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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

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Running title: *Lactobacillus pentosus* in packaged olives

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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 GTG_5, 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

141 *3.1. Physicochemical and microbiological changes during packaged olive storage*

142 Salt (~4.75 g/100 mL) and pH (~4.0) values were kept quite stable during the
143 study (63 days), without significant differences among sampling times, while the
144 combined and titratable acidity showed a slightly increasing trend (Fig. 1). Firmness
145 slightly decreased from an initial 6.0 value to a final 5.0 kN/100 g after two months
146 (Fig. 2), showing a high variability in measurements which did not allow establishing
147 significant statistical differences. The initial total sugar content in brines was ~7.5 g/L
148 and was composed of glucose (4.8 g/L), fructose (1.5 g/L), mannitol (0.8 g/L) and
149 sucrose (0.4 g/L). Total sugar concentrations significantly decreased throughout shelf
150 life and reached ~5.0 g/L after two months, being glucose the only sugar whose level
151 decreased (Fig. 2), because, as usual, the simple sugars are the first to be consumed by
152 microorganisms. The L^* colour parameter significantly increased ($p<0.05$) from an
153 initial 56 to final 59 value at the end of storage. The a^* colour parameter also rose from
154 0.5 to 3.5, which means a significant loss of green colour. Hue angle (h_{ab}) decreased
155 from 88º (initial) to 85º (final), indicating the browning of fruits. The packing brine's
156 was also browning progressively (B_d increased from the initial 0.25 to final 0.35) (Fig.
157 3). Therefore, the main physicochemical changes that compromised the stability and
158 quality of the product were: loss of green colour in fruits, a decrease of firmness,
159 browning of brines and glucose reduction. From Figs. 1-3 can be deduced that the 20th
160 day was critical in the evolution of many of the physicochemical parameters.

161 *Enterobacteriaceae* were only found on olives after packaging (1st day) at very
162 low population levels (<2.2 $\log_{10}$ CFU/g). The sequencing of small-subunit 16 rRNA
163 gene of two isolates obtained from fruits showed that both belonged to the same species,
164 *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 10^{th} 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 63^{rd} 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±0.84% 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

189 The dendrogram generated with the pattern profiles of the 65 lactobacilli isolates
190 obtained from commercially packaged olives (Fig. 6) showed six major biotypes that
191 were differentiated below reproducibility of the technique (85.1%). In practice, all
192 isolates obtained from the initial sampling point formed two groups were clearly
193 separated from the rest (cluster V and VI) sharing a 62.1% similarity between them.
194 Sequencing analysis of small-subunit 16 rRNA gene and further confirmation by
195 multiplex PCR assay based on *recA* gene (amplification 318 bp), allowed identification
196 of isolates B-I-4 and O-I-3 (belonging to clusters V and VI, respectively) as *L.*
197 *plantarum* species (Table 1). Both DNA fingerprint profiles were not found later and at
198 middle and final sampling points, in either brines or olives, while other four different
199 genotypes were noted (clusters I, II, III and IV). Representative isolates of these groups
200 were subjected, as in the previous case, to molecular identification; but, in this instance,
201 the study of *recA* gene (amplification 218 bp) allowed assignation of isolates O-M-6, O-
202 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
203 assignation to clusters). Therefore, the strains of *L. plantarum* found after packaging
204 were displaced by the rest of genotypes of *L. pentosus* throughout shelf life. A total of
205 10 lactobacilli were also obtained exclusively from fruits with visible evidence of
206 spoilage. These 10 isolates (labelled with the letter A in Fig. 6) were distributed in 3
207 biotypes: I (6 isolates), II (3 isolates) and III (1 isolate), all of them belonging to *L.*
208 *pentosus* species. In clusters, I and III were also present other isolates obtained from the
209 middle, and final samplings in both brines and non-altered fruits: but, on the contrary,
210 all isolates obtained from cluster II were exclusively related to spoilt fruits.

211 The dendrogram generated using the patterns profile of the 20 yeast isolates
212 obtained after packaging (then this group of microorganism disappeared) (Fig. 7)
213 differentiated a total of 9 major groups below reproducibility of the technique (80.5%).

214 Hence, there is a greater number of biotypes in yeast population compared to that of
215 LAB. The clusters with a larger number of isolates were I, IV, VI and VIII, with three
216 isolates each one. As in the previous case, isolates from different clusters were
217 randomly selected for further identification by sequencing of D1/D2 domains of 26S
218 gene and Blast analysis. *Candida tropicalis* was the predominant species in clusters II,
219 III, IV, V and VI (a total of 10 isolates), while *Candida parapsilosis* was present in
220 clusters VII and VIII (6 isolates) and *Lodderomyces elongisporus* in clusters I and IX (4
221 isolates). The percentage of identity with previously published sequences in NCBI
222 GenBank database was always high (>99%) (see Table 1).

223 **4. Discussion**

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

233 The use of the new packaging conditions has also altered the physicochemical
234 characteristics of the packages. Respect to previous studies, now NaCl concentration is
235 lower, the fruit firmness has decreased, but the titratable acidity, L^*, a^* and sugar
236 concentration have increased (Arroyo-López et al., 2005, 2006b, 2009). Alves et al.
237 (2015) also have reported a considerable residual sugar concentration and an increase in

238 the *a** colour parameter during storage of cracked green table olives from Maçanilha
239 cultivar in the presence of sorbic and benzoic acids. A loss of fresh appearance, together
240 with the presence of high levels of reducing sugars, limit considerably the shelf life of
241 packaged cracked olives (Alves et al., 2015; Arroyo-López et al., 2005). However,
242 darkening of brines in the present study was lower than those reported by Alves et al.
243 (2015) for Maçanilha green cracked olives.

244 In this study, *Enterobacteriaceae* were only detected at the first sampling time (1
245 day after packaging) and at low population levels. Therefore, they are not related to the
246 spoilage. Their strong inhibition was possibly due to the low pH (~4.0) prevailing
247 during all the shelf life period. The disappearance of these microorganisms during the
248 first week after olive packaging has also been reported by other authors (Arroyo-López
249 et al., 2005, 2009; Bautista-Gallego et al., 2011; Alves et al., 2015). The species found,
250 *E. gergoviae*, has been related to different human diseases outbreaks (Batt & Tortorello,
251 2014) and has also been isolated from diverse vegetables marketed in Spain and
252 Germany (Schwaiger, Helmke, Hölzel, & Bauer, 2011; Falomir, Rico, & Gozalbo,
253 2013) but, as far as we know, this is the first time that has been isolated from table
254 olives. *E. gergoviae* presence in packaged *Aloreña de Málaga* reinforces the need for
255 maintaining low pH levels in this brine mixture, to ensure the final product safety. Also,
256 industry should wait for at least 24 h before bringing the packages to market to ensure
257 the inhibition of this species.

258 Another difference found in this work with respect to the previous finding is
259 related to yeast populations. In the past, the yeast population during storage of *Aloreña*
260 *de Málaga* olives was reduced in only 1.5 $\log_{10}$ cycle (from 5.0 to 3.5 $\log_{10}$ CFU/mL
261 after 67 days (Arroyo López et al., 2005). However, in other cases, a marked yeast

262 increase was even noticed reaching population levels up to 6 $\log_{10}$ CFU/mL; they were
263 mainly caused by the presence of *Saccharomyces cerevisiae, Issatchenkia occidentalis,*
264 *Geotrichum candidum, Zygosaccharomyces bailli, Candida diddensiae* and *Candida*
265 *holmii* species (Arroyo López et al., 2006a, 2006b, 2009; Bautista-Gallego et al., 2011).
266 In the presence of sorbic and benzoic acids, Alves et al. (2015) reported a reduction of
267 the yeast population (from approximately 4 to 2 $\log_{10}$ CFU/mL) in packed cracked
268 green table olives from Maçanilha cultivar. However, in this study, there was a faster
269 and complete inhibition of the yeast population from the 10^{th} day onward due to the
270 higher concentrations of potassium sorbate and sodium benzoate currently applied in
271 packaging, which have a strong inhibitory effect on table olive related yeasts (Arroyo-
272 López, Bautista-Gallego, Durán-Quintana, & Garrifo-Fernández, 2008). The species
273 found initially were *Lodderomyces elongisporus, Candida parapsilosis* and especially
274 *Candida tropicalis,* the latter strongly associated with table olive processing (Arroyo-
275 López et al., 2012). However, *L. elongisporus* (synonym *Saccharomyces elongisporus*)
276 is frequently related to soft drinks and concentrated juices (Kurtzman, Fell, &
277 Boekhout, 2011). Because of phenotypic similarities, *L. elongisporus* had been
278 considered the teleomorph state of *C. parapsilosis.* In any case, there is a close
279 taxonomic relationship among *C. tropicalis, L. elongisporus* and *C. parapsilosis* species
280 (Kurtzman et al., 2011).

281 The presence of higher sugar concentrations, lower salt contents, and yeast
282 inhibition has favoured in this survey the growth of LAB. The survival of LAB during
283 storage of diverse green table olives is well documented (Casado, Higínio-Sánchez, De
284 Castro, Rejano, Beato, & Montaño, 2011; Blana, Polymeneas, Tassou, & Panagou,
285 2016). Because of the high identity value (>99%) shared between *L. plantarum* and *L.*
286 *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.

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437 **Figure Legends**

438 *Figure 1*. Evolution of the main physicochemical parameters of brines (pH, salt,
439 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 GTG_5 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.

Microbial group	**Isolate reference**	**Cluster number**	***Matching nucleotides/ identity%**	****Closest relative species**	*****Identification based in *recA* gene product (bp)**
LAB	O-M-6	I	765 bp / 99%	*Lactobacillus plantarum* | gi|758840967|CP010528.1	218 bp / *Lactobacillus pentosus*
	O-A-5	I	446 bp / 100%	*Lactobacillus plantarum* | gi|746590652|KM657203.1	218 bp / *Lactobacillus pentosus*
	O-A-2	II	796 bp / 100%	*Lactobacillus plantarum* | gi|675277874|KJ917253.1	218 bp / *Lactobacillus pentosus*
	B-F-5	III	1000 bp / 99%	*Lactobacillus plantarum* | gi|749389291|CP005942.2	218 bp / *Lactobacillus pentosus*
	O-A-7	III	346 bp / 99%	*Lactobacillus plantarum* | gi|675277874|KJ917253.1	218 bp / *Lactobacillus pentosus*
	B-M-9	IV	974 bp / 100%	*Lactobacillus plantarum* | gi|749389291|CP005942.2	218 bp / *Lactobacillus pentosus*
	B-I-4	V	783 bp / 100%	*Lactobacillus plantarum* | gi|732665802|KM577184.1	318 bp / *Lactobacillus plantarum*
	O-I-3	VI	989 bp / 99%	*Lactobacillus plantarum* | gi|731188889|KM507561.1	318 bp / *Lactobacillus plantarum*
Enterobacteriaceae	F-I-1		647 bp / 100%	*Enterobacter gergoviae* | gi|404211719|JX567313.1	
	F-I-2		461 bp / 99%	*Enterobacter gergoviae* | gi|359803192|AB682278.1	
Yeast	B3	I	526 bp / 100%	*Lodderomyces elongisporus* | gi|588284414|KF935228.1	
	O7	II	552 bp / 100%	*Candida tropicalis* | gi|736603388|KP064125.1	
	O2	III	544 bp / 100%	*Candida tropicalis* | gi|736603388|KP064125.1	
	O4	IV	537 bp / 99%	*Candida tropicalis* | gi|657234362|KF359928.1	
	B1	V	557 bp / 99%	*Candida tropicalis* | gi|170676492|EU543680.1	
	B2	VI	541 bp / 99%	*Candida tropicalis* | gi|736603388|KP064125.1	
	O8	VII	543 bp / 100%	*Candida parapsilosis* | gi|672941186|KJ817165.1	
	B6	VIII	546 bp / 100%	*Candida parapsilosis* | gi|672941186|KJ817165.1	
	B5	IX	547 bp / 100%	*Lodderomyces elongisporus* | gi|588284414|KF935228.1	

* 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 1

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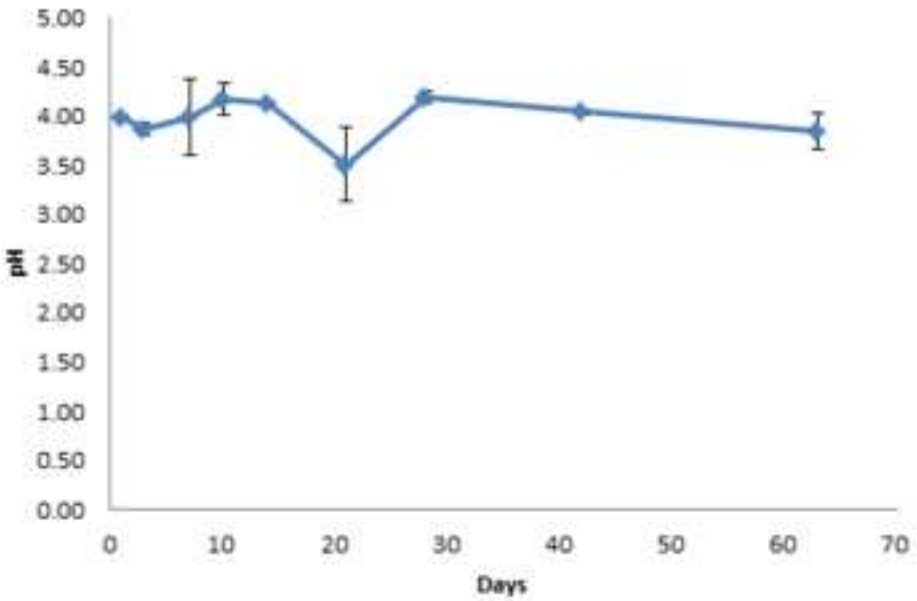

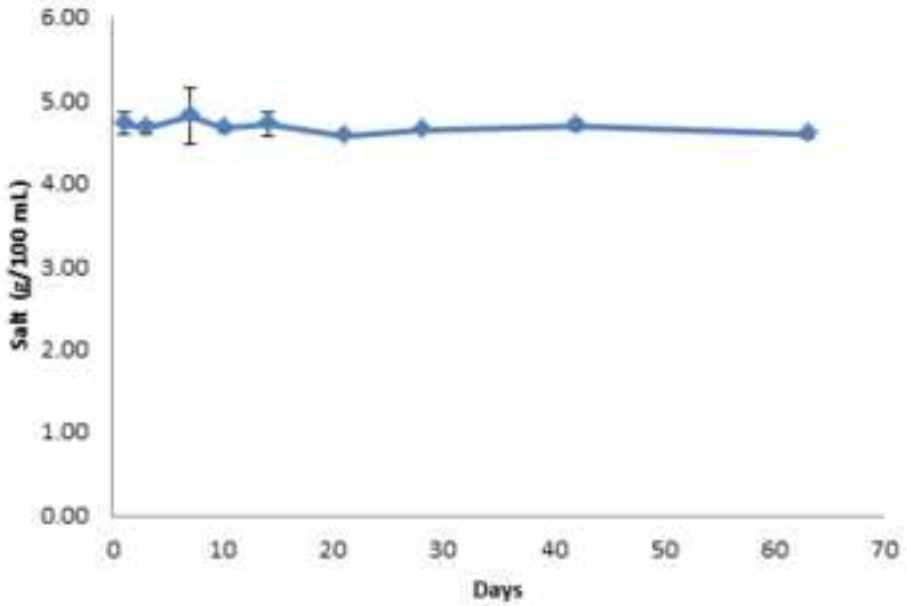

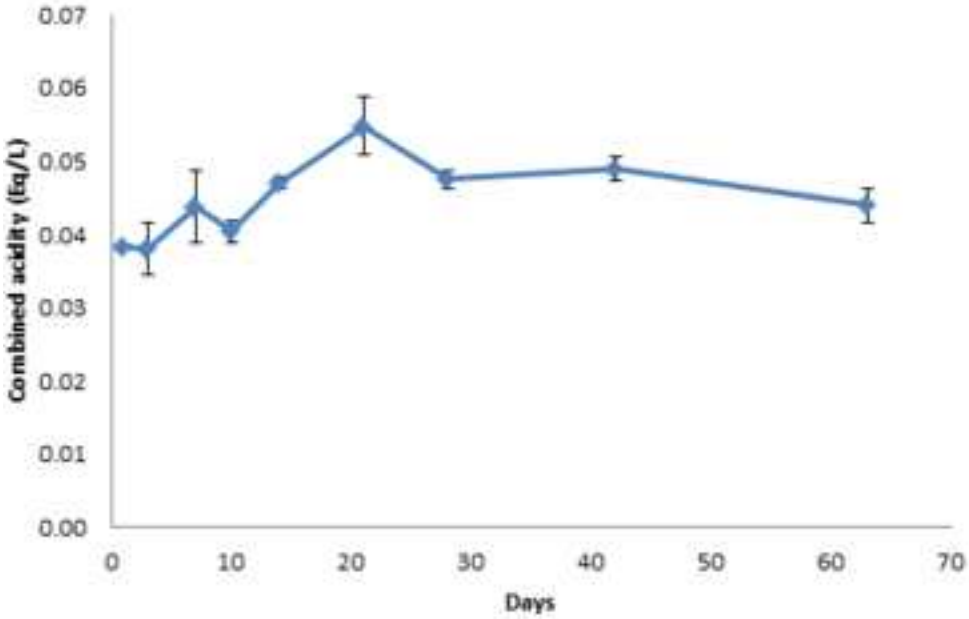

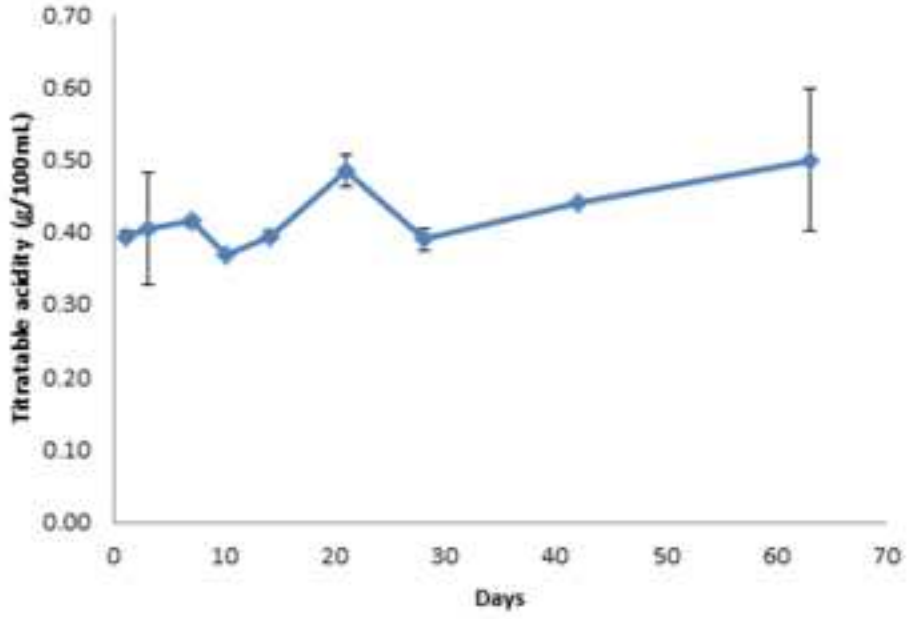

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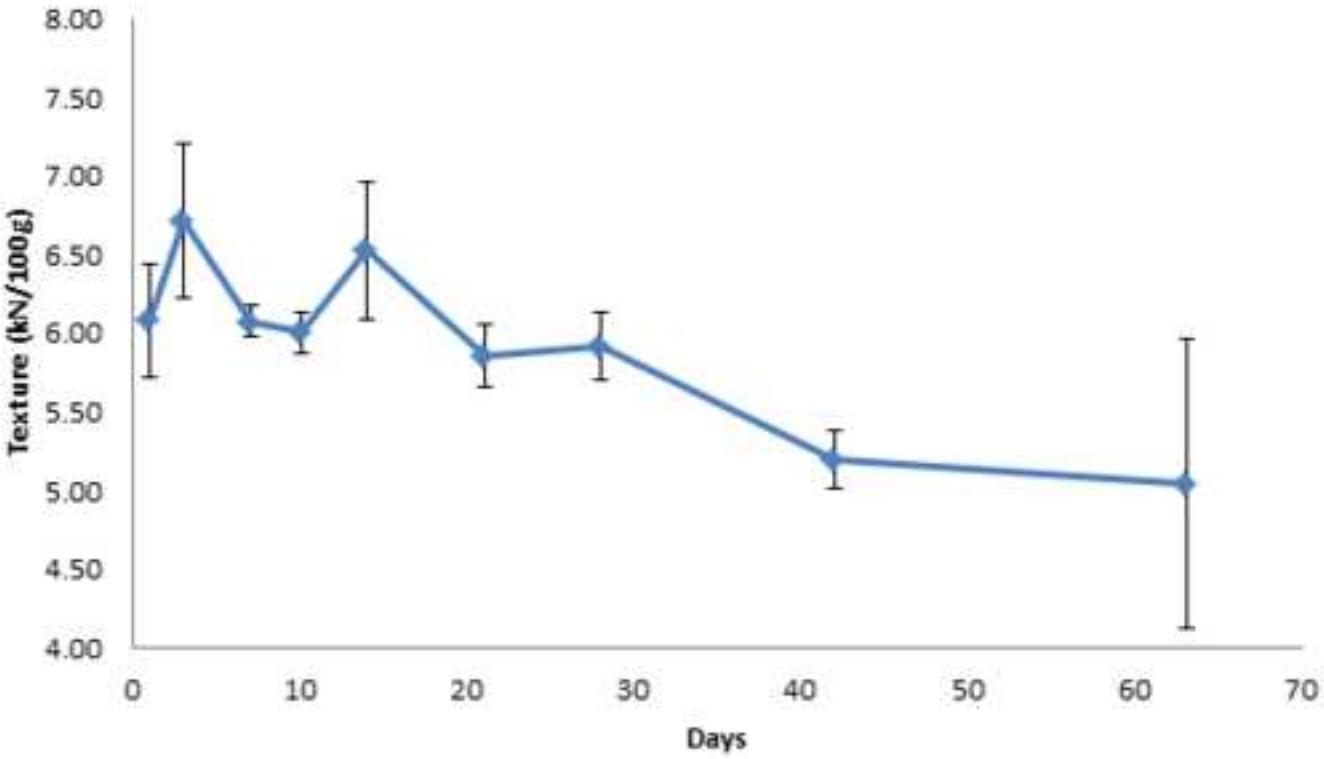

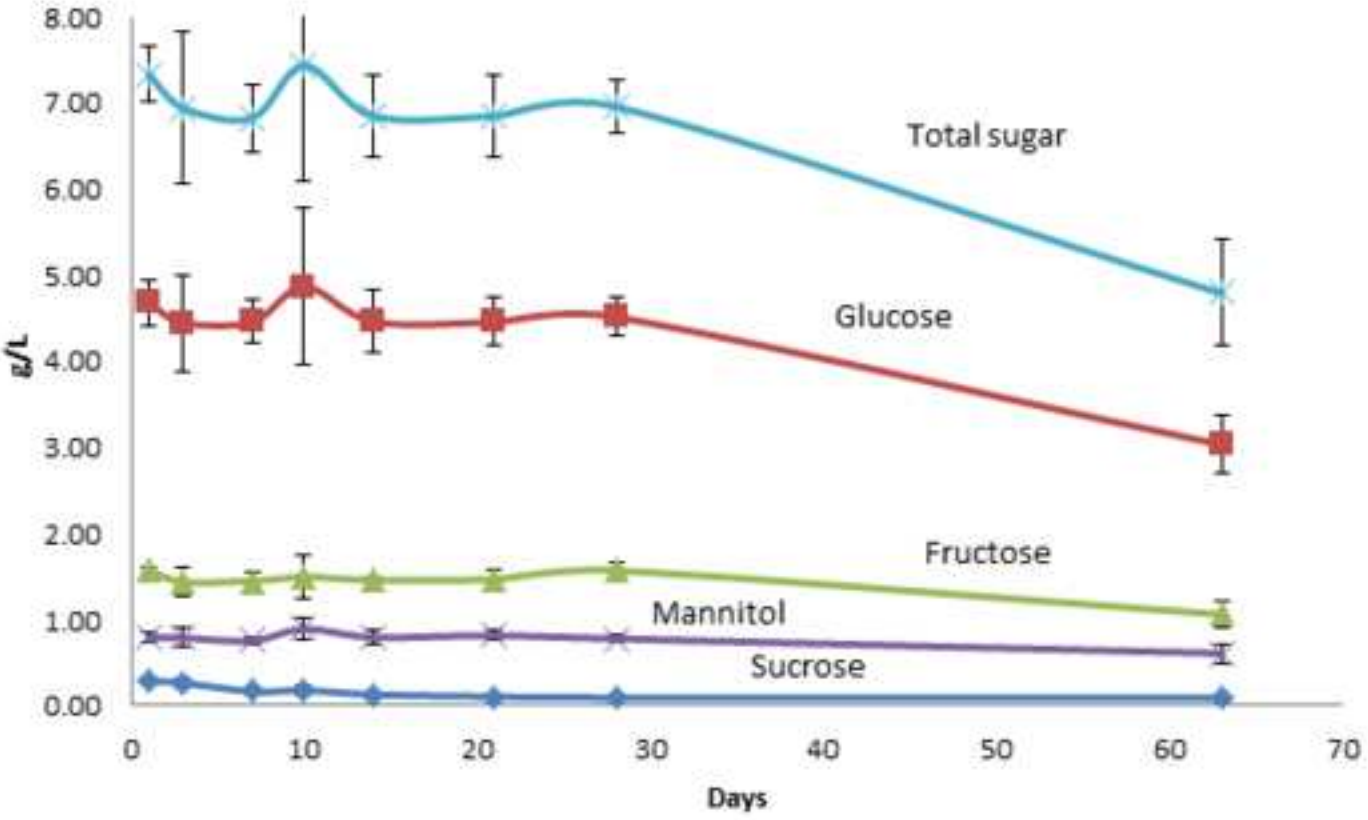

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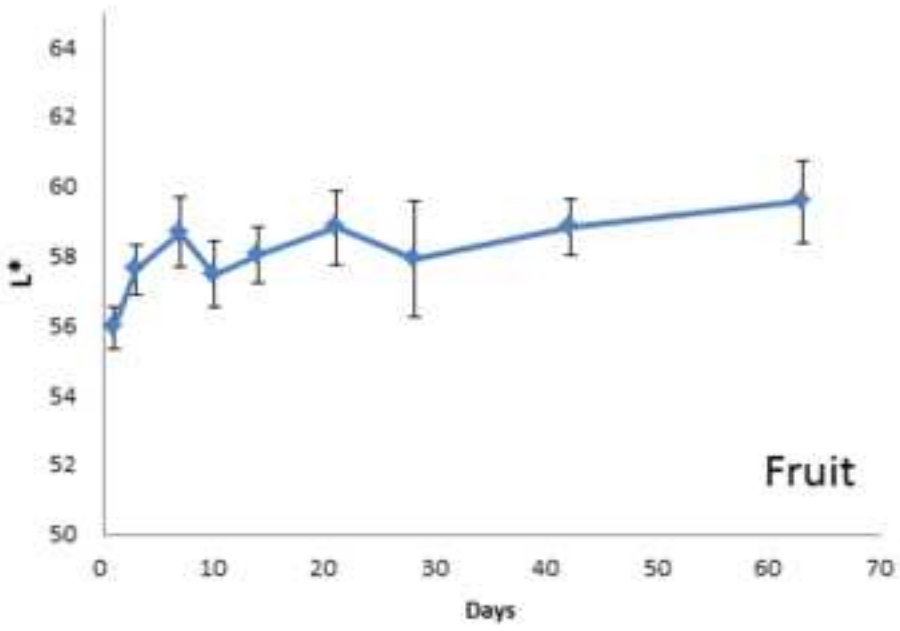

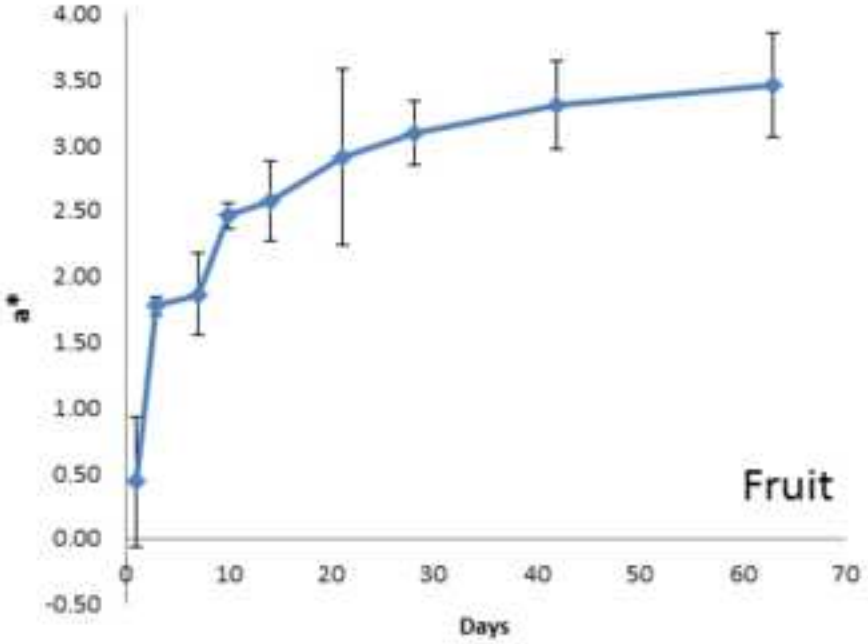

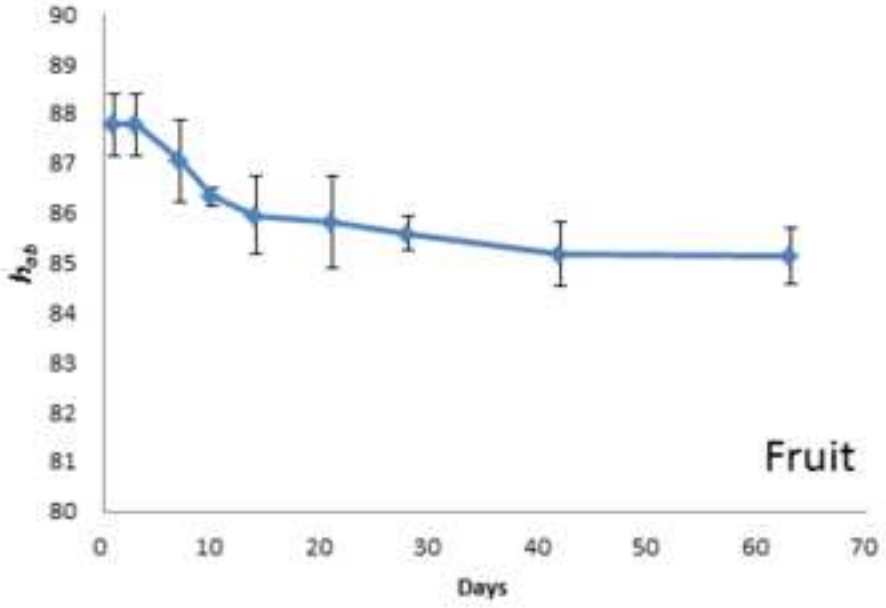

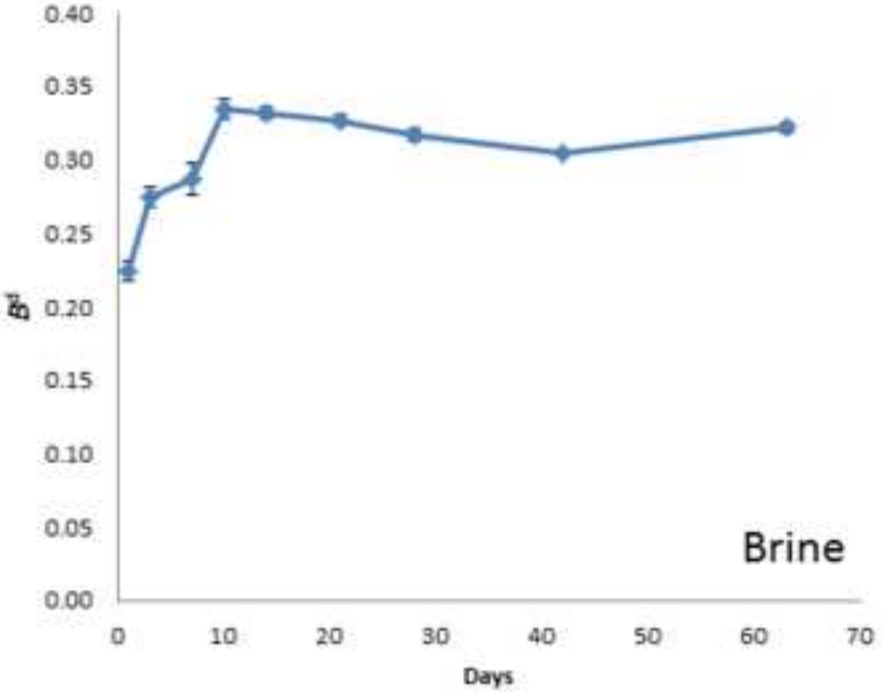

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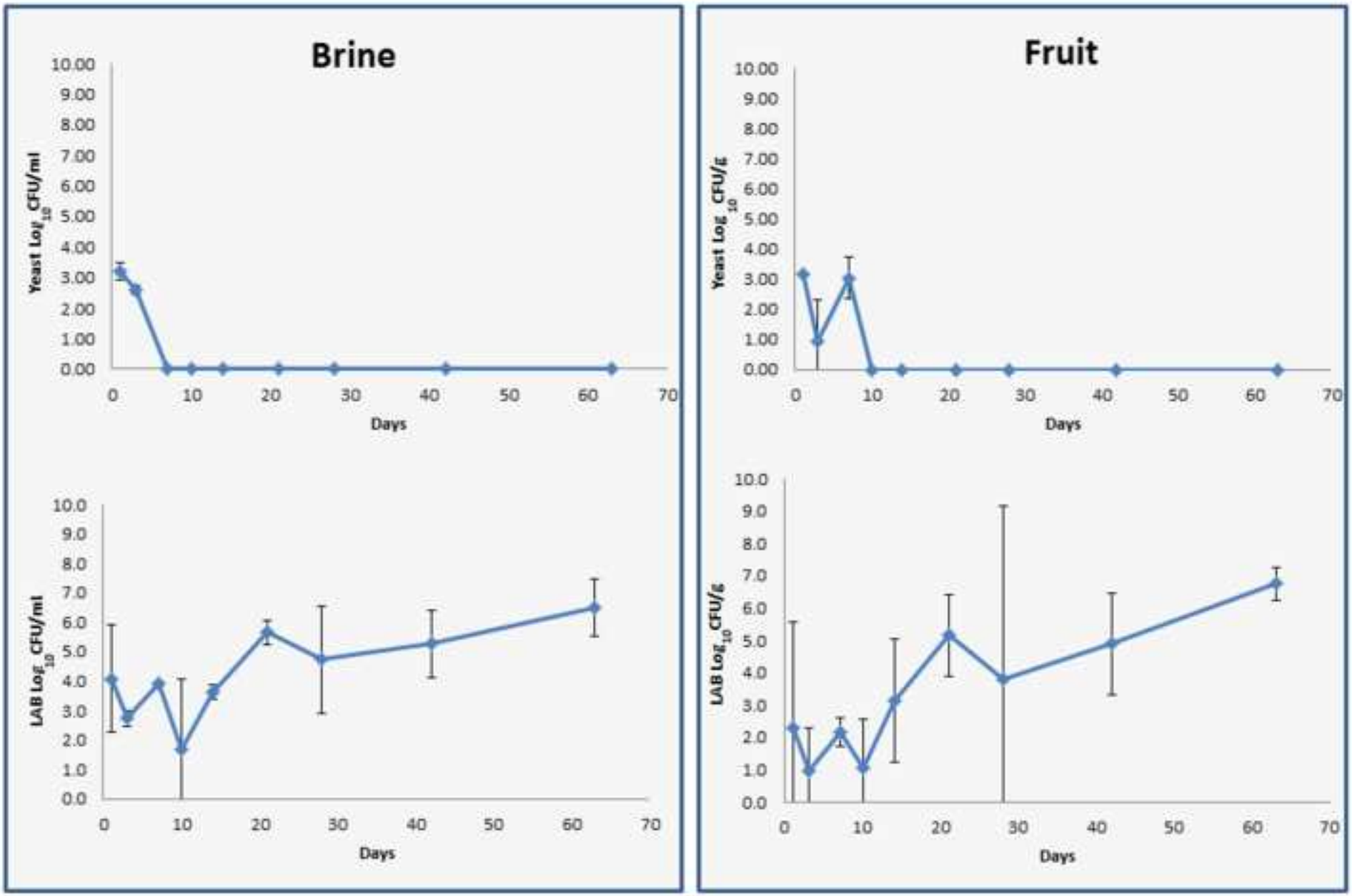

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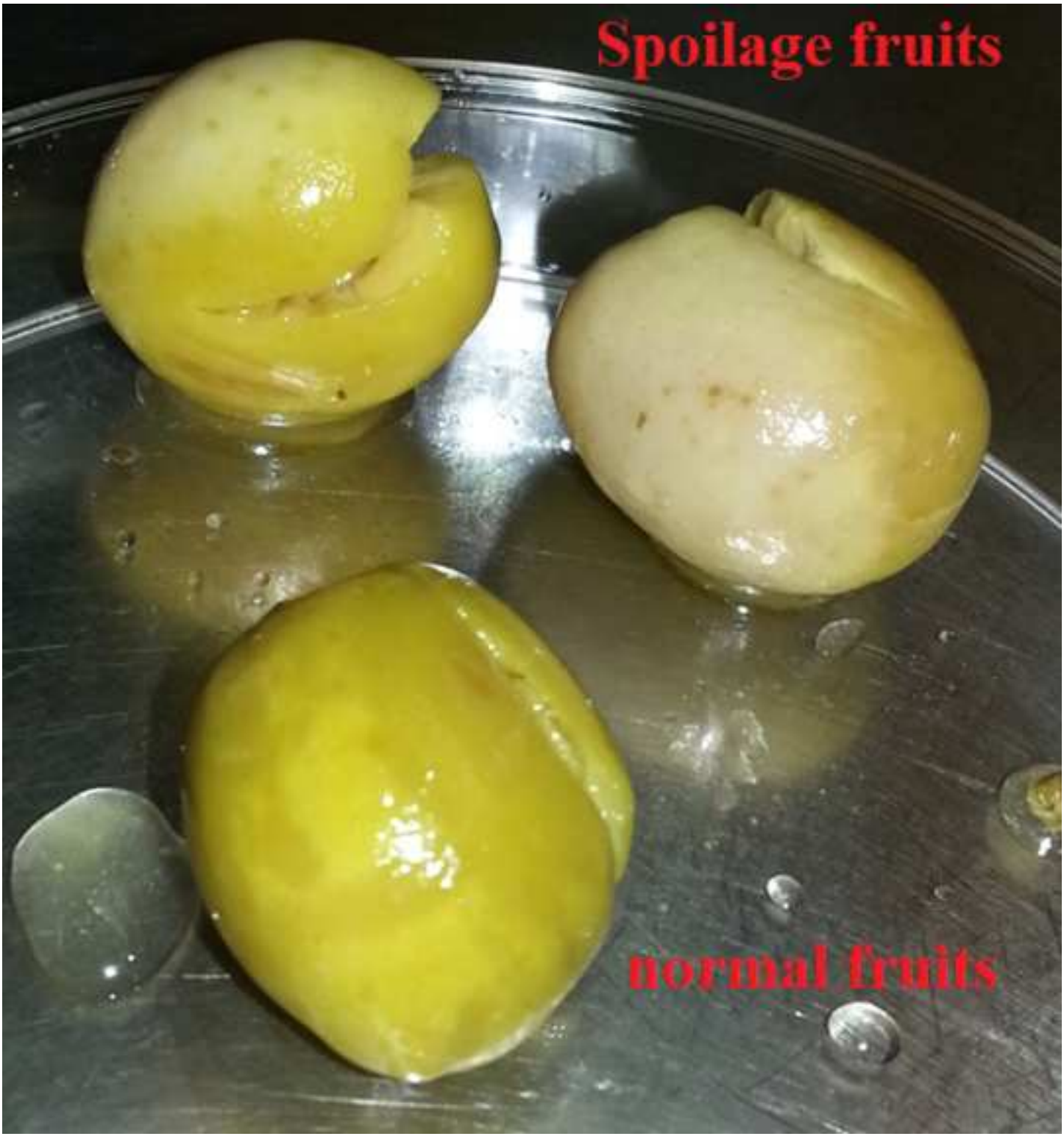

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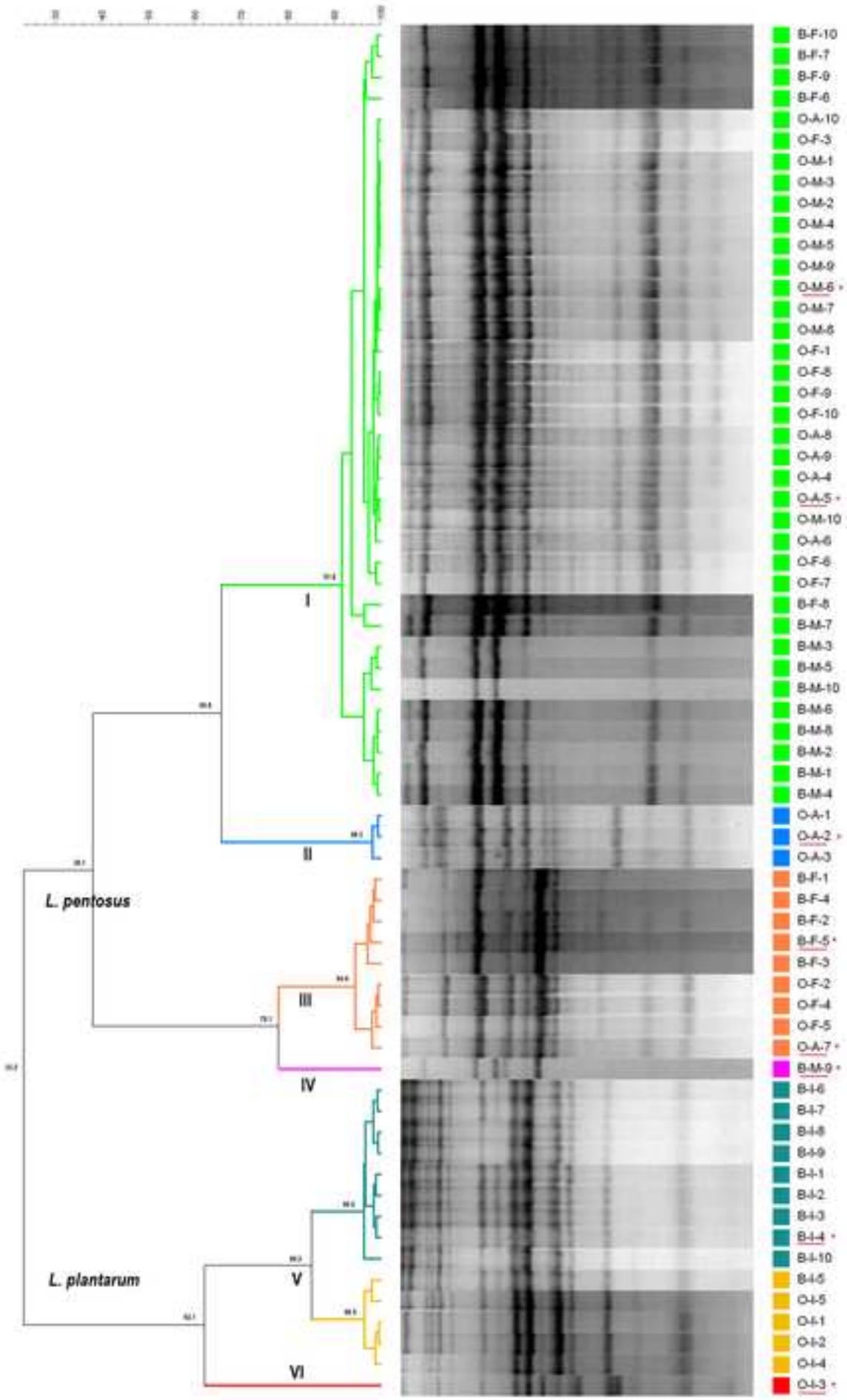

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