**Effect of post-fermentation storage on Spanish-style green Manzanilla olives**

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**Abstract**

The aim of the present study was to determine the physicochemical, microbiological, and sensory changes during the post-fermentation storage phase of Spanish-style green Manzanilla olives*.* The storage phase (achieved mainly by increasing NaCl up to ~90 g/L) caused: i) an overall significant titratable acidity decrease and combined acidity increase, ii) a degradation of texture of fruits, iii) a considerable decrease in the lactic acid bacteria population, and iv) not significant changes in the yeast population. The later packing of olives improved the colour index of the fruits, but did not prevent texture degradation. The multivariate statistical analysis of their sensory scores segregated inoculated fermented olives from spontaneous process, indicating that the fermentation profiles of olives may persist even after the post-fermentation storage.

*Key words:*Table olive, Lactic acid bacteria, Packaging, Post-fermentation storage, Principal component analysis, Yeasts.

**1. Introduction**

The worldwide table olive production for the 2010/2011 season was approximately 2,440,000 tonnes, with the Spanish-style green table olives as the most common elaboration (IOC, 2013). This process consists of subjecting fruits to a lye treatment (20-35 g/L NaOH), followed by washing with tap water and brining in a ~110 g/L NaCl solution where fruits usually undergo spontaneous lactic acid fermentation.

In green olives, regardless of the type of fermentation (spontaneous or inoculated), after the microbial population reaches a maximum, a later decline phase is usually observed which coincides with a depletion of nutrients and an accumulation of inhibitory compounds. Then, at industrial scale, fermented olives must be preserved while waiting for the consumer’s demand. This post-fermentation bulk storage phase is usually achieved by increasing the salt content from approximately 50 g/L up to 80-90 g/L NaCl, provided that the pH (≤4.3) and titratable acidity (~10 g/L) are appropriate. Thereby, olives are safely stored for long periods of time (Garrido Fernández, Fernández-Díez & Adams, 1997). This salt increase may influence the LAB and yeasts survival during this period. According to Romero-Gil et al. (2013), a salt value above 50 g/L could affect the survival of microorganisms during olive processing (Romero Gil et al. 2013). However, the effect of this phase (characterized by a sharp salt increase) on microbial survival and on the table olive sensorial characteristics has received scarce (if any) attention so far.

 The main goals of this work were to determine, for the first time, the influence of the post-fermentation storage on the: a) physicochemical, b) microbiological (in both cover brines and olive surface), and c) sensory characteristics (after packing) of Spanish-style green table olives. Results from this work may contribute to a better understanding of the effects of the industrial bulk storage phase on olive quality and microbial survival.

**2. Materials and methods**

*2.1. Post-fermentation storage conditions*

Manzanilla fruits, previously fermented (in duplicate) according to the Spanish-style in spontaneous (F1) and inoculated processes with 4 *Lactobacillus pentosus* strains (F2, LAB2; F3, LAB3; F4, LAB4; and F5, LAB5) were used in the present study. The strains were selected because of their multifunctional characteristics (Bautista-Gallego et al., 2013) and ability to form biofilms (Arroyo-López et al., 2012). The fermentative process is described elsewhere (Rodriguez-Gómez et al., 2013).

To mimic the industrial post-fermentation bulk storage of fermented Spanish-style green table olives, 700 g of fermented olives were transferred to plastic containers of 1.3 L capacity and covered with their own fermentation brines (660 mL), increasing the salt concentration in brine up to ~ 90 g/L NaCl. The containers were covered with lids and then stored for 3 months at room temperature which rose progressively from ~17ºC to ~30ºC.

*2.2. Packing of stored olives*

After 3 months of post-fermentation storage, 175 g of olives were introduced into A314 jars (314 mL volume, 75 mm diameter x 103.5 mm high) and covered with 145 mL of new fresh brine. This brine had the adequate concentrations of salt and lactic acid to reach the following equilibrium levels: 50 g/L NaCl and 5 g/L titratable acidity, expressed as lactic acid. The jars were then placed in a cold room for 1 month at 6-8 ºC to obtain the mentioned equilibrium while minimizing any other changes and then the olives were sensory analyzed.

*2.3. Physicochemical analyses*

The analysis of cover brines (pH, salt, titratable and combined acidity) were carried out using the standard methods developed for table olives (Garrido-Fernández et al., 1997). The instrumental surface colour and firmness determinations of fruits were carried out according to methods described elsewhere (Rodríguez Gómez et al., 2013).

*2.4. Microbial analysis*

Brine samples were taken at different times (0, 1, 2, and 3 months) throughout storage and diluted, if necessary, in a sterile saline solution (9 g/L NaCl). Then, they were plated using a Spiral System model dwScientific (Dow Whitley Sci. Ltd., Shirpley, UK) on appropriate media. *Enterobacteriaceae*, LAB and yeasts in brine were selectively grown and counted according to Bautista Gallego et al. (2010). The microorganisms adhered to olive epidermis were determined following the enzymatic protocol developed by Böckelmann, Szewzyk and Grohmann (2003) for the detachment of biofilms.

*2.5. Sensory analysis*

This was conducted in individual booths under controlled conditions of light, temperature and humidity by a panel of 11 members of experienced judges from the staff of the Food Biotechnology Department of Instituto de la Grasa (CSIC, Seville, Spain). Previously, olives were classified according to the Method of Sensory Analysis of Table Olives (IOC, 2010) and then subjected to descriptive analysis, using descriptors corresponding to gustatory sensations (acidity, salty, bitter) and kinaesthetic sensations (hardness, fibrousness, crunchiness). The methodology and panel performance have been described in detail elsewhere (Moreno-Baquero, Bautista-Gallego, Garrido-Fernández & López-López, 2012).

*2.6. Chemometric analysis*

Centred within assessors average scores were subjected to Principal Component Analysis (PCA), using Statistica 7.0 software package (StatSoft Inc, Tulsa, USA)and XLSTAT for excel.

**3. Results and discussion**

*3.1. Physicochemical changes during post-fermentation storage*

The trend observed in the evolution of pH, salt, titratable and combined acidity between the initial and the end of storage (3 months) were very similar and did not show significant differences among treatments. For this reason, they were lumped together (Table 1). Intentionally added NaCl at the onset of post-fermentation storage increased its concentration from 45 g/L up to 90 g/L, fact that gave the stored fermented olives great stability because prevented spoilage, in spite of the rise in environmental temperature (13 ºC). This behaviour is in agreement with industrial practices (Garrido Fernández et al., 1997).

There were significant decreases in titratable acidity and significant increases in combined acidity during the post-fermentation storage. This might have been caused due to a progression of the flesh-brine equilibrium process, or by the consumption of acids by the diverse aerobic yeast species in brine (Garrido Fernandez et al., 1997). In any case, these changes did not produce a significant change in pH (Table 1).

*3.2. Microbial changes during post-fermentation storage*

*Enterobacteriaceae* were never found. There was always a significant LAB population decrease with time in the cover brine of all treatments (Figure 1, upper panel). In the spontaneous and LAB2 treatments (F1 and F2), the LAB population reduction was about 1.5 log10 cycles after 3 months of storage. Intermediate reductions were noticed in treatments F4 and F5, with a LAB decrease of about 2 log10 cycles, while the lowest final population was found in F3 treatment. This general reduction behaviour may be caused by the high NaCl concentration used during the post-fermentation storage because the inhibitory effect of salt on LAB (Romero-Gil et al., 2013).

Yeast plate counts in the cover brines were initially lower than those of LAB but their changes were not systematic and were affected by high variability, possible due to their uncontrolled evolution during the whole process (Figure 1, lower panel). Apparently, there was a trend for an initial population decrease (except in F5) followed by an increase at the end of storage, probably due to their adaptation to saline conditions, the decrease in the LAB populations, or to the higher summer temperatures. Overall, yeast populations did not show marked changes during the storage period.

Initial counts on the olive surface were higher for both LAB and yeast populations (Figure 2). For LAB, a general significant (except in F2 treatment) decrease (1-2 log10 cycles) was noticed throughout the storage process (Figure 2, upper panel). LAB populations after 3 months of storage were lower than at the initiation of the process in all treatments and fairly similar; they ranged between 5-6 log10 CFU/olive. The behaviour of yeasts on the olive surface was variable (Figure 2, lower panel) and only slight changes were noticed (except in treatment F4). Overall, it can be stated that the storage phase had a limited effect on yeasts, possibly due to the higher resistance to salt of this organisms with respect to lactobacilli(Romero-Gil et al., 2013).

*3.3. Instrumental colour and firmness changes during the post-fermentation storage and packing*

Colour index (*Ci*) of fruits was similar in F2 and F4 throughout storage and packing while there were improvements in F1 and F5 and a decrease in F3 after packing but differences did not exceed two units. Then, in practice, the changes in this and other parameters were scarce and the values of treatments can be combined. An overall test for the statistical comparison of initial, at the end of the post-fermentation storage, and packing colour parameters is shown in Table 2. No statistically significant changes (p<0.05) in the *Ci*, *L*\*, *b\**, and *chroma* were observed during storage. However, there was an increase in greenness (*a\**) and in the *hue* angle. These changes can be related to the so called “curing” effect observed in many fermented products. Packing caused a significant increases in *L\** (packed olives were lighter than stored ones), *b\** (packed olives improved yellowness), and *chroma* (packed olives showed a more saturated colour).

Firmness of fruits decreased during storage (Figure 3) but the differences were only significant between F2 and F5 treatments. However, samples had final firmness above 1000 N/100 g pitted olives, which is the lowest limit recommended for marketable products (Sánchez et al., 1997). Then, all the olives from these experiments had appropriate firmness.

*3.4. Sensory analysis*

The packed products, after equilibrium, were assessed by a formal panel. Median and robust standard deviations are shown in Table 3. No median value for the most frequently perceived defect of “negative sensations” (“abnormal fermentation” and “other defects”) exceeded the 3.0 value threshold (IOC, 2010). Therefore, after 3 months of storage in high salt concentration and one month packing, all green table olives analyzed (F1-F5) were classified as “extra” commercial category (IOC, 2010).

With respect to the other perceptions (Table 3), the Kruskal-Wallis test, applied to their medians, showed that there were only significant differences among treatments for hardness. The spontaneous (F1) had a significantly (p=0.0007) lower score than F2 and F3 treatments (Table 3), in agreement with the lowest average values in shear compression measurements found in the spontaneous fermentation (Figure 3).

*3.5. Multivariate analysis*

Apparently, an overall assessment based on individual attributes is difficult. Therefore, the application of multivariate analysis methods could be convenient. To achieve this analysis, the scores of the gustative and kinaesthetic sensations were considered continuous variables from an unstructured scale, were centred with respect to assessors and, finally, averaged by panel tasters (Table 4).

A first analysis on the panel performance showed no significant differences among assessors for any of the analyzed attributes (salty, p=0.2243; bitter, p=0.1451; acid, p=0.4320; hardness, p=0.6919; fibrousness, p=0.8552; crunchiness, p=0.2264) and therefore the centred average may be representative of the overall panel performance.

To find a clear relationship among attributes and treatments, the results were subjected to PCA. Three significant (higher than 1) eigen-values were found, which accounted for 98.04% of the variance. Factor 1 accounted for 54.57% of the variance and was related to the kinaesthetic sensations (correlations with hardness, fibrousness, and crunchiness of 0.955, 0.901, 0.955, respectively); Factor 2 accounted for 28.13% of the variance and was mainly linked to bitter (0.876) and acid (-0.749). Factor 3 explained 15.34% of the variance and was associated to salty (0.669).

The biplots graph (Figure 4) showed a close relationship among all the kinaesthetic sensations, which is in agreement with previous work (Moreno-Baquero et al., 2012). On the contrary, bitter and acidic were almost orthogonal (not related) and had an opposite link with Factor 2. The relationship among these gustative sensations and kinaesthetic sensations was moderate.

Factor 1 segregated the treatments into two groups (Figure 4); treatments F3 and F4 were located on the right while F2, F5 and F1 (spontaneous) were put on the left. The segregation of these two groups can be attributed to kinaesthetic sensations (because of the link of these with Factor 1). Factor 2 produced a further differentiation among groups F1 and F2 (on the upper-left quadrant) and F5 (in the lower-right quadrant) as well as between treatments F3 and F4. The graph can also relate treatments with specific attributes. Thereby, treatments F3 and F4 are strongly correlated to the kinaesthetic sensations and represent treatments with high hardness, fibrousness and crunchiness scores (Table 3); in addition, treatment F3 is characterized by marked bitterness while treatment F4 is strongly associated to high acidic scores. On the opposite side, treatments F2 and F1 are mainly characterized by their low scores in the kinaesthetic sensations, although treatment F2 was also linked to bitterness. Finally, treatment F5 was located alone in the lower-left quadrant and showed lower than average texture scores, fairly low bitter but high acidic and salty scores. F3, fermented with LAB3 strain, was the treatment that led to the highest overall kinaesthetic sensations, showing the highest hardness, fibrousness, and crunchiness scores. F1 (spontaneous) followed a particularly different trend with respect to all the other inoculated treatments, indicating that spontaneous fermentations usually led to low scores for kinaesthetic sensations but had average bitterness and acidity values. Hurtado, Reguant, Bordons, & Rozès (2010) have also found that inoculation influenced the sensory attributes of the fermented green natural olives.

**4. Conclusion**

The storage for 3 months of previously fermented olives caused an overall significant titratable acidity decrease and a combined acidity increase in the brines throughout storage as well as a considerable decrease in LAB populations in both cover brines and on the olive surface. Post-fermentation storage also led to slight changes in the colour parameters of fruits, but caused a marked degradation in texture (although fruits still maintained appropriate levels for market). All stored and packed olives were classified as “extra” commercial category, but multivariate analysis showed that previously inoculated olives were clearly different from the spontaneous fermented fruits.

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