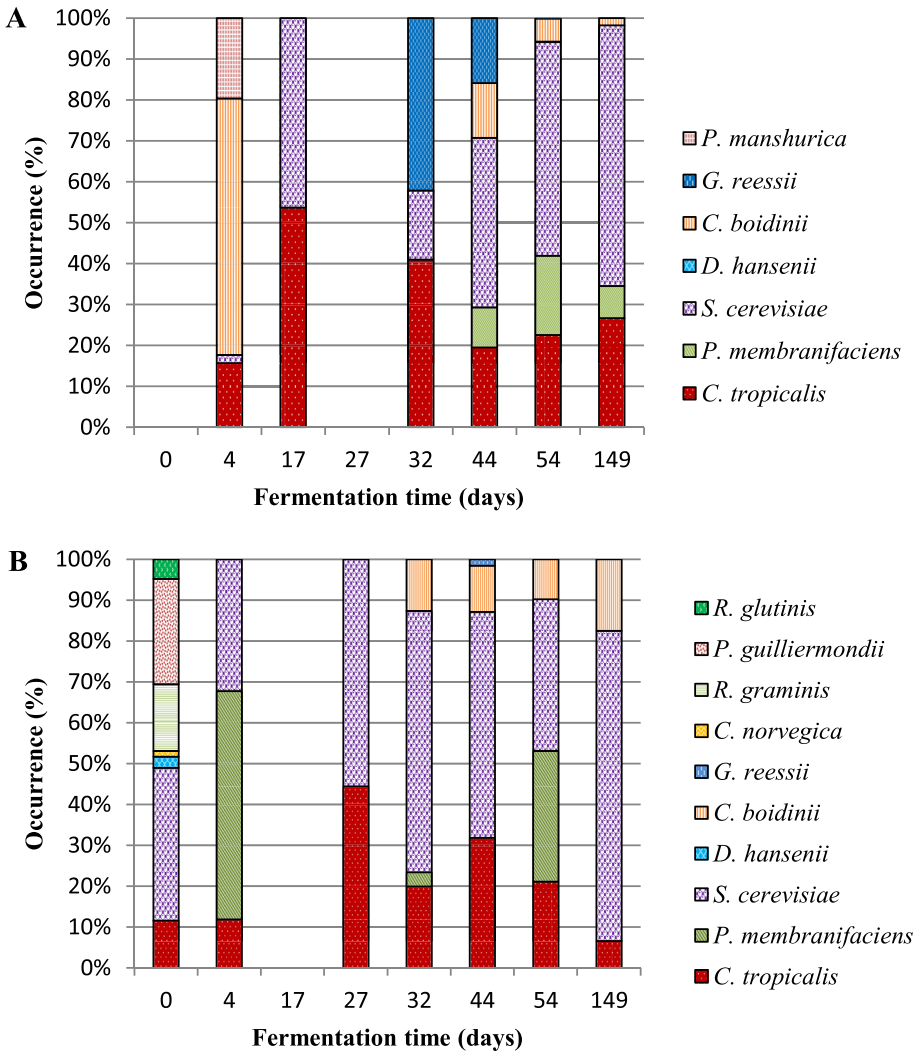

Fig. 3. Frequency of occurrence of each yeast species in brine (A) and olive pulp (B) along fermentation.

(−0.44, $p < 0.001$), *S. cerevisiae* (−0.47, $p < 0.001$) and *P. membranifaciens* (−0.55, $p < 0.001$) were negatively affected by the pH. This indicates a higher sensibility of these species to pH when compared to the others yeast species identified; and their growth seemed to be favored by low pH values.

In conclusion, a great diversity and variability of yeast species was found over the spontaneous fermentation of PDO green table olives *Negrinha de Freixo*. The yeast community present in brine was similar to the olive pulp, and significantly influenced by the pH of the brine. The most probable sources of yeasts in the fermentation of this table olive are the fruits themselves and the containers in which the olives were fermented. Future studies should be focused in the standardization of the fermentation process by developing starter cultures. This will avoid variations in the quality and flavor of the product and consequently will improve its safety.

Table 2
Similarity of yeast community between brine and olive pulp over fermentation.

Indexes	Brine *vs* olive pulp					Total
	Fermentation time (days)					
	4	32	44	54	149	
Jaccard	0.4	0.4	0.8	0.7	0.8	0.5
Sørensen	0.6	0.6	0.9	0.8	0.9	0.7
Bray–Curtis	0.9	0.7	0.4	0.3	0.4	0.2

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.10.003.

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