PAPER

EFFECT OF DIFFERENT STORAGE CONDITIONS ON THE SHELF LIFE OF NATURAL GREEN TABLE OLIVES

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ABSTRACT

The aim of this research is to study the effects of different storage conditions on Spanish (alkaline debittering) and natural (directly brined) green olives. Laboratory-processed olives were stored in 6% brine or in a vacuum bag without brine, at 6 or 20°C. After 18 months, natural olives showed higher microbial and olive oil stability than NaOH-treated olives. The lower pH (<4.80) and higher total phenol content (0.2 g/100 g wet pulp) influenced positively the long shelf life of natural olives. The packaging in 6% NaCl brine and in a vacuum bag stored at 20°C gave better performance, while growth of psychrophilic spoilage bacteria occurred at cold temperature.

Keywords: microbial count, natural green olives, oil oxidation, phenols, sensory profile

1. INTRODUCTION

World consumption of table olives (TO) continues to increase, passing from 957 to 2,480 thousand tones in the period from 1990/91–2014/15 (IOOC, 2017). Major producer countries are located in the Mediterranean basin (Spain, Greece, Italy, Turkey, Tunisia, Morocco, Egypt, and Algeria). Green Spanish-style, natural black Greek-style and black ripe Californian are the most popular TO types; however, many other typologies are produced according to local traditions (ERCOLINI *et al*., 2006). In fact, TO types can vary depending on olive fruit kind (green or black olives), debittering method (chemical or biochemical), fermentation conditions (temperature, levels salt, and type of acid) and finally, canning (SANCHEZ *et al*., 2006).

Debittering and fermentation are the main steps of the table olive process. The first is fundamental to remove natural bitterness, while the fermentation is important to prevent microbial spoilage. The typical strong bitter and pungent taste of green olives is due to the high presence of oleuropein, a secoiridoid containing elenolic acid glucoside linked to hydroxytyrosol (AMBRA *et al.*, 2017; GHANBARI *et al*., 2012). In the chemical process, such as the Spanish-style method, olives are debittered in a few hours through a bath in a sodium hydroxide solution that hydrolyzes oleuropein quickly into less bitter compounds (CHAROENPRASERT and MITCHELL, 2012); subsequently, olives are water-washed repeatedly and then brined in 8-10% NaCl where a spontaneous lactic acid fermentation occurs in the following months (APONTE *et al*., 2010). Olive fermentation is caused by indigenous microbiota, including lactic acid bacteria (LAB) and/or yeasts, which transform the sugars in lactic acid with a consequential decrease in pH; in addition, substances inhibiting undesirable microorganisms are produced (BEVILACQUA *et al*., 2009). Moreover, several microbial strains are able to hydrolyse oleuropein contributing to olive debittering (IORIZZO *et al*., 2016; SERVILI *et al*., 2006). IOOC (2017) indicates the natural olives as "green olives, olives turning colour or black olives placed directly in brine in which they undergo complete or partial fermentation, preserved or not by the addition of acidifying agents". Therefore, raw olives are placed in water or brine as long as required for the spontaneous loss of bitterness. In this case, oleuropeinolytic bacterial/yeast strains degrade oleuropein in synergy with specialized enzymes naturally occurring in the olives (RAMIREZ *et al*., 2017a; RAMIREZ *et al*., 2016). However, olive debittering is slow and several months are needed to obtain a satisfactory good-tasting product (SANCHEZ *et al*., 2006).

In recent years, demand of organic and natural foods has become trendy (ROZIN *et al*., 2004). In this contest, the demand for natural TO risen since they have become popular and appreciated for the nutritional value and distinctive sensory characteristics. Several studies on natural TO have been carried out focusing on the content of bioactive components (CHAROENPRASERT and MITCHELL, 2012; SAKOUHI *et al*., 2008) and microbial characterization (BLEVE *et al*., 2015; PEREIRA *et al*., 2008). Moreover, also new systems to accelerate olive debittering with biotechnological approaches as an alternative to chemical treatment have been tried obtaining positive results by using enzymes (DE LEONARDIS *et al*., 2016), selected LAB (PERRICONE *et al*., 2013; HURTADO *et al*., 2010), batches in modified brine (RAMIREZ *et al*., 2017b) and vacuum impregnation (TAMER *et al*., 2013).

In this study, several chemical, microbial and sensory parameters have been tested in green olives laboratory-processed through both the Spanish-style and natural methods and stored for a period of time 18 months, under different conditions. Specifically, the effects of storing in brine were compared with those in vacuum bags, without brine, in order to evaluate the possibility to reduce the salt, potentially harmful to health. Finally, considering that in the supermarkets the packaged olives are frequently placed in

refrigerator, the effects of cold temperatures have been studied. The results could be useful to the improvement of natural olives, in olive packaging and conservation systems.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents were of analytical or HPLC grade. Gallic acid, hydroxytyrosol and oleuropein were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). All the media and the supplements for the microbiological analysis, unless otherwise stated, were from Oxoid, Basingstoke, Hampshire, UK.

2.2. Olive processing and storage conditions

About 10 kg of green or yellow-green olive fruits were handpicked from 'Cazzarella' trees, a dual-purpose autochthonous cultivar grown in the Molise region (Italy). At the laboratory, the olive batch was split and processed as described synthetically in Table 1.

Table 1. Olive sample typologies.

1. Raw olives:	*fresh untreated olives.*
2. B1-NaOH / B1-Nat:	*olives brined in 6% NaCl solution (first brining) and stored in the dark under 20 °C constant temperature for 9 months.*
3. B2-NaOH / B2-Nat:	*olives of point 2 brined in 6% NaCl fresh solution (second brining) and stored in the dark under 20 °C constant temperature for other 9 months.*
4. C-NaOH / C-Nat:	*olives of point 2 packaged without brine in vacuum bags and stored in a refrigerator under 6 °C temperature (C = cold) for other 9 months.*
5. T-NaOH / T-Nat:	*olives of point 2 packaged without brine in vacuum bags and stored in the dark under 20 °C constant temperature (T = tempered) for other 9 months.*

-NaOH: processed by Spanish method;
-Nat: natural processed.

In the preparation of the B1-NaOH sample, olives were placed into a 2% NaOH solution for 16 hours (ratio olive/lye 1:1.3, w/v); olives were washed repeatedly with water until neutral pH and brined into 6% NaCl solution (ratio olive/brine 1:1.3, w/v) in three different closed glass jars. In the case of the B1-Nat sample, the olives were immersed directly into 6% NaCl solution (ratio olive/brine 1:1.3, w/v); three weeks after (21 days), the brine was renewed with a fresh 6% NaCl solution. All jars were stored in the dark under 20°C constant temperature for 9 months. Thus, the olives were water-washed repeatedly, analyzed, repackaged and stored for another 9 months as described in Table 1 (B2-, C-, T-).

2.3. Analytical determinations

Moisture and total fat were determined on the pulp separated manually from the stone and homogenized. Moisture was determined at 105°C on about 5 g of pulp; successively, oil content was measured in dry pulp by using Soxhlet extraction with 40-60°C petroleum ether. Lactic acid, pH and total phenol were determined on both olive pulp and brine.

Brine was filtered through a 0.45 μm PVDF syringe-filter before assaying. As for the pulp, about 20 g of fresh pulp was dispersed in 60 mL of distilled water (1:3, w/v) and the flask was put into a sonicator bath for 20 minutes at room temperature by filtering the aqueous phase on paper. The pH measurement was performed through a pH-meter, while the lactic acid was measured by titration with 0.1 M NaOH up to pH 7.0. Free acidity and fatty acid composition were determined on the oil extracted at cold temperatures from 100 g of olive pulp by following the methods described in PASQUALONE *et al*. (2014). The free acidity (expressed as g oleic acid per 100 g pulp olive) was measured following the olive oil European Union Commission (1991), while the fatty acid composition was determined by gas-chromatographic analysis as described in DE LEONARDIS and MACCIOLA (2012). *p*-anisidine value was determined on the cold extracted oil according to AOCS (1998). Phenol extraction from the olive pulp, determination of total phenols and the HPLC analysis with UV detector were carried out in the same conditions described in DE LEONARDIS *et al*. (2016). Hydroxytyrosol (Hy), dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) and oleuropein (OLE) were monitored and each of them was quantified through a hydroxytyrosol calibration curve derived from a plot of area counts versus concentration. Hy-compounds were calculated as the sum of Hy, HyEDA and OLE expressed as mg/100 g wet pulp olives as Hy equivalent.

2.4. Microbial counts

Microbiological analysis was carried out on the olive pulp according to the method reported in IORIZZO *et al*. (2016) with modifications. For each sample, about 30 g of olives were homogenized in 0.9% NaCl (w/v) for 1 min in a Stomacher bag (Bag Mixer-400). Pulp homogenates were diluted serially and plated in triplicate by using the following growth media and incubation conditions: Plate Count Agar (PCA) at 30°C (72 h) and 15°C (5 days) for total mesophilic and psychrotrophic aerobic bacteria; Violet Red Bile Glucose Agar (VRBGA) at 37°C for 24 h for *Enterobacteriaceae*; MRS Agar, added with 0.17 g/L of cycloheximide (Sigma-Aldrich Co, St. Louis, MO, USA), at 30°C for 4 days in anaerobiosis (Anaerogen kit, Oxoid, Basingstoke, United Kingdom) for lactic acid bacteria (LAB); YPD agar (1% yeast extract, 2% peptone, 2% glucose and 2% agar) supplemented with 100 mg/L chloramphenicol at 28°C for 72 h for yeasts; *Pseudomonas* Agar Base (PAB), added with CFC selective supplement, at 30°C for 48 h for *Pseudomonas* spp.

2.5. Sensory analysis

Sensory profile was evaluated according to Galán-Soldevilla and Pérez-Cacho (2012), with modifications. The panel group was composed of 15 untrained volunteers selected and led by one olive oil expert taster. Training and alignment of panel and sensory profile sheet was developed by using similar commercial products. Nine attributes (defects, fruit odor, salty, bitter, acid, firmness, fibrous, crunchy and overall opinion) were selected and evaluated through a seven-point hedonic scale (7: like extremely; 6: like very much; 5: like slightly; 4: neither like nor dislike; 3: dislike slightly; 2: dislike very much; 1 = dislike extremely). Value zero was given for absent attribute. During the panel training, panel leader held a discussion on the descriptors and a consensus lexicon was developed about the following nine attributes (GALÁN-SOLDEVILLA and PÉREZ-CACHO, 2012): a) defects (presence of abnormal negative odor/aroma); b) gustatory sensations (salty: typical taste produced by sodium chloride aqueous solutions; bitter: basic taste produced by diluted aqueous solutions of caffeine: acid: basic taste produced by aqueous solutions of substances like citric acid); d) fruity odor (odor/aroma characteristic of fresh olives); e) kinesthetic sensations (firmness: mechanical property of texture related to the strength

required to attain a certain penetration of the olive; fibrous: geometrical property of texture related to the perception of strands oriented in the same direction; crunchy: mechanical property of texture related to the cohesion and strength necessary to break an olive with teeth); f) overall judgment (general grade of appreciation).

2.6. Elaboration data

Each jar was analyzed independently in duplicate (six independent replicates for every sample), by calculating mean and standard deviation. Means obtained were compared through ANOVA (Duncan's multiple range post hoc tests at $p<0.05$) by using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Basic olive pulp characteristics

Processing method strongly affects the final characteristics of table olive (SANCHEZ *et al*., 2006) and the data reported in Table 2 confirm this evidence.
Olive water content was initially 69.4%. Overall, water content in the processed olives was generally higher in the olives treated with NaOH than in the natural-processed (Nat). B1-NaOH and B2-NaOH samples, stored in the brine, kept the original moisture (69.6 and 72.6%, respectively), while the matching B1-Nat and B2-Nat samples showed a lower value (63.7 and 67.2%, respectively). However, the observed differences were not statistically significant. Conversely, significant moisture reduction was observed in the olives stored in the vacuum bags. Specifically, water content was 60.1, 59.0, 53.0 % in the C-Nat, T-NaOH and T-Nat samples, respectively.
An increase of lactic acid and a decrease of pH expected as result of the microbial activity. All the NaOH-treated olives had about one-higher-point of pH than the natural olives. More specifically, pH values were 5.17, 5.39, 5.56 and 5.73 in B1-NaOH, B2-NaOH, C-NaOH and T-NaOH, respectively. These high pH values are potentially harmful being not fit to inhibit growth of spoilage microorganisms. Actually, brine acid characteristics of the NaOH treated samples (Table 2) were below the requirements for trade according to the IOOC (2004) that has a set the minimum brine pH value at 4.1; however, addition of acidity regulators is permitted.
The pH values of Nat olive pulp were 4.62, 4.42, 4.80 and 4.75 in B1-Nat, B2-Nat, C-Nat and T-Nat, respectively. The pH value was obviously related to lactic acid, which was found in the Nat olives more-than-three-times higher than NaOH-treated olives. According to MARSILIO *et al*. (2005), lye treatment and subsequent water washing presumably caused sugar and nutrient loss from olive pulp giving rise to insufficient acidification in the NaOH-treated samples. In the set group of Nat olives, the maximum and minimum values of lactic acid were C-Nat (0.24%) and B2-Nat (0.11%), respectively. These values are in line with literature (DE LEONARDIS *et al*., 2016; APONTE *et al*., 2012).

3.2. Phenolic content

Many studies have evidenced a close relationship between processing method and content/composition of phenols in table olives (AMBRA *et al*., 2017; CHAROENPRASERT and MITCHELL, 2012; BLEKAS *et al*., 2002).
In Table 3 phenolic compound evolution is given only for the Nat olives due to the phenol content in the NaOH-treated olives was negligible.

Table 2. Analytical characteristics of the pulp olive samples and relative canned brine.

	Pulp olive			Brine	
	Moisture %	**pH**	**Lactic acid %**	**pH**	**Lactic acid g/l**
Raw olives	69.4(4.3)^{a,b}	5.67(0.06)^{a}	nd	7.02(0.06)^{a}	n.d.
Stored in the first brine					
B1-NaOH	69.6(5.2)^{a,b}	5.17(0.38)^{b,c,d}	0.02(0.00)^{a}	5.05(0.27)^{b}	0.4(0.0)^{a}
B1-Nat	63.7(5.3)^{b}	4.62(0.34)^{b,c}	0.22(0.02)^{b}	4.26(0.16)^{c}	2.5(0.2)^{b}
Stored in the second brine					
B2-NaOH	72.6(4.2)^{a,b}	5.39(0.41)^{b,c,d}	0.03(0.00)^{a}	5.02(0.25)^{b}	0.5(0.0)^{a}
B2-Nat	67.2(4.1)^{a,b}	4.42(0.26)^{b}	0.11(0.01)^{c}	4.10(0.22)^{c}	2.5(0.2)^{b}
Stored in vacuum bags at 6°C					
C-NaOH	69.1(3.8)^{a,b}	5.56(0.29)^{c,d}	0.07(0.03)^{a}		
C-Nat	60.1(3.3)^{b}	4.80(0.31)^{b,c,d}	0.24(0.02)^{b}		
Stored in vacuum bags at 20°C					
T-NaOH	59.0(3.6)^{b}	5.73(0.29)^{d}	0.03(0.00)^{a}		
T-Nat	53.0(3.2)^{b}	4.75(0.26)^{b,c,d}	0.19(0.03)^{b}		

Values are means (standard deviation) of six independent replicates; letters on the column point out significant difference at $p<0.05$.
-NaOH: lye processed; -Nat: natural processed; n.d. = not detected.

Table 3. Total phenol and hydroxytyrosol (Hy) compounds obtained in pulp olives and related brine (natural olives).

	Pulp olives		Brine
	Total phenols % GAE	**Hy-compounds mg/100g as HyE**	**Total phenols mg/L GAE**
Raw olives	1.19(0.08)^{a}	199(13)^{a}	
B1-Nat	0.21(0.01)^{b}	53(4)^{b}	1,791(102)^{a}
B2-Nat	0.14(0.01)^{b}	25(1)^{c}	1,006(132)^{b}
C-Nat	0.20(0.01)^{b}	47(3)^{b}	
T-Nat	0.20(0.01)^{b}	62(4)^{b}	

Values are means (standard deviation) of six independent replicates and different letters on the column point out significant difference ($p<0.05$).

Specifically, Nat olive samples showed total phenol values dropped from 1.19 (raw olives) to about 0.20 g/100g (Nat processed olives) as GAE (gallic acid equivalent), respectively. Although the reduction of the starting phenol content is a coveted result from an organoleptic point of view, presence of residual phenols may be positive from a nutritional point of view, since several phenols are recognized as antioxidant and healthy substances (D'ANTUONO *et al*., 2016; DE LEONARDIS *et al*., 2013; DE LEONARDIS *et al*., 2008; SANCHEZ *et al*., 2006).
At the end of the first brining (B1-Nat), Hy-compound content was 53 mg per 100 g olives and insignificant changes were observed in C-Nat (47 mg/100 g) and T-Nat (62 mg/100g); conversely, Hy-compounds halved during the second brining (B2-Nat, 25 mg/100g).

The European Union Commission (2012) recently approved a health claim for extra virgin olive oil stating that 'daily intake of 5 mg of hydroxytyrosol and its derivatives is able to prevent low density lipoprotein (LDL) oxidation'. Therefore, Nat olives may be claimed as a source of phenols having health-promoting properties. However, the Hy-compound values reported in Table 3 were certainly underestimated because other hydroxytyrosol linked compounds, known to be present in olives (AMBRA *et al.*, 2017; CHAROENPRASERT and MITCHELL, 2012), have been omitted in this study for purposes of simplification of the model.
According some studies (RAMIREZ *et al.*, 2017a; Ramirez *et al.*, 2016; DE LEONARDIS *et al.*, 2015), biological and enzymatic oleuropein depletion may occur through a rapid transformation in Hy-EDA and subsequent hydrolysis with gradual Hy release. A similar pathway occurred also in the Nat olives, as noted in the graph of Fig. 1.

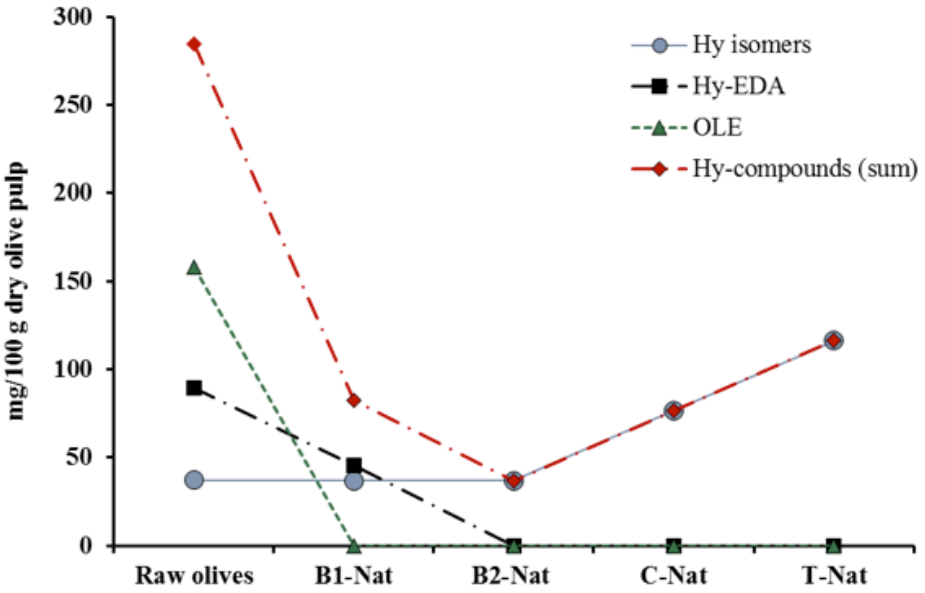

Figure 1. Changes in the table olives of the monitored phenolic compounds, expressed in mg (Hy equivalent) per 100 g dry olive pulp.

Raw olive pulp contained 37, 89 and 158 mg/100 g in dry matter (d.m.) of Hy, Hy-EDA and OLE, respectively. After 21 days of brining (when the first brine was replaced, see Material and Methods), the olive phenol profile was changed in 73, 167 and 22 mg/100 g d.m. of Hy, Hy-EDA and OLE, respectively (data not shown). Hy-EDA is a recognized antimicrobial compound inhibiting the growth of lactic bacteria (MEDINA *et al.*, 2007). After 9 of months brining (B1-Nat), OLE was completely depleted, while Hy-EDA was 45 mg/100 g d.m. Successively, Hy-EDA was undetectable in sample B2-Nat, C-Nat and T-Nat in which only Hy was found in the amounts of 37, 77 and 116 mg/100 g d.m., respectively.

3.3. Lipid fraction

The characteristics of lipid fraction of the raw olives and samples stored for 18 months are given in Table 4.

Table 4. Changes of lipid fraction of olives samples.

	Raw olives	NaOH-processed olives			Nat-processed olives		
		B2-NaOH	C-NaOH	T-NaOH	B2-Nat	C-Nat	T-NAT
Total oil % w.m.	18.4 (1.1)^{a}	19.5 (1.2)^{a}	19.3 (1.2)^{a}	17.5 (1.1)^{a}	22.1 (1.3)^{b}	22.2 (1.3)^{b}	21.6 (1.3)^{a,b}
Total oil % d.m.	26.3 (1.6)^{a}	26.8 (1.6)^{a}	28.2 (1.7)^{a}	29.3 (1.8)^{a}	33.0 (2.2)^{b}	36.5 (2.2)^{c}	40.5 (2.5)^{d}
Free acidity %	0.5 (0.0)^{a}	11.2 (0.7)^{b}	15.3 (0.9)^{c}	13.7 (0.8)^{d}	2.8 (0.2)^{e}	5.5 (0.3)^{f,a}	3.4 (0.2)^{e}
p-Anisidine value	5.9(0.3)^{a}	33.5(1.7)^{e}	30.7(1.6)^{d}	31.6(1.7)^{d,e}	12.8(0.7)^{b}	29.2(1.5)^{d}	23.1(1.2)^{c}
Fatty acids %							
C16:0	16.5 (1.0)^{a}	23.7 (1.4)^{b,c}	24.4 (1.5)^{b}	21.6 (1.3)^{c,d}	17.0 (1.0)^{a}	19.9 (1.2)^{d}	18.2 (1.1)^{a,d}
C16:1	1.4 (0.1)	1.4 (0.1)	1.3 (0.1)	1.2 (0.0)	1.3 (0.1)	1.4 (0.2)	1.3 (0.1)
C18:0	2.9 (0.2)^{a}	4.2 (0.3)^{b}	4.6 (0.3)^{b}	4.5 (0.3)^{b}	3.4 (0.2)^{c}	3.9 (0.3)^{b,c}	3.8 (0.2)^{c}
C18:1	65.5 (4.0)^{a}	60.5 (3.7)^{a,b}	57.1 (3.5)^{b}	60.0 (3.6)^{a,b}	64.5 (3.9)^{a}	63.0 (3.8)^{a,b}	64.3 (2.7)^{a,b}
C18:2	11.6 (0.7)^{a}	4.2 (0.3)^{b}	3.2 (0.2)^{c}	5.1 (0.2)^{d}	11.3 (0.5)^{a}	8.2 (0.3)^{e}	9.3 (0.3)^{f}
C18:3	0.6 (0.0)^{a}	0.1 (0.0)^{b}	0.1 (0.0)^{b}	0.2 (0.0)^{b}	0.6 (0.1)^{a}	0.3 (0.1)^{a}	0.4 (0.1)^{a}
C20:0	0.5 (0.0)^{a}	0.7 (0.0)^{b}	0.8 (0.0)^{b}	0.7 (0.0)^{b}	0.5 (0.0)^{a}	0.6 (0.0)^{a}	0.6 (0.0)^{a}
C20:1	0.2 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.2 (0.0)	0.2 (0.0)
C22:0	0.1 (0.0)^{a}	2.9 (0.7)^{b}	4.8 (0.2)^{b}	3.8 (0.6)^{b}	0.3 (0.0)^{c}	1.2 (0.5)^{c}	0.7 (0.3)^{c}
C22:1	0.0^{a}	1.5 (0.6)^{b}	2.7 (0.2)^{b}	1.9 (0.8)^{b}	0.1 (0.0)^{a}	0.7 (0.2)^{c}	0.3 (0.3)^{c}

Values are means (standard deviation) of six independent replicates and different letters on the lines point out a significant difference ($p<0.05$).

A different oil content was observed between NaOH- and Nat-processed olives. Apparently, total oil content increased in the Nat-olives, especially considering total oil on dry matter. TAMER *et al*. (2013) have found that alkali treatment caused oil loss from the olives. Moreover, the advanced fermentation status (with large sugar consumption) and the reduced lipolytic activity (with low fat consumption) could explain the apparent increase of oil in the Nat-olives (Table 4). Unfortunately, we did not have enough data to establish a possible link between oil increase and microbial counts and types (Table 5). According to what was observed in previous studies (PASQUALONE *et al*., 2014; LOPEZ *et al*., 2011), a higher hydrolytic and oxidative oil degradation was observed in the NaOH-*vs* Nat-olives. Specifically, a considerable increase of free acidity was evident in B2-NaOH, C-NaOH and T-NaOH with values of 11.2, 15.3 and 13.7% on oil extracted, respectively; conversely, free acidity was less than 5.5% for the Nat-olives. Formation of free fatty acids could be catalysed by the lipases contained in the fruits or synthesized by the environmental microflora. A positive effect of low temperature on the triacylglycerol hydrolysis was evident due to the high free acidity values found in C-NaOH (15.3%) and C-Nat (5.5%). Similarly, oil oxidation was higher in the cold temperature stored olives, as the *p*-anisidine values show (Table 4). As known, *p*-anisidine assay measures secondary lipid oxidation compounds; the highest *p*-anisidine values were obtained in the oils extracted from the B2-NaOH, C-NaOH, T-NaOH and C-Nat olives.

A fatty acid composition of raw olives (Table 4) was in agreement with the literature (MALHEIRO *et al*., 2012). Oleic acid was the main fatty acid (65.5%), followed by palmitic acid (16.5%) and linoleic acid (11.6%). In order to make Table 4 more readable, the C17:0,

C17:1 and C24:0 fatty acids (found in equal or less amount 0.1%) were deliberately omitted; however, these fatty acids did not change during the storage of the olives.
Significant modifications of fatty acid composition occurred especially in the NaOH-olives, specifically, a reduction of linoleic and linolenic acid was registered in the B2-NaOH, C-NaOH, T-NaOH samples; in the same samples a decrease of oleic acid and an increase of palmitic and stearic acid were obtained. It is known that polyunsaturated fatty acids were more affected by oxidation and therefore decreased at a greater extent (CAPONIO *et al*., 2003; DE LEONARDIS and MACCIOLA, 2012). In the Nat-olives, especially in B2-Nat and T-Nat, the olive residual phenols have limited oil oxidation, as evidenced by the minor changes of *p*-anisidine and fatty acid profile. Unexpectedly, significant increase of the peaks corresponding to behenic (C22:0) and erucic (C22:1) fatty acids were found in the lipid fraction of stored olives. Generally, these long chain fatty acids are present in olive oil in amounts lower than 0.2%, as the data of raw olive oil shows (Table 4). In the NaOH treated olives, C22:0 and C22:1 were found in amounts higher than 2.9 and 1.5%, respectively. In particular, lipid fraction of the C-NaOH sample showed the highest content (C22:0 = 4.8%; C22:1 = 2.7%). Conversely, in the Nat-olives percentage of C22:0 and C22:1 was lower than NaOH olive oil counterpart, but higher than raw olive oil. We have no explanation for this and similar results are missing in literature. Perhaps, it is reasonable to hypothesise a microbial origin of C22:0 and C22:1 in the olives, by considering that few oleaginous microorganisms are able to synthesize long chain fatty acids (EL BIALY *et al*., 2011; RATLEDGE, 2004).

3.4. Sensory analysis

Graphical presentations of the obtained sensory evaluation (average values) are shown in Fig. 2.
The panel did not evaluate color; however, NaOH-olives were overall lightly yellow-green, while Nat-olives were dark gray-green. In general, sensory profile of NaOH-olives was very different from that of Nat-olives; these differences were amplified during the second storage. In general, 'overall opinion' was more positive for Nat- than NaOH- olive samples. The B1-NaOH obtained an 'overall opinion' of 4 points convincing us to continue the storage work for this sample despite its high pH (Table 2). However, at the end of study, B2-NaOH and T-NaOH showed perceptible sensory defects and loss of firmness, fibrous and crunchy, whereas C-NaOH was not evaluated due to its pronounced smell of rotten caused by an abnormal fermentation. In addition, salty was the prevailing, if not unique, taste in the tasted NaOH olives. Conversely, taste of Nat-olives was more complex and well-balanced between salty, bitter and acid tastes. Salty in the brined B1-Nat and B2-Nat was imperceptible, while bitter taste was perceived clearly in all Nat-olives. Effectively, bitter taste is a distinctive but pleasant flavor of the natural-style olives (LANZA and AMORUSO, 2016). The intensity of the bitter taste remained essentially unchanged between the B1-Nat, C-Nat and T-Nat samples, while it decreased in B2-Nat. Finally, a positive fruit odor was perceived clearly up to 18 storage months only in the Nat-olives.

3.5. Microbiological analysis

Fermentation of both NaOH- and Nat-olives occurred spontaneously without adding any starter culture. The microbial count of raw olives highlighted presence of yeasts (log 7.3 CFU/g), LAB (log 2.0 CFU/g) and *Enterobacteriaceae* (log 2.0 CFU/g).
Heperkan (2013) reports that microbiota of olives include principally yeasts and lactic acid bacteria (LAB), members of *Enterobacteriaceae*, *Clostridium*, *Pseudomonas*, *Staphylococcus*, and

occasionally moulds. The processing method impacts microbial dynamics affecting greatly quality and shelf life of the TO (DE ANGELIS *et al.*, 2015). Generally, indirectly brined green olives yeasts become the predominant population; however, a correct adding of LAB starter may improve lactic fermentation performance (CAMPUS *et al.*, 2017; DE LEONARDIS *et al.*, 2016; PERRICONE *et al.*, 2013; CORSETTI *et al.*, 2012).

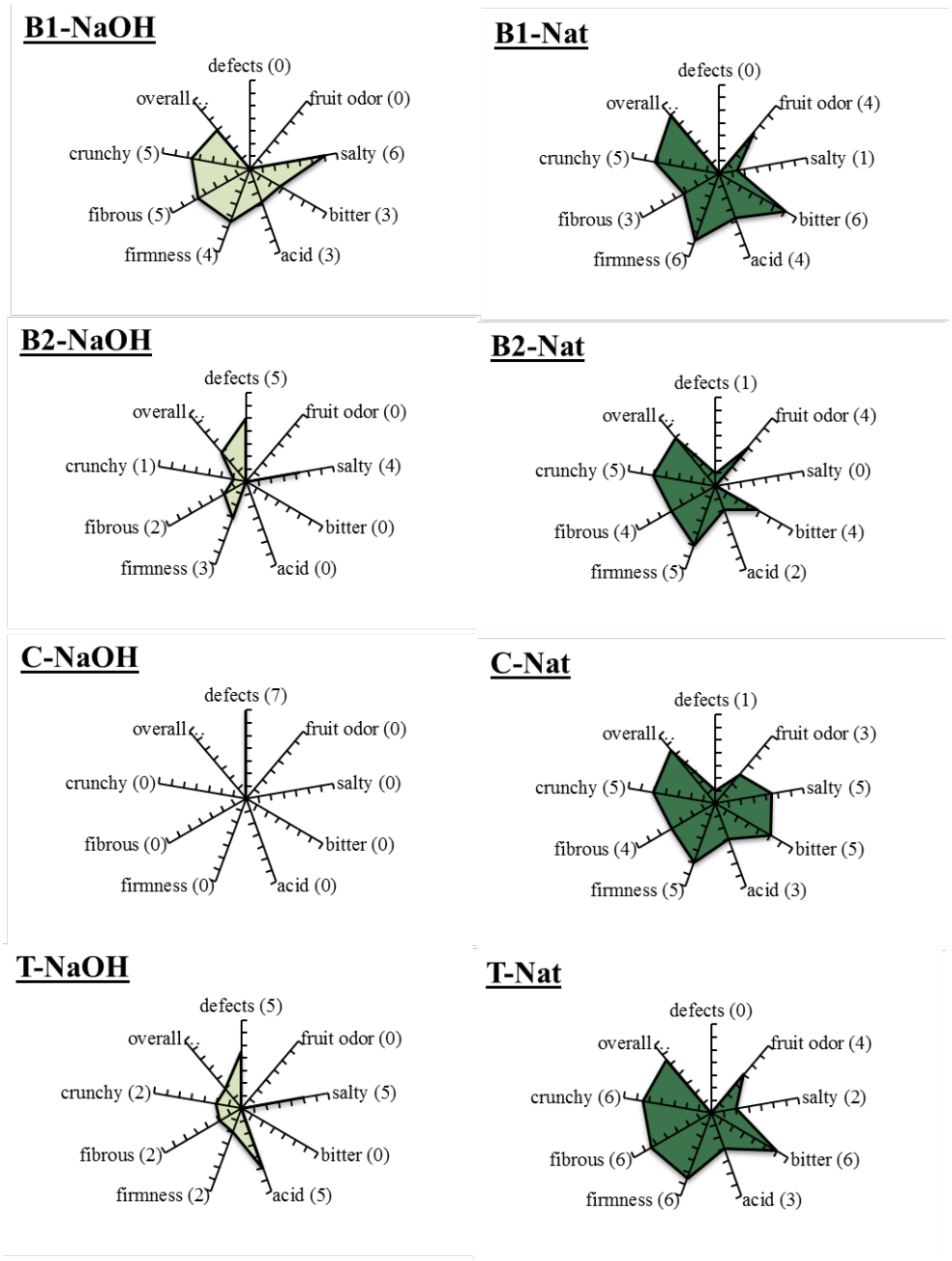

Figure 2. Sensory profile rings of the table olives.

The mean of microbial count and standard deviation of the microorganisms searched in the olive pulp are given in Table 5. The most relevant result was absence of detectable microorganisms in B1-Nat and B2-Nat, apart from yeast cells in B1-Nat (7.1 log CFU/g);

conversely, in all other samples LAB and yeasts coexisted until the end of study (Table 5). In addition, neither *Enterobacteriaceae* nor *Pseudomonas* spp. were found in B2-Nat, C-Nat e T-Nat. Therefore, low pH (Table 2) and high phenol level (Table 3) influenced the microbial profile of B1-Nat affecting positively the subsequent storage of the Nat-olives. Generally, the *Enterobacteriaceae* spp., eliminated during fermentation, are not detected at the end of the process (HEPERKAN, 2013). Nevertheless, *Enterobacteriaceae* cells were found in all NaOH-olives ranging from 3.3 to 4.8 log CFU/g (Table 5). Certainly, in these samples, high pH values and lack of phenols have favoured the development of *Enterobacteriaceae* and *Pseudomonas* spp., which have contributed to the spoilage of B2-NaOH, C-NaOH and T-NaOH samples. Moreover, in C-NaOH sample, *Enterobacteriaceae* and *Pseudomonas* spp. were not inhibited at cold temperature. Indeed, low temperature storage has penalized the populations of yeast and LAB favouring the growth of psychrophilic bacteria, which caused lipolysis, oil oxidation (Table 4), pectolytic action and, putrefaction (Fig. 2). Moreover, also for the Nat-olives, the conservation at cold temperature was proven to be less effective than that in brine and under temperate conditions.

Table 5. Cell count (log CFU/g) of microorganisms determined on pulp olives.

	Media					
	YPD	**MRS**	**PCA (at 30°C)**	**PCA (at 6°C)**	**VRBGA**	**PCFC**
NaOH processed olives						
B1-NaOH	6.20 (0.10)^{a}	4.90 (0.10)^{a}	6.10 (0.20)^{a}	6.13 (0.12)^{a}	3.50 (0.20)^{a}	4.10 (0.20)^{a}
B2-NaOH	5.47 (0.15)^{c}	4.00 (0.20)^{b}	5.00 (0.10)^{b,c}	4.90 (0.20)^{b}	4.80 (0.20)^{b}	0.00 (0.00)
C-NaOH	6.13 (0.15)^{a}	5.47 (0.15)^{c}	6.10 (0.30)^{a}	5.07 (0.15)^{b}	3.30 (0.20)^{a}	3.80 (0.20)^{b}
T-NaOH	5.90 (0.20)^{a}	4.67 (0.25)^{a}	5.70 (0.20)^{d}	5.60 (0.20)^{d}	4.03 (0.15)^{c}	4.60 (0.30)^{c}
Nat processed olives						
B1-Nat	7.10 (0.20)^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
B2-Nat	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C-Nat	4.60 (0.10)^{d}	4.83 (0.59)^{a}	4.70 (0.10)^{c}	4.00 (0.10)^{c}	n.d.	n.d.
T-Nat	5.17 (0.35)^{c}	4.50 (0.40)^{a}	5.20 (0.40)^{b}	n.d.	n.d.	n.d.

Values are means (standard deviation) of three independent replicates; letters on the column point out significant difference at $p<0.05$.
n.d. = not detected.

4. CONCLUSIONS

After 18 months of storage, natural green olives showed good nutritional features (hydroxytyrosol, unchanged fatty acid profile), organoleptic identity, microbial safety, low oil hydrolysis and oxidation. Conversely, after 9 months of storage, pH values below the requirements for the trade were obtained in the NaOH-treated olives. Natural olives preserved high total phenols (0.2 g/100 g wet pulp) and a significant level of the Hy-compounds determined in this study. It is reasonable to suppose that residual phenols have influenced positively polyunsaturated fatty acid preservation and, together with the low pH level, have inhibited the growth of *Enterobacteriaceae* and *Pseudomonas* spp.
Therefore, the slow olive debittering of natural olives was counterbalanced by a prolonged shelf life. The packaging in 6% NaCl renewed brine or in vacuum bag, under a storing

temperature of 20°C, gave the best results, while conservation at cold temperature proved to favor the growth of psychrophilic spoilage bacteria.

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