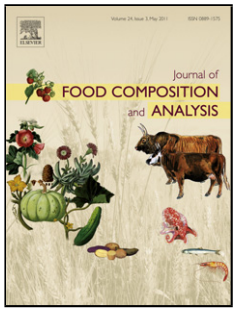

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PII: S0889-1575(18)30800-7
DOI: https://doi.org/10.1016/j.jfca.2018.08.008
Reference: YJFCA 3129

To appear in:

Received date: 15-6-2018
Revised date: 20-8-2018
Accepted date: 30-8-2018

Please cite this article as: { https://doi.org/

Original Research Article

Composition of health-promoting phenolic compounds in two extra-virgin olive oils and diversity of associated yeasts

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Highlights:

1) Tyrosol, hydroxytyrosol, oleocanthal and oleacein were determined in two Tuscan EVOOs
2) The two EVOOs showed different contents of oleocanthal and oleacein
3) During storage the contents of the phenols tyrosol and hydroxytyrosol increased
4) Oils differing in phenols levels showed different yeast community composition
5) Cluster analysis revealed important changes in yeast diversity during storage

Abstract

Extra virgin olive oil (EVOO), a basic component of the Mediterranean diet, is an important functional food, for its content in health-promoting compounds, showing antioxidant, anti-inflammatory and antiproliferative activities. Here, two Tuscan EVOOs were analyzed for the occurrence and concentrations of health-promoting phenols, such as tyrosol and hydroxytyrosol and the secoiridoid derivatives, oleocanthal and oleacein. Independently of the milling period, the two EVOOs showed different contents of oleocanthal and oleacein. During storage, the contents of oleocanthal and oleacein decreased, while those of simple phenols increased. In all oil samples oleacein displayed a higher rate of reduction than oleocanthal. Multivariate analyses of the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles, revealed the occurrence and diversity of oil-borne yeast communities, which differed in the two EVOOs. Sequences of excised DGGE bands identified *Candida adriatica, Eremothecium coryli* and *Lachancea fermentati* as the main components of the oil-borne yeast community. Our work detected, for the first time, differences in the content of tyrosol, hydroxytyrosol, oleocanthal and oleacein between the two Tuscan EVOOs analyzed, consistent with the differences found in yeast community composition. Further studies could confirm whether oil-borne yeasts may affect the composition of health-promoting oil phenolic compounds.

Keywords: Extra-virgin olive oil; Tyrosol; Hydroxytyrosol; Oleocanthal; Oleacein; Oil-borne yeasts; Polymerase Chain Reaction-Denaturating Gradient Gel Electrophoresis (PCR-DGGE); *Candida adriatica*; Food analysis; Food composition.

1. Introduction

Olive oil represents the most important vegetable oil used for human nutrition in the Mediterranean area and is gaining popularity worldwide as a basic component of the Mediterranean diet, due to its well-known nutritional and nutraceutical value (Cicerale et al., 2012). Its specific health-promoting properties have been officially recognized by the European Food Safety Authority (EFSA), with a health claim that attributed the protection of blood lipids from oxidative stress to extra virgin olive oil (EVOO) phenols, in particular hydroxytyrosol and its derivatives (EFSA, 2011). The family of phenolic compounds characteristic of EVOO comprises simple phenols, tyrosol and hydroxytyrosol, and secoiridoid derivatives, oleocanthal and oleacein (Fig. 1) (Karkoula et al., 2014; Antonini et al., 2015).

Phenols have several biological activities, such as the depletion of oxidized low-density lipoproteins (Visioli et al., 1995), antioxidant activity (Visioli et al., 2002) and protection against neurodegenerative diseases associated with aging (Casamenti & Stefani, 2017). In particular, oleocanthal, responsible for the pungent and irritating sensation associated with the ingestion of EVOO (Barbieri et al., 2015), displayed an anti-inflammatory activity linked to the inhibition of two subtypes of cyclooxygenases (COX 1 and COX 2) comparable to that of the well-known anti-inflammatory drug ibuprofen (Beauchamp et al., 2005). Furthermore, oleocanthal reduced lipopolysaccharide induced nitric oxide synthase expression and nitric oxide production in the ATDC-5 murine chondrogenic and in murine macrophages J774 cell lines in a dose-dependent manner, showing a potential activity for the treatment of inflammatory degenerative joint diseases (Iacono et al., 2010; Scotece et al., 2012). In addition, oleocanthal was active against peptic ulcer-

related microorganisms (Romero et al., 2007) and some types of cancer (Fabiani, 2016; Fogli et al., 2016; Cusimano et al., 2017). Oleacein, exhibited anti-inflammatory properties due to its 5-lipoxygenase inhibitory effect (Vougogiannopoulou et al., 2014), and showed angiotensin converting enzyme (ACE) inhibitory activity (Czerwińska et al., 2012).

The occurrence and concentrations of such beneficial phenolic compounds in olive oil depends on diverse factors, such as olive cultivar, fruit ripening state and harvest season, pedoclimatic conditions, irrigation and agronomic techniques, oil processing, preservation and storage conditions (Servili et al., 2004; 2007; Caruso et al., 2014).

Furthermore, recent findings revealed that the concentrations of these compounds in olive oil may be affected by the presence and transformation activity of oil-borne microbiota originating from olives' carposphere and migrating into olive oil during processing in the mill (Ciafardini et al., 2004; Koidis et al., 2008).

Here, for the first time, a culture-independent method, PCR-denaturating gradient gel electrophoresis (PCR-DGGE), was used to investigate the occurrence and diversity of yeast communities in two Tuscan EVOOs. The quantitative composition of health-promoting phenols, such as the simple compounds tyrosol and hydroxytyrosol and the secoiridoid derivatives, oleocanthal and oleacein, was also assessed.

2. Material and methods

2.1. Oil sampling

The two EVOOs analyzed (hereafter A and B), originating from Tuscany, were cold extracted from washed and defoliated olives which were mechanically crushed, the paste kneaded, the oil extracted by centrifugation and separated from water and pomace. The oil, without any further refining process, was collected directly from the producers in October 2015 (first olive milling) and in December 2015 (second olive milling) and stored in 5-L metal cans at ambient temperature in the

Laboratory of Microbiology, DiSAAA-a, until analysed. Chemical analyses were carried out on three replicate samples taken after remixing the whole oil mass, at 7 time points, from October 2015 to April 2016 (first olive milling) and from December 2015 to June 2016 (second olive milling). Molecular analyses of oil microbial communities were performed on two replicate samples, along three-time points during oil storage, at time 0 and after 4 and 7 months of storage. Chemical analyses were carried out immediately after sample collection, while molecular analyses were performed at the end of the study, on 15-mL samples collected simultaneously and preserved at –20 °C.

2.2. Analytical reagents

Solvents used for the purification procedure, high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analyses were purchased from Sigma-Aldrich (Sigma Aldrich srl, Milan, Italy): acetonitrile (99.8% purity, certified ACS reagent grade), hexane (≥ 97% purity, certified ACS reagent grade), methanol (99.8% purity, certified ACS reagent grade), water (HPLC Plus, EMD Millipore), ethyl acetate (99.8% purity, certified ACS reagent grade), chloroform (≥ 99.8% purity, certified ACS reagent grade), acetic acid (≥ 99.8% purity, certified ACS reagent grade). Solvent evaporation was carried out under vacuum using a rotating evaporator Strike 300 (Steroglass srl, Perugia, Italy). Silica gel flash chromatography was performed using silica gel 60Å (0.040–0.063 mm; Sigma Aldrich srl, Milan, Italy). Thin layer chromatography (TLC) analysis was carried out on Merck aluminum silica gel (60 F254; Sigma Aldrich srl, Milan Italy), visualized under a Spectroline CM-10 UV lamp (λ = 254 nm) (Spectroline, Westbury, NY) or by spraying with a 10% solution of phosphomolybdic acid in absolute ethanol (Sigma Aldrich srl, Milan, Italy). Preparative TLC (Prep TLC) purification was performed using either 2 mm (20 × 10) and 1 mm (10 × 10) glass-backed sheets precoated with silica gel 60 F254 purchased from Sigma Aldrich. Tyrosol (≥ 99.5% purity, certified ACS reagent grade), hydroxytyrosol (≥ 98.0% purity, certified ACS

reagent grade) as analytical standards and *p*-hydroxyphenylacetic acid (≥ 98.0% purity, certified ACS reagent grade) as internal standard were purchased from Sigma-Aldrich.

2.3. Standard preparation

Oleocanthal and oleacein were obtained, as pure standards, by direct extraction and purification from virgin olive oil, using a slightly modified method developed in our previous study (Fogli et al., 2016) for the extraction and purification of oleocanthal from olive oil. Briefly, a mixture of about 212 g of virgin olive oil with *n*-hexane (848 mL) and acetonitrile (1325 mL) was homogenized using a vortex mixer. After centrifugation at 1800 *g* for 5 min, at 25 °C, the acetonitrile phase was collected and evaporated under reduced pressure, to afford a crude residue, which was purified by column chromatography using the mobile phase shown in Table 1.

Fractions 75–97 (89.3 mg) and fractions 104–148 (45.2 mg) containing oleocanthal and oleacein, respectively, were subjected to further purification, by preparative TLC using *n*-hexane/AcOEt 6:4 as mobile phase. The corresponding zones were extracted from the stationary phase using AcOEt under sonication for 15 min, then the suspension was filtered, and the solvent evaporated under reduced pressure, giving oleocanthal (15.0 mg) and oleacein (9.0 mg). Identification and purity of the extracted compound were based on ^{1}H NMR, and HPLC analyses. The ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker AVANCE IIITM 400 spectrometer operating at 400 MHz (Bruker Corporation, Billerica, MA).

HPLC analysis was performed using a HPLC instrument (Beckman, Ramsey, MN) equipped with a System Gold Solvent Delivery module (Pumps) 125, System Gold UV/Vis Detector 166, set to 278 nm. Analyses, in accordance with the method previously reported (Margari & Tsabolatidou, 2015), were performed on a Phenomenex Gemini reverse-phase C18 column (250 × 4.6 mm, 5 µm particle size; Phenomenex, Castel Maggiore, Italy). The mobile phase was a mixture of H_2O/AcOH (97.5:2.5 *v/v*) (**A**) and MeOH/ACN (1:1 *v/v*) (**B**). The elution gradient started from 5% eluent **B** and

reached 100% **B** after 65 min at flow rate 1 mL min^{-1}. The injected volume was 30.0 µL. The analyses revealed that oleocanthal and oleacein had purity >95%.

2.4. Olive oil extraction and quantification of the phenolic compounds

A mixture of about 3.0 g of EVOO with *n*-hexane (12.0 mL) and acetonitrile (15.0 mL) was homogenized using a vortex mixer. After centrifugation at 1800 *g* for 5 min, at 25 °C, the acetonitrile phase was collected and evaporated under reduced pressure, to afford a residue. A mixture of methanol/water (1:1 *v/v*) was added to residue for the HPLC injection. Phenolic compounds were identified by comparing retention times and UV absorbance spectra with those of authentic standards, and quantified at 279 nm using *p*-hydroxyphenylacetic acid as the internal standard, according to the method previously reported (Margari & Tsabolatidou, 2015). For each standard compound, the calibration curve was built and the detection limits (LOD) and quantification (LOQ) were estimated (Table 2). Sample concentrations were determined by linear regression. Determination coefficients (r^2) for each of the calibration curves were >0.999 (Table 2).

2.5 Statistical analysis

Statistical significance of differences between phenolic compounds levels was determined by one-way ANOVA with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). Tukey's post-test was used for multiple comparisons. *P*-values < 0.3 were considered to be significant. Data are expressed as means ± SEM of three independent experiments, each performed in duplicate.

2.6. DNA extraction from oil samples and PCR amplification

DNA was extracted from 3 mL oil samples by using "Power Soil DNA Isolation kit" (MO BIO Laboratories, Inc., Cabru S.A.S. Arcore, Italy) according to the manufacturer's protocol and stored at –20 °C until further analyses. For the analysis of yeast populations, an approximately 250 bp long

fragment of D1/D2 region of the large sub-unit (LSU) rRNA gene was amplified as previously reported (Palla et al., 2017).

2.7. DGGE and profile analyses

For the DGGE analysis, amplicons were separated in 8% (*w/v*) polyacrylamide gels with a 25–60% urea-formamide gradient, using the DCode™ Universal Mutation Detection System (Bio-Rad, Milan, Italy). A composite mix of fungal 26S rDNA gene fragments from *Saccharomyces cerevisiae* ATCC 32167, *Kazachstania exigua* DBVPG 6956, *Dekkera bruxellensis* IMA 1L, *Kazachstania humilis* DBVPG 6754, *Candida boidinii* IMA 18S and *Geotrichum candidum* IMA F23 were added as reference DGGE markers (M). Gels were run and visualized as described in Agnolucci et al. (2013). DGGE profiles were digitally processed with BioNumerics software version 7.5 (Applied Maths, St-Martens-Latem, Belgium), and analyzed as reported in Turrini et al. (2017). A position tolerance and optimization of 0.5 and 0.5%, respectively, were used. DGGE profiles were also analyzed using non-metric multidimensional scaling analysis (NMDS) performed from a data matrix based on presence/absence of bands (Bray-Curtis coefficient). The significance of data was assessed by the one-way ANOSIM method (analysis of similarities; 999 permutations) using the software PAleontological STatistics Version 3.0 (Hammer et al., 2001) with oil type as variability factor.

2.8. DGGE band sequencing

The main bands of DGGE profiles were excised from the gels for sequencing at the Eurofins Genomics MWG Operon (Ebersberg, Germany) as reported in Palla et al. (2017). Sequences were analyzed using BLAST on the NCBI web (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The related sequences were collected and aligned using MUSCLE (Edgar, 2004a, b), and phylogenetic trees were constructed using the Neighbor-Joining method based on Kimura's 2-parameter model (Kimura, 1980) in Mega 6 software (http://www.megasoftware.net/) with 1000 bootstrap replicates.

The sequences were submitted to the European Nucleotide Archive under the accession numbers from LT963318 to LT963347.

3. Results and discussion

3.1 Quantification of phenolic compounds

Olive oil samples were analyzed for their phenolic compounds content, in particular of tyrosol and hydroxytyrosol and the corresponding secoiridoid derivatives oleocanthal and oleacein. The results are reported in Tables 3 and 4 (first and second milling, respectively). The chemical data corresponding to the time points of molecular analyses (time 0, 4 and 7 months of storage) are reported in Figs. S1 and S2 (oil A and B, respectively). As shown in Tables 3 and 4 and in Figs. S1 and S2, in fresh oil (time 0) the amounts of both total and single phenols, oleocanthal, oleacein, tyrosol and hydroxytyrosol, varied in the two EVOOs, consistently with previous findings (Owen et al., 2000; Karkoula et al., 2012; Caporaso et al., 2015). In particular, independently of the milling period, oil A showed a higher content of oleocanthal compared with oil B, which showed the highest content of oleacein (Tables 3 and 4, Figs. S1 and S2). Furthermore, in both oils A and B, the concentration of secoiridoid compounds, oleocanthal and oleacein, was higher than that of the simple phenols which was very low, in accordance with the literature (Mazzotti et al., 2012, Caporaso et al., 2015). Overall, the concentration of phenols was higher than 250 ppm, consistent with the European health claim (EFSA, 2011).

The concentration of phenolic compounds in the two EVOOs changed during storage. In particular, while the contents of oleocanthal and oleacein tended to decrease, those of simple phenols, tyrosol and hydroxytyrosol, increased, in agreement with previous findings (Montedoro et al., 1993; Pagliarini et al., 2000). The increase in the content of these simple phenolics may be ascribed to the hydrolytic degradation of the corresponding secoiridoid derivatives (Krichene et al., 2015; Migliorini et al., 2013).

In all oil samples oleacein displayed a higher rate of reduction than oleocanthal, confirming the data obtained by Karkoula et al. (2012). Indeed, the reduction of oleacein was about 75%, while the content of oleocanthal was reduced by 60% for both oils, during storage (seven months) and for both millings (Tables 3 and 4, Figs. S1 and S2).

3.2. Yeast community diversity

A DNA fragment of approximately 250 bp of the partial D1/D2 domain of 26S rRNA gene was successfully amplified from all oil samples. DGGE analyses of PCR products showed distinctive patterns, characterized by intense and clearly defined fragments. The microbial community composition was studied by cluster analysis of DGGE profiles (Fig. 2). Consistently with chemical findings, the dendrogram clearly separated the microbial populations of the two oils, with a similarity of only 41%. It is interesting to note that the cluster corresponding to oil A was further split into two sub-clusters (52% similarity), where the samples collected at time 0 grouped separately from those collected after 4 and 7 months storage (Fig. 2). The latter communities were further separated, on the basis of olive milling time, into two sub-clusters (55% similarity), whose samples were highly similar (82% and 81%), irrespective of the storage time (Fig. 2). Data from cluster analysis were confirmed by the NMDS analysis of the DGGE profiles (Fig. 3). ANOSIM revealed significant differences in the microbial community composition of the two EVOOs (R = 0.962, p = 0.0001). Our findings cannot be compared with other works, as this is the first assessment of olive oil microbiota by molecular methods and in particular by PCR-DGGE analyses. However, the important changes in microbial community composition found in A olive oil samples between time 0 and times 4 and 7, as revealed by cluster analysis, are consistent with previous data obtained using a culture-dependent approach, that found strong variations during the first month of storage (Ciafardini et al., 2004). Such trend may be ascribed to the selective action exerted by the olive oil ecological niche, representing a stressful environment for the yeast community, which may

be negatively affected by the absence of nutrients like sugars and proteins and a high content of total phenolics.

In order to identify the major yeast species characterizing the two EVOOs, PCR-DGGE bands were excised, sequenced and affiliated to species by using BLAST and phylogenetic trees analyses. Sequences were affiliated with the yeast or yeast-like species *Aureobasidium pullulans*, *Candida adriatica*, *Eremothecium coryli*, *Eremothecium cymbalariae*, *Geotrichum candidum*, *Lachancea fermentati*, *Ogataea phyllophila*, and with the fungal species *Cladosporium cladosporioides*, *Pseudophaeomoniella oleae* and *Pseudophaeomoniella oleicola*. In addition, sequences affiliated to soil-borne fungi, such as *Racocetra alborosea*, *Scutellospora gilmorei* and *Scutellospora pellucida* were found (Fig. 4).

As to the occurrence of the different species, sequences affiliated to the yeasts *E. coryli*, *L. fermentati* were retrieved from oil B (Fig. 4), confirming previous reports on the isolation of *L. fermentati* from crushed paste and pomaces in Tuscany, as well as in wastewater from continuous olive mills in Southern Italy and Spain (Romo-Sánchez et al., 2010; Mari et al., 2016). By contrast, the yeasts *A. pullulans*, *C. adriatica*, *E. coryli*, *E. cymbalariae*, *G. candidum*, *O. phyllophila* were retrieved in A olive oil (Fig. 4). Among such yeast species, *C. adriatica* was previously isolated from olive oil in Italy, Croatia and Slovenia (Čadež et al., 2012; Mari et al., 2016). The presence of *A. pullulans* confirms previous data on its isolation from washed olives (Deshpande et al., 1992; Mari et al., 2016) and its occurrence in a broad range of habitats, including the phyllosphere, i.e. the above-ground portion of olive plants representing a habitat for microorganisms (Gostinčar et al., 2014). Its ability to produce a vast array of enzymes, including lipase (Gostinčar et al., 2014), could be relevant in the olive oil niche, although we cannot speculate on its functional significance. *G. candidum* is a ubiquitous yeast, previously reported to occur in olive paste and olive pomace (Baffi et al., 2012), able to produce the enzyme lipase, capable of hydrolyzing oleic acid esters, and recently proposed as a tool for partial hydrolysis of oils to produce nutraceutical diacylglycerols (Laguerre et al., 2017). Such compounds have shown nutraceutical properties, in particular for their

ability to reduce the amount of lipid stored in adipose tissue (Yuan et al., 2010). *E. cymbalariae* and *E. coryli* are plant pathogens causing fruit rotting "yeast spot disease" on diverse plant species, such as cotton and tomato (Miyao et al., 2000), probably occurring as casual contaminants, as well as *O. phyllophila*, a phylloplane yeast (Koowadjanakul et al., 2011).

4. Conclusions

Here, two Tuscan EVOOs revealing different contents of health-promoting phenolic compounds (oleocanthal, oleacein, tyrosol and hydroxytyrosol) showed also significant differences in yeast community composition, as detected by a culture-independent method, PCR-DGGE. While in the past olive oil was considered an unsuitable habitat for microorganisms, scientists are currently aware that it is a privileged niche for yeasts, originating from olives' carposphere and migrating into olive oil during processing in the mill. Here, for the first time, using PCR-DGGE, we detected the presence of a large community of yeast species, which are able to condition the physicochemical and sensorial characteristics of olive oil, through the production of a vast array of enzymes (Ciafardini and Zullo, 2018). Further studies could answer the intriguing question as to whether single or complex consortia of oil-borne yeasts may play a role in the transformation and/or production of health-promoting oil phenolic compounds.

Funding

Funding was provided by University of Pisa grants (Fondi di Ateneo).

Author Contributions

Experiments were conceived by M.G. and M.M. Chemical investigations were carried out by M.D., C.M., S.B., and M.M., who wrote also the relevant part of the manuscript; microbiological investigations were carried out by M.P., C.C., M.A. and M.G., who wrote also the relevant part of the manuscript.

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FIGURE CAPTIONS

Figure 1. Chemical structure of major health-promoting phenolic compounds present in extra virgin olive oil.

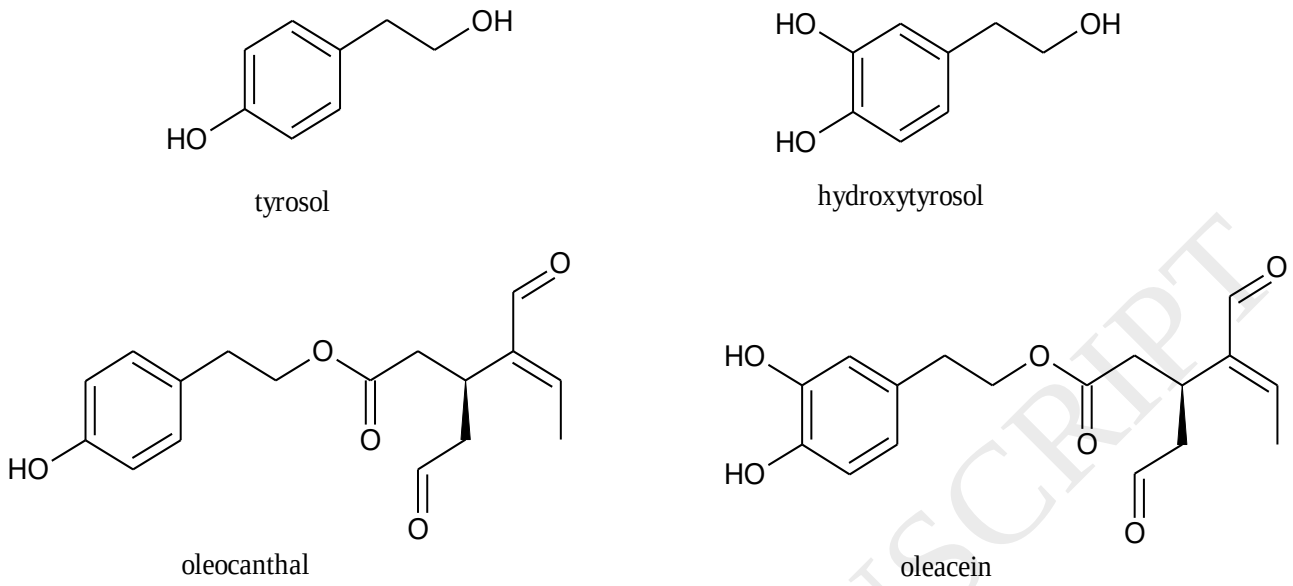
Figure 2. Dendrogram obtained from UPGMA analysis, using Dice's coefficient, of DGGE profiles of microorganisms associated with extra virgin olive oils A and B, collected during the 1st (empty) and the 2nd (full) milling, at time 0 (square) and after 4 (circle) and 7 (triangle) months of storage.

Figure 3. Non-metric multidimensional scaling (NMDS) plot of yeast DGGE profile analysis using Bray-Curtis coefficient. Each point on the plot represents yeast communities associated with extra

virgin olive oils A and B, collected during the 1st (empty) and the 2nd (full) milling, at time 0 (square) and after 4 (circle) and 7 (triangle) months of storage. The stress value is 0.2, the ANOSIM values (*R*) indicates significant differences between the two olive oils (0.962, $p = 0.0001$).

Figure 4. Affiliation of the sequences retrieved from DGGE gel bands with the existing sequences of the partial D1/D2 region of the large sub-unit rRNA gene. Phylogenetic analysis was inferred by using the Neighbor-Joining method based on Kimura's 2-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA6. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their corresponding band number and their accession number. Colored symbols indicate oil samples, A (full) and B (empty), analyzed at time 0 (square) and after 4 (circle) and 7 (triangle) months of storage.

FIGURES



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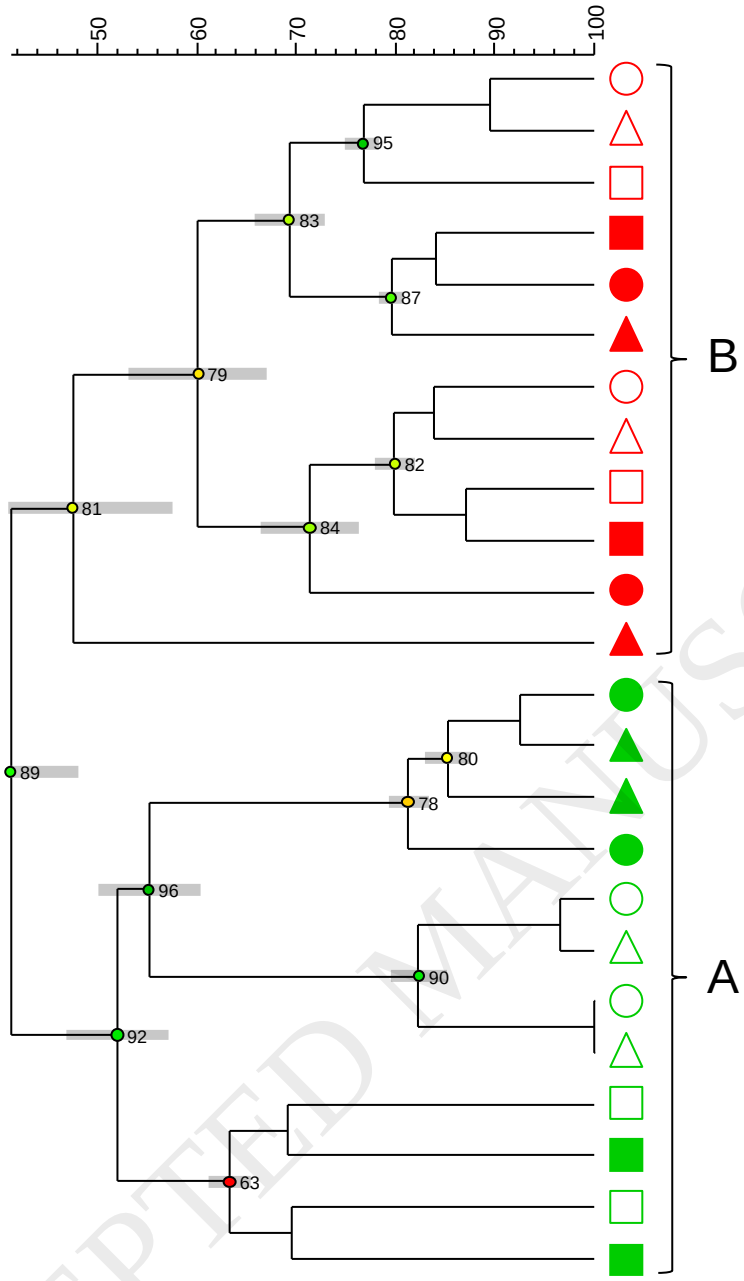

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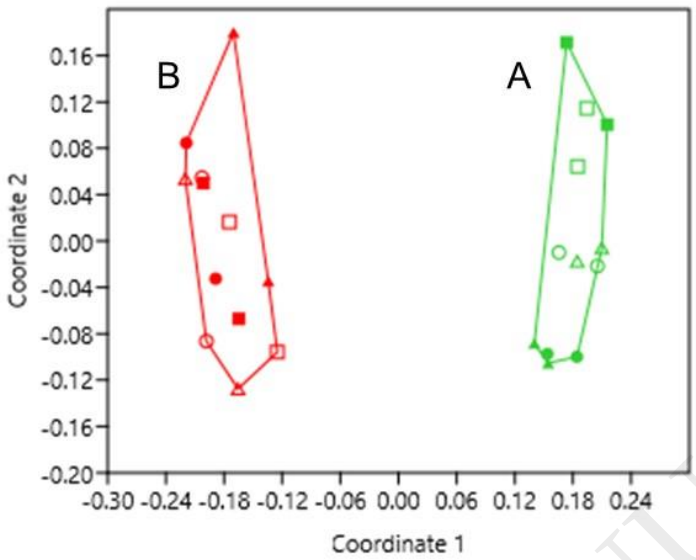

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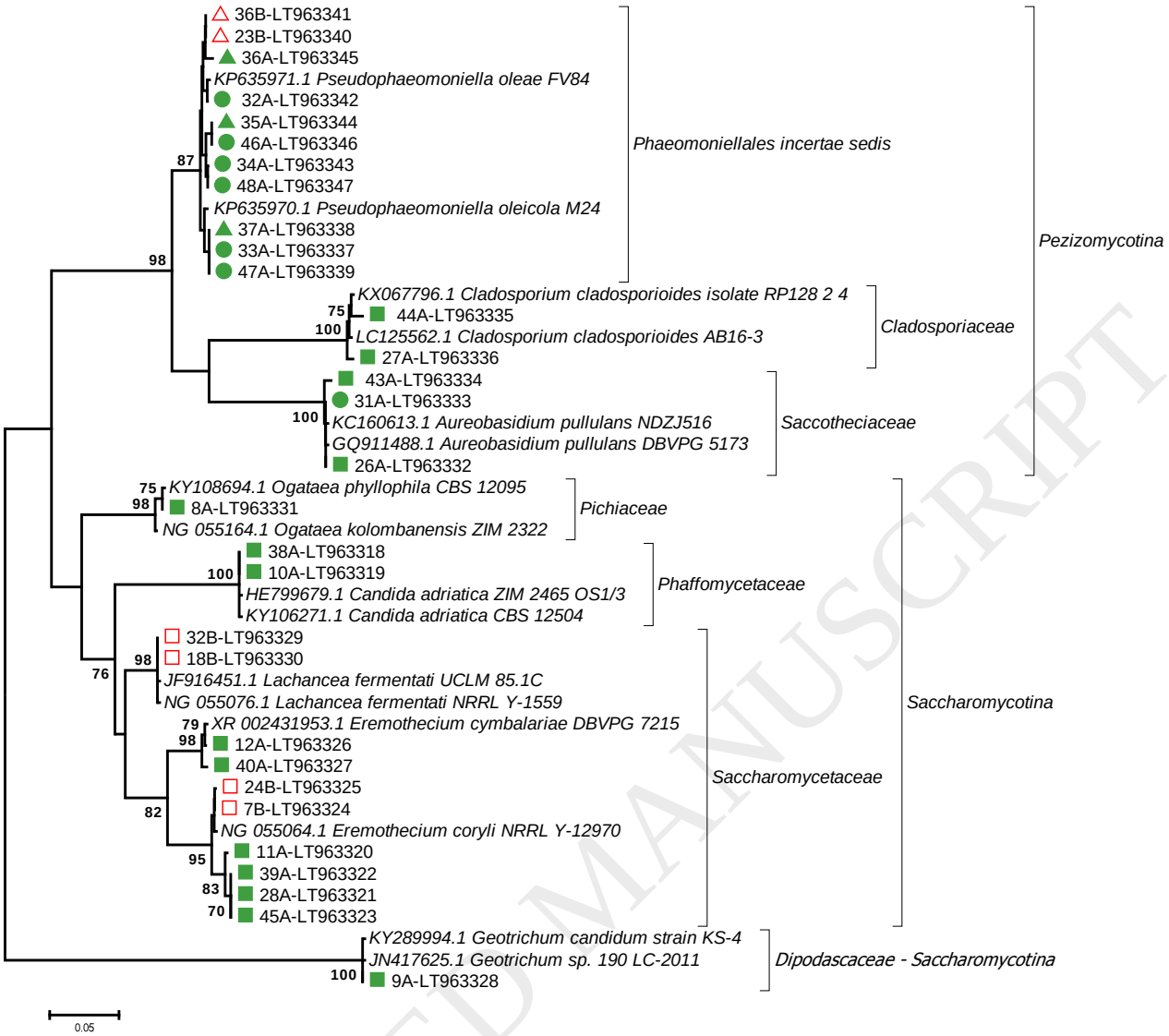

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TABLES

Table 1. Details of mobile phase related to the chromatographic method utilized.

mobile phase	fractions	volume (mL)
100% $CHCl_3$	1	500
95:5% $CHCl_3$/AcOEt	2–14	200
90:10% $CHCl_3$/AcOEt	15–27	200
85:15% $CHCl_3$/AcOEt	28–40	200
80:20% $CHCl_3$/AcOEt	41–53	200
75:25% $CHCl_3$/AcOEt	54-66	200
70:30% $CHCl_3$/AcOEt	67–79	200
65:35% $CHCl_3$/AcOEt	80–92	200
60:40% $CHCl_3$/AcOEt	93–105	200
55:45% $CHCl_3$/AcOEt	106–108	200
50:50% $CHCl_3$/AcOEt	109–145	600
0:100% AcOEt	146–170	400

Table 2. Calibration curve, determination coefficient (r^2), limits of detection (LOD) and quantification (LOQ) for each standard phenolic compounds.

Compounds	Calibration curves	r^2	LOQ (mg/kg)	LOD (mg/kg)
tyrosol	$Y = 1.026X + 0.04172$	0.9997	0.10	0.40
hydroxytyrosol	$Y = 1.883X + 0.04403$	0.9996	0.10	1.00
oleocanthal	$Y = 0.4088X + 0.008184$	0.9998	0.90	5.20
oleacein	$Y = 0.3158X + 0.005722$	0.9999	0.50	1.60

Table 3. Concentrations of phenolic compounds in two different extra virgin olive oils (A and B), obtained from the first milling and stored from October 2015 to April 2016[a]

time	olive oil	tyrosol	hydroxytyrosol	oleacein	oleocanthal
October	B	4.1±0.05	1.0±0.05	466±22.4	256±7.3
October	A	7.1±0.3	1.3±0.2	159±7.2	400±24.6
November	B	4.2±0.3	1.3±0.1	408±6.8	251±21.2
November	A	9.4±0.2	2.4±0.05	156±11.8	373±21.6
December	B	5.7±0.1	2.3±0.3	407±22.4	245±16.3
December	A	14.1±0.5	3.2±0.05	127±6.2	296±18.9
January	B	13.8±0.1	4.4±0.1	261±4.9	199±11.4
January	A	35.7±1.5	8.4±0.5	95.8±8.3	262±25.3
February	B	20.8±0.3	6.6±0.2	206±11.7	163.9±7.2
February	A	57.3±0.9	12.6±0.5	69.3±1.7	189±10.1
March	B	28.9±0.9	9.4±0.2	162±12.7	141±5.9
March	A	81.0±1.3	21.6±0.1	63.3±0.7	153±1.2
April	B	37.5±0.8	10.9±0.3	129±8.2	101±7.2
April	A	86.7±1.2	22.6±0.5	48.9±3.7	130±13

[a]Phenolic compounds are expressed in ppm

Table 4. Concentrations of phenolic compounds in two different extra virgin olive oils (A and B), obtained from the second milling and stored from December 2015 to June 2016[a]

Time	olive oil	tyrosol	hydroxytyrosol	oleacein	oleocanthal
December	B	8.5±0.7	2.8±0.1	249±21.7	203±14.8
December	A	4.4±0.3	0.3±0.03	108±9.9	371±26.0
January	B	9.3±0.4	4.0±0.6	226±5.2	198±7.5
January	A	7.4±0.9	1.2±0.05	88.2±4.6	331±20.0
February	B	11.9±0.3	5.3±0.2	198±4.9	191±15.6
February	A	12.1±0.9	2.3±0.2	66.1±6.2	293±17.9
March	B	17.1±0.8	8.5±0.3	190±6.4	189±4.2
March	A	16.9±0.2	3.3±0.1	48.4±2.1	234±21.3
April	B	17.6±0.5	8.6±0.8	121±2.4	133±4.4
April	A	52.1±1.3	11.3±0.4	23.8±0.6	108±7.9
May	B	26.1±1.4	9.4±0.3	107±7.8	117±10.3
May	A	55.2±0.6	11.4±0.5	22.7±1.5	90.1±4.2
June	B	35.0±0.8	10.7±0.3	71.7±5.0	91.1±5.1
June	A	68.0±1.4	11.4±0.4	14.2±0.5	87.1±4.7

[a]Phenolic compounds are expressed in ppm