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ORIGINAL ARTICLE

Effects of irrigation treatments on the quality of table olives produced with the Greek-style process

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Abstract The irrigation of olive orchards is commonly applied to produce table olives with optimal size. No data have been published on the microbiological quality of drupes from irrigated olive groves during fermentation. The trials T100 and T50 (receiving a water amount equivalent to 100 % and 50 % of the required amount, respectively) and control T0 (rainfed trial) were monitored during two consecutive years. The results showed a significant increase of equatorial diameter and flesh:pit ratio of irrigated drupes. The decrease of pH and the numbers of lactic acid bacteria (LAB) registered for the irrigated trials during the fermentation were more consistent than those displayed by control T0. *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus coryniformis*, and *Pediococcus pentosaceus* for LAB and *Candida boidinii*, *Candida diddensiae*, and *Wickerhamomyces anomalus* for yeasts were isolated at the highest concentrations. The global sensory acceptance was better for irrigated trials than control T0. The statistical multivariate analysis showed that the effect of irrigation was independent of the seasonal variability and it clearly distinguished the T100 and T50 trials from rainfed control. Irrigation improves significantly the quality of table olives. Interestingly, all explorative multivariate analyses showed low dissimilarity between irrigated trials; thus, the thesis T50 represents an effective approach to save water in olive orchards without compromising the quality of table olives.

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Introduction

Traditionally, olive trees are not watered (Caruso and Di Marco 1982), but due to the growing interest of table olive producers, the majority of olive groves are going to be replaced by intensive cultivation systems (Vossen 2007), subjected to periodic irrigation. The irrigation of table olive trees has several positive effects: it improves vegetative and reproductive growth, increases the size and uniformity of drupes, and reduces fruit drop and alternate bearing (Lavee et al. 1990; Michelakis 1990; Patumi et al. 2002).

Generally, the irrigation scheduling is established following the indications of the Food and Agriculture Organization of the United Nations (FAO), applying the estimated evapotranspiration (100 % ETc) based on the fully replenishing soil water extraction (Allen et al. 1998). Proietti and Antognozzi (1996) studied the effect of irrigation on the vegetative and productive activities of trees, as well as on the quality of table olives belonging to the cultivar Ascolana. Those authors registered a significant increase of weight, volume, and flesh:pit ratio. Recently, other authors (Patumi et al. 2002; Tovar et al. 2002; Marsilio et al. 2006; García et al. 2014) reported that drupes from irrigated olive trees are characterized by lower contents in phenolic compounds than those from not irrigated trees, evidencing an effect of the irrigation on the chemical composition of the drupes. However, the effects of different regulated deficit irrigation (RDI) strategies applied to olive orchards have been evaluated, so far, on the drupes for oil extraction rather than table olive production (Goldhamer 1999; Iniesta et al. 2009). Following this trend, a large number

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of scientific papers have been published on the quality of olive oils produced from drupes obtained from trees subjected to irrigation (Pastor et al. 1998; Gómez-Rico et al. 2006, 2007; Servili et al. 2007), but no information on fermented table olives is available.

Although Marsilio et al. (2006) monitored some aspects of table olives produced from drupes grown under RDI and produced in accordance to Greek-style, no data have been provided on the microbiological quality during fermentation in terms of concentration of spoilage and potential pathogenic microorganisms, as well as diversity of pro-technological microorganisms, in particular lactic acid bacteria (LAB), but also yeasts. Aponte et al. (2012) monitored the microbial populations of two Spanish-like fermentations of green table olives of the cultivar "Nocellara del Belice", coming from irrigated and not irrigated fields in order to isolate LAB strains with relevant technological properties, but registered that yeast and staphylococcal populations were higher in the production carried out with olives harvested from not irrigated fields.

In recent years, the use of selected LAB strains (Aponte et al. 2012; Martorana et al. 2016) and innovative methods to ferment table olives (Martorana et al. 2015) is growing, but the spontaneous fermentation method is still widely applied to produce olives at the industrial level. The spontaneous fermentation takes place thanks to the indigenous olive surface microflora consisting mainly of Gram-negative bacteria (Enterobacteriaceae), LAB, and yeasts. Several spoilage microorganisms belonging to the genera *Pseudomonas* and *Staphylococcus* (Heperkan 2013) could also be encountered during the olive manufacturing process. When the pH decreases to 5.0, consistent levels of LAB are commonly found. LAB coexist with yeasts that become dominant in the last stage of fermentation and influence the sensory characteristics of the final product (Garrido et al. 1995; Garrido Fernández et al. 1997; Arroyo-López et al. 2008). However, an overgrowth of fermentative yeasts could produce a vigorous production of CO_2 that could damage drupe texture (Fernández-Díez et al. 1985).

The aim of this work was to register the effects of different irrigation treatments on the carpological, physico-chemical, microbiological, and sensory characteristics of Greek-style table olives. The work was carried out using the cv. Nocellara del Belice, due to its importance in Sicily.

Materials and methods

Plant material, growing conditions, and experimental design

The experimental production of table olives was performed with drupes of "Nocellara del Belice" cultivar. The study was carried out during two consecutive olive crop seasons (2013–2014) in a 13-ha section of a commercial olive orchard located in Castelvetrano (37°41′0″N 12°47′35″E; Trapani, Sicily, Italy). Adult trees (about 60 years old) spaced 5 × 7 m were included in the experimental design.

The experimental plan included the following three trials: two RDI treatments were managed in order to supply, respectively, 100 % (trial T100) and 50 % (trial T50) of the ETc.; the trial T0 did not receive water. From July to September, trees managed with trials T100 and T50 received 1500 m^3/ha and 750 m^3/ha of water, respectively. Irrigation was performed weekly, starting soon after the pit hardening (July) occurred and ending in autumn, when the rain season started.

In accordance to FAO methodology (Allen et al. 1998), 100 % ETc was based on the fully replenishing soil water extraction. The ETc was calculated applying the method proposed by Doorenbos and Pruitt (1977): ETc = ETo × Kc × Kr, where a crop coefficient (Kc), representing the ratio of crop evapotranspiration (ETc) to reference evapotranspiration (ETo), is adjusted by the crop cover (Kr) (Fereres and Castel 1981). Kc suggested for olive trees growing under the conditions reigning in Castelvetrano (from 0.66 to 0.77) was estimated considering 30 years of climatic data (Servizio Informativo Agrometeorologico Siciliano, SIAS). Irrigation was supplied with a sub-irrigation pipe system. Two lateral pipes wings (0.50 m depth) which support micro-emitters (4 L/h), spacing each other along the pipes, were installed per tree row.

A randomized complete-block design was used with three blocks per treatment and two trees per block. Irrigation scheduling was controlled with the measurements of six trees per treatment (two per block) during the growing season.

Table olive processing and sample collection

Olive drupes were manually harvested at the suitable maturity stage for table olive processing and immediately transported to the manufacturer located in Castelvetrano (Italy), where they were mixed separately for each trial. Amounts of 150 kg of drupes for each trial were transferred into vats of 180 L volume, filled with 30 L of brine composed of NaCl 9 % (w/v), and subjected to spontaneous fermentation. The brine salt concentration was constantly maintained at the initial level by periodical addition of coarse salt. The fermentation of all trials was carried out at room temperature for 220 days and was monitored periodically. Samples of brine (about 50 mL) were collected at 4, 11, 24, 41, 80, 120, 160, and 220 days of fermentation. The experiment was performed in triplicate (three vats per trial) in two consecutive olive crop seasons (2013 and 2014).

Carpological measurement, pH monitoring, and microbiological analyses

Olive drupes from single trees were selected in commercial calibers (diameter bigger than 19 mm). For quality

assessment, about 2.5 kg of table olives (corresponding to about 500 drupes) per treatment was randomly selected and the weight of flesh and stone was measured using a digital scale (ORMA mod. BC, Italy). The equatorial diameter (ED) of the olives was measured using a digital caliper (SAMA Tools, Italy).

The pH of brines was measured using the pH meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain), calibrated with three standard solutions at pH 4.0, 7.0, and 9.2.

The cell densities of LAB, yeasts, Enterobacteriaceae, pseudomonads, staphylococci, and coagulase-positive staphylococci (CPS) were determined as reported by Martorana et al. (2015) after decimal dilutions of brines prepared in Ringer's solution (Sigma-Aldrich, Milan, Italy).

Isolation, grouping, and genotypic differentiation of LAB

At least four colonies with identical aspect were collected from the highest dilutions of cell suspension inoculated on Man–Rogosa–Sharpe (MRS) agar for all the morphologies of the presumptive LAB. The isolates were purified by successive sub-culturing on the same medium and the purity was checked microscopically to ascertain a unique cell morphology per culture. Gram-positive (determined by the Gregersen KOH method) and catalase-negative (determined in the presence of H_2O_2 5 %, v/v) were stored in broth containing 20 % (v/v) glycerol at −80 °C until further experimentations. LAB were initially subjected to a phenotypic grouping based on cell morphology and disposition, growth at 15 and 45 °C, and metabolism type, testing the ability to produce CO_2 from glucose. The last assay was carried out with the same growth media used for isolation without citrate from which certain LAB produce gas. The obligate homofermentative metabolism was determined by the absence of growth in the presence of a mixture of pentose carbohydrates (xylose, arabinose, and ribose; 8 g/L each) in place of glucose. Sub-grouping of cocci included also the growth at pH 9.6 and in the presence of 6.5 % (w/v) NaCl.

DNA from LAB isolates was extracted using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Presumptive LAB were grouped by random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis in a 25-μL reaction mix using a single primer M13 as previously described by Gaglio et al. (2014). The identification at the species level was performed by multiplex PCR analysis of the *rec*A gene with species-specific primers for *Lactobacillus pentosus*, *Lactobacillus plantarum*, and *Lactobacillus paraplantarum*, according to the protocol described by Torriani et al. (2001). One representative culture for each multiplex cluster and all strains that did not show amplification by multiplex PCR analysis were analyzed by 16S rRNA gene sequencing, as described by Weisburg et al. (1991). DNA sequencing reactions were performed at Eurofins Genomics S.r.l. (Milan, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov and those available at EzTaxon located at http://www.ezbiocloud.net/eztaxon.

Isolation and genotypic characterization of yeasts

At least five colonies per morphology on agar plates were randomly collected from dichloran rose bengal chloramphenicol (DRBC) medium, and after purification onto the same medium of isolation, the cultures were identified genotypically.

DNA extraction was performed as reported above. All isolates were preliminary grouped by restriction fragment length polymorphism (RFLP) analysis of the region

Table 1 Carpological characteristics of olive drupes

Treatment	Fruit equatorial diameter (mm)	Fruit weight (g)	Pit weight (g)	Flesh:pit ratio
T100	21.97 ± 0.18^{b}	6.85 ± 0.06^{b}	0.93 ± 0.05^{a}	6.49 ± 0.14^{c}
T50	21.39 ± 0.16^{b}	6.48 ± 0.11^{b}	1.03 ± 0.03^{a}	5.73 ± 0.19^{b}
T0	20.25 ± 0.11^{a}	5.54 ± 0.07^{a}	0.94 ± 0.04^{a}	4.87 ± 0.09^{a}
Statistical significance[§]	*	*	N.S.	*

The results indicate mean values ± standard error of three measurements (carried out in triplicate for two olive crop seasons)

T100 irrigated trial performed with 100 % of the ETc, T50 irrigated trial performed with 50 % of the ETc, T0 rainfed trial

[§] Data within a column followed by the same letter are not significantly different according to Tukey's test. *p*-value: $p \leq 0.05$; N.S., not significant; *significant

spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene, as reported by Esteve-Zarzoso et al. (1999). One isolate per group was identified at the species level by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. The D1/D2

Table 2 Values of pH and microbial concentrations of brine samples

	Days of fermentation								
	0	4	11	24	41	80	120	160	220
pH									
T100	7.0 ± 0.1[a]	6.6 ± 0.1[a,b]	5.0 ± 0.1[b]	4.2 ± 0.0[b]	4.0 ± 0.1[b]	3.9 ± 0.1[b]	3.9 ± 0.0[b]	3.9 ± 0.0[a]	3.8 ± 0.0[a]
T50	7.0 ± 0.1[a]	6.6 ± 0.1[b]	4.9 ± 0.1[b]	4.3 ± 0.1[b]	4.1 ± 0.1[b]	3.9 ± 0.0[b]	3.9 ± 0.1[a]	3.9 ± 0.1[a]	3.9 ± 0.1[a]
T0	7.2 ± 0.0[a]	6.9 ± 0.1[a]	5.7 ± 0.0[a]	4.9 ± 0.1[a]	4.5 ± 0.1[a]	4.3 ± 0.0[a]	4.3 ± 0.0[a]	4.1 ± 0.1[a]	3.9 ± 0.1[a]
Statistical significance[§]	N.S.	*	*	*	*.	*	*	N.S.	N.S.
MRS									
T100	1.7 ± 0.1[a]	2.2 ± 0.4[a]	2.1 ± 0.5[a]	1.9 ± 0.1[a]	1.7 ± 0.1[a]	1.0 ± 0.6[a]	1.4 ± 0.4[a]	3.5 ± 0.5[a]	4.6 ± 0.2[b]
T50	1.1 ± 0.1[b]	1.0 ± 0.1[b]	2.0 ± 0.1[a]	1.1 ± 0.2[b]	1.1 ± 0.1[b]	1.2 ± 0.4[a]	1.1 ± 0.5[a]	3.3 ± 0.4[a]	6.0 ± 0.2[a,b]
T0	1.7 ± 0.2[a]	1.0 ± 0.2[b]	1.1 ± 0.2[b]	1.0 ± 0.1[b]	1.0 ± 0.2[b]	1.0 ± 0.2[a]	1.2 ± 0.2[a]	1.5 ± 0.1[b]	6.2 ± 0.1[a]
Statistical significance[§]	*	*	*	*	*	N.S.	N.S.	*	*
DRBC									
T100	3.7 ± 0.1[a]	5.0 ± 0.1[a]	3.9 ± 0.4 [a,b]	2.6 ± 0.1[a]	1.6 ± 0.1[a]	1.2 ± 0.1[b]	1.2 ± 0.7[a]	5.0 ± 0.4[a]	6.1 ± 0.4[a]
T50	3.8 ± 0.3[a]	5.4 ± 0.3[a]	3.5 ± 0.3 [b]	2.6 ± 0.3[a]	2.2 ± 0.1[a]	2.1 ± 0.2[a]	2.4 ± 0.4[a]	5.5 ± 0.5[a]	6.2 ± 0.2[a]
T0	3.9 ± 0.7[a]	5.1 ± 0.6[a]	5.3 ± 0.4[a]	2.5 ± 0.7[a]	1.3 ± 0.7[a]	1.0 ± 0.2[b]	1.6 ± 0.3[a]	5.5 ± 0.7[a]	6.0 ± 0.1[a]
Statistical significance[§]	N.S.	N.S.	*	N.S.	N.S.	*	N.S.	N.S.	N.S.
VRBGA									
T100	1.0 ± 0.2[a]	2.7 ± 0.6 [a,b]	2.4 ± 0.4[a]	1.2 ± 0.4[a]	n.d.	n.d.	n.d.	n.d.	n.d.
T50	1.2 ± 0.2[a]	2.3 ± 0.3[b]	2.3 ± 0.3[a]	1.5 ± 0.1[a]	n.d.	n.d.	n.d.	n.d.	n.d.
T0	1.3 ± 0.3[a]	3.9 ± 0.2[a]	3.6 ± 0.8[a]	2.0 ± 0.3[a]	n.d.	n.d.	n.d.	n.d.	n.d.
Statistical significance[§]	N.S.	*	N.S.	N.S.					
PAB									
T100	n.d.	1.3 ± 0.1[a]	3.7 ± 0.4[a]	3.9 ± 0.8[a]	4.6 ± 0.1[a]	1.9 ± 0.4[b]	n.d.	n.d.	n.d.
T50	n.d.	1.7 ± 0.2[a]	3.9 ± 0.7[a]	4.0 ± 0.3[a]	4.8 ± 0.5[a]	2.8 ± 0.6 [a,b]	n.d.	n.d.	n.d.
T0	n.d.	2.1 ± 0.7[a]	5.0 ± 0.2[a]	4.3 ± 0.1[a]	5.5 ± 0.8[a]	3.9 ± 0.1[a]	1.3 ± 0.1	1.1 ± 0.0	n.d.
Statistical significance[§]		N.S.	N.S.	N.S.	N.S.	*	*	*	
BP									
T100	n.d.	1.5 ± 0.3[a]	3.9 ± 0.4[a]	4.2 ± 0.2[a]	4.8 ± 0.4[a]	2.2 ± 0.1[c]	n.d.	n.d.	n.d.
T50	n.d.	1.3 ± 0.3[a]	3.7 ± 0.4[a]	4.1 ± 0.5[a]	4.9 ± 0.6[a]	3.1 ± 0.2[b]	n.d.	n.d.	n.d.
T0	n.d.	1.9 ± 0.1[a]	4.2 ± 0.6[a]	4.8 ± 0.4[a]	5.4 ± 0.4[a]	4.5 ± 0.4[a]	2.1 ± 0.4	1.1 ± 0.7	n.d.
Statistical significance[§]		N.S.	N.S.	N.S.	N.S.	*	*	*	
PCA									
T100	4.3 ± 0.7[a]	5.3 ± 0.2[a]	5.2 ± 0.2[a]	3.1 ± 0.2[a]	2.4 ± 0.3[a]	2.1 ± 0.1[a]	2.1 ± 0.2[a]	4.5 ± 0.1[a]	6.2 ± 0.7[a]
T50	3.8 ± 0.4[a]	5.6 ± 0.2[a]	4.0 ± 0.5[a]	2.9 ± 0.3[a]	1.2 ± 0.6[a]	0.9 ± 0.1[b]	1.0 ± 0.4[b]	5.6 ± 0.9[a]	6.3 ± 0.5[a]
T0	3.5 ± 0.2[a]	5.2 ± 0.4[a]	4.9 ± 0.5[a]	2.2 ± 0.2[b]	1.6 ± 0.4[a]	1.4 ± 0.3[b]	2.0 ± 0.2[a]	5.6 ± 0.5[a]	6.1 ± 0.1[a]
Statistical significance[§]	N.S.	N.S.	N.S.	*	N.S.	*	*	N.S.	N.S.

The results of microbial loads are expressed as Log colony-forming units (CFU)/mL and indicate the mean values ± standard deviation of three plate counts (carried out in triplicate for two olive crop seasons)

T100 irrigation supplied 100 % of the ETc, T50 irrigation supplied 50 % of the ETc, T0 rainfed trial, MRS Man–Rogosa–Sharpe agar for mesophilic rod LAB, DRBC dichloran rose bengal chloramphenicol agar for total yeasts and filamentous fungi, VRBGA violet red bile glucose agar for Enterobacteriaceae, PAB *Pseudomonas* agar base for pseudomonads, BP Baird–Parker agar base for staphylococci, n.d. not detected (value < detection limit of method)

[§] Data within a column followed by the same letter are not significantly different according to Tukey's test. *p*-value: $p \leq 0.05$; N.S., not significant; *significant

region was amplified and the PCR products were visualized as described by Francesca et al. (2014). The reaction of DNA sequencing and the identities of the sequences were determined as reported above.

Sensory evaluation

Evaluation of the sensory profiles of the experimental olives was performed using a descriptive method (UNI 10957 2003), as reported by Martorana et al. (2016). A total of 16 descriptors were included in the analysis for the external aspect (green color intensity and brightness), odor (green olive aroma, complexity, and off-odours), taste (crispness, easy stone that means easy pit detachment from the flesh, juicy, sweet, sour, bitter, salt, astringent, and complexity), flavours and overall satisfaction. The olive samples were randomly evaluated by assigning a score between 1.00 (absence of sensation) and 9.00 (extremely intense) in individual booths under incandescent white light.

Sensory tests were performed with three evaluations for each trial. Each evaluation was carried out in different test sessions at the same time of day. Assessors conducted no more than three tests per day, lasting a maximum of 1 h.

Statistical and explorative multivariate analysis

Data from carpological, physico-chemical, microbiological, and sensory analyses were investigated using a generalized linear model (GLM) based on the analysis of variance (ANOVA) model that included effects of olive crop seasons (2013 and 2014 years) and irrigation (100 %, 50 %, and 0 % of the ETc.), as well as the interaction between olive crop seasons and irrigation strategies. The post-hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to p-values of < 0.05.

In order to represent graphically the values of the microbial counts, a heat map clustered analysis (HMCA), based on double hierarchical dendrogram with heat map plot, was employed to represent the individual content values contained in the data matrix as colors. The values of counts for microbial groups were depicted by color intensity from yellow (lowest concentration) to red (highest concentration). Heat map analysis of the volatile levels was performed using the autoscaled data. Graphical construction was achieved by using XLSTAT software version 7.5.2 (Addinsoft, New York, USA) for Excel.

In addition, an explorative multivariate analysis was employed to investigate the relationships among data obtained from the different measurements. Principal component analysis (PCAn) was employed to investigate the relationships among samples. The input matrix used for PCAn consisted of the total area under the growth/decline curves of total mesophilic count, LAB, yeasts, enterobacteria, pseudomonads, CPS, as well as pH values (Bautista-Gallego et al. 2011; Blana et al. 2014). Areas were calculated by integration using the OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA). In addition, other relevant indexes of pH and microbial changes were taken into account as follows: maximum and minimum values of pH, maximum and minimum values of microbial populations (Bautista-Gallego et al. 2011). The number of principal factors was selected according to the Kaiser criterion (Jolliffe 1986) and only factors with eigenvalues higher than 1.00 were retained. All data were preliminary evaluated by using Bartlett's sphericity test (Dillon and Goldstein 1984; Mazzei et al. 2010) in order to check the statistically significant difference among samples within each dataset. Statistical

Table 3 Molecular identification of lactic acid bacteria (LAB) species during the table olive production

Species	Strain	Isolation source (day of sampling)	Size of multiplex PCR[a] amplicon	% similarity[b] (accession no. of closest relative) by:		Sequence length (bp)	Accession no.	No. of isolates (% relative abundance)
				BLAST	EzTaxon			
Lactobacillus coryniformis	AMol-2	T100 (120)	n.a.	99 (KF994925.1)	98.72 (KCTC3535^T)	1484	KU240501	123 (8.9)
Lactobacillus pentosus	AMol-1	T50 (11)	218	99 (KT215616.1)	99.86 (JCM 1558^T)	1476	KU240502	677 (48.9)
Lactobacillus plantarum	AMol-4	T100 (4)	318	100 (KT268296.1)	99.80 (ATCC14917^T)	1488	KU240503	387 (27.9)
Pediococcus pentosaceus	AMol-3	T0 (80)	n.a.	99 (KP119818.1)	99.79 (DSM 20336^T)	1442	KU240504	198 (14.3)

The results refer to mean values for two olive crop seasons

Abbreviations: T100 irrigation supplied 100 % of the ETc, T50 irrigation supplied 50 % of the ETc, T0 rainfed trial, *n.a.* not amplified

[a] Results obtained by multiplex PCR analysis of the *rec*A gene with species-specific primers for *Lactobacillus pentosus*, *Lactobacillus. plantarum*, and *Lactobacillus paraplantarum* (Torriani et al. 2001)

[b] Results obtained by the 16S rRNA sequence search

Table 4 Molecular identification of yeasts isolated during the table olive production

Species	Strain	Isolation source (day of sampling)	R.P.	5.8S-ITS PCR	Size of restriction fragments			% similarity[a] (accession no. of closest relative) by:	Accession No.	No. of isolates (% relative abundance)
					*Cfo*I	*Hae*III	*Hin*fI			
Candida boidinii	NFol-1	T0 (41)	I	700	325 + 300	690	400	99 (KC442246.1)	KU240505	622 (33.2)
Candida diddensiae	NFol-4	T100 (4)	II	695	290 + 180 + 140 + 70	455 + 152 + 80	345	99 (U45750.1)	KU240506	474 (25.3)
Candida membranifaciens	NFol-5	T100 (41)	III	650	310 + 300	400 + 160 + 60	340	99 (EF362752.1)	KU240507	161 (8.6)
Kluyveromyces marxianus	NFol-2	T50 (120)	IV	780	290 + 208 + 185 + 87	640 + 85	300 + 190 + 140 + 85	99 (FJ896140.1)	KU240508	178 (9.5)
Pichia kudriavzevii	NFol-3	T50 (80)	V	560	240 + 190 + 90	400 + 115	240 + 155 + 170	99 (JQ419945.1)	KU240509	138 (7.4)
Wickerhamomyces anomalus	NFol-6	T100 (120)	VI	600	600	540	295	99 (KM246030.1)	KU240510	301 (16.0)

The results refer to mean values for two olive crop seasons

All values for the 5.8S-ITS PCR and 26S PCR and restriction fragments are given in bp

T100 irrigation supplied 100 % of the ETc, T50 irrigation supplied 50 % of the ETc, T0 rainfed trial, R.P. restriction profile

[a] According to BlastN search of D1/D2 26S rRNA gene sequences in the NCBI database

data processing and graphical construction were achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and XLSTAT software version 7.5.2 (Addinsoft, New York, USA) for Excel.

Results

Carpological, physico-chemical, and microbiological analyses

Data from the measurements of equatorial diameter, weight, and pit of drupes as well the flesh:pit ratio are reported in Table 1. Experimental treatments mainly affected the equatorial diameter, weight, and flesh:pit ratio, which resulted higher than those registered for the control. Furthermore, no significant differences were found between trials T100 and T50.

The physico-chemical and microbiological characteristics of brine samples collected during the olive manufacturing are reported in Table 2. The pH ranged between 7.1 (day 0) and 3.9 (day 220) for all trials. In particular, trials T100 and T50 showed the highest decrease of pH within the first 41 days of fermentation; from day 160 onwards, no significant differences among trials were registered.

The microbial groups most often represented on untreated olives were yeasts and total mesophilic bacteria [about 4.0 Log colony-forming units (CFU)/g on average], while LAB were almost two orders of magnitude lower. Members of the Enterobacteriaceae family reached highest levels of 1 Log CFU/g. Staphylococci and pseudomonads were found at undetectable levels.

During the fermentation, yeasts reached counts higher than LAB throughout the process and in all trials.

The presence of LAB observed in trial T0 was more limited than that registered for the other two trials during the entire period of monitoring. In particular, the levels of LAB were mainly estimated in trials T100 and T50, and they showed the highest concentrations at the end of the process, between days 160 (about 3.5 Log CFU/mL) and 200 (about 6.2 Log CFU/mL).

The Enterobacteriaceae population was detectable until day 24 of the process in all trials, and the highest concentration was estimated for trial T0. Staphylococci and *Pseudomonas* spp. populations reached the highest concentrations (between 4.6 and 5.5 Log CFU/mL) at day 41 in all trials. Both microbial groups were detected until day 80 for trials T100 and T50 and disappeared from trial T0 after 160 days.

Identification and distribution of LAB and yeasts

A total of 2111 colonies were collected from the highest plated dilutions of cell suspensions. The cultures showing a rod shape morphology of the cells typical of lactobacilli were better investigated. A total of 1385 isolates were considered presumptive LAB, as being Gram-positive and catalase-negative. All these cultures were able to grow at 15 °C, unable to develop at 45 °C, and were facultatively homofermentative because of their inability to generate CO_2 from glucose and their growth in the presence of pentose carbohydrates. Owing to the high number of isolates, about 40 % of the cultures (selected to be representative of the isolation source, colony

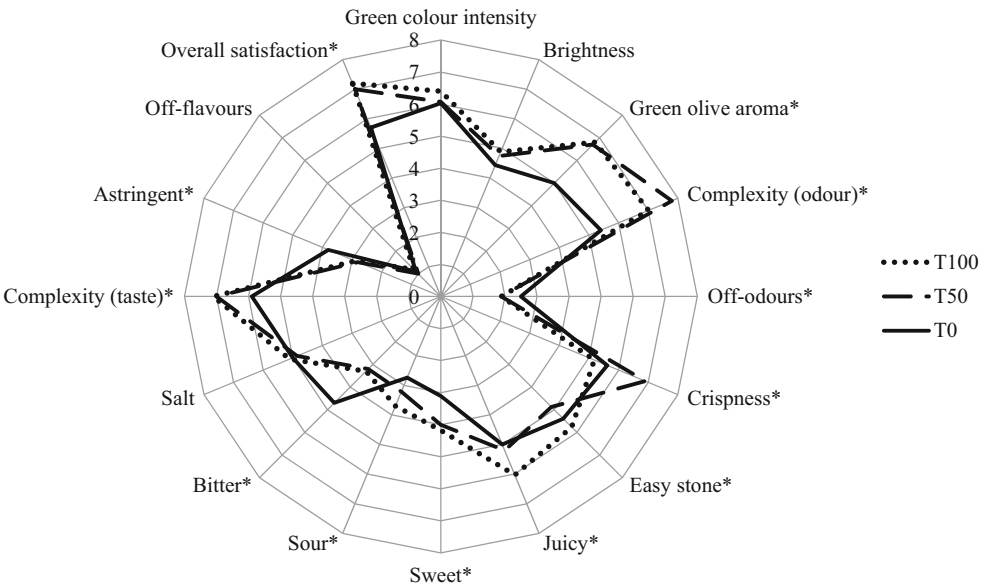


Fig. 1 Evaluation of sensory attributes of table olives at 220 days of fermentation

Results indicate mean values of three replicate per each trials (carried out in triplicate for two olive crop seasons)
Abbreviations: T100, irrigation supplied 100 % of ETc; T50, irrigation supplied 50 % of ETc; T0, rainfed trial
* indicate significant differences among experimental trials for the same sample ($p < 0.05$)

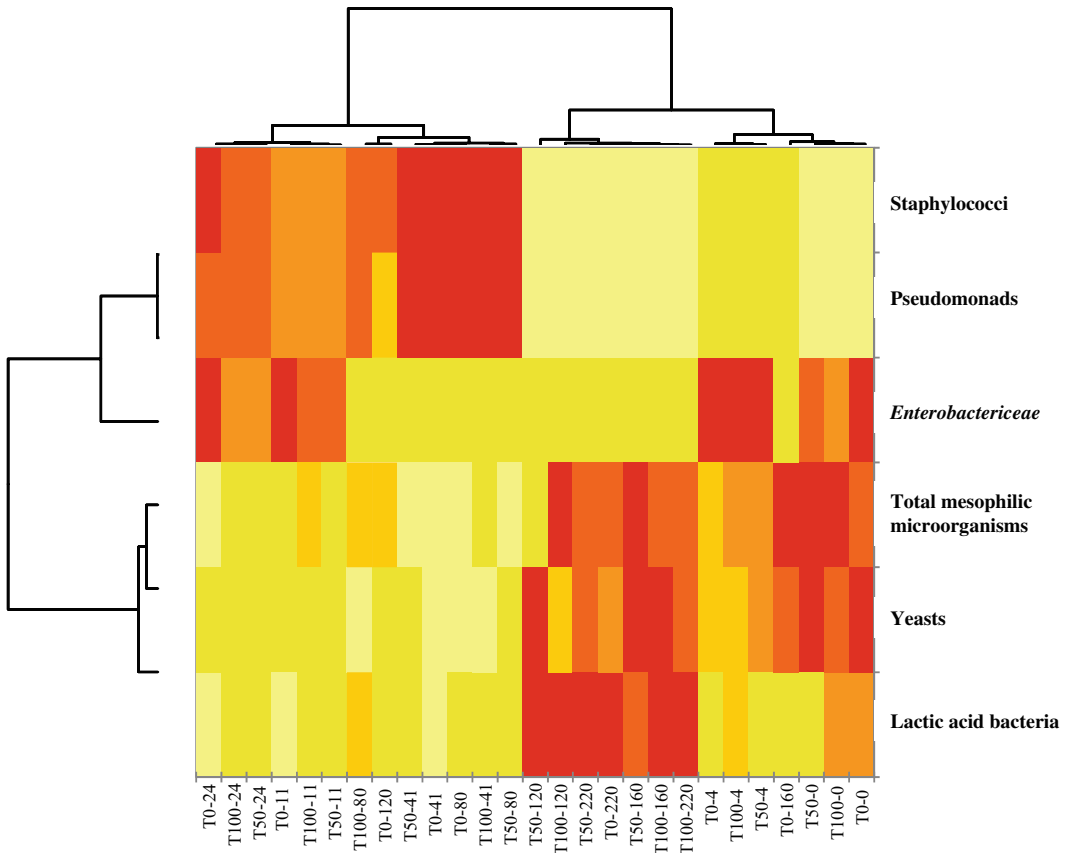
morphology, experimental trial, and year of production) were grouped on the basis of the RAPD-PCR profiles and subsequently identified at the species level by multiplex PCR analysis. This analysis revealed the presence of *L. pentosus* and *L. plantarum* (Table 3). Furthermore, the identification process continued by using 16S rRNA gene sequencing, which confirmed the presence of *L. pentosus* and *L. plantarum*, and revealed the presence of *Lactobacillus coryniformis* and *Pediococcus pentosaceus* among the bacteria resulted negative to the multiplex PCR specific for the *L. plantarum* group. The species *L. plantarum* and *L. pentosus* were mainly found during the fermentation of the trials T100 and T50. *Lactobacillus coryniformis* and *P. pentosaceus* were isolated only in T50 (day 120) and T0 (day 80).

The yeast population that characterized the olive manufacturing process was also subjected to molecular investigation. A total of 1874 yeast colonies were collected from DRBC agar. Based on colony and cell morphology, 671 isolates were subjected to molecular identification. After restriction analysis of the 5.8S-ITS region, the isolates were clustered into six groups (Table 4). Sequencing of the D1/D2 domain of the 26S rRNA gene identified six species: *Candida boidinii*, *Candida diddensiae*, *Candida membranifaciens*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, and *Wickerhamomyces anomalus*.

With regards to the distribution of yeast species, *C. diddensiae*, *C. membranifaciens*, and *W. anomalus* were isolated mainly from the olives of the trials T100 and T50; the latter samples showed the highest diversity of yeast because *P. kudriavzevii* and *K. marxianus* were also found. Trial T0 showed very limited yeast diversity, since only *C. boidinii* was identified.

Sensory evaluation

The results of the sensory analysis are reported in Fig. 1. Significant differences among the three trials were registered for green olive aroma, acid, bitter, firmness, crunchiness, and overall descriptors. In particular, both irrigated trials T100 and T50 showed the highest scores for green olive aroma, odor and flavor complexity, crispness, sweet, and overall



Results indicate mean values of three replicate per each trials (carried out in triplicate for two olive crop seasons)
Abbreviations: T100, irrigation supplied 100 % of ETc; T50, irrigation supplied 50 % of ETc; T0, rainfed trial
The values for microbial groups are depicted by color intensity from yellow (lowest concentration) to red (highest concentration). Clusters based on the distance of the samples along the X-axis and the microbial groups along the Y-axis are indicated in the upper and left sections of the figure, respectively. Codes (T0, T50 and T100) correspond to experimental trials. The numbers associated to each code correspond to day of fermentation per each experimental trials

Fig. 2 Microbial population distribution among samples. The double hierarchical dendrogram is based on values of microbiological counts. The heat map plot depicts the relative percentage of each microbial group (variables clustering on the Y-axis) within each sample (X-axis clustering)

satisfaction. In contrast, the highest values of acid, bitter, firmness, and crunchiness were found for the olives obtained from the rainfed trial.

Statistical and explorative multivariate analysis

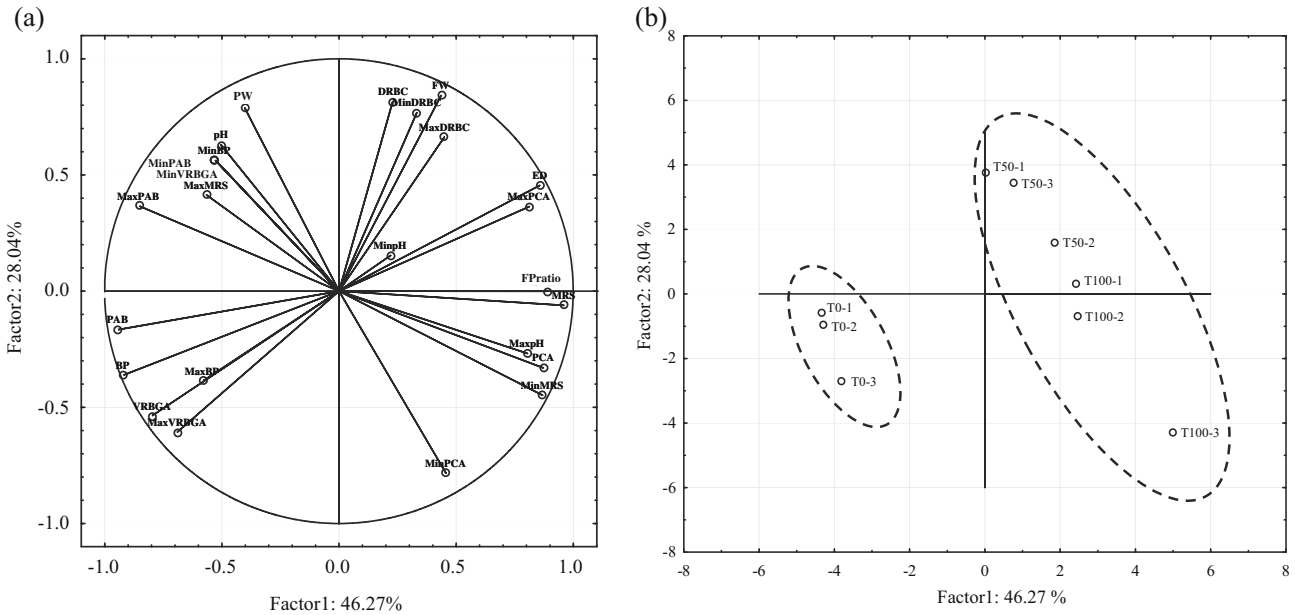
The statistical analysis was performed mainly to show significant differences among data collected during the two olive crop seasons. The results obtained from a GLM based on the ANOVA model showed no significant effect between crop seasons and the dependent variables associated to carpological, physico-chemical, and microbiological data.

The HMCA (Fig. 2) clearly separated LAB, yeasts, and total mesophilic bacteria from members of the Enterobacteriaceae family, pseudomonads, and staphylococci as an effect of the irrigation. At the same time, the HMCA grouped samples in two mega-clusters characterized from different relative abundances of microbial populations. One mega-cluster (mainly represented by trials T100 and T50) showed the highest concentration of LAB (between days 120 and 220) and Enterobacteriaceae (between days 0 and 4). In contrast, the second mega-cluster (mainly represented by trial T0) was characterized by the highest relative abundances of pseudomonads and staphylococci.

In order to better analyze the differences among trials, the results obtained by monitoring microbial counts, pH values, and carpological characteristics of drupes were also subjected to PCAn. This type of analysis condensed the information from 25 variables into a restricted number of factors. The score and loading plots are visualized in Fig. 3a, b, respectively. Trial T0 resulted in being significantly separated from the other trials along factor 1 that explained 46.27 % of the total variance. Trials T50 and T100 were also represented onto different quadrants of the score plot but only along factor 2 that explained 28.04 % of the total variance. The 25 variables were expressed as a linear combination of two factors in a loading plot (Fig. 3a) that explained 74.31 % of the total variance. In detail, the loading plot showed that factor 1 has been more closely related to the variables MRS (0.96), BP (−0.92), and PAB (−0.94). Furthermore, factor 2 was mainly correlated to the variables FW (0.84), DRBC (0.82), MaxVRBGA (−0.61), and MinPCA (−0.78).

Discussion

The aim of the present study was to evaluate the effect of different amounts of water supplied by irrigation on the quality of green table olives belonging to cv. Nocellara del Belice. According to the scientific literature, several studies evaluated the physiological response and performance of olive trees grown under RDI (d'Andria et al. 2009), as well as the overall



Results indicate mean values of three replicate per each trials (carried out in triplicate for two olive crop seasons)
Abbreviations: MRS,Man–Rogosa–Sharpe agar for mesophilic rod LAB; DRBC, dichloran rose bengal chloramphenicol agar for total yeasts and filamentous fungi; VRBGA, violet red bile glucose agar for Enterobacteriaceae; PAB, pseudomonas agar base for pseudomonads. 'Max'and 'Min' correspond to the maximum and minimum values of pH and microbial populations detected on the corresponding medium used for counts; ED, equatiorial diameter of fruit; PW, pit weight, FP, flesh:pit ratio
The codes (T0, T50, and T100) correspond to the experimental trials. The numbers associated to each code correspond to replicates per experimental trial Ann Microbiol

Fig. 3 Principal component analysis (PCA) analysis based on the values of carpological, physico-chemical, and microbiological data (**a**: loading plot; **b**: score plot)

development and composition of drupes (Chaves et al. 2010). In our study, within the carpological results, the irrigation mainly affected the equatorial diameter and flesh:pit ratio of fruits, which resulted significantly higher in the trials subjected to irrigation. Interestingly, drupes of trial T50 showed values of carpological measurements similar to that registered for trial T100. Cano-Lamadrid et al. (2015) also observed the highest equatorial diameter in fruits submitted to irrigation during stone hardening. Similar results were reported by Proietti and Antognozzi (1996) and Aponte et al. (2012) for olives harvested from irrigated trees. Regarding the influence of the irrigation regimes on the microbiology of the process of table olive transformation, only the work of Aponte et al. (2012) is available in the literature. However, in that work, table olives were produced according to the Sevillian-style, which includes the addition of a starter culture. No data have been published for the Greek-style table olive production system that involves a spontaneous fermentation determined by the indigenous microbiota.

In accordance to our results on pH changes, Marsilio et al. (2006) reported a greater decrease of pH in fermenting brines of olives collected from irrigated trees than those from the rainfed trial. The pH values are of paramount importance for the growth of microbial populations, and the pH significantly affects the development of spoilage and potentially pathogenic bacteria. In our study, the concentrations of spoilage microorganisms were in agreement with those estimated by Aponte et al. (2012). Enterobacteriaceae, pseudomonads, and staphylococci were more abundant in the trial that was not irrigated and the highest concentrations were found at the beginning of the manufacturing process. The high counts of xerophilic groups such as staphylococci could be related to the supply of the deficit irrigation and the consequent changes in the chemical composition in the leaves (Chehab et al. 2009) and drupes Aponte et al. (2012).

In our study, although yeast concentrations were mainly higher than LAB during brine fermentation, the trials T100 and T50 were characterized by an optimal dynamics of concentration of these microbial populations (Aponte et al. 2012; Martorana et al. 2015); thus, an appropriate decrease of pH was registered throughout olive manufacturing. Bleve et al. (2015) demonstrated that the spontaneous fermentations of Conservolea and Kalamàta black olives were driven by yeasts during the first half of the process and, after that, by yeasts and LAB until the end of the process.

In our study, fermented table olives from the trials T100 and T50 showed, overall, the highest score of sensory overall satisfaction from assessors. Cano-Lamadrid et al. (2015) also observed positive effects on the quality and consumers' satisfaction degree of table olives from moderate irrigation. In contrast, Aponte et al. (2012) did not show significant differences among samples coming from irrigated and not irrigated groves, a result imputable to the action of the LAB strains added as starter culture.

The explorative multivariate analysis, widely applied in table olive production (Bautista-Gallego et al. 2011; Rodríguez-Gómez et al. 2012a, b, 2013; Martorana et al. 2015, 2016), clearly showed a significant effect of the irrigation on the final products.

Conclusions

In conclusion, our work provided an overview on the microbial ecology of olives collected from trees subjected to two regulated deficit irrigation (RDI) regimes and a rainfed treatment. In comparison to the rainfed trial, the use of irrigation favored carpological features of drupes and the development of lactic acid bacteria (LAB) during the manufacturing process, in particular *L. pentosus* and *L. plantarum*. All aspects of the composition of the irrigated olives were in agreement with those reported for the commercial standards, and spoilage microorganisms and undesired sensory aspects were not affected by RDI treatments. In addition, fermented olives from trial T50 were characterized by scores of sensory attributes similar to that of trial T100, as well as with the highest sensory overall satisfaction degree.

Interestingly, the statistical analysis showed that the effect of irrigation was independent from the seasonal variability.

These results showed that a reduced irrigation allows the production of table olives with characteristics highly similar to those obtained with the full irrigation. Thus, this study showed a strategy for an efficient water saving during olive tree cultivation without negative effects on the final quality of the table olives produced with the cv. "Nocellara del Belice".

Although this research was undertaken in triplicate and during two consecutive season crops, further investigations with other cultivars and with different RDI regimes are being prepared to extend the knowledge of the effect of irrigation on the quality of the resulting fermented products.

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