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Changes in chemical and microbiological parameters of some varieties of Sicily olives during natural fermentation

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RESUMEN

Variaciones de los parámetros químicos y microbiológicos durante la fermentación natural en salmuera de aceitunas de variedades sicilianas

Los cambios y las características de algunas variedades de aceitunas han sido examinados durante la fermentación natural. Las variedades estudiadas fueron las típicas de Sicilia. Las determinaciones carpológicas revelan una buena calidad de los frutos. Las características químicas (azúcares, acidez, fenoles y materia seca) han sido estudiadas. El cloruro de sodio en la salmuera se aumentó gradualmente del 5% al 6% después de 60-75 días y hasta 7% después de 90 días. Las aceitunas fueron colocadas en la salmuera acidificada a pH 4. Una evolución uniforme del pH se observó para todas las aceitunas, alcanzándose un pH de equilibrio inferior a 4,5 unidades. La evolución de la acidez en las salmueras estuvo de acuerdo con la evolución del pH. La concentración de los polifenoles en la pulpa de las aceitunas se determinó durante el período de fermentación. En las aceitunas "Tonda iblea", "Nocellara etnea" y "Moresca" la disminución de los polifenoles ha sido más rápida. La desaparición de la oleuropeina ha sido seguida midiendo la concentración de hidroxitirosol en las salmueras. Durante el período de fermentación han sido medidas las concentraciones de bacterias lácticas, bacterias mesófilas, levaduras y mohos.

PALABRAS-CLAVE: Aceitunas de mesa - Acidez - Desarrollo microbiológico - Fenoles - Salmuera.

SUMMARY

Changes in chemical and microbiological parameters of some varieties of Sicily olives during natural fermentation

The changes and features of some olive cultivars during natural fermentation in brine were examined. The cultivars were typical of Sicily: "Nocellara messinese", "Nocellara etnea", "Moresca", "Ogliarola" and "Tonda iblea". The carpological data revealed their good quality. Chemical characteristics such as reducing sugars, titratable acidity, total phenols and dry matter were also investigated. The physical and chemical changes of olives and brines throughout the process time were monitored. Sodium chloride concentration was gradually increased from 5% (w/v) to 6% after 60-75 days and 7% after 90. The olives were put in brine acidified up to pH = 4. The salt concentration and pH value were necessary to favor lactic fermentation. An uniform trend of pH was measured for all the olive cultivars: a stable pH under 4.5 value was measured. The course of titratable acidity measured in fermentation brines was in agreement with the pH trend. The concentration of total phenols in flesh olives through the brine storage and fermentation is reported and the reduction of total phenols during the brine fermentation was fast in "Tonda iblea", "Nocellara etnea" and "Moresca" olives. To measure the degree of oleuropein disappearance, the course of hydroxytyrosol in brine was monitored. Throughout the brining period, the growth of microbial populations was revealed on selective media: lactic acid bacteria, mesophilic microorganisms, yeasts and moulds.

KEY-WORDS: Acidity - Brining - Microbial growth - Phenols - Table olives.

1. INTRODUCTION

Olive fruit is an important product in Mediterranean agriculture. With some exception, southern Italy olives are normally used for oil production. Traditionally only in some areas, the olive fruit is used to prepare a valuable foodstuff. Sicily is an important olive producing area, and a considerable amount of these fruits is used in table olive preparations. The natural style is one of the preferred methods by which olives are processed in Sicily; this preparation is also called untreated table olives. Olives can be harvested as green or pigmented ripening and placed in brine at a variable salt concentration from 8 to 12%. The production of untreated table olives is a spontaneous, traditional acid fermentation based on an empirical process that relies upon microorganisms present in the raw material and in the processing environment. In homemade preparations, the fermentation is not well controlled and only salt concentration is responsible for the safety and correct preparation of it.

Several factors can affect the growth of native microflora. Sodium chloride concentration and pH of the brine are the main control parameters acting during fermentation (Garrido Fernandez *et al.,* 1997). The polyphenols in fruit inhibit Lactic Acid Bacteria (LAB) growth, the presence of oleuropein and related compounds posses a specific inhibitory effect (Juven and Henis, 1970; Ruiz Barba *et al*., 1990; 1993).

Spontaneous olive fermentation has shown some problems concerning the acidity of the medium: the pH value of brine is always quite

higher than safety limits (4.5) with serious risks that may occur. The growth of LAB in table olive preparation is aided by mainly a correct pH at low value, good hygiene, and proper salt concentration. So LAB provide the amount of lactic acid needed for preservation. In some cases, acid is not produced in adequate amounts for the preservation of olives, and spoilage occurs through subsequent contamination by other microorganisms. A lot of literature is available on Spanish-style green olives applied to different cultivars, but only scarce references are present for a natural style of processing. The natural fermentation, as previously reported, is particularly influenced by the characteristics of the olive cultivar, so investigation on different olive productions is necessary. Some researchers have studied particular and typical productions. Piga et *al*., (2001) reported problems with the quality improvement of natural style green table olives of Sardinian cultivars. Other researchers reported observations on the natural fermentation in brine of the "Aloreña" cultivar (García Garcia *et al*., 1992), they observed the changes in the main physico-chemical characteristics of brines and the evolution of microbial population of *Lactobacillus plantarum*, *Pediococcus* spp. and yeasts.

In this study the nature of fermentation of the more important typical Sicilian olives was observed ("Nocellara messinese", "Nocellara etnea", "Moresca", "Ogliarola" and "Tonda iblea") with respect to table olive production. Chemical and microbiological parameters were measured during the process with the main objective of evaluating the availability of the olives for processing.

2. MATERIALS AND METHODS

2.1. Plant material

The following olive cultivars were used for the experiments: "Nocellara Messinese", "Nocellara etnea", "Tonda Iblea", "Moresca" and "Ogliarola". The first two cultivars were collected in a green stage, while the others at a full pigmented with the typical black color for "Moresca" and "Ogliarola" and intense brown for "Tonda iblea". Fruits were harvested between the end of September and the first week of November, at a maturity stage suitable for processing, and immediately transported to the laboratory, where only fruits without or with minimal peel defects were selected. Calibration by weight was performed in order to have uniform fruit callipers.

Carpological analyses were carried out on 50 fruits randomly sampled from the entire amount.

2.2. Olive processing

After washing with tap water, the olives were put into 35 litre plastic containers (three fermentation vessels for each thesis) filled with freshly prepared 5% NaCl brine and acidified with 0.5 mL/L lactic acid (90%, Fluka) up to pH 4.0. Olives were brined with a fruit/brine ratio of 1. During the period of fermentation, salt concentration was gradually increased up to 7% (w/v) with salt powder. The olives were maintained at ambient temperature.

2.3. Chemical analyses

The pH and free acidity of brine samples were measured using a Metrhom pHmeter. Free acidity was determined by titring up to pH 8 with 0.1 N NaOH and expressed as mmol/L of brine.

Chloride content in brine was measured by titration with $AgNO_3$.

Total polyphenols were extracted from olive flesh after blending of 50 g at temperature near 0°C (ice bath). The extraction method was that reported by Amiot *et al*. (1986) and measured spectrophotometrically at 725 nm after reaction with the Folin-Ciocalteu's reagent, and expressed as mg/kg of gallic acid by means of a calibration plot using pure gallic acid as standard at different concentrations.

Reducing sugars of starting material were determined by titration of Fehling reagents. The dry matter content was assessed by oven drying at 105°C.

An amount of olive pulp (5 g) was extracted with water and free acidity was obtained by titration with NaOH and phenolphthalein as indicator.

HPLC analyses of phenol fraction of the brine were achieved by directly injecting the brine in the chromatographic system. The HPLC apparatus consisted of a Waters 625 pump, and a Waters 486 spectrophotometer detector set at 280 nm. A Nova-Pak C18 column (4 μm, 300 x 3.9 mm) was used. Separation was achieved by elution gradient using an initial composition of 95% of A solution (water acidified with 2% acetic acid) and 5% of B solution (methanol). After 10 min of isocratic conditions, the concentration of B solution was increased to 20% in 10 min and to 50% in another 20 min. A flux of 0.6 cm^3/min was used.

The phenolic compounds were identified by comparing retention time with pure oleuropein (Extrasynthese) and hydroxytyrosol obtained by acid hydrolysis of oleuropein. The response factor of hydroxytyrosol was considered the same of tyrosol.

All the analyses were performed in triplicate for each sample.

2.4. Microbiological analyses

During fermentation, the enumeration of different microbial populations in brine using selective media was effected.

The viable mesophilic counts in brine were estimated on Plate Count Agar Standard (Oxoid) incubated at 32°C for 48 h.

The lactic acid bacteria amount was estimated on MRS Agar (Oxoid) added with 50 mg/L of Nystatin (Sigma) at 32°C for 48 h in anaerobic condition.

The population of yeasts and moulds was estimated on Glucose Chloramphenicol Agar (YGC, BioMérieux) at 25°C for 48 h.

The analyses were done in triplicate and the plates were subjected to microbiological numbering by CFU counting.

2.5. Data analyses

Statistica software was used for data processing. One-way analysis of variance was used to test the effects of cultivar on the measured factors. Duncan's multiple range test was used to compare means when a significant variation was highlighted by analysis of variance.

3. RESULTS AND DISCUSSION

3.1. Carpological and composition characteristics of the olive cultivar

The carpological parameters of the olives are reported in table 1. Carpological data reveal that cultivars are suitable for processing as table olives. Only "Ogliarola" was classified, according to the IOOC (2000), as medium weight fruits (from 2 to 4 g). "Nocellara etnea", "Nocellara messinese", "Moresca" and "Tonda iblea" were high weight fruits (from 4 to 6 g), and "Moresca" was more than 7 g of weight. Other technological parameters, such as the flesh to pit ratio, were quite significant for all of them. Based on the classification proposed by Brighigna (1998), all the cultivars studied had a flesh/pit > 5 (flesh percentage 84-85%) and so considered very good for table olives. Very interesting was "Moresca" which showed a whole weight higher than 7 grams and a flesh to pit ratio higher than 7, so a high quantity of edible part (88%) was considered.

The data of the chemical characteristics of the olives are given in table 2. Sugars are the most important components with respect to the olive fermentation process, the availability of this fermentable substrate is a requirement reported by Garrido Fernandez *et al*. (1997) for good fermentation. In the tested olives of "Nocellara etnea", the reducing sugars were higher than 5%, in the other samples these components were lower than 4%, with the lowest measured in "Ogliarola" (3%). Titrable acidity of the pulp, mainly represented by organic acids, is important because of the buffering capacity of these components during fermentation. "Moresca" showed the highest amount (75 mmol/kg), while "Tonda iblea" the smallest one. The phenol components, reported as total phenols and measured by means of the Folin-Ciocaleu's reagent, ranged from 4183 mg/kg in "Moresca" flesh to 6323 of the "Ogliarola". This low amount could be explained by the full ripening for "Moresca" and "Tonda iblea". "Ogliarola" olives were collected at a stage of complete pigmentation, but a high quantity of total phenol substances was measured. In addition, dry matter content was not explained by the ripening stage of the olives.

3.2. Evolution of the brining process

When olives are placed in brine, they were subjected to physical and chemical changes that

Table 1
Carpological characteristics of olive fruits. Mean ± Standard Deviation

	lenght (mm)	diameter (mm)	fruit (g)	stone (g)	flesh (g)	flesh/pit
Nocellara etnea	27,54 ± 2,50	19,62 ± 1,70	5,70 ± 0,76	0,86 ± 0,20	4,84 ± 0,68	5,82 ± 1,16
Nocellara messinese	25,10 ± 2,35	19,59 ± 1,73	5,46 ± 1,26	0,87 ± 0,16	4,59 ± 1,17	5,33 ± 1,43
Moresca	27,89 ± 1,70	22,35 ± 1,11	7,54 ± 0,97	0,94 ± 0,16	6,60 ± 0,85	7,10 ± 0,83
Ogliarola	21,51 ± 2,32	16,53 ± 1,28	3,77 ± 1,25	0,55 ± 0,18	3,22 ± 1,10	5,91 ± 1,12
Tonda iblea	26,89 ± 1,88	20,37 ± 1,51	6,09 ± 1,03	0,91 ± 0,18	5,18 ± 0,94	5,82 ± 1,25

Table 2
Compositional characteristics of starting olive fruits

	reducing sugars (%)	titrable acidity (mmol/kg of flesh)	total polyphenols (mg of gallic acid/kg of flesh)	dry matter (%)
Nocellara etnea	5,13	65,69	5291	29,12
Nocellara messinese	3,98	64,15	6005	40,8
Moresca	3,47	75,65	4183	23,05
Ogliarola	3	66,52	6323	46,06
Tonda iblea	3,88	47,13	4323	21,9

modified the fruits and their brine solution. The water soluble components are withdrawn from olive flesh due to diffusion of these components into brine. This process is influenced by the salt concentration. The sugars diffused in brine are being used by microorganisms and converted to organic acids. Changes in chemical components are related to concomitant microbiological populations developed during spontaneous fermentation.

In figure 1, the salt concentration in brines during fermentation is reported. From this the initial concentration decrease was observed, it is a result of the equilibrium between olives and brine. Subsequent salt additions are necessary. In this study the concentration was gradually increased from 5% (w/v) to 6% after 60-75 days and 7% after 90. The influence of the initial salt content was observed by some researchers (Duran Quintana *et al*., 1999; Leal-Sanchez *et al*., 2003). Lactic acid bacteria, the most important agents of the olive fermentation, are sensitive to salt in brine. So a first period at low salt concentration permits a good growth of them.

The acidity level of brine is of fundamental importance in the process. The olives were put in brine acidified at a pH of 4 to prevent low acidity value at the beginning of the process that easily enhanced the growth of Gram-negative bacteria. These bacteria are responsible for the olives' gas-pocket and putrid fermentation. The initial correction of pH by lactic acid was beneficial in maintaining the preparation in safe conditions. In figure 2, the evolution of the pH in the brines during the fermentation of the olive cultivars is reported. With the exception for the first days in "Ogliarola" and "Tonda iblea" brines, an uniform trend was measured for all the olive cultivars. The two "Nocellara" showed the lower pH level under 4 value. In the fermentation brine of all olive cultivars the pH was always under 4.5 and safe to avoid alterations through the observed period (270 days), but some of them showed a slight increasing trend in the last (after 150 days).

In figure 3, the course of titrable acidity measured in fermentation brines is reported. According to the pH trend, this parameter showed an increase during the observed period in all the fermentations. The "Nocellara messinese" "Ogliarola" and "Tonda iblea" showed the greatest increase at the beginning of the process: all of them reached 75% of the final value before three months of brining. A constant increase through the period was measured in the brine of "Nocellara etnea". "Moresca" showed a decrease in the final time of fermentation, this fact could be related to the pH increase and an acid consuming activity could be realized in the fermentation vessels of this olive cultivar. Despite to the high titrable acidity of the "Moresca" olive starting material, a different buffering ability of olive cultivar could be responsible for the acidic character behaviour of the brines. Some unusual trends were observed for "Moresca" from 180 to 240 days. This could be due to the buffer capability of the acidic pool in brine. "Ogliarola" and "Tonda iblea" showed a pH increase after 2 days; this was reported at a level lower than 4.5 by lactic acid addition.

The concentration of total phenols in flesh through the brine storage and fermentation is shown in table 3. Normally equilibrium of polyphenol concentration between brine and flesh

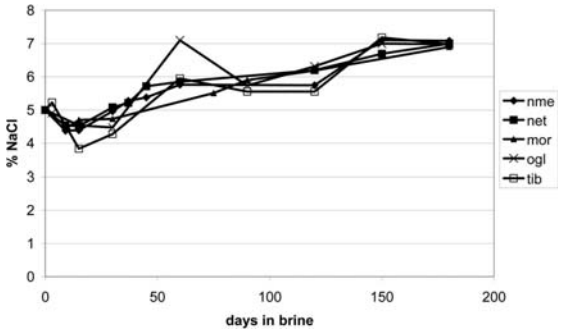

Figure 1
Salt concentration (%) in brines during fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

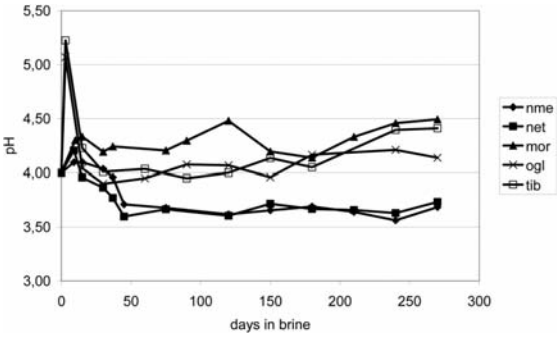

Figure 2
Changes in pH value in brine during natural fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

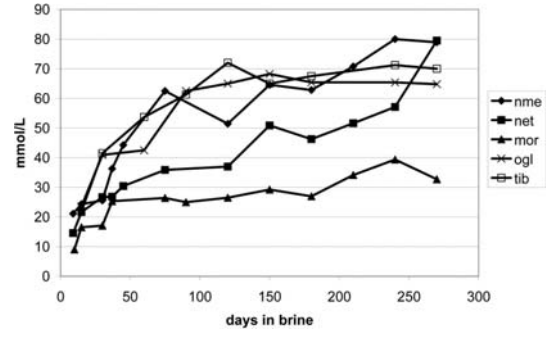

Figure 3
Changes in free acidity in brine (mmol/L) during natural fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

Table 3
Total phenol content in flesh olives (reported as mg of gallic acid/ kg of flesh) and percentage depletion respecting the initial content during the brining period. Percentage reduction within each cultivar followed by different letters differ significantly at P ≤ 0.05 according to Duncan's multiple range test. n.s.: not significant

days	Nocellara etnea		Nocellara messinese		Moresca		Ogliarola		Tonda iblea	
	mean	% respect initial	mean	% respect initial	mean	% respect initial	mean	% respect initial	mean	% respect initial
0	5291	100,00	6005	100,00	4183	100,00	6323	100,00	4323	100,00
15	4027	76,11 b	5429	90,41 a	3280	78,42 ab	5641	89,21 a	2898	67,05 a
30	3409	64,44 b	4878	81,23 a	3193	76,33 ab	5149	81,44 a	2660	61,54 a
120	3616	68,35 ab	3669	61,11 b	2567	61,37 b	4618	73,03 a	1996	46,17 b
180	3382	63,92 a	3539	58,95 ab	2296	54,91 ab	3465	54,8 ab	1966	45,48 b
270	3152	59,58 n.s.	2937	48,91 n.s.	1872	44,75 n.s.	2916	46,13 n.s.	1892	43,78 n.s.

was reached at third – fourth month and depended on the salt concentration of the medium. Diffusion of phenolic compounds into the brine is closely related to the permeability of the olive skin. The cultivar characteristic is important in fermentation for amount and type of phenols and their ability to diffuse outside the fruit. Among the olives studied, it is possible to observe a different behaviour in them. In table 3 the standardized reduction of total phenols during fermentation in brine is also reported. "Tonda iblea", "Nocellara etnea" and "Moresca" olives experienced a significant decline in the first phases (up to 30 days). At the end of fermentation, all of the olive cultivars took a similar course. The greater speed of phenol diffusion in the first brining phases could be explained by the content of water in the drupe. In fact, "Tonda iblea", "Nocellara etnea" and "Moresca" olives had a content of dry matter lower than 30% while for "Nocellara messinese" and "Ogliarola" it was higher than 40%. The equilibrium could be reached faster due to the high amount of juice inside the drupe. After a long brining period, the percent of total phenols decrease was similar for all the olive cultivars with the exception of "Nocellara etnea". The latter showed a decrease of total phenols of 40% after 270 days in brine, while in the other olive cultivars 50% of phenols was residue in flesh. "Tonda iblea" showed decrease of 50% of the initial phenol content after 120 days unlike the other varieties, which can be observed in table 3. The olives of this cultivar had the lowest content of dry matter and a low consistency of the flesh, these factors could be responsible for a fast diffusion in brine of the phenol substances. "Ogliarola" had the highest amount of dry matter; this olive cultivar showed the slowest decrease of total phenols: after 120 days, only 27% of the initial phenol content had disappeared.

Oleuropein, the main bitter glucoside compound present in olives is an ester of elenolic acid and hydroxytyrosol. It can be enzymatically hydrolyzed and Marsilio and Lanza (1998) reported a two-step pathway for oleuropein metabolism. The first step is the hydrolysis of glycoside linkage by β-glucosidase with formation of oleuropein-aglicone. In the second step, the aglicone is hydrolyzed to elenolic acid and hydroxytyrosol. The last is an unstable compound that diffuses in brine. To measure the degree of oleuropein disappearance, the hydroxytyrosol in brine was monitored: a high amount of hydroxytyrosol was related to a high hydrolytic effect on oleuropein. This compound is oxidable for which it can disappear and in a long period, its marker character is not valid. About to the presence of hydroxytyrosol in brine, the observed olive cultivars manifested particular properties. As can be seen in figure 4, the "Nocellara etnea" brine was at the lowest level of this compound for the whole period of fermentation. After 15 days, hydroxytyrosol was 25 mg/L and it became 155 mg/L after 270 days. The marker compound in the brines of the other cultivars showed a fast increase in the first 150 days. In some cases, as in "Tonda iblea", after that time it decreased. In every sample, after 60 days the product of oleuropein hydrolysis was higher than 150 mg/L and in the brines of "Nocellara messinese" and "Tonda iblea" higher than 200 mg/L.

Figures 5-7 show the growth curves of the microbial populations revealed on selective media. Lactic acid bacteria (LAB) were evaluated on an MRS medium added with nystatin, the related trend is reported in figure 5. The LAB count in "Ogliarola"

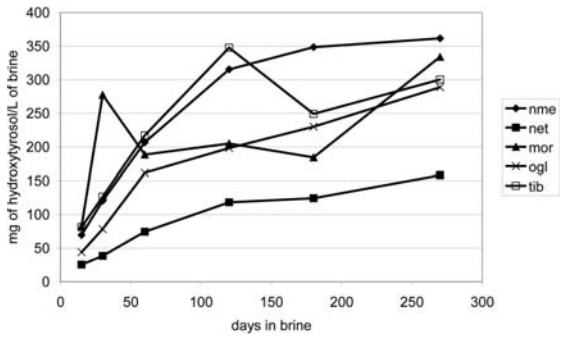

Figure 4
Changes in hydroxytyrosol in brine (mg/L) during natural fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

brines was significantly different from the counts of the other cultivars; this is particularly evident after 120 days when the LAB population tended to completely disappear. In the other cultivars, this microorganisms ranged from a $\log_{10}$ of 6 to 7. The tendency was stable over a long period and after 200 days, they tended to decrease, no statistically significant differences were observed.

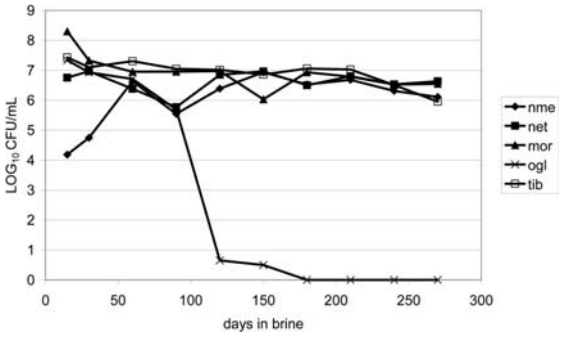

Figure 5
Changes in population of Lactic Acid Bacteria in brine ($\log_{10}$ CFU/mL) during natural fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

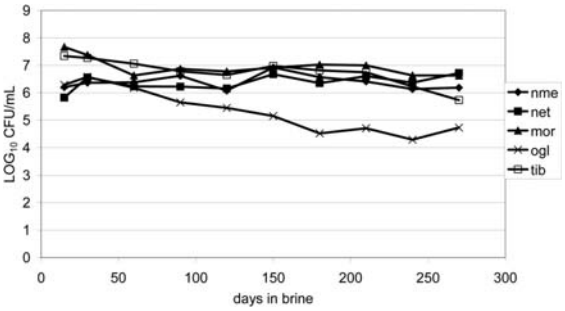

Figure 6
Changes in population of mesophilic bacteria in brine ($\log_{10}$ CFU/mL) during natural fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

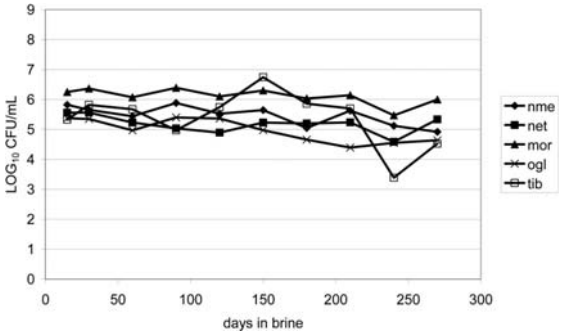

Figure 7
Changes in population of yeasts and moulds in brine ($\log_{10}$ CFU/mL) during natural fermentation of the olive of the cultivars studied. nme: Nocellara messinese; net: Nocellara etnea; mor: Moresca; ogl: Ogliarola; tib: Tonda iblea.

The mesophilic bacteria growth was reported in figure 6. Also with these bacteria, significant differences could be observed. As for lactic population, "Ogliarola" showed a decreasing trend, the mesophilic counts ranged from a count of 6.5 $\log_{10}$ CFU/mL to near 4 $\log_{10}$ CFU/mL after 220 days. "Nocellara messinese" brines showed a constant presence throughout the period. "Moresca" showed the highest counts.

Figure 7 shows the changes in the yeast and mould populations, which grew in all procedures of the experiment. In general, yeasts coexisted with LAB throughout the whole preservation period. Their counts were lower than those of the LAB through the most active fermentation period and their presence was stable. In all cases, a decrease throughout the process was observed, with a tendency toward stabilization and maintenance of a moderate presence. Two behaviors could be seen: "Moresca" brines with the highest count, more or less of 6 $\log_{10}$ CFU/mL, and "Ogliarola" in agreement with the lowest count.

4. CONCLUSIONS

The olive cultivars showed different fermentation behaviours when directly brined. These differences could be considered due to chemical, physical and biological cultivar characteristics. At the end of fermentation, all of the olive cultivars showed suitability for consumption evaluated by untrained table olive consumers.

A safe pH was developed and maintained in the brines of "Nocellara messinese" and "Nocellara etnea", so a proper environment could be created for lactic acid bacteria growth. "Moresca" brines showed the highest amount of mesophilic bacteria population and yeast and mould throughout the brining period so an initial high contamination of resistant microorganisms could be assumed. In all cases, the counts were similar to those measured in other olive cultivars. The sequence of appearance of microbial populations in olive brines might be related to certain characteristics of the cultivar, such as abundance of vitamins and nutrients, high sugar content, which could facilitate the growth of microorganisms. So a more complete knowledge of the olive cultivars is necessary to bring the fermentation to a successful condition.

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