**Effect of packaging and storage conditions on microbial survival, physicochemical characteristics and colour of** **non-thermally preserved green Spanish-style Manzanilla olives**

F. Rodríguez-Gómez, V. Romero-Gil, F.N. Arroyo-López\*, J. Bautista Gallego, P. García-García & A. Garrido-Fernández

Food Biotechnology Department. Instituto de la Grasa (Agencia Estatal Consejo Superior de Investigaciones Científicas, CSIC). University Pablo de Olavide, Building 46. Ctra. Utrera, km 1, 41013 Seville, Spain.

**Running title:** Study of table olive storage conditions

**\*Corresponding author:** Francisco Noé Arroyo-López, Ph.D. Tel: +34 954 611 550 ext 142; e-mail: fnarroyo@cica.es

**Abstract**

This work studies the effect of packaging material (glass jar or plastic pouches), olive environment (covered with brine, under N2 atmosphere or vacuum) and storage temperature (7, 13, and 22 C) on the microbial survival, brine characteristics and colour of packaged fermented olives (Manzanilla cv.) subjected to preservation only by pH and NaCl levels. The highest lactic acid bacteria counts on olive epidermis (6.2-6.5 log10 CFU/olive) were found in fruits stored in glass jar and plastic pouches at 22 C which, on the contrary, showed the lowest yeast population levels (5.2-6.5 log10 CFU/olive) due to the opposed growth trend observed between bacteria and yeasts. The pH increase in the packages with brine limited the product shelf life to about 2 months but without brine the shelf life could be longer. Changes in colour parameters did not affect the product’s shelf life. The verification of the high lactic acid bacteria on the olive surface, particularly for plastic pouches with brine, and under N2 atmosphere at room temperature, constitutes an essential step for producing probiotic table olives.

**Keywords***:*Lactic acid bacteria; Packaging; Shelf life; Table olive; Yeasts.

**1. Introduction**

Probiotics are the live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). Different species of *Lactobacillus* and *Bifidobacterium* have been the main microorganisms used with this aim (Boyle & Tang, 2006). Usually exogenous probiotic bacteria are incorporated into dairy products; however, at present, there is an increasing trend for using many other food systems as carriers due to the increasing intolerance of population to lactose (Ranadheera, Baines & Adams, 2010).

Verganović et al. (2011) improved sauerkraut production with the probiotic strains *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. Irish Brassica was also recently prepared as a probiotic vegetable by Jaiswall, Gupta & Abu-Channam (2012). Table olives have been explicitly proposed for delivering probiotic bacteria to humans (Lavermicocca, Rossi, Russo & Srirajaskanthan, 2010) based on the adhesion and survival of *Lactobacillus* and *Bifidobacterium* species on these fruits (Lavermicocca et al., 2005) and on the adhesion on the olive surface of the human origin *Lactobacillus paracasei* IMPC2.1 (De Bellis, Valerio, Sisto, Lonigro & Lavermicocca, 2010). The formation of LAB-yeast biofilms on abiotic and biotic surfaces during green Spanish-style olive fermentation (spontaneous or inoculated) is also documented (Arroyo-López et al., 2012; Domínguez-Manzano et al., 2012). Recently, a review on fermented plant material as carriers and sources of potentially probiotic lactic acid bacteria (LAB) has been published (Peres, Peres, Hernández-Mendoza & Malcata, 2012). All these works support the possibility of using fermented vegetables, and especially table olives, as a probiotic carrier.

Pasteurization is currently the most widely applied procedure to stabilize green Spanish-style table olives (Sánchez-Gómez, García-García & Garrido-Fernández, 2013) but it would not be applicable in the case of putative probiotic table olive production which requires microbial survival after processing. Such an objective may be achieved by using appropriate physicochemical conditions, provided the olives were adequately cured (Borbolla y Alcala & González Pellisso, 1972). Recommended packaging conditions are: 5.0 g NaCl/100 mL brine, 0.5 g titratable acidity/100 mL brine and around 25 mEq/L combined acidity (Garrido Fernández et al., 1997). However, a post-fermentation curing process might not be practical in the case of potential probiotic table olives because it may reduce the microbial load on the olive biofilm (Rodríguez Gómez et al., 2013). Therefore, it is important to investigate the application of such an approach to not-completely-cured olives. The results are necessary for the proper design of the new processing conditions for putative probiotic table olives.

The present study aims to investigate the effect of the packaging material (glass jar and plastic pouches), olive environment (brine, inert atmosphere and vacuum), and storage temperature (7, 13, and 22 ºC) on the survival of LAB and yeast populations on the olive surface. The effects on the microbial and physicochemical changes in brine (essentials for olive preservation) and olive colour are also reported.

**2. Materials and methods**

*2.1. Starting olives*

The fruits used in the present study were from the Manzanilla cultivar fermented according to Spanish-style during the 2010/2011 season. They were inoculated with the putative probiotic LAB starter cultures *Lactobacillus pentosus* TOMC-LAB2 and TOMC-LAB4, selected because of their appropriate behaviour when used as starter cultures, ability to form biofilms (Arroyo-López et al., 2012) and good *in vitro* apparent probiotic characteristics (Bautista-Gallego et al., 2013). On the contrary, yeasts were always allowed to grow spontaneously during fermentation. Details on the fermentation processes can be found elsewhere (Rodriguez-Gómez et al., 2013). The fermented olives were mixed and the microbial population on the olive surface molecularly characterized (data not shown). It was mainly composed of LAB2 (86%) and LAB4 (10%) strains, among the LAB species, and *Geotrichum candidum* (56%), *Candida sorbosa* (30%) and *Pichia galeiformis* (14%) among the yeasts. Before packaging, the olives were washed with sterile water to remove the microbial load not adhered to the olive epidermis.

*2.2. Packaging treatments*

 For packaging, glass jar (G) and plastic pouches (P) were used. In the case of plastic pouches, a high barrier polyester and polyethylene film with a thickness of 109 µm and oxygen permeability of less than 8.5 cm3/m2/24h (SP Group, Villarrubia, Spain) was used. For the glass jar, the containers were filled with 125 g of olives and 125 mL of sterile brine (B). Packaging in plastic pouches included several alternatives: i) olives (75 g) covered, as in the glass jar, with sterile brine (75 mL); ii) olives packaged without brine and under N2 atmosphere, which was achieved by substituting the initial air with N2 (only 30% of the air volume removed) (N); and iii) olives packaged without brine and under vacuum (V) (achieved by reducing the initial air volume inside the pouches to 1%). Plastic pouches in all the experiments were sealed with the same machine (Tecnotrip mod EVT7G, Terrassa, Spain).

The packaging brine had an initial salt concentration of 5.7 g NaCl/100mL (without acid) and, prior to use, was sterilized by autoclaving (15 min at 121°C and 15 psi). When olives were packaged without brine, the fruits were previously equilibrated in glass jar for 20 days in a cold room (7 ºC), using the same brine mentioned above.

The packaged olives were stored at three different temperatures (7, 13, and 22 ºC, to cover from refrigeration to room temperature) and ten samples from each treatment (combination of packaging material and storage temperature) were analysed periodically for 3 months. For the study of effects and interactions of variables, these data were grouped according to the type of analysis.

*2.3. Physicochemical analyses*

The analyses of cover brines for pH, salt concentration, titratable acidity, and combined acidity were carried out using standard methods (Garrido-Fernández, Fernández-Díaz & Adams, 1997). All the analyses per sample were performed in duplicate and the average recorded. The instrumental surface colour of the fruits was measured using a BYK-Gardner Model 9000 Colour View Spectrophotometer (Silver Spring, MD, USA) and expressed in terms of the CIE *L\*, a\*, b\** parameters and *Colour Index* (*CI*). *CI* was calculated according to formula: *CI* = (-2·R560+R590+4·R635)/3, where R stands for the reflectance at 560, 590 and 635 nm, respectively. Results were the average of 10 measurements.

*2.4. Microbiological analysis*

Brine samples were diluted, if necessary, in a sterile saline solution (0.9 g/100 mL NaCl) and plated using a Spiral System model dwScientific (Dow Whitley Scientific Limited, England). *Enterobacteriaceae* were counted on Crystal-violet Neutral-Red bile glucose (VRBD) agar (Merck, Darmstadt, Germany), LAB were spread onto de Man-Rogosa and Sharpe (MRS) agar (Oxoid) supplemented with sodium azide (Sigma, St. Luis, USA), and yeasts were grown on a yeast–malt–peptone–glucose medium (YM) agar (Difco, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulphate as selective agents for yeasts. The plates were incubated at 30 ºC for 24 (*Enterobacteriaceae)* or 72 h (yeasts and LAB) and counted using a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system. Brine counts were expressed as log10 CFU/mL. To determine the microorganisms adhered to the olive epidermis, the enzymatic protocol developed by Böckelmann, Szewzyk & Grohmann (2003) for the detachment of biofilms was followed. Briefly, fruits from each packaging vessel were randomly taken and washed for 1 h with 250 mL of a sterile PBS (8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na2HPO4, 0.24 g/L KH2PO4, pH finally adjusted to 7.4 with HCl 1M) buffer solution. Then, the olives were transferred to 50 mL of a PBS solution with the following enzymes: 14.8 mg/L lipase (L3126), 12.8 mg/L β-galactosidase (G-5160) and 21 µL/L α-glucosidase (G-0660) (Sigma-Aldrich, St. Louis, USA). To achieve biofilm disintegration and removal of the adhered cells, the fruits were incubated at 30 ºC in this enzyme cocktail with slight shaking (150 rpm). After 12 h, the olives were removed and the resulting suspension was centrifuged at 9,000 x *g* for 10 min at 4 ºC. Finally, the pellet was re-suspended in 2 mL of PBS and spread onto the different culture media described above. Olive counts were expressed as log10 CFU per olive (average weight and surface: 4.08±0.46 g and 10.99±1.01 cm2, respectively; n=50).

*2.5. Statistical data analysis*

The experimental design consisted of a complete factorial design of packaging conditions (4 levels) and storage temperature (3 levels), with ten replicates for each treatment. Periodically, samples were analysed by duplicate and the average recorded. The effects of packaging conditions and storage temperature were estimated by ANOVA analysis. Comparisons among variable levels within factors were obtained by Least Significant Difference (LSD) for a probability level of p<0.05. Data analysis was carried out using Statistica 8.0 software package (StatSoft Inc, Tulsa, USA).

**3. Results**

*3.1 Changes of the microbial population in olive biofilm*

*Enterobacteriaceae* was never found in the olive biofilm; therefore, the comments are limited exclusively to the changes in LAB and yeast populations. Due to the general approach used in this work, only the overall count changes in their respective microbial groups are shown.

The LAB and yeast survival in the olive biofilm during packaging (previously formed during fermentation process) was affected by the packaging conditions and sampling time (Figures 1 and 2). The highest average LAB populations were found on the olives packaged in plastic pouches under N2 atmosphere (PN) or covered with brine (PB) (Figure 1, panel A) while the lowest average count was detected on the olives from plastic pouches under vacuum (PV) but without significant difference with respect to those in glass jars with brine (GB). Yeast populations were similar in GB, PB and PV, but significantly increased on the olives packaged in PN (Figure 1, panel B). The effect of the storage conditions significantly increased and decreased the average LAB and yeast populations, respectively, as the temperature increased (Figure 1, panels C and D). The opposed trend followed by both groups of microorganisms may indicate a competition between them for survival, with LAB prevailing at 22 C.

The LAB population in the olive biofilm significantly decreased after packaging and reached the lowest average at the 2nd month of sampling (Figure 2, panel A); later, the LAB counts increased significantly (p<0.05) again but remained below the initial level. This first decrease may be due to the LAB release into the brine after packaging as deduced by the simultaneous LAB increase in the surrounding brine (see below). The progressive reduction in nutrients and the stressing effect of the packaging conditions may also have contributed to the LAB population decrease in the olive biofilm. The yeast population was progressively inhibited with time (Figure 2, panel B), although the differences between the initial counts and that at the first month or between the second and third month were not significantly different; however, the difference between the counts from the first two and the last two samplings were significantly different. The causes of this decrease could have also been similar to those mentioned above for LAB. Regardless of packaging conditions, the interaction temperature\*sampling period on the microbial populations in the biofilm was significant (Figure 2, panels C and D). Temperature caused significant differences at selected sampling points, particularly at the 1st and 2nd months after packaging; this means that, at 22 C, the average LAB populations were the highest while in the olives stored at 7 °C (Figure 2, panel C) they were the lowest. The changes for yeasts with respect to temperature were opposed to those of LAB and the curve of yeast counts from olives at 22 ºC was always the lowest.

*3.2 Changes in the microbial populations of brines* *(GB and PB treatments)*

The packaging material had a significant effect on the LAB population in the brines which was higher in GB than in PB (Figure 3, panel A). The opposite occurred with yeasts, whose counts were significantly higher in PB than in GB (Figure 3, panel B). The effect of temperature on LAB population in brine (Figure 3, panel C) was initially similar to that on the olive surface, except that counts at 22 °C were lower (although not statistically significant) than at 13 °C. However, the effect of temperature on yeasts in brine (Figure 3, panel D) was similar to that observed on the olive surface (Figure 1, panel D), decreasing progressively to reach the lowest significant population at 22°C. With respect to the changes in LAB in brine (Figure 4, panel A), there was a decrease from the first to the second sampling period with a recovery at the third.

In the LAB and yeast population in brine there were also several significant interactions. In the case of packaging material\*temperature (Figure 4, panel B), the yeast population was always higher in the brines from PB but decreased as the temperature increased; in this way, the lowest level was observed at 22 °C in GB; in PB, the trend was also rather similar and the level at this temperature was significantly lower than that at 7 °C whereas in GB, the difference was significant with respect to the counts at 7 and 13 °C. The interaction packaging material\*sampling period on LAB and yeast populations in brine were also significant (Figure 4, panels C and D); the overall trend observed for LAB vs. time was mimicked in the interaction but the initial decrease led to significantly lower populations in PB in the first sampling period (Figure 4, panel C) but not in the other samplings. The yeast presence was also affected by the interaction packaging material\*sampling period (Figure 4, panel D) with the populations in PB brines being always higher than in GB without significant differences among sampling periods; however, the counts in GB were lower in the last sampling with respect to the first.

*3.3. Effect of the packaging treatments on the physicochemical characteristics*

During the study, the concentrations of NaCl in brine solutions from GB oscillated between 4.89 and 5.10 g/100mL, with a standard error of 0.07 g/100mL, while in the brine from PB, the salt content ranged from 4.99 to 5.07 g/100mL brine, with a standard error of 0.08 g/100mL brine. The values of titratable acidities (expressed as lactic acid) in the brines from GB and PB ranged from 0.54 (standard error, 0.01) to 0.56 (0.02) g/100mL and from 0.46 (0.01) to 0.50 (0.01) g/100mL, respectively. Therefore, the results indicate that the NaCl equilibrium expected (5.0 g NaCl/100mL) was properly achieved and that its levels were stable during the 3 month period of the experiment. However, the titratable acidity (expected value of 0.5 g/100mL) was consistently higher in the GB than in PB brines. In GB, the averages were above this level and the changes were scant but in PB the levels were always lower than in GB and the average consistently had a non-significant decreasing trend as temperature and time increased, which led to 0.46 g/100 mL after three month storage at 22 ºC.

The changes in pH in the brines were similar (Figure 5), regardless of the packaging material. There was a practical stabilization of pH during the first 2 months but, later, it increased, particularly at 22 °C, regardless of the packaging materials (Figure 5, panels A and B). On the contrary, the combined acidity followed an opposite evolution (Figure 5, panels C and D) and, apart from the increase after 2 months in PB (Figure 5, panel D), significantly decreased, at all temperatures, by the end of the storage period.

With respect to colour, changes in the CIE *L\** parameter (luminance) were affected by the packaging treatments (Figure 6, panel A), temperature (Figure 6, panel B) and sampling time (Figure 7, panel A). In general, high *L\** values are associated with better (more typical) colour. Therefore, the best olive colour was obtained in GB (Figure 6, panel A), followed by PB or PN and, finally, PV. The CIE *L\** values improved as storage temperature was higher; as a result, olives stored at 22° C had the highest *L\** (better colour) while those maintained at 7 °C had the lowest value (Figure 6, panel B). *L\** values decreased after packaging, due to packaging operations (visual sorting, washing and filling of containers), but had a significant improvement after 2 months storage with only a slight (non-significant) decrease at the third sampling period (Figure 7, panel A). Changes in *a\** colour parameter (greenness) were never significant. In GB, *a\** ranged between 3.19 and 3.28 with a standard error of 0.21. In PB, the average value of *a\** was about 3.25, while in olives without brine the values ranged from 3.32 to 3.66 in PN and 3.50 in PV; the standard error in these cases was 0.24. The CIE *b\** parameter was affected only by the packaging treatment (Figure 6, panel C) and sampling time (Figure 7, panel B). The values of *b\** decreased (lower yellowness, which means poorer colour) as the container was more permeable to oxygen due to the subsequent oxidation process on the olive surface. The best results were obtained in GB, although results from PB were not significantly worse (only slightly lower yellowness); however, packaging in the absence of liquid caused greater deterioration, particularly in PV where the lowest *b\** values, and subsequently the poorest colours, were observed (Figure 6, panel C). The values of *b\** increased after packaging (Figure 7, panel B) but decreased (improved) over time, although the changes were very limited (less than 1.0 unit). The trend observed in the *CI* was similar to that above mentioned for the CIE *L\** parameter and their values decreased (olives became browner) after packaging, due to olive handling operations; its final recovery was limited (Figure 7, panel C). The best *CI* (more yellow typical colour) was found in olives from GB and PN (Figure 6, panel D).

**4. Discussion**

Traditionally, the microbial population in table olives has been monitored in the cover brines. However, the change in the microbial load on the fruits, which is the fraction ingested by consumers, is essential information for the development of potential probiotic olives. The results from this study may represent an approximation of the estimation of LAB and yeasts that could be carried by future probiotic olive products. Because of the packaging of these olives was directly carried out after fermentation, the LAB and yeast loads on the fruits were quite relevant. In general, there were decreasing trends for LAB and yeasts on the olive surface during storage, with a partial recovery after two months; on the contrary, in brine the decrease mainly affected yeasts in GB while in PB the LAB/yeast ratio was 6.5/5.5, fairly higher than in GB, possibly due to the oxygen permeability of the plastic material. This may have favoured the survival of more facultative or oxidative yeasts able to use substrates in an oxygen restricted environment.

The LAB survival depended on packaging characteristics (average maximum in PB and PN, 6.3-6.4 log10 CFU/olive) and on storage temperature (with an average highest population, 6.6 log10 CFU/olive at 22 ºC). Furthermore, at 22 °C, the lowest LAB counts on olives during most of the packaging time ranged between 6.26 and 6.56 log10 CFU/olive, an interesting load for this potential probiotic product, bearing in mind the protective effect that the olive matrix has shown for LAB survival throughout the gastrointestinal tract (Arroyo López et al., 2014). Therefore, provided the organisms were a proper probiotic LAB, it is very likely that olives packaged in PB or in PN (or in GB with only a slightly lower load) and stored at room temperature could be good carriers of potential functional bacteria.

In this study, the LAB population has always been accompanied by yeasts whose populations, although always decreased as storage time progressed, had average of 6.0 log10 CFU/olive but always above 5.2 log10 CFU/olive. It is probable that the simultaneous presences of both groups of microorganisms are an essential element for the development of probiotic olives. In fact, they have always been found in the olive biofilms (Arroyo López et al., 2012; Domínguez Manzano et al., 2012). In dairy products, the microbiology includes a wide range of LAB species, in addition to the added probiotic, but also adventitious yeasts, which may increase or interfere with the growth or survival of bacteria (Suharja, Henriksson & Lui, 2014). Furthermore, particular species of *Saccharomyces cerevisiae* have been proven to have probiotic properties, although the mechanism of action is not well understood yet (Etienne-Mesmin et al., 2011). The stabilization of probiotic products at ambient temperature by the simultaneous use of LAB and yeasts is an interesting challenge because the cold chain represents major costs. This study has shown that, when the interest is focused on the preservation of a putative probiotic LAB population, 22 ºC would be the storage temperature of choice. On the contrary, 7 ºC would be more appropriate when the maximum yeast counts on the olives would be of interest.

Therefore, the results from this work reveal that the selection of not only the potential LAB but also the yeast strains could lead to the preparation of a functional food with a dual probiotic characteristic. Furthermore, it could be packaged in simple common packages (glass jar or plastic pouches, under N2 if desired) and distributed at room temperature. The balance LAB/yeasts in table olives can be modified to produce the most convenient properties by favouring the appropriate microbial group. The development of products which include simultaneously probiotic LAB and yeasts, as suggested in this work, may, in addition to the individual therapeutic effects, enhance viability and synergy with respect to them.

With respect to physicochemical characteristics, the increase in pH and decrease in combined acidity during the product storage has been usually attributed to the use of free or combined acids by yeasts, which was not compensated in PB with the possible production of lactic acid by LAB. The acid consumption was more evident in the PB brine where titratable acidity decreased also, possibly due to a higher yeast activity (Garrido Fernández et al., 1997). However, the conditions traditionally used for green olive stabilization were efficient for about 2 months in treatments using cover solutions because, after that time, pH increased and, with it, the risk of product deterioration. As this change characterizes the initial stages of important olive malodorous spoilage (Garrido Fernandez et al., 1997), the effect represents a clear limitation on the shelf life of these treatments. Therefore, the potential probiotic product packaged in brine, using the equilibrium conditions applied in this work, could have a maximum shelf life of about 2 months, regardless of temperature. However, olives in plastic pouches, particularly under N2, showed stable physicochemical conditions and did not show any visual spoilage evidence after 3 months; thus, they might have a longer shelf life. In other products like yoghurt and cheese it has been observed that, apart from the probiotic strain, other environmental conditions (dissolved oxygen, permeation of oxygen through packages, pH or organic acids) may have important effects on microbial survival (Karimi, Mortazavian & Da Cruz, 2011).

Colour is an attribute of marked importance in the green Spanish-style presentation. The changes observed in this work were limited to CIE *L\** and *b\** parameters as well as *CI*. In green Spanish-style olives, there are no scales for *L\**, *a\** and *b\** parameters; however, as higher are the values of *L\** and *CI* (above 25) or the lower those of *a\** and *b\** the more typical (best) is the colour. Packaging operations (exposure to air) deteriorated the initial colour of the fermented fruits because the levels of the three colour parameters were worse after 1 month storage, but there was a progressive improvement with time which was greater as temperature increased. In this way, *L\** and *b\** after 3 months packaging had more favourable values than after 1 month. Overall, the best results were observed in olives from GB (highest *L*\* and lowest *b\** values) but PB or PN also showed good results with respect to *L\** (higher values). The colour improvement after the second packaging month could have been due to the favourable effect of pH and titratable acidity on colour parameters (Garrido Fernández et al., 1997) or even to the oxygen depletion due to the microbial activity. Furthermore, the effects increased as the storage temperature was increased. In the absence of brine, olives from PN had an initial protection (because of the *CI* level preservation) due to the oxygen removal but had a very reduced later recovery; on the contrary, olives from PV showed the worse behaviour possibly due to the increasing oxidation caused by oxygen permeation through the packaging material which could have also been aggravated as the temperature was higher. Hence, with regards to colour, glass jar, and pouches with brine or under N2 could be appropriate packages. The storage at room temperature caused a convenient improvement of luminance (*L\**).

**5. Conclusions**

The highest LAB population survival in biofilms during storage of packaged green Spanish-style olives was observed when storing the packages at 22 C; the levels were more relevant in plastic pouches with brine or under N2, followed by glass jars in brine and plastic pouches under vacuum. Therefore, the best conditions for potential probiotic table olive packaging and storage would be: i) plastic pouches under N2, stored at 22 ºC, with a possible shelf life of 3 months; ii) glass jar in brine, stored at 22 ºC, with 2 months shelf life. The evolution in the colour parameters did not affect the shelf life of the potential probiotic olive products since room temperature even caused a certain improvement with time in the first two cases. On the contrary, the changes in the physicochemical characteristics, particularly the increase in pH in packages containing brine, limited the shelf life of certain products. In any case, the LAB populations after 2 months storage ranged between 6.2-6.5 log10 CFU/olive at 22 C. Therefore, this work has demonstrated the simultaneous survival of LAB and yeasts (forming biofilms) on the surface of packaged green Spanish-style olives allowing for the preparation of potential LAB probiotic olives to be distributed at room temperature. This study, together with the characteristic fruit composition, opens the possibility for the development of synbiotic/functional table olives.

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**Figure Captions**

*Figure 1*. Un-weighted mean microbial population values on olives and significant differences obtained by ANOVA, regardless of storage time. A) effect of packaging material and characteristics on LAB; B) packaging material and characteristics on yeasts; C) effect of temperature on LAB; D) effect of temperature on yeasts. GB, packaging in glass jar with brine; PB, packaging in plastic pouches with brine; PN, packaging in plastic pouches under N2 atmosphere; PV, packaging in plastic pouches in vacuum. Vertical bars denote 0.95 confidence intervals for un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test. Average (±SE) initial counts: LAB, 7.06(±0.09) log10 CFU/olive; yeasts, 6.51(±0.12) log10 CFU/olive.

*Figure 2*. Un-weighted mean microbial population values on olives and significant differences obtained by ANOVA. A) effect of sampling period on LAB; B) sampling period on yeasts; C) effect of interaction temperature\*sampling period on LAB; D) effect of interaction temperature\*sampling period on yeasts. Legends of temperature: . Vertical bars denote 0.95 confidence intervals for un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test.

*Figure 3*. Un-weighted mean microbial population values on olives and significant differences obtained by ANOVA. A) effect of packaging material on LAB; B) packaging material on yeast; C) effect of temperature on LAB; D) effect of temperature on yeast. GB, packaging in glass jar with brine; PB, packaging in plastic pouches with brine. Vertical bars denote 0.95 confidence intervals of un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test. Average (±SE) initial counts: LAB, 7.06 (±0.09) log10 CFU/olive; yeasts, 6.51 (±0.12) log10 CFU/olive.

*Figure 4*. Un-weighted mean population values in brine and significant differences obtained by ANOVA. A) effect of sampling period on LAB; B) effect of interaction packaging material\*temperature on yeasts; C) interaction packaging material\*sampling period on LAB; D) interaction packaging material\*sampling period on yeasts. . Vertical bars denote 0.95 confidence intervals for un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test. Average (±SE) initial counts: LAB, 7.06 (±0.09) log10 CFU/olive; yeasts, 6.51 (±0.12) log10 CFU/olive.

*Figure 5*. Changes (un-weighted means) in brine during the storage of the potential functional olives: A) effect of the interaction temperature\*sampling period on pH in olives packaged in glass jar with brine; B) effect of the interaction temperature\*sampling period on pH in brines packaged in plastic pouches; C) effect of the interaction temperature\*sampling period on combined acidity in olives packaged in glass jar with brine; D) effect of the interaction temperature\*sampling period on combined acidity in olives packaged in plastic pouches with brine. Legends of temperature: . Vertical bars denote 0.95 confidence intervals for un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test.

*Figure 6.* Unweighted mean values of colour parameters and significant differences obtained by ANOVA. A) effect of packaging material and characteristics on *L\**; B) effect of temperature on *L\**; C) effect of packaging materials and characteristics on *b\**; D) effect of packaging material and characteristics on Colour Index. GB, packaging in glass jar with brine; PB, packaging in plastic pouches with brine; PN, packaging in plastic pouches under N2 atmosphere; PV, packaging in plastic pouches in vacuum. Vertical bars denote 0.95 confidence intervals of the un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test. Average (±SE) initial values: *L\**, 49.77(0.26); *b\**, 34.38 (0.46); colour index 24.66 (0.32).

*Figure 7.* Un-weighted mean values of colour parameters and significant differences obtained by ANOVA. A) effect of sampling period on *L\**; B) effect of sampling period on *b\**; C) effect of sampling period on colour index. Vertical bars denote 0.95 confidence intervals of the un-weighted means. Bars with different letters are significantly different at p0.05, according to the LSD test.