The Activity of Healthy Olive Microbiota during Virgin Olive Oil Extraction Influences Oil Chemical Composition

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MAY 2011

Impact Factor: 2.91 · DOI: 10.1021/jf200642s · Source: PubMed

4 AUTHORS, INCLUDING:

Stefania Vichi
University of Barcelona
53 PUBLICATIONS **1,206** CITATIONS
SEE PROFILE

Agusti Romero
IRTA Institute of Agrifood Research and Tech…
70 PUBLICATIONS **357** CITATIONS
SEE PROFILE

Josep Caixach
Spanish National Research Council
147 PUBLICATIONS **2,863** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

The Activity of Healthy Olive Microbiota during Virgin Olive Oil Extraction Influences Oil Chemical Composition

Stefania Vichi,*[,†] Agustí Romero,[‡] Joan Tous,[‡] and Josep Caixach[†]

[†]Laboratory of Mass Spectrometry, IDAEA-CSIC, c. Jordi Girona, 18-26, E-08034, Barcelona, Spain

[‡]Olive Production, Oil Processing and Nut Trees, IRTA (Research & Technology, Food & Agriculture), Mas de Bover Crta. de Reus, El Morell 43120 Constantí (Tarragona), Spain

ABSTRACT: The activity of olive microbiota during the oil extraction process could be a critical point for virgin olive oil quality. With the aim to evaluate the role of microbiological activity during the virgin olive oil extraction process, just before oil extraction freshly collected healthy olive fruits were immersed in contaminated water from an olive mill washing tank. The oils extracted were then compared with control samples from the same batch of hand-picked olives. The presence of lactic and enteric bacteria, fungi and *Pseudomonas* on the surface of olives was proved to be much higher in washed than in control olives, with increments in cfu/g between 2 and 3 orders of magnitude. The biogenesis of volatile compounds and the extraction of olive polyphenols and pigments were significantly influenced by the microbiological profile of olives even without any previous storage. In most cases the effect of olive microbiota on oil characteristics was greater than the effect exerted by malaxation time and temperature. Oils from microbiologically contaminated olives showed lower amounts of C5 volatiles and higher levels of C6 volatiles from the lipoxygenase pathway and some fermentation products. On the other hand, a decrease of chlorophylls, pheophytins, xanthophylls and the ratio chlorophyll/pheophytin was observed in these oils. Likewise, the microbiological activity during oil extraction led to significantly lower amounts of polyphenols, in particular of oleuropein derivatives. These differences in olive oil chemical composition were reflected in oil sensory characteristics by the decrease of the green and bitter attributes and by the modification of the oil color chromatic ordinates.

KEYWORDS: virgin olive oil, microbiota, malaxation, volatiles, polyphenols, pigments, sensory

INTRODUCTION

The effect of microorganisms on virgin olive oil quality and characteristics has been always considered as a consequence of olive fruit deterioration during preprocessing storage.[1−5] Quite recently, the effect of residual microorganisms in virgin olive oil during its storage has been reported,[6−9] revealing the importance of hygienic−sanitary aspects in the virgin olive oil production process. Few references of spontaneous microbiota of fresh olives intended for oil production are available, and they report it to be mainly represented by yeasts, filamentous fungi and lactic acid bacteria.[2,10−12] Enzymatic activities of yeasts found in spontaneous olive microbiota and in virgin olive oil have been evaluated and reported to comprise β-glucanase, β-glucosidase, peroxidase and in some cases lipase and cellulase activities.[6,7,9,11] In addition to fungi, olives' spontaneous bacteria and their enzymatic activity, which to the best of our knowledge has not yet been studied, should be taken into account. A recent assay inoculating olive fruit with *Lactobacillus* species to improve virgin olive oil phenolic fraction[13] demonstrates that microbiological activity occurring during the extraction process can significantly influence virgin olive oil features.

In view of these results, the activity of olive microbiota during the oil extraction process could be a critical point for virgin olive oil quality if hygienic practices prior to oil extraction are not fulfilled. With this concern, the reports on the hygienic quality of water used in olive mills for olive washing prior to grinding, with biochemical oxygen demand (BOD) between 0.12 and 2 g O_2/L,[14,15] indicate that the risk of fruit cross-contamination is quite high.

As far as we know, the role played by olive microbiota during the oil extraction process is currently not taken into account. The aim of the present study was to evaluate the effect of microbiological activity during virgin olive oil extraction process on oil chemical, physical and sensory characteristics. For this scope, two batches of oils were extracted from microbiologically contaminated and control olives, at different malaxation conditions. Quality indices, volatile compounds, polyphenols, pigments and sensory characteristics of these virgin olive oils were compared and evaluated as a function of microbiological activity at different malaxation times and temperatures.

MATERIALS AND METHODS

Chemicals. Hexanal, 1-hexanol, (*E*)-2-hexenol, (*Z*)-3-hexenol, (*E*)-2-hexenal, hexyl acetate, hexenyl acetate, 3-methyl-1-butanal, 1-penten-3-one, acetic acid, hexanoic acid, hexyl acetate, (*Z*)-3-hexenyl acetate, nonanal, 3-methylbutanol, phenylethyl alcohol, ethylbenzene, 4-methyl-2-pentanol, *p*-hydroxyphenylacetic acid, *o*-coumaric acid, chlorophyll a and b and lutein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pheophytin a and b were obtained by acidification with hydrochloric acid according to Sievers and Hynninen.[16]

Reagents and Materials. Chloroform, acetic acid, ethanol, diethyl ether, cyclooctane of spectrophotometric grade, sodium chloride and sodium hydroxide were from Panreac (Barcelona, Spain). Methanol, acetonitrile,

Received: February 15, 2011
Accepted: April 9, 2011
Revised: April 5, 2011
Published: April 20, 2011

acetone (LiChrosolv) and hexane (Suprasolv) were from Merck (Darmstadt, Germany); ethyl acetate (Pestanal) was from Riedel de Haen (Seelze, Germany). Violet Red Bile Glucose Agar (VRBG), cetrimide agar, MRS agar, Sabouraud glucose-chloramphenicol agar, yeast extract, casein peptone and Sharpe agar were supplied by Oxoid (Basingstoke, Hampshire, England). Mannitol, cycloheximide, nisin, ammonium acetate and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

SPME fiber divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm, 2 cm long (DVB/CAR/PDMS) and SPE cartridges (3 mL), packed with diol-phase (500 mg), were from Supelco (Bellefonte, PA, USA).

Olive Fruits and Oil Extraction. In order to evaluate the effect of olive microbiota on virgin oil characteristics at different malaxation conditions, a multilevel factorial experimental design was performed consisting of 8 experiments performed in duplicate. Three experimental factors were tested at two levels: olive microbiological contamination (control-C_0 and contaminated-C_1), malaxation temperature (27 and 35 °C) and malaxation time (30 and 60 min).

Healthy olives of the Arbequina variety (24 kg) were handpicked at IRTA-Mas de Bover (Constantí, Spain) with a maturity index (MI) of 3, according to the "Estación de Olivicultura de Jaén".[17] Immediately after collection and just before oil extraction, 12 kg of olives were washed with bidistilled water (C_0), while, in order to simulate olive mill conditions, the remnant 12 kg were immersed during 3 min in contaminated water coming from an olive mill washing tank (olives:water 1:1 w/v)(C_1). Both batches of olives were immediately processed by a pilot extraction plant Abencor (Comercial Abengoa S.A., Sevilla, Spain) equipped with a hammer crusher, a paste beater and a pulp centrifuge. Olives from the two batches were processed in duplicate by carrying out the malaxation step at 27 and 35 °C and during 30 and 60 min. Two sets of virgin olive oils were obtained from contaminated and control olives and then decanted, transferred into dark glass bottles and stored in the dark at 4 °C until the analyses.

Virgin Olive Oil Quality Indices. Free acidity, coefficients of specific extinction at 232 and 270 nm (K_{232} and K_{270}) of VOO samples obtained from the assay were determined in analytical duplicate according to EC regulation UE 796/2002.[18] Unfortunately, the data concerning the peroxide values of samples were irremediably lost. The sensory analysis of the same samples was carried out according to Regulations UE 796/2002[18] and UE 640/2008[19] by the Official Tasting Panel of Virgin Olive Oils of Catalonia, which relies on IOOC and ISO 17025 accreditation. Global sensory punctuation, intensity of sensory defects and positive attribute were assessed and expressed as median of the panelists' scores.

HS-SPME-GC/MS Analysis of Volatile Compounds. Virgin olive oil volatiles were analyzed as reported by Vichi et al.[20] Briefly, 2 g of oil spiked with 4-methyl-2-pentanol (internal standard) was weighed into a 10 mL vial fitted with a silicone septum and placed into a silicone oil bath at 40 °C where the oil was maintained under magnetic stirring (700 rpm). After 10 min of sample conditioning, a DVB/CAR/PDMS fiber was exposed during 30 min to the sample headspace and immediately desorbed in the gas chromatograph injector. Each extraction was performed in duplicate.

Identification of compounds was performed by gas chromatography coupled to ion trap mass selective spectrometry using a ThermoFinnigan Trace GC equipped with a Polaris (Thermo Electron Corporation, Waltham, MA). Analytes were separated on a Supelcowax-10 (Supelco, Bellefonte, PA) 30 m × 0.25 mm i.d., 0.25 μm film thickness. Column temperature was held at 40 °C for 5 min, increased to 200 at 4 °C/min. The injector temperature was 250 °C, and the time of desorption of the fiber into the injection port was fixed at 5 min. Helium was the carrier gas, at constant flow of 1.2 mL/min. The temperature of the ion source was 200 °C and the transfer line, 275 °C. Mass spectra were recorded with a scan time of 0.54 s; electron energy and the emission current were 70 eV and 250 μA, respectively.

Table 1. Microbiological Profile of the Olives' Surface[a]

	cfu/g			
	Pseudomonas	enteric bacteria	lactic bacteria	fungi (yeasts and molds)
control (C_0)[b]	<30	<30	795	2880
contaminated (C_1)[c]	9825	40725	50325	104250

[a] Values are means of three replicates. [b] Control olives, washed with bidistilled water prior to extraction. [c] Olives washed in microbiologically contaminated water coming from the washing tank of an olive mill.

GC−MS analysis in the complete scanning mode (SCAN) in the 35−350 m/z range allowed the identification of compounds in olive oil samples. The data was processed using Xcalibur 2.0. Identification of compounds was carried out by comparison of their mass spectra and retention times with those of standard compounds or with those available in mass spectrum library NIST 2.0 and in the literature, respectively. Non-isothermal Kovats retention indices, using the definition of Van den Dool and Kratz, were calculated and compared with those available in the literature. Response factors of volatile compounds were calculated using a calibration curve, by analyzing deodorized sunflower oil with different concentrations of reference compounds in the range 0.01−5 mg/kg. The internal standard (4-methyl-2-pentanol) concentration in the samples was maintained at 1.5 mg/kg.

SPE-HPLC/DAD Analysis of Polyphenols. Phenolic compounds were determined according to Mateos et al.[21] Briefly, 2.5 g of oil spiked with 0.025 mg of *p*-hydroxyphenylacetic acid and 0.005 mg of *o*-coumaric acid were dissolved in 6 mL of hexane and loaded on a diol-bonded phase cartridge previously conditioned with 6 mL of methanol and 6 mL of hexane. After washing with 6 mL of hexane and 4 mL of hexane:ethyl acetate 90:10 v/v, phenolic compounds were eluted with 10 mL of methanol. After evaporation at room temperature the residue was redissolved in 0.5 mL of methanol:water 1:1.

HPLC analysis was performed on an Agilent 1200 liquid chromatographic system equipped with a diode array UV detector. A Luna C18(2) column (4.6 mm i.d. × 250 mm; particle size 5 μm) (Phenomenex, Torrance, CA), coupled to a security guard C18 4 × 3.0 mm (Phenomenex), was used. Elution was performed at a flow rate of 1.0 mL/min, using as mobile phase a mixture of water/acetic acid (97:3, v/v) (solvent A) and methanol/acetonitrile (50:50 v/v) (solvent B). The solvent gradient changed according to the following conditions: from 95% (A)−5% (B) to 70% (A)−30% (B) in 25 min; 65% (A)−35% (B) in 10 min; 60% (A)−40% (B) in 5 min; 30% (A)−70% (B) in 10 min; and 100% (B) in 5 min, followed by 5 min of maintenance. Chromatograms were acquired at 240, 280, and 335 nm. Quantification was performed using the response factors calculated by Mateos et al.[22]

SPE-HPLC/DAD and UV Analysis of Chlorophylls and Carotenoids. Virgin olive oils chlorophylls and carotenoids were isolated by solid phase extraction using diol bonded phase cartridges as reported by Mateos et al.[22] One gram of oil was dissolved in 4 mL of hexane and loaded on SPE cartridges previously conditioned with 6 mL of methanol and 6 mL of hexane. Sample loading solvent and a subsequent washing fraction with 5 mL of hexane were collected into a volumetric flask. The amount of β-carotene, retained by the hexane phase, was measured after volume adjustment to 10 mL using the coefficient of extinction = 2592. The column was then eluted with 3 mL of acetone, and the solvent was evaporated at room temperature. The residue was redissolved in 0.3 mL of acetone.

HPLC analyses were carried out on an Agilent 1200 liquid chromatographic system equipped with a diode array UV detector. A Luna C18(2) column (4.6 mm i.d. × 250 mm; particle size 5 μm) (Phenomenex, Torrance, CA), coupled to a security guard C18 4 × 3.0 mm

Table 2. Chemical Quality Indices and Sensory Characteristics of Virgin Olive Oils Obtained from Microbiologically Contaminated and Control Olives by Applying Different Malaxation Temperatures and Times[a]

	control (C_0)[b]				contaminated (C_1)[c]				effect of factors[d]					
	27 °C[e]		35 °C[f]		27 °C		35 °C		A[g]		B[h]		C[i]	
	30[j]	60[k]	30	60	30	60	30	60	C_0	C_1	27	35	30	60
quality indices														
free acidity (g of oleic acid/kg of oil)	0.25	0.10	0.25	0.55	0.10	0.30	0.10	0.15						
K_{232}	1.69	1.67	1.64	1.57	1.32	1.75	1.83	1.60						
K_{270}	0.13	0.11	0.12	0.13	0.11	0.10	0.10	0.11						
color														
a^*	−11.9 b	−14.6 c	−14.2 c	−14.2 c	−9.5 a	−9.4 a	−12.2 b	−13.5 c		*	*			*
b^*	77.1 c	89.8 a	82.8 abc	90.1 a	64.6 d	68.5 d	79.3 bc	85.8 ab	*			*		*
L^*	84.8	87.1	87.6	87.3	85.0	83.1	85.7	87.9						
taste														
bitter	4.6 cd	4.6 cd	5.4 a	4.8 bc	4.2 d	4.2 d	5.1 ab	4.2 d	*			*	*	
pungent	4.9	4.9	5.4	5.1	5.0	5.0	5.0	4.7						
astringent	2.5 c	2.6 bc	3.1 a	2.5 c	2.9 ab	2.5 c	3.1 a	2.3 c						*
flavor														
fruity	6.0 abc	6.3 ab	6.4 a	5.9 bc	6.2 abc	5.9 bc	6.2 abc	5.8 bc					*	
green	4.2 a	4.1 ab	4.2 a	4.0 ab	4.1 ab	3.7 c	4.0 ab	3.9 bc	*				*	
apple	0.0	0.0	0.7	0.4	1.2	0.0	0.0	0.0						
ripe fruit[l]	16 b	18 b	7 b	19 b	26 ab	44 a	7 b	26 ab		*	*			*
unripe fruit[l]	91	100	94	94	100	94	94	88						

[a] Processing conditions showing significantly higher values for each dependent variable are indicated by *, and different letters in the same row indicate significant differences ($p < 0.05$). [b] Oils from control olives, washed with bidistilled water prior to extraction. [c] Oils from olives washed in microbiologically contaminated water. [d] Significant differences in quality and sensory indices due to each factor. [e] Oils obtained with a malaxation temperature of 27 °C. [f] Oils obtained with a malaxation temperature of 35 °C. [g] Microbiological charge factor. [h] Malaxation temperature factor. [i] Malaxation time factor. [j] Oils obtained with a malaxation time of 30 min. [k] Oils obtained with a malaxation time of 60 min. [l] Expressed as percent of sensory assessors able to perceive the attribute.

(Phenomenex) was used. Elution was performed at a flow rate of 1.0 mL/min at room temperature, using as the mobile phase a mixture (8:2 v/v) of methanol/water containing 0.025% ammonium acetate and 0.05% triethylamine as phase A and methanol/acetone (1:1 v/v) as phase B. The solvent gradient changed according to the following conditions: from 75% (A)−25% (B) to 50% (A)−50% (B) in 10 and 2.5 min of maintenance; 20% (A)−80% (B) in 1.5 and 2 min of maintenance; and 100% (B) in 5 min, followed by 10 min of maintenance. Quantification of pigments was carried out by the external standard method using calibration curves in the range 0.5−5 mg/kg.

Color Measurement. A spectrophotometer Minolta CN3500D (Osaka, Japan) was used to assess the oil color, and the CIELAB colorimetric system was applied. The oil color is expressed as chromatic ordinates a^*, b^* and L^*. Measurements were made with five replicates for each oil sample.

Microbiological Profile of Olives. The viable-culturable cell number in the olives' surface was determined as previously reported.[5] Briefly, a suspension of 50 g of olives was prepared in 100 mL of sterile water with 0.9% NaCl. After 5 min in the ultrasound bath, the suspension was serially diluted in 0.9% NaCl, and 100 μL of appropriate dilutions were plated in triplicate. Fungi were evaluated on Sabouraud glucose-chloramfenicol agar; lactic acid bacteria on MRS agar; enteric bacteria on Violet Red Bile Glucose Agar and *Pseudomonas* on Cetrimide agar. The plates were incubated at 30 °C during 3−5 days, and viable counts were expressed as cfu/g olive.

Statistical Analysis. The multilevel factorial experimental design was performed using the package "Statgraphics Plus 5.1". In order to evaluate the influence of the tested experimental factors and their interactions on virgin olive oil characteristics, the results of the experimental design, evaluated at a 5% significance level, were analyzed by a standardized Pareto diagram, which displays a frequency histogram where the length of each bar on the chart is proportional to the absolute value of its associated estimated effect or the standardized effect. The standardized effect is the estimated effect divided by its standard error, which is equivalent to computing a t-statistic for each effect. Bars that extend beyond the vertical line in the Pareto diagram correspond to effects that are statistically significant at the 95% confidence level.

Factorial ANOVA was carried out to assess the effect of microbiological charge, malaxation time and temperature on virgin olive oil characteristics. Fisher's LSDs (least significant differences) were applied to establish the differences between each group of samples.

RESULTS AND DISCUSSION

Microbiological Profile of Olive Fruit Surface. With the aim to evaluate the role of microbiological activity during the virgin olive oil extraction process, and to reproduce real industrial conditions, just before oil extraction at a laboratory scale, freshly hand-picked healthy olive fruits were immersed in contaminated water from an olive mill preprocessing washing tank and compared with control olives from the same batch but washed with bidistilled water. The presence of fungi and bacteria on the surface of treated olives was evaluated and proved to be much higher than in control olives (Table 1).

Table 3. Concentration (mg/kg) of Volatile Compounds in Virgin Olive Oils Obtained from Microbiologically Contaminated and Control Olives by Applying Different Malaxation Temperatures and Times[a]

		control (C_0)[b]				contaminated (C_1)[c]				effect of factors[d]					
		27 °C[e]		35 °C[f]		27 °C		35 °C		A[g]		B[h]		C[i]	
	RI[j]	30[k]	60[l]	30	60	30	60	30	60	C_0	C_1	27	35	30	60
methyl acetate[m,n]	829	0.12 abc	0.12 abc	0.13 abc	0.14 a	0.09 c	0.13 ab	0.10 c	0.15 a						*
ethyl acetate	889	0.048 bc	0.049 bc	0.066 a	0.062 ab	0.040 c	0.044 bc	0.056 ab	0.063 ab				*		
3-methylbutanal	912	0.022 a	0.022 a	0.023 ab	0.017 bc	0.015 c	0.014 c	0.015 c	0.015 c	*					
sum of 3-pentanone and pentanal[m,o]	985	0.028 bc	0.044 a	0.030 b	0.042 a	0.019 d	0.045 a	0.024 cd	0.041 a	*					*
1-penten-3-one	1019	0.33 b	0.38 a	0.29 b	0.24 c	0.21 cd	0.24 c	0.24 c	0.17 d	*		*			
ethylbenzene	1120	0.023 b	0.023 b	0.021 b	0.025 b	0.028 ab	0.034 a	0.027 ab	0.030 ab		*				
hexanal	1089	0.76 c	0.76 c	0.75 c	0.83 c	1.41 a	1.42 a	1.14 b	1.06 b		*	*			
2- or 3-methylbutyl acetate[m,p]	1128	0.016 b	0.020 b	0.019 b	0.018 b	0.027 ab	0.034 a	0.019 b	0.021 b		*				
(*E*)-2-pentenal[m,q]	1140	0.051 bc	0.059 a	0.053 ab	0.047 cd	0.040 d	0.052 bc	0.044	0.048 bc	*					*
1-penten-3-ol[m,o]	1163	0.13 bc	0.15 a	0.13 bc	0.14 ab	0.11 d	0.12 cd	0.12 cd	0.13 bc	*					
2- and 3-methylbutanol	1215	0.10 d	0.10 d	0.14 ab	0.11 cd	0.12 bc	0.14 ab	0.15 a	0.15 a		*		*		
(*E*)-2-hexenal	1230	6.2 de	6.0 de	5.5 e	5.8 e	7.9 ab	8.3 a	7.0 bcd	7.2 bc		*	*			
(*E*)-β-ocimene[m,r]	1250	0.11 d	0.13 b	0.10 d	0.13 b	0.11 cd	0.14 a	0.10 d	0.12 bc						*
hexyl acetate	1281	0.18 de	0.19 cde	0.23 a	0.16 e	0.21 abc	0.21 abc	0.21 ab	0.19 bcd		*			*	
(*E*)-4,8-dimethyl-1,3,7-nonatriene[m,r]	1310	0.020 abc	0.023 ab	0.018 abc	0.020 abc	0.017 c	0.025 a	0.015 c	0.019 abc						*
(*E*)-2-pentenol[m,q]	1323	0.043 cd	0.073 a	0.051 bc	0.061 ab	0.033 d	0.054 ab	0.031 d	0.044 cd	*					*
(*Z*)-2-pentenol	1332	0.36 ab	0.39 a	0.37 ab	0.37 ab	0.32 c	0.35 bc	0.33 c	0.38 ab	*					*
1-hexanol	1362	0.44 cd	0.41 ef	0.46 bc	0.40 f	0.48 ab	0.45 c	0.49 a	0.42 de		*			*	
(*Z*)-3-hexenol	1394	0.66 bc	0.58 d	0.56 d	0.62 cd	0.72 ab	0.75 a	0.62 cd	0.61 cd		*	*			
nonanal	1402	0.13 d	0.18 bcd	0.14 cd	0.17 bcd	0.15 cd	0.23 ab	0.21 bc	0.27 a		*				*
(*E*)-2-hexenol	1417	0.15 bc	0.14 cd	0.11 d	0.13 cd	0.18 b	0.21 a	0.14 c	0.16 bc		*	*			
acetic acid	1448	0.090 bc	0.080 bc	0.099 abc	0.113 ab	0.080 bc	0.070 c	0.099 abc	0.123 a				*		
hexanoic acid	1839	0.18 bc	0.17 cd	0.13 d	0.14 d	0.25 a	0.21 ab	0.19 bc	0.21 b		*	*			
benzenemethanol[m,s]	1889	0.009 c	0.008 c	0.010 c	0.009 c	0.013 b	0.018 a	0.014 b	0.013 b		*				
benzeneethanol	1926	0.009 e	0.008 e	0.010 de	0.010 de	0.011 cd	0.016 a	0.013 b	0.013 bc		*				
sum of pentene dimers[m,q]		2.2 ba	2.1 b	2.0 b	2.4 ab	2.0 b	2.4 a	2.3 ab	2.4 ab						*
sum LOX C5		0.91 b	1.04 a	0.89 bc	0.86 bc	0.71 e	0.82 cd	0.77 de	0.78 de	*		*			*
sum LOX C6 Ald		7.0 cd	6.7 d	6.3 d	6.6 d	9.3 ab	9.7 a	8.2 bc	8.3 bc		*	*			
sum LOX C6 Alc		1.2 bc	1.1 cd	1.1 cd	1.1 cd	1.4 a	1.4 a	1.3 b	1.2 bc		*	*			
sum LOX L		1.4 e	1.4 e	1.5 de	1.4 e	2.1 a	2.1 a	1.9 bc	1.7 cd		*	*			
sum LOX Ln		7.6 cde	7.3 de	6.8 e	7.1 e	9.4 ab	9.9 a	8.4 bc	8.6 bc		*	*			
LOXLn/LOXL		5.5 a	5.4 ab	4.7 cd	5.1 ab	4.5 d	4.8 cd	4.5 d	5.1 ab	*					*
LOXC6Ald/LOXC6Alc		5.6 d	6.0 bcd	5.6 d	5.8 cd	6.7 ab	6.9 a	6.6 abc	6.9 a		*				

[a] The table only shows compounds that according to the factorial analysis of variance were significantly influenced by the experimental factors. Processing conditions showing significantly higher values for each dependent variable are indicated by *, and different letters in the same row indicate significant differences ($p < 0.05$). [b] Oils from control olives, washed with bidistilled water prior to extraction. [c] Oils from olives washed in microbiologically contaminated water. [d] Significant differences in volatiles concentration due to each factor. [e] Oils obtained with a malaxation temperature of 27 °C. [f] Oils obtained with a malaxation temperature of 35 °C. [g] Microbiological charge factor. [h] Malaxation temperature factor. [i] Malaxation time factor. [j] Kováts indices on Supelcowax capillary column. [k] Oils obtained with a malaxation time of 30 min. [l] Oils obtained with a malaxation time of 60 min. [m] Tentatively identified by mass spectrum and linear retention index. [n] Quantified using the response factor of ethyl acetate. [o] Quantified using the response factor of 1-penten-3-one. [p] Quantified using the response factor of methylbutanol. [q] Quantified using the response factor of (*Z*)-2-pentenol. [r] Expressed as mg/kg equivalents of 4-methyl-2-pentanol (IS). [s] Quantified using the response factor of benzeneethanol.

Virgin Olive Oil Quality Indices. Quality indices and sensory characteristics of virgin olive oils obtained from microbiologically contaminated and control olives are reported in Table 2. No significant differences in free acidity and specific extinctions at 232 and 270 nm were observed between virgin olive oils from olives with different microbiological charge. In both cases, free acidity and specific extinctions at 232 and 270 nm maintained well below the limits fixed for EVOO category by the EU

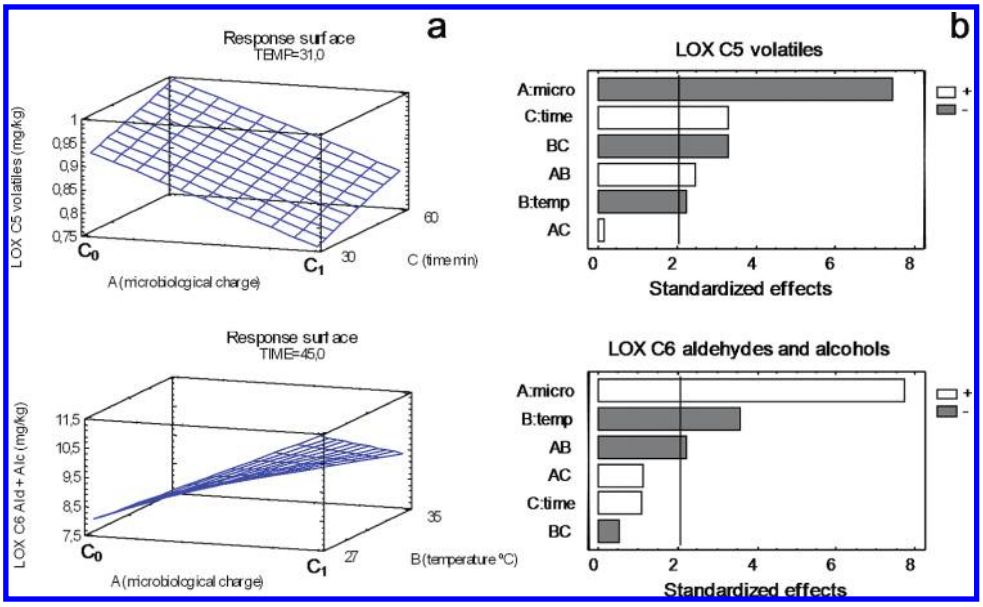

Figure 1. (a) Estimated response surfaces of Lox C5 oxygenated and Lox C6 compounds (mg/kg) as a function of olives microbiological charge (C_0-C_1) and malaxation time (30−60 min) or temperature (27−35 °C), respectively. (b) Pareto diagram showing the influence of experimental factors and interactions on the concentration of Lox C5 and C6 compounds: *A*, microbiological charge; *B*, malaxation temperature; *C*, malaxation time. White bars (+) indicate that a factor is proportional to the concentration of the volatiles; gray bars (−) indicate that a factor is inversely proportional to the concentration of volatiles.

Table 4. Concentration (mg/kg) of Chlorophylls, Pheophytins, Xanthophylls and *β*-Carotene in Virgin Olive Oils Obtained from Microbiologically Contaminated and Control Olives by Applying Different Malaxation Temperatures and Times[a]

	control (C_0)[b]				contaminated (C_1)[c]				effect of factors[d]					
	27 °C[e]		35 °C[f]		27 °C		35 °C		*A*[g]		*B*[h]		*C*[i]	
	30[j]	60[k]	30	60	30	60	30	60	C_0	C_1	27	35	30	60
chlorophyll A	2.9 b	4.4 a	4.4 a	5.2 a	0.8 c	1.2 c	1.5 c	1.5 c	*			*		*
chlorophyll A′	0.8 ab	1.2 a	0.9 a	0.4 d	0.3 d	0.4 bc	0.2 d	0.2 d	*					
chlorophyll B	0.4 cd	0.7 b	0.6 b	0.8 a	0.2 f	0.3 ef	0.4 de	0.6 bc	*			*		*
pheophytin A	2.6 bc	3.5 b	3.8 b	5.4 a	1.4 c	1.5 c	3.7 b	5.2 a	*			*		*
pheophytin A′	0.5 c	0.7 b	0.8 b	1.0 a	0.3 c	0.3 cd	0.7 b	1.0 a	*			*		*
pheophytin B	2 b	5 a	3 b	1 b	2 b	3 b	2 b	1 b	*					
lutein	3.7 de	4.6 bc	4.2 bcd	5.0 a	2.8 f	3.4 e	4.1 cd	4.8 ab	*			*		*
lutein isomer[l]	0.37 bc	0.51 a	0.43 abc	0.49 a	0.19 d	0.25 cd	0.33 bc	0.51 ab	*			*		*
lutein isomer′[l]	0.23 c	0.31 a	0.25 bc	0.30 a	0.18 d	0.22 c	0.24 c	0.29 ab	*			*		*
antheroxanthin[l]	0.83 bc	0.70 bc	0.97 ab	1.35 a	0.46 d	0.45 d	1.03 ab	1.31 a				*		
mutatoxanthin[l]	0.44 c	0.54 b	0.46 c	0.63 a	0.33 d	0.34 d	0.46 c	0.60 ab	*			*		*
β-cryptoxanthin[l]	0.06 d	0.09 ab	0.08 bc	0.11 a	0.03 e	0.06 d	0.07 cd	0.09 ab	*			*		*
β-carotene	0.9 cd	1.3 a	1.0 bcd	1.4 a	0.7 d	1.1 abc	1.2 ab	1.4 a				*		*
sum chlorophylls	4.1 b	6.3 a	5.9 a	6.3 a	1.4 d	1.9 cd	2.1 cd	2.4 c	*			*		
sum pheophytins	3.6 c	4.8 bc	5.0 b	7.0 a	2.0 d	2.3 d	5.1 bc	6.9 a	*			*		*
sum xanthophylls	5.6 de	6.7 bc	6.4 cd	7.9 a	4.0 f	4.8 ef	6.2 cd	7.6 ab	*			*		*
chlorophylls/ pheophytins	1.1 ab	1.3 a	1.2 a	0.9 ab	0.7 bc	0.8 ab	0.4 c	0.3 c	*		*			

[a] The table only shows compounds that according to the factorial analysis of variance were significantly influenced by the experimental factors. Processing conditions showing significantly higher values for each dependent variable are indicated by *, and different letters in the same row indicate significant differences ($p < 0.05$). [b] Oils from control olives, washed with bidistilled water prior to extraction. [c] Oils from olives washed in microbiologically contaminated water. [d] Significant differences in pigments concentration due to each factor. [e] Oils obtained with a malaxation temperature of 27 °C. [f] Oils obtained with a malaxation temperature of 35 °C. [g] Microbiological charge factor. [h] Malaxation temperature factor. [i] Malaxation time factor. [j] Oils obtained with a malaxation time of 30 min. [k] Oils obtained with a malaxation time of 60 min. [l] Quantified using the response factor of lutein.

dx.doi.org/10.1021/jf200642s |*J. Agric. Food Chem.* 2011, 59, 4705–4714

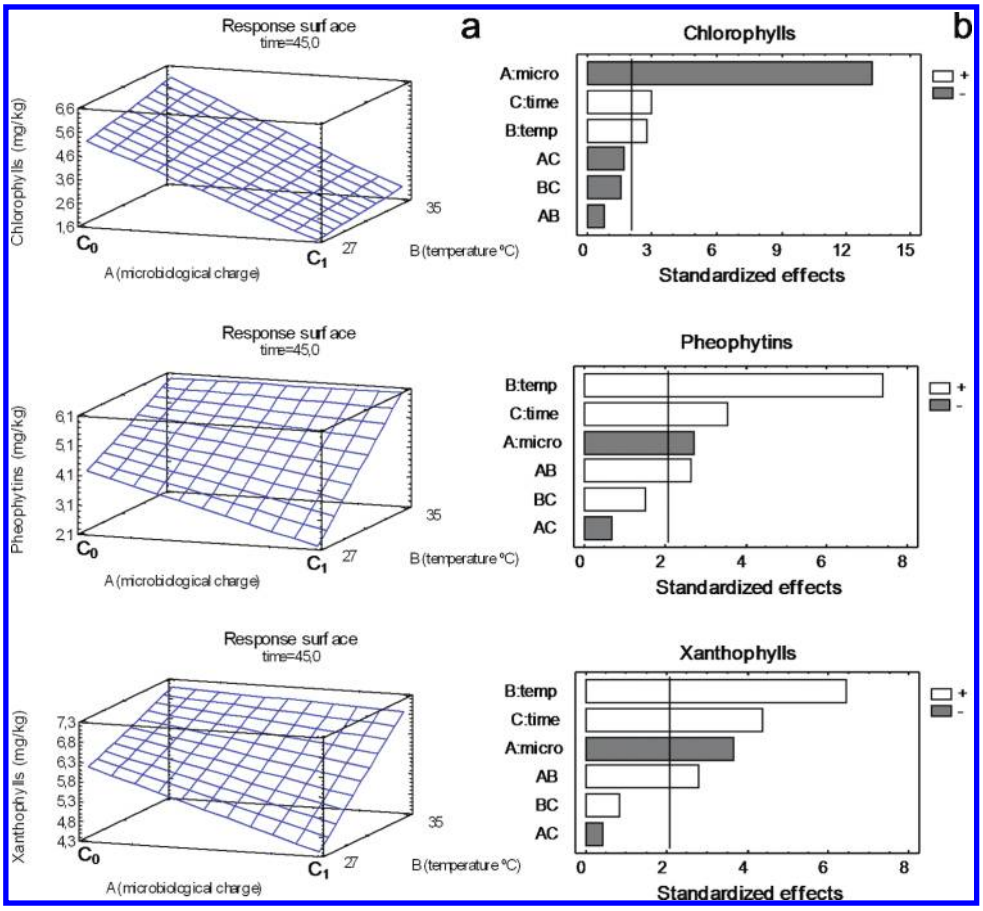

Figure 2. (a) Estimated response surfaces of chlorophylls, pheophytins and xanthophylls (mg/kg) as a function of olives microbiological charge (C_0-C_1) and malaxation temperature (27−35 °C), respectively. (b) Pareto diagram showing the influence of experimental factors and interactions on the concentration of chlorophylls, pheophytins and xanthophylls: *A*, microbiological charge; *B*, malaxation temperature; *C*, malaxation time. White bars (+) indicate that a factor is proportional to the concentration of the pigments; gray bars (−) indicate that a factor is inversely proportional to the concentration of pigments.

regulations.[18,19] Likewise, the median of sensory defect was in all cases 0 and the median of the fruity attribute was always >0, as required for the classification of oils in the extra virgin category.[18,19]

Volatile Compounds. Microbiological activity during the extraction process significantly influenced the composition of the olive oil volatile fraction (Table 3). In particular, oxygenated C5 volatile compounds formed by β-scission of alkoxy radicals produced after lipoxygenase (LOX) action on linolenic acid (Ln)[23] were present in lower amounts in oils from contaminated olives, as illustrated by the response surface graphic (Figure 1 a), while pentene dimers, seven C10 hydrocarbons originated by the same metabolic route after pentene radical coupling,[23] were only influenced by the malaxation time (Table 3). The negative effect of microbiota on C5 compound was more marked when malaxation was carried out at 27 °C (Figure 1 b), resulting in a significant positive interaction between microbiological profile and temperature evidenced by the standardized Pareto diagram (Figure 1 b).

Conversely, both C6 aldehydes and alcohols produced by the LOX pathway (LOX C6 Ald and LOX C6 Alc) as a result of hydroperoxyde lyase (HPL) and alcohol dehydrogenase (ADH) action were promoted by the higher presence of microorganisms during oil extraction. The ratio LOXC6Ald/LOXC6Alc reveals a slightly higher positive effect of olive microbiota activity on aldehydic compounds (Table 3). In particular, the influence of microbiological activity on LOX C6 Ald and LOX C6 Alc was more pronounced when malaxation was carried out at lower temperature (27 °C), as can be noticed in Figure 1 a. The microbiological activity at this malaxation temperature seems to favor in general the formation of LOXL, with the consequent decrease of the ratio LOXLn/LOXL (Table 3). Regarding the C6 esters produced by alcohol acyltransferase (AAT) catalysis, only the one proceeding from linoleic acid (L) was significantly increased by the olives' microbiological charge (Table 3).

As expected, malaxation time and temperature also influenced the biogenesis of volatiles regardless the microbiological profile of olives. The lowest malaxation temperature (27 °C), closer to the optimum temperature of LOX and HPL,[24] promoted the production of C6 compounds from LOX pathway (Table 3). Moreover, the malaxation time exerted a negative effect on the production of hexanol and its corresponding acetyl ester, in agreement with previous results reported for other olive varieties.[25] Conversely, except 1-penten-3-one, the majority of C5 volatiles were not influenced by malaxation temperature, while several of them were more abundant in oils with a longer malaxation time, as previously observed in oils obtained from other olive varieties.[25−27] However, the magnitude of the microbiological activity effect on virgin olive oil features in comparison with other processing conditions is worthy of mention.

Table 5. Concentration (mg/kg) of Polyphenols in Virgin Olive Oils Obtained from Microbiologically Contaminated and Control Olives by Applying Different Malaxation Temperatures and Times[a]

	control (C_0)[b]				contaminated (C_1)[c]				effect of factors[d]					
	27 °C[e]		35 °C[f]		25 °C		35 °C		A[g]		B[h]		C[i]	
phenolic compds[j]	30[k]	60[l]	30	60	30	60	30	60	C_0	C_1	27	35	30	60
3,4-DHPEA	0.58 bc	0.76 a	0.43 c	0.87 a	0.49 c	0.52 bc	0.44 c	0.71 ab	*					*
3,4-DHPEA-EDA	408 c	368 cd	483 a	475 ab	340 d	286 e	417 bc	318 de	*			*	*	
3,4-DHPEA-EA	22 c	19 c	33 a	28 b	21 c	21 c	37 a	21 c				*	*	
elenolic acid	111 abc	102 bc	145 a	71 cd	131 ab	111 abc	116 abc	54 d					*	
p-HPEA	0.32 bc	0.36 bc	0.28 c	0.50 a	0.38 b	0.51 a	0.35 bc	0.45 a		*				*
p-HPEA-EA	2.45 ab	2.93 ab	2.88 ab	2.35 b	3.05 ab	3.17 ab	3.39 a	3.20 ab		*				
apigenin	0.27 a	0.19 bc	0.19 bc	0.17 c	0.29 a	0.27 a	0.25 ab	0.17 c		*	*		*	
luteolin	0.69 ab	0.44 d	0.53 cd	0.41 d	0.80 a	0.59 bc	0.52 cd	0.41 d			*		*	
vanillic acid	1.68 bc	1.39 de	1.31 e	1.77 b	2.40 a	1.60 c	1.83 b	1.48 cd		*	*		*	

[a] Quantification was carried out using the response factors determined by Mateos et al.[22] The table only shows compounds that according to the factorial analysis of variance were significantly influenced by the experimental factors. Processing conditions showing significantly higher values for each dependent variable are indicated by *, and different letters in the same row indicate significant differences ($p < 0.05$). [b] Oils from control olives, washed with bidistilled water prior to extraction. [c] Oils from olives washed in microbiologically contaminated water. [d] Significant differences in concentration of phenols due to each factor. [e] Oils obtained with a malaxation temperature of 27 °C. [f] Oils obtained with a malaxation temperature of 35 °C. [g] Microbiological charge factor. [h] Malaxation temperature factor. [i] Malaxation time factor. [j] 3,4-DHPEA, 3,4-dihydroxyphenylethanol (hydroxytyrosol); 3,4-DHPEA-EDA, 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (dialdehydic form of oleuropein aglycon); 3,4-DHPEA-EA, 3,4-dihydroxyphenylethanol-elenolic acid (oleuropein aglycon); *p*-HPEA, hydroxyphenylethanol (tyrosol); *p*-HPEA-EA, hydroxyphenylethanol-elenolic acid (ligstroside aglycon). [k] Oils obtained with a malaxation time of 30 min. [l] Oils obtained with a malaxation time of 60 min.

As illustrated by the standardized Pareto diagrams showing the degree of influence of each factor on the concentration of LOX C6 and C5 compounds (Figure 1 b), the microbiological activity during oil extraction showed a considerably greater effect than malaxation temperature and time on the biogenesis of the principal olive oil volatiles.

Moreover, although no significant differences were found in oils' specific extinctions at 232 and 270 nm, the oxidation marker nonanal was found to increase in oils from microbiologically contaminated olives, in particular at higher malaxation temperature (Table 3). The ability of this volatile marker to detect olive oil oxidation at earlier stages than do spectrophotometric absorbances has been already reported.[28] These results may suggest that microbiological contamination of olives could imply an increase of oxidative reactions in the oil.

Finally, some volatiles known to be fermentation products were more abundant in oils from contaminated olives: benzenemethanol, benzeneethanol, 3- and 2-methylbutanol and methylbutyl acetate. Likewise, the amounts of the aromatic hydrocarbon ethylbenzene, known as ubiquitous environmental contaminant, were significantly higher in these oils, corroborating the hypothesis of its biogenic origin.[29] On the other hand, the formation of acetic acid and ethyl acetate was favored by the higher malaxation temperature, regardless of the olives' microbiota profile, while hydrocarbons (terpenes and pentene dimers) were only influenced by the malaxation time, presenting higher levels in oils with 60 min of malaxation (Table 3).

The main repercussion in oils from microbiologically contaminated olives of the volatile composition on the sensory profile was the decrease of the green attribute, possibly due to the reduction of 1-penten-3-one and other C5 endogenous volatiles,[30,31] and the increase of the percent of assessors able to detect the ripe fruit note in oils (Table 2).

Although the implication of any specific microbial enzyme at this stage would be speculative due to the scarce characterization of olive microbiota, the exposed results indicate that microbiological enzyme activities could be implicated in the biogenesis of olive oil volatiles.

Pigments. The activity of olive microbiota during oil extraction involved a significant decrease in the content of chlorophylls, pheophytins and xanthophylls (Table 4). It is noteworthy that chlorophyll content was lower in oils from contaminated olives independently of the malaxation conditions applied, while the content of pheophytins and xanthophylls was lower in these samples only when malaxation was performed at 27 °C (Figure 2 a), in agreement with the biogenesis of LOX C6 volatiles. This affected the chlorophyll/pheophytin ratio, which was significantly lower in oils from contaminated olives obtained by malaxating the paste at 35 °C (Table 4).

Moreover, according to published data, the accumulation of chlorophyllic compounds and carotenoids in olive oil was significantly favored by higher malaxation temperature,[32] and time (Table 4), but the microbiological profile of olives was by far the factor with the highest degree of influence on concentration of chlorophylls (Figure 2 b). On the other hand, pheophytins and xanthophylls were mainly influenced by malaxation temperature, followed by malaxation time and olive microbiological profile (Figure 2 b), while β-carotene concentration was not significantly influenced by microbiological contamination of olives (Table 4).

The activity of microbiota on olive pigments was reflected by olive oil chromatic ordinates a^* and b^* (Table 2). Both greenness (negative a^* values) and yellowness (positive b^* values) were lower in oils from contaminated olives, but this effect was more marked when malaxation was carried out at a temperature of 27 °C, in agreement with pigment behavior. Also malaxation temperature and time influenced olive oil color; greenness and yellowness were higher when malaxation was performed at 35 °C during 60 min (Table 2). Conversely, lightness (L) was not affected by any of the factors tested. A relationship between

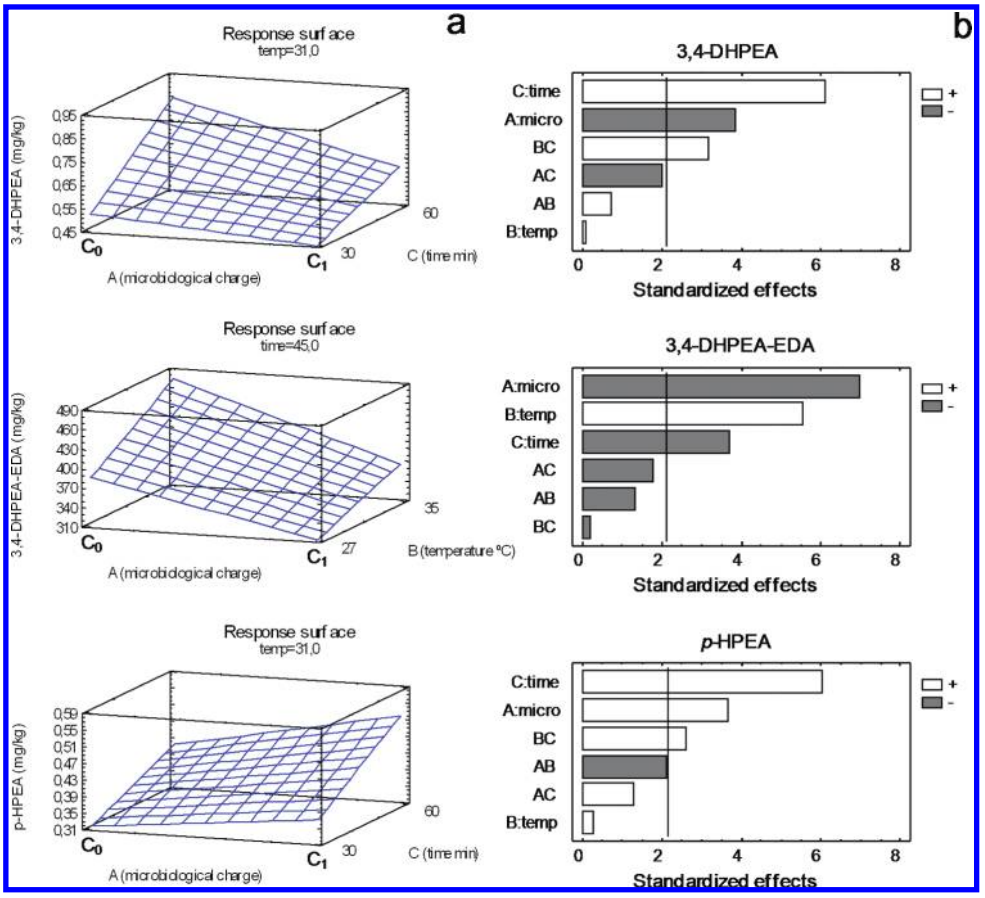

Figure 3. (a) Estimated response surfaces of 3,4-DHPEA, 3,4-DHPEA-EDA and *p*-HPEA (mg/kg) as a function of olives microbiological charge (C_0-C_1), malaxation time (30−60 min) or temperature (27−35 °C), respectively. (b) Pareto diagram showing the influence of each experimental factor and interactions on the concentration of 3,4-DHPEA, 3,4-DHPEA-EDA and *p*-HPEA: *A*, microbiological charge; *B*, malaxation temperature; *C*, malaxation time. White bars (+) indicate that a factor is proportional to the concentration of the polyphenols; gray bars (−) indicate that a factor is inversely proportional to the concentration of the polyphenols.

chromatic ordinates and the major pigments was observed. In particular, b^* values showed the best correlation with the xanthophyll fraction ($r = 0.891$, $p < 0.05$) while a^* values were better correlated with the pheophytin fraction ($r = 0.756$, $p < 0.05$), as previously reported.[33]

Previous studies report that degradation of chlorophyllic and carotenoid pigments may be a direct or indirect consequence of enzymatic oxidation. In these studies, carried out in wheat or in a model system, the decrease in xanthophylls, chlorophyll and carotenoids has been attributed to the direct or indirect action of endogenous lipoxygenase, peroxidase and polyphenol oxidase.[34,35] Specific studies on virgin olive oil would be necessary to determine whether the decrease of pigments observed in oils from microbiologically contaminated olives could be due to the presence of oxidants formed by enzymatic action.

Polyphenols. Microbiological contamination of olives involved a significant reduction in the amount of oleuropein derivatives such as hydroxytyrosol (3,4-DHPEA) and the dialdehydic form of oleuropein aglycon (3,4-DHPEA-EDA) in the oil (Table 5). The dialdehydic form of ligstroside aglycon (*p*-HPEA-EDA) showed the same trend as 3,4-DHPEA-EDA, but the differences were not statistically significant (data not shown). Conversely, a positive effect of olives' microbiological charge was noticed in the concentration of *p*-HPEA, apigenin and vanillic acid in the oil (Table 5). Moreover, the presence of olive biophenols in the oil was in general favored by a short malaxation time, except for phenolic alcohols 3,4-DHPEA and *p*-HPEA, which in accord with previous studies[26,27] were more abundant after 60 min of malaxation. Likewise, the malaxation temperature selectively influenced the extraction of polyphenols; some *o*-diphenols were more abundant in oils extracted at 35 °C, as previously reported,[27,32,36] while flavonoid extraction was favored in oils extracted at 27 °C (Table 5). Surface response graphics allow a better appreciation of the extent of the modifications induced on olive oil phenols by microbiological charge (Figure 3 a).

Malaxation time was the factor showing the main effect on phenolic alcohols, as shown in Figure 3 b, followed by the effect of olive microbiological charge. On the contrary, olive microbiota activity showed the largest effect on the concentration of 3,4-DHPEA-EDA in the oil.

The lower concentration of some of the main *o*-diphenols in oils from contaminated olives seems to be in accordance with the lower resistance to oxidation suggested for these oils by their higher levels of nonanal, especially at higher malaxation temperatures (Table 3).

As expected, polyphenol concentration was related to olive oil sensory attributes. Oil bitterness and astringency were significantly correlated to *o*-diphenols ($p < 0.001$, $r = 0.852$; $p < 0.05$, $r = 0.504$, respectively), and in particular to 3,4-DHPEA-EDA

($p < 0.001$, $r = 0.845$; $p < 0.05$, $r = 0.490$, respectively). Nevertheless, only oil bitterness was significantly influenced by the experimental factors tested, and, similarly to 3,4-DHPEA-EDA concentration, it was reduced by microbiota activity and longer malaxation time and favored by a lower malaxation temperature (Table 2).

In conclusion, olive microbiota activity during the oil extraction process significantly influenced the biogenesis of volatile compounds and the extraction of olive polyphenols and pigments, even without any previous storage. In most cases the effect of olive microbiota on oil characteristics was greater than the effect exerted by malaxation time and temperature. Oils from microbiologically contaminated olives presented lower levels of LOX C5 volatiles, chlorophylls, pheophytins, xanthophylls and *o*-diphenols, and higher amounts of LOX C6 volatiles, tyrosol and flavonoids. The effect of olive microbiota on LOX derived volatiles and pigments such as pheophytins and xanthophyll was more pronounced when malaxation was performed at 27 °C, leading us to hypothesize the involvement of enzymatic reactions favored by this temperature. These modifications in virgin olive oil minor compounds were reflected by oil sensory characteristics, namely, color, taste and flavor. Olive microbiological contamination was accompanied by a decrease in oil greenness and yellowness, and in bitter and green attributes, and by an increase of the ripe fruit note.

Although modifications of oil characteristics should depend on the specific olives' microbiological profile, the present study evidences for the first time the ability of olive microbiota in simulated olive mill conditions to influence oil features, even without any previous storage of fruits. The results obtained demonstrate the significance of hygienic−sanitary aspects in the virgin olive oil production process, as well as the need for a more in-depth knowledge about olive spontaneous microorganisms and their enzymatic activities.

AUTHOR INFORMATION

Corresponding Author

*Phone: +34 93 4006100. Fax: +34 93 2045904. E-mail: stefania.vichi@idaea.csic.es.

Funding Sources

This study was supported by the Generalitat de Catalunya, through the Beatriu de Pinós program.

ACKNOWLEDGMENT

The authors are grateful to the Official Tasting Panel of Virgin Olive Oils of Catalonia for the sensory evaluation of samples.

REFERENCES

(1) Angerosa, F.; Di Giacinto, L.; Solinas, M. Influenza dello stoccaggio in massa delle olive sull'aroma degli oli di risulta: valutazione del difetto di "riscaldo" mediante analisi HPLC e GLC dei componenti volatili. *Riv. Merceol.* **1990**, *29*, 275–294.

(2) Angerosa, F.; Lanza, B.; Marsilio, V. Biogenesis of "fusty" defect in virgin olive oils. *Grasas Aceites* **1996**, *47*, 142–150.

(3) Angerosa, F.; Lanza, B.; Marsilio, V.; Cumitini, S. Olive oil off-odour compounds produced by *Aspergillus* and *Penicillium*. *Acta Hortic.* **1999**, *474*, 695–699.

(4) Vichi, S.; Romero, A.; Tous, J.; López-Tamames, E.; Buxaderas, S. Determination of volatile phenols in virgin olive oils and their sensory significance. *J. Chromatogr. A* **2008**, *1211*, 1–7.

(5) Vichi, S.; Romero, A.; Gallardo-Chacón, J.; Tous, J.; López-Tamames, E.; Buxaderas, S. Influence of olives' storage conditions on the formation of volatile phenols and their role in off-odor formation in the oil. *J. Agric. Food Chem.* **2009**, *57*, 1449–1455.

(6) Ciafardini, C.; Zullo, B. A. Microbiological activity in stored olive oil. *Int. J. Food Microbiol.* **2002**, *75*, 111–118.

(7) Ciafardini, C.; Zullo, B. A.; Iride, A. Lipase production by yeasts from extra virgin olive oil. *Food Microbiol.* **2006**, *23*, 60–67.

(8) Ciafardini, C.; Zullo, B. A.; Cioccia, G.; Iride, A. Lipolytic activity of *Williopsis californica* and *Saccharomyces cerevisiae* in extra virgin olive oil. *Int. J. Food Microbiol.* **2006**, *107*, 27–32.

(9) Zullo, B. A.; Ciafardini, C. Lipolytic yeasts distribution in commercial extra virgin olive oil. *Food Microbiol.* **2008**, *25*, 970–977.

(10) Rodriguez de la Borbolla y Alcalá, J. M.; Gómez-Herrera, C.; Gonzalez-Cancho, F.; Fernández-Díez, M. J. Conservación de las aceitunas de molino. *Grasas Aceites* **1958**, *9*, 18–30.

(11) Romo-Sánchez, S.; Alves-Baffi, M.; Arévalo-Villena, M; Úbeda-Iranzo, J.; Briones-Pérez, A. Yeast biodiversity from oleic ecosystems: Study of their biotechnological properties. *Food Microbiol.* **2010**, *27*, 487–492.

(12) Alves-Baffi, M.; Romo-Sánchez, S.; Úbeda-Iranzo, J. B.; Briones-Pérez, A. M. Molecular identification of filamentous fungi from olive phyllosphere and investigation of their biotechnological properties. *New Biotechnol.* **2009**, *25*, S66. Abstracts of the 14th European Congress on Biotechnology, Barcelona, Spain, 13−16 September 2009.

(13) Kachouri, F.; Hamdi, M. Use of *Lactobacillus plantarum* in olive oil process and improvement of phenolic compounds content. *J. Food Eng.* **2006**, *77*, 746–752.

(14) Amaral, C.; Lucas, M. S.; Coutinho, J.; Crespí, A. L.; Anjos, M. R.; Pais, C. Microbiological and physicochemical characterization of olive mill wastewaters from a continuous olive mill in Northeastern Portugal. *Bioresour. Technol.* **2008**, *99*, 7215–7223.

(15) Borja, R.; Raposo, F.; Rincón, B. Tecnología de tratamiento de los efluentes líquidos y residuos sólidos resultantes del proceso de elaboración del aceite de oliva por centrifugación en dos fases. *Grasas Aceites* **2006**, *57*, 32–46.

(16) Sievers, G.; Hynninen, P. H. Thin-layer chromatography of chlorophylls and their derivatives on cellulose layers. *J. Chromatogr. A* **1977**, *134*, 359–364.

(17) Uceda, M.; Hermoso, M. La calidad del aceite de oliva. In *El cultivo del olivo*; Barranco, D., Fernández-Escobar, R., Rallo, L., Eds.; Mundi Prensa: Madrid, Spain, 2001; pp 589−614.

(18) European Commission, *Off. J. Eur. Communities*, May 6, Commission Regulation (EC) No 796/2002 of 6 May 2002 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis and the additional notes in the Annex to Council Regulation (EEC) No 2658/87 on the tariff and statistical nomenclature and on the Common Customs Tariff.

(19) European Commission, *Off. J. Eur. Communities*, July 4, Commission Regulation (EC) No 640/2008 of 4 July 2008 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis.

(20) Vichi, S.; Castellote, A. I.; Pizzale, L.; Conte, L. S.; Buxaderas, S.; Lopez-Tamames, E. Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas Chromatography with mass spectrometric and flame ionization detection. *J. Chromatogr. A* **2003**, *983*, 19–33.

(21) Mateos, R.; Espartero, J. L.; Trujillo, M.; Ríos, J. J.; León-Camacho, M; Alcudia, F.; Cert, A. Determination of Phenols, Flavones, and Lignans in Virgin Olive Oils by Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Ultraviolet Detection. *J. Agric. Food Chem.* **2001**, *49*, 2185–2192.

(22) Mateos, R.; García-Mesa, J. A. Rapid and quantitative extraction method for the determination of chlorophylls and carotenoids in olive oil by high-performance liquid chromatography. *Anal. Bioanal. Chem.* **2006**, *385*, 1247–1254.

(23) Angerosa, F.; Camera, L.; d'Alessandro, N.; Mellerio, G. Characterization of seven new hydrocarbon compounds present in the aroma of virgin olive oil. *J. Agric. Food Chem.* **1998**, *46*, 648–653.

(24) Salas, J. J.; Sánchez, J. The decrease of virgin olive oil flavor produced by high malaxation temperature is due to inactivation of hydroperoxide lyase. *J. Agric. Food Chem.* **1999**, *47*, 809–812.

(25) Angerosa, F.; D'Alessandro, N.; Basti, C. Vito. Biogeneration of volatile compounds in virgin olive oil: their evolution in relation to malaxation time. *J. Agric. Food Chem.* **1998**, *46*, 2940–2944.

(26) Angerosa, F.; Mostallino, R.; Basti, C.; Vito, R. Infuence of malaxation temperature and time on the quality of virgin olive oils. *Food Chem.* **2001**, *72*, 19–28.

(27) Kalua, C. M.; Bedgood, D. R.; Bishop, A. G.; Prenzler, P. D. Changes in volatile and phenolic compounds with malaxation time and temperature during virgin olive oil production. *J. Agric. Food Chem.* **2006**, *54*, 7641–7651.

(28) Vichi, S.; Pizzale, L.; Conte, L. S.; Buxaderas, S.; López-Tamames, E. Solid-phase microextraction in the analysis of virgin olive oil volatile fraction: modifications induced by oxidation and suitable markers of oxidative status. *J. Agric. Food Chem.* **2003**, *51*, 6564–6571.

(29) Biedermann, M.; Grob, K.; Morchio, G. On the origin of benzene, toluene, ethylbenzene and xylene in extra virgin olive oil. *Z. Lebensm.-Unters. Forsch.* **1995**, *200*, 266–272.

(30) Angerosa, F.; Mostallino, R.; Basti, C.; Vito, R. Virgin olive oil odour notes: theirrelationship with volatile compounds from the lipoxygenase pathway and secoiridoid compounds. *Food Chem.* **2000**, *68*, 283–287.

(31) Angerosa, F. Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 639–660.

(32) Ranalli, A.; Malfatti, A.; Lucera, L.; Contento, S.; Sotiriou, E. Effects of processing techniques on the natural colourings and the other functional constituents in virgin olive oil. *Food Res. Int.* **2005**, *38* 873–878.

(33) Mínguez-Mosquera, M. I.; Rejano-Navarro, L.; Gandul-Rojas, B.; Sanchez-Gomez, A. H.; Garrido-Fernandez, J. Color-Pigment Correlation in Virgin Olive Oil. *JAOCS* **1991**, *68*, 332–336.

(34) Schoefs, B. Chlorophyll and carotenoid analysis in food products. A practical case-by-case view. *Trends Anal. Chem.* **2003**, *22*335–339.

(35) Jarén-Galán, M.; Carmona-Ramón, C.; Mínguez-Mosquera, M. I. Interaction between chloroplast pigments and lipoxygenase enzymatic extract of olives. *J. Agric. Food Chem.* **1999**, *47*, 2671–2677.

(36) Gómez-Rico, A.; Inarejos-garcía, A. M.; Salvador, M. D.; Fregapane, G. Effect of malaxation conditions on phenol and volatile profiles in olive paste and the corresponding virgin olive oils (*Olea europaea L.* Cv. Cornicabra). *J. Agric. Food Chem.* **2009**, *57*, 3587–3595.