Research Highlights

* This study reports a new spoilage in olive packages and its characterization.

* The genotypes of the microbial populations were determined by molecular techniques.

* Enterobacteriaceae, yeasts and LAB were initially present, but only LAB counts increased.

* Packaging conditions favour L. pentosus growth against the initial L. plantarum.

* A specific biotype of L. pentosus was predominant in the spoilt product.
**Lactobacillus pentosus** is the dominant species in spoilt packaged **Aloreña de Málaga** table olives

V. Romero-Gil, F. Rodríguez-Gómez, A. Garrido-Fernández, P. García-García, and F.N. Arroyo-López*

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

**Running title:** *Lactobacillus pentosus* in packaged olives

*Corresponding author:* Francisco Noé Arroyo-López, Ph.D. Tel. +34954611550 ext 142. e-mail: fnarroyo@cica.es
Abstract

The present study focused on investigating a peculiar spoilage of traditional *Aloreña de Málaga* table olives characterized by the formation of whitish and soft regions on the olive surface. To determine its causes, the main microbiological and physicochemical changes in 50 commercial packages were monitored until the alteration in appearance (63 days). Colour and firmness of fruits deteriorated progressively in the packaged olives during storage. Sugar in brines (7.5 g/L) remained stable during the first month and then decreased gradually (5.0 g/L) while, in parallel, the lactic acid bacteria population and titratable acidity increased. After two months of storage, evidence of spoilage was noticed, coinciding with the maximal lactic acid bacteria populations in fruit (>6.5 log_{10} CFU/g). The spoilage affected ~25% of the fruits within packages. The microbial species detected in the product after packaging were *Enterobacter gergoviae* and *Lactobacillus plantarum* among bacteria, and *Candida tropicalis*, *Candida parapsilosis*, and *Lodderomyces elongisporus* among the yeasts while *Lactobacillus pentosus* was the dominant species in the spoilt packages. A specific biotype of *L. pentosus* was only detected in the damaged fruits. Further studies will be made to confirm the association between the spoilage and the presence of this *L. pentosus* biotype.

**Keywords:** *Enterobacter gergoviae*; Packaged olives; Molecular techniques; Shelf life; Yeast.
1. Introduction

Table olives have a great importance in the diet and culture of many Mediterranean countries. Green Spanish-style, Greek naturally black and ripe Californian styles are the most popular commercial preparations (Garrido-Fernández, Fernández-Díez, & Adams, 1997). However, in the last years, consumers are purchasing more traditional and natural homemade seasoned olives.

_Aloreña de Málaga_ table olive is a traditional green olive preparation from Guadalhorce Valley (Málaga, Spain) with a Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). This olive variety has unique features, related to the production area, which make them quite different from others: its fruits are characterized by an excellent flesh-to-stone ratio, a green–yellow colour, a crispy firmness, and a peculiar mild bitter taste. Due to its low-to-moderate concentrations of oleuropein, the processing does not include alkaline debittering (López-López & Garrido-Fernández, 2006). The manufacturing process is carried out by small and medium enterprises placed in, or very close to, the region of production.

For the elaboration of traditional PDO _Aloreña de Málaga_, fruits are cracked after harvesting, and brined in a 10-11% NaCl solution for at least 20 days. Then, the olives are seasoned with pepper, fennel, thyme and garlic, and packaged according to demand (López-López & Garrido-Fernández, 2006).

The stabilization of packaged natural cracked green olives is difficult due to the high residual sugar content and to the subsequent risk of post-fermentation by microorganisms, causing gas formation and top package leakages (Arroyo-López, Romero, Durán-Quintana, López-López, García-García, & Garrido-Fernández, 2005; Arroyo-López et al., 2009). For preventing these problems and extend the shelf life,
nowadays, the industries use a combination of different preservatives (sorbate, benzoate, citric, acetic and lactic acid). The goal of all these preservatives is primarily the inhibition of yeasts but not particular attention is played to lactic acid bacteria (LAB) also common in these products (Arroyo-López et al., 2005; Arroyo-López, Durán-Quintana, Ruiz-Barba, Querol, & Garrido-Fernández, 2006a; Arroyo-López et al., 2009; Bautista-Gallego, Arroyo-López, Romero-Gil, Rodríguez-Gómez, & Garrido-Fernández, 2011; Abriouel, Benomar, Gálvez, & Perez Pulido, 2014). Albeit the role played by LAB species during olive fermentation is positive producing lactic acid through sugar consumption and the consequent pH decrease (Hurtado, Requant, Bordons, & Rozes, 2012), their presence during packaging could jeopardize its stability (Johanningsmeier & McFeeters 2013; Montaño, Higinio-Sánchez, Casado, Beato, & de Castro, 2013; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). The pasteurization of seasoned olives is not an option due to its negative effect on the typical green colour, the development of cooked taste, and weird off-flavours from condiments. Other technologies like high hydrostatic pressure (Abriouel et al., 2014), application of ozone (Arroyo-López, Durán-Quintana, & Garrido-Fernández, 2006b), the use of zinc chloride (Bautista-Gallego et al., 2011), or sorbic and benzoic acids (Alves, Esteves, & Quintas, 2015) have also been studied, although a complete stabilization of the final product has not been fully achieved yet.

The aim of this work was to investigate the cause of a new spoilage, which appears during storage of these cracked seasoned olives. Particularly, molecular techniques (DNA-fingerprinting and sequencing) and bioinformatics analysis were used to identify the microbial groups present in the spoilt product. The results of this study may be useful for the development of new integral preservation methods for improving the stability, quality and safety of seasoned olives in general.
2. Material and methods

2.1 Sampling of commercially packaged olives

This survey was carried out with Aloreña de Málaga fruits previously fermented in brine for 20 days (the traditional style) without the addition of any starter culture, and then packaged by an unique industry in the Guadalhorce Valley (Málaga, Spain). Polyethylene terephthalate (PET) packages (1.6 L volume) were filled with 0.9 kg of olives, 16 g of seasoning material (a mixture of diced garlic, pepper strips, and small pieces of fennel, and thyme) and 0.7 L of cover brine (5.5% NaCl, 0.3% citric acid, 0.2% potassium sorbate, 0.1% sodium benzoate, 0.1% ascorbic acid and 0.08% lactic acid, expressed as g/100 mL). Packages were kept at room temperature (23±2°C) and the lot (a total of 50 commercial packages) sampled (in duplicate and without replacement) at 1, 3, 7, 10, 14, 21, 28, 42 and 63 days. At this last time, spoilage was noticed in all packages of the lot causing the end of the study. To determine the frequency of fruits affected by the alteration, approximately 27% of the olives included in duplicated packages (n=98 from a total of 360 fruits) were removed (54 and 44, respectively) photographed, and classified (0, absence of alteration; 1, presence of alteration). As described below, 10 lactobacilli were isolated from fruits with alteration, while other 10 were obtained from unaltered fruits.

2.2. Physicochemical and microbiological analyses

The analyses of olive brine for pH, titratable acidity, combined acidity, and NaCl were carried out using the routine methods described by Garrido-Fernández et al. (1997). Firmness and surface colour of fruits followed methods described elsewhere (Bautista Gallego et al., 2011), determining the CIE parameters: \( L^* \) (lightness), \( a^* \)
(freshness, negative values indicate green while positive values are related to red tones), and \( b^* \) (negative values indicate blue and positive values associated to yellowish). The darkness of brine packing \( (B_d) \) was estimated according to Montaño, Sánchez-Gómez, & Rejano (1988). Individual reducing sugars (glucose, fructose, sucrose and mannitol) were determined by HPLC according to the methods developed by Sánchez, De Castro, Rejano, & Montaño (2000).

For the isolation of the *Enterobacteriaceae*, yeasts and LAB populations in both brine and olive samples, a culture-dependent approach was used according to methods described by Rodríguez-Gómez, Romero-Gil, Arroyo-López, Bautista-Gallego, García-García, & Garrido-Fernández (2015). Counts were expressed as \( \log_{10} \) CFU/mL for brines or \( \log_{10} \) CFU/g for olives.

2.3. **Characterization and identification of microbial populations**

A total of 20 yeast and 65 LAB isolates were obtained from brines or olives of commercially packaged olives at different sampling times as well as from fruits with visible spoilage evidence at the end of the experiment. Isolates were labelled with different letters as a function of their origin (B, brine; O, non-spoilt olives; A, altered fruits) and sampling time (I, initial 1 day; M, middle 28 days; F, final 63 days). Two isolates of *Enterobacteriaceae* were also obtained from fruits just after packaging and directly destined for sequencing analysis. The yeast and LAB isolates were genotypically characterized by DNA-based typing techniques such as RAPD-PCR and rep-PCR with primer M13 and GTG5, respectively (Gevers, Huys, & Swings, 2001; Tofalo et al., 2009). The resulting fingerprints were digitally captured and analysed with the BioNumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). The similarity between digitalized profiles was calculated using the Pearson product-
moment correlation coefficient. Dendrograms were obtained using the UPGMA clustering algorithm. *Candida boidinii* TOMC-Y5, *Wickerhamomyces anomalus* TOMC-Y2 and TOMC-Y20, *Saccharomyces cerevisiae* TOMC-Y4 and TOMC-Y30, *Pichia galeiformis* TOMC-Y8 and TOMC-Y27 for the yeasts, and *Lactobacillus pentosus* TOMC-LAB2, TOMC-LAB3 and TOMC-LAB4, *Lactobacillus plantarum* TOMC-LAB8 and TOMC-LAB9 for the LAB, were used as internal control to determine the reproducibility of the techniques. All these microorganisms were obtained from the Table Olive Microorganisms Collection (Instituto de la Grasa, Seville, Spain). A reproducibility of 80.5% for yeasts (RAPD-PCR), and 85.1% for bacteria (rep-PCR) was obtained (data not shown). Then, one representative isolate from different clusters obtained above cut-points was selected for molecular identification using sequencing of D1/D2 domains of the 26S rDNA gene with primers NL1 and NL4 for yeasts (Kurtzman & Robnett, 1998) and small-subunit 16 rRNA gene with universal primers 27F and 1492R for bacteria (Barrangou, Yoon, Breidt, Fleming, & Klaenhammer, 2002). Percentage of identity with available sequences obtained from NCBI GenBank database was deduced from Blast analysis. For discrimination between *L. pentosus* and *L. plantarum* species, multiplex PCR assay based on recA gene was used (Torriani, Felis, & Dellaglio, 2001).

2.4. Statistical data analysis

Graph, mean and standard deviations for the different physicochemical and microbiological parameters were obtained from duplicated packages at each sample time using the One-way ANOVA module of Statistica 7.1 software package (Statsoft Inc., Tulsa, USA).

3. Results
3.1. Physicochemical and microbiological changes during packaged olive storage

Salt (~4.75 g/100 mL) and pH (~4.0) values were kept quite stable during the study (63 days), without significant differences among sampling times, while the combined and titratable acidity showed a slightly increasing trend (Fig. 1). Firmness slightly decreased from an initial 6.0 value to a final 5.0 kN/100 g after two months (Fig. 2), showing a high variability in measurements which did not allow establishing significant statistical differences. The initial total sugar content in brines was ~7.5 g/L and was composed of glucose (4.8 g/L), fructose (1.5 g/L), mannitol (0.8 g/L) and sucrose (0.4 g/L). Total sugar concentrations significantly decreased throughout shelf life and reached ~5.0 g/L after two months, being glucose the only sugar whose level decreased (Fig. 2), because, as usual, the simple sugars are the first to be consumed by microorganisms. The L* colour parameter significantly increased (p<0.05) from an initial 56 to final 59 value at the end of storage. The a* colour parameter also rose from 0.5 to 3.5, which means a significant loss of green colour. Hue angle (h_ab) decreased from 88º (initial) to 85º (final), indicating the browning of fruits. The packing brine’s was also browning progressively (B_d increased from the initial 0.25 to final 0.35) (Fig. 3). Therefore, the main physicochemical changes that compromised the stability and quality of the product were: loss of green colour in fruits, a decrease of firmness, browning of brines and glucose reduction. From Figs. 1-3 can be deduced that the 20th day was critical in the evolution of many of the physicochemical parameters.

Enterobacteriaceae were only found on olives after packaging (1st day) at very low population levels (<2.2 log_{10} CFU/g). The sequencing of small-subunit 16 rRNA gene of two isolates obtained from fruits showed that both belonged to the same species, Enterobacter gergoviae (Table 1). Yeasts and LAB were the main microbial groups
detected in both brines and fruits during shelf life, although with completely different evolutions. Changes in yeasts in both brines and olives during storage were quite similar (Fig. 4). They were found at population levels around $3.2 \log_{10}$ CFU in both brines (per mL) and fruits (per g); but, after the first week of storage had a sharp decline, and they were not detected from the 10th day onwards (Fig. 4, upper panels). Apparently, the presence of preservatives (sorbate and benzoate) resulted in a reduction in the numbers of these microorganisms after approximately 10 days. The behaviour of LAB was completely different compared to yeasts (Fig. 4, lower panels). After packaging their population levels were $4.1 \log_{10}$ CFU/mL (brine) and $2.3 \log_{10}$ CFU/g (fruits), which were stable during the first 15 days. However, after this time, the population increased progressively and reached after two months levels close to $6.5 \log_{10}$ CFU/mL (brine) or $6.8 \log_{10}$ CFU/g (olives), significantly ($p<0.05$) higher than those found initially in the case of fruits samples. It must notice the differences sometimes observed between replicated measurements, as usual in natural and cracked samples obtained from different packages of the lot. This high variability did not allow establishing significant statistical differences in certain sampling times.

3.2. Evidence of spoilage

At the 63rd day of study, all packages of the lot showed clear evidence of spoilage that affected the fruits’ product appearance. This coincided with the LAB maximum population and forced the termination of the study. The spoilage (Fig. 5) affected to $25.6\pm0.84\%$ of fruits ($n=98$) within a package. The appearance of the spoilage (whitish and soft olive parts close to the cracked border) was associated with the presence of a more viscous, browning and mucous brine (personal observation).

3.3. DNA-fingerprinting and identification of microbial populations
The dendrogram generated with the pattern profiles of the 65 lactobacilli isolates obtained from commercially packaged olives (Fig. 6) showed six major biotypes that were differentiated below reproducibility of the technique (85.1%). In practice, all isolates obtained from the initial sampling point formed two groups were clearly separated from the rest (cluster V and VI) sharing a 62.1% similarity between them. Sequencing analysis of small-subunit 16 rRNA gene and further confirmation by multiplex PCR assay based on recA gene (amplification 318 bp), allowed identification of isolates B-I-4 and O-I-3 (belonging to clusters V and VI, respectively) as L. plantarum species (Table 1). Both DNA fingerprint profiles were not found later and at middle and final sampling points, in either brines or olives, while other four different genotypes were noted (clusters I, II, III and IV). Representative isolates of these groups were subjected, as in the previous case, to molecular identification; but, in this instance, the study of recA gene (amplification 218 bp) allowed assignation of isolates O-M-6, O-A-5, O-A-2, B-F-5, O-A-7 and B-M-9 to L. pentosus species (see Table 1 or Fig. 6 for assignation to clusters). Therefore, the strains of L. plantarum found after packaging were displaced by the rest of genotypes of L. pentosus throughout shelf life. A total of 10 lactobacilli were also obtained exclusively from fruits with visible evidence of spoilage. These 10 isolates (labelled with the letter A in Fig. 6) were distributed in 3 biotypes: I (6 isolates), II (3 isolates) and III (1 isolate), all of them belonging to L. pentosus species. In clusters, I and III were also present other isolates obtained from the middle, and final samplings in both brines and non-altered fruits: but, on the contrary, all isolates obtained from cluster II were exclusively related to spoilt fruits.

The dendrogram generated using the patterns profile of the 20 yeast isolates obtained after packaging (then this group of microorganism disappeared) (Fig. 7) differentiated a total of 9 major groups below reproducibility of the technique (80.5%).
Hence, there is a greater number of biotypes in yeast population compared to that of LAB. The clusters with a larger number of isolates were I, IV, VI and VIII, with three isolates each one. As in the previous case, isolates from different clusters were randomly selected for further identification by sequencing of D1/D2 domains of 26S gene and Blast analysis. *Candida tropicalis* was the predominant species in clusters II, III, IV, V and VI (a total of 10 isolates), while *Candida parapsilosis* was present in clusters VII and VIII (6 isolates) and *Lodderomyces elongisporus* in clusters I and IX (4 isolates). The percentage of identity with previously published sequences in NCBI GenBank database was always high (>99%) (see Table 1).

4. Discussion

The shelf life of traditional packaged *Aloreña de Málaga* table olives for a period similar to that noticed in this work was determined in previous studies (Arroyo-López et al., 2005, 2006b, 2009). However, during the last decade, the industry has considerably modified the conditions of the packaging brine using new combinations and higher concentrations of preservatives compared to the past. The more stringent packaging conditions have prevented the gas production and container swelling spoilages (related to yeast growth), but on the contrary, have favoured apparition of a new type of spoilage not described before characterized by the formation of whitish and soft regions on the olive surface.

The use of the new packaging conditions has also altered the physicochemical characteristics of the packages. Respect to previous studies, now NaCl concentration is lower, the fruit firmness has decreased, but the titratable acidity, $L^*$, $a^*$ and sugar concentration have increased (Arroyo-López et al., 2005, 2006b, 2009). Alves et al. (2015) also have reported a considerable residual sugar concentration and an increase in
the $a^*$ colour parameter during storage of cracked green table olives from Maçanilha cultivar in the presence of sorbic and benzoic acids. A loss of fresh appearance, together with the presence of high levels of reducing sugars, limit considerably the shelf life of packaged cracked olives (Alves et al., 2015; Arroyo-López et al., 2005). However, darkening of brines in the present study was lower than those reported by Alves et al. (2015) for Maçanilha green cracked olives.

In this study, Enterobacteriaceae were only detected at the first sampling time (1 day after packaging) and at low population levels. Therefore, they are not related to the spoilage. Their strong inhibition was possibly due to the low pH (~4.0) prevailing during all the shelf life period. The disappearance of these microorganisms during the first week after olive packaging has also been reported by other authors (Arroyo-López et al., 2005, 2009; Bautista-Gallego et al., 2011; Alves et al., 2015). The species found, E. gergoviae, has been related to different human diseases outbreaks (Batt & Tortorello, 2014) and has also been isolated from diverse vegetables marketed in Spain and Germany (Schwaiger, Helmke, Hölzel, & Bauer, 2011; Falomir, Rico, & Gozalbo, 2013) but, as far as we know, this is the first time that has been isolated from table olives. E. gergoviae presence in packaged Aloreña de Málaga reinforces the need for maintaining low pH levels in this brine mixture, to ensure the final product safety. Also, industry should wait for at least 24 h before bringing the packages to market to ensure the inhibition of this species.

Another difference found in this work with respect to the previous finding is related to yeast populations. In the past, the yeast population during storage of Aloreña de Málaga olives was reduced in only 1.5 log$_{10}$ cycle (from 5.0 to 3.5 log$_{10}$ CFU/mL after 67 days (Arroyo López et al., 2005). However, in other cases, a marked yeast
increase was even noticed reaching population levels up to 6 log_{10} CFU/mL; they were mainly caused by the presence of *Saccharomyces cerevisiae, Issatchenka occidentalis, Geotrichum candidum, Zygosaccharomyces bailli, Candida diddensiae* and *Candida holmii* species (Arroyo López et al., 2006a, 2006b, 2009; Bautista-Gallego et al., 2011).

In the presence of sorbic and benzoic acids, Alves et al. (2015) reported a reduction of the yeast population (from approximately 4 to 2 log_{10} CFU/mL) in packed cracked green table olives from Maçanilha cultivar. However, in this study, there was a faster and complete inhibition of the yeast population from the 10th day onward due to the higher concentrations of potassium sorbate and sodium benzoate currently applied in packaging, which have a strong inhibitory effect on table olive related yeasts (Arroyo-López, Bautista-Gallego, Durán-Quintana, & Garrifo-Fernández, 2008). The species found initially were *Lodderomyces elongisporus, Candida parapsilosis* and especially *Candida tropicalis*, the latter strongly associated with table olive processing (Arroyo-López et al., 2012). However, *L. elongisporus* (synonym *Saccharomyces elongisporus*) is frequently related to soft drinks and concentrated juices (Kurtzman, Fell, & Boekhout, 2011). Because of phenotypic similarities, *L. elongisporus* had been considered the teleomorph state of *C. parapsilosis*. In any case, there is a close taxonomic relationship among *C. tropicalis, L. elongisporus* and *C. parapsilosis* species (Kurtzman et al., 2011).

The presence of higher sugar concentrations, lower salt contents, and yeast inhibition has favoured in this survey the growth of LAB. The survival of LAB during storage of diverse green table olives is well documented (Casado, Higínio-Sánchez, De Castro, Rejano, Beato, & Montaño, 2011; Blana, Polymeneas, Tassou, & Panagou, 2016). Because of the high identity value (>99%) shared between *L. plantarum* and *L. pentosus* in 16S ribosomal DNA sequences (Collins et al., 1991), all selected isolates
for sequencing were identified in the first step as *L. plantarum* species (see Table 1). However, multiplex PCR assay based on *recA* gene permitted the discrimination between isolates belonging to *L. plantarum* and *L. pentosus*, which identified *L. plantarum* as the species dominant after packaging, being displaced by *L. pentosus* in the middle and final storage times. This way, *L. plantarum* was not present when the alteration was noticed. Blana et al. (2016) also found a better adaptation of *L. pentosus* than *L. plantarum* to survive under olive packaging conditions of Halkidiki variety. Both species have already been identified using molecular methods from packaged *Aloreña de Málaga* table olives by Bautista-Gallego et al. (2011). A specific biotype (II) of *L. pentosus* was present only in the spoilt fruits (and possible a general overpopulation of the species) and could be associated with the spoilage detected at the end of storage. Their presence could also be linked to the “ropy” and the browning aspect of the surrounding brine, possibly due to the production of extracellular polysaccharides, although this point should be proved in further studies. Diverse LAB species have been characterized to metabolize organic acids and preservatives into undesirable compounds in foods and cause clouding and spoilage in the packaged low acidified vegetables, meats and foods in general (Johanningsmeier & McFeeters 2013; Pothakos et al., 2015). In particular, Pothakos et al. (2015) mention the importance of the LAB characterization at strain level in the spoilt product, because significant distinction among biotypes is substantiated by studies determining spoilage potential as a strain-specific trait. In this context, Montaño et al. (2013) identified a strain of *L. pentosus* (L6) from table olive processing, with the ability to remove sorbate from the medium and compromise the packed product stability.

5. Conclusions
The present study reports for the first time the presence of a new type of spoilage affecting fruit quality and stability of directly brined table olives. Enterobacteriaceae and yeasts were absent during most of the storage time and were unlikely to be associated with the spoilage. The NaCl, sugar content and yeast inhibition in brine may have favoured the dynamic of the LAB population, which changed throughout shelf life from being predominantly L. plantarum (after packaging) to L. pentosus (at the end of the study). Therefore, the alteration, apparently, was caused by an increasing higher population of LAB, which overpopulation coincided with the product spoilage (after 2 months packaging) and, particularly, with the presence of a specific biotype (II) of L. pentosus isolated from spoilt olives. However, more controlled experiments are required to confirm the association between this microorganism and the unique spoilage observed in this study, as well as the origin of these organisms (spices, fermentation process, industrial equipment, etc.). These results emphasise the need for developing more appropriate preservatives and packaging conditions for this presentation and green directly brined olives in general.

Acknowledgments

The research leading to these results has received funding from Junta de Andalucía Government through the PrediAlo project (AGR-7755: www.predialo.science.com.es). FNAL wishes to express thanks to the Spanish government for his RyC postdoctoral research contract while VRG would like to thank ceiA3, Spanish Government, Bank of Santander, IG-CSIC and ‘Aloreña de Málaga’ Olive Manufacturing Association for her pre-doctoral fellowship.

References


**Figure Legends**

*Figure 1.* Evolution of the main physicochemical parameters of brines (pH, salt, combined and titratable acidity) during shelf life (0-63 days) of traditional PDO Aloreña
de Málaga table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n=2).

Figure 2. Evolution of the firmness of fruits and sugar content in brine (total and individual) during shelf life (0-63 days) of traditional PDO Aloreña de Málaga table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n=2).

Figure 3. Evolution of the colour parameters of fruits ($L^*$, luminosity; $a^*$, green-fresh; $h_{ab}$, hue angle) and brines ($B_d$, brine darkness) during shelf life (0-63 days) of traditional PDO Aloreña de Málaga table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n=2).

Figure 4. Lactic acid bacteria and yeast counts in both brines and fruits during shelf life (0-63 days) of traditional PDO Aloreña de Málaga table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n=2).

Figure 5. An example of fruits with spoilage evidence in traditional PDO Aloreña de Málaga table olives. The presence of this spoilage ($63^{rd}$ day) in all packages marked the end of the shelf life experiment.

Figure 6. Dendrogram generated after cluster analysis of the digitalized rep-PCR fingerprints with a GTG3 primer of 65 lactobacilli isolates. They were obtained from brines (B) or olives (O) at the initial sampling time (I, 1 day), middle (M, 28 days) and end shelf life (F, 63 days) as well as in fruits with evidence of spoilage (A). 1-10 is the number of the isolate, obtained from the different sampling periods. Clustering parameters: 0.5% optimization and 0.0% curve smoothing. Isolates selected for sequencing from different clusters below 85.1% similarity (reproducibility of technique) are marked with an asterisk (*).
Figure 7. Dendrogram generated after cluster analysis of the digitalized RAPD-PCR fingerprints with an M13 primer of 20 yeast isolates obtained from brines (B, 1-10) or olives (O, 1-10) after packaging (0 days). Clustering parameters: 0.5% optimization and 0.0% curve smoothing. Isolates selected for sequencing from different clusters below 80.5% similarity (reproducibility of technique) are marked with an asterisk (*).
Table 1. Microbial isolates obtained from different clusters of the DNA-fingerprinting analysis from PDO Aloreña de Málaga table olive packages and subjected to further molecular identification.

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Isolate reference</th>
<th>Cluster number</th>
<th>*Matching nucleotides/identity %</th>
<th>**Closest relative species</th>
<th>***Identification based in <em>recA</em> gene product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>O-M-6</td>
<td>I</td>
<td>765 bp / 99%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O-A-5</td>
<td>I</td>
<td>446 bp / 100%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O-A-2</td>
<td>II</td>
<td>796 bp / 100%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B-F-5</td>
<td>III</td>
<td>1000 bp / 99%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O-A-7</td>
<td>III</td>
<td>346 bp / 99%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B-M-9</td>
<td>IV</td>
<td>974 bp / 100%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B-I-4</td>
<td>V</td>
<td>783 bp / 100%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O-I-3</td>
<td>VI</td>
<td>989 bp / 99%</td>
<td><em>Lactobacillus plantarum</em></td>
<td>gi</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>F-I-1</td>
<td></td>
<td>647 bp / 100%</td>
<td><em>Enterobacter gergoviae</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>F-I-2</td>
<td></td>
<td>461 bp / 99%</td>
<td><em>Enterobacter gergoviae</em></td>
<td>gi</td>
</tr>
<tr>
<td>Yeast</td>
<td>B3</td>
<td>I</td>
<td>526 bp / 100%</td>
<td><em>Lodderomyces elongisporus</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O7</td>
<td>II</td>
<td>552 bp / 100%</td>
<td><em>Candida tropicalis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td>III</td>
<td>544 bp / 100%</td>
<td><em>Candida tropicalis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O4</td>
<td>IV</td>
<td>537 bp / 99%</td>
<td><em>Candida tropicalis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>V</td>
<td>557 bp / 99%</td>
<td><em>Candida tropicalis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>VI</td>
<td>541 bp / 99%</td>
<td><em>Candida tropicalis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>O8</td>
<td>VII</td>
<td>543 bp / 100%</td>
<td><em>Candida parapsilosis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>VIII</td>
<td>546 bp / 100%</td>
<td><em>Candida parapsilosis</em></td>
<td>gi</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>IX</td>
<td>547 bp / 100%</td>
<td><em>Lodderomyces elongisporus</em></td>
<td>gi</td>
</tr>
</tbody>
</table>

* Sequence identity of the D1/D2 domains of the 26S ribosomal gene for yeasts, and small-subunit 16S rRNA gene for bacteria.
** Accession number for nucleotide sequences and closest related species found in the NCBI GenBank database.
*** Final identification for lactobacilli deduced from multiplex PCR assay based on *recA* gene (Torriani et al. 2001).
Figure 4
Click here to download high resolution image