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Controlled fermentation of Moroccan picholine green olives by oleuropein-degrading Lactobacilli strains

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SUMMARY: The control of the spontaneous fermentation process of un-debittered Moroccan Picholine green olives was undertaken basing the inoculation with two lactobacilli strains (*Lactobacillus plantarum* S175 and *Lactobacillus pentosus* S100). These strains, previously selected in our laboratory for their oleuropein-degrading capacity, were inoculated in olives brined at 5% of NaCl, and then incubated at 30 °C. The physico-chemical parameters (pH, free acidity, reducing sugars, sodium chloride, oleuropein and its hydrolysis products), and the microbiological parameters (mesophilic aerobic bacteria, coliforms, *Staphylococcus*, lactic acid bacteria and yeasts and moulds), were regularly analyzed during the fermentation time. The results obtained showed the effectiveness of the lactic acid bacteria strains to develop suitable oleuropein biodegradation and controlled lactic fermentation processes more than the un-inoculated olives (control). This result was confirmed by the rapid elimination of coliforms and staphylococcus, the accumulation of hydroxytyrosol as a result of oleuropein biodegradation, and a drastic reduction in spoiled olives with good quality fermented olives.

KEYWORDS: *Fermentation; Green olives; Lactobacillus; Oleuropein; Starter*

RESUMEN: ***Fermentación controlada de aceitunas verdes picholine marroquí mediante cepas de lactobacilos degradantes de oleuropeína***. Se llevó a cabo el control del proceso de fermentación espontánea de aceitunas verdes sin desamargar picholine marroquí basado en la inoculación con dos cepas de lactobacilos (*Lactobacillus plantarum* S175 y *Lactobacillus pentosus* S100). Estas cepas, seleccionadas previamente en nuestro laboratorio por su capacidad de degradar a la oleuropeína, se inocularon en las aceitunas en salmuera al 5 % de NaCl, y después se incubaron a 30 °C. Los parámetros físico-químicos (pH, acidez libre, reducción de azúcares, cloruro sódico, oleuropeína y sus productos de hidrólisis) y los parámetros microbiológicos (bacterias aerobias mesófilas, coliformes, estafilococos, bacterias lácticas y levaduras y mohos), fueron analizados regularmente durante el tiempo de fermentación. Los resultados obtenidos mostraron la eficacia de las cepas de bacterias lácticas para desarrollar una adecuada biodegradación de la oleuropeina y los procesos de fermentación láctica controlados más que en el caso de las aceitunas no inoculadas (control). Estos resultados fueron confirmados por la rápida eliminación de coliformes y estafilococos, por la acumulación de hidroxitirosol como resultado de biodegradación de la oleuropeína, y por ladrástica reducción de aceitunas estropeadas y por la buena calidad de las aceitunas fermentadas.

***PALABRAS CLAVE*:** *Aceitunas; Fermentación; Iniciadores; Lactobacillus; Oleuropeína*

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1. INTRODUCTION

The natural lactic fermentation of green olives is a complex process. It is mainly based on a spontaneous fermentation characterized by its long duration and frequently associated with high spoilage incidence in olives, and leads to an end product with varying quality (Fernandez-Diez *et al*., 1985). The fermentation process is naturally associated with the development of various microorganisms. The lactic acid bacteria (LAB) and yeasts considered as beneficial microorganisms are highly desired to assure the fermentation process of olives which consequently leads to their transformation to edible products, in comparison to the role played by undesirable microbiota, composed of enterobacteria, *Bacillus* and moulds, known for their involvement in various olive spoilages (Fernández-Diez *et al*., 1985, Garrido-Fernández *et al*., 1997, Arroyo-López *et al*., 2012, Hurtado *et al*., 2012).The control of the natural olive fermentation process with selected LAB strains is of great importance to reduce olive spoilage and to improve the organoleptic properties of the end product.

Green olives are not edible without the elimination of bitterness, due to their natural polyphenols, mainly oleuropein. This polyphenol is known for its antimicrobial activity against various microorganisms, particularly LAB (Juven B. *et al*., 1968, Fleming and Etchells, 1967, Fleming *et al*., 1973, Furneri *et al*., 2002, Ruiz-Barba *et al*., 1990, Ruiz-Barba *et al*., 1991, Ruiz-Barba *et al*., 1993, Landete *et al*., 2008, Medina *et al*., 2009, Rodríguez *et al*., 2009) which is highly desired to assure the natural lactic fermentation of green olives. The main LAB involved in this process is composed of *L. plantarum, L. pentosus, L. brevis, Leuconostoc* and *Pediococcus* (Durán Quintana *et al*., 1997, Rodríguez *et al*., 2009). Among these species *Lactobacillus plantarum* and *L. pentosus* were reported as effective starters to allow for a controlled fermentation of table olives (Lamzira *et al*., 2005, Marsilio *et al*., 2005, Panagou *et al*., 2008, Sabatini *et al*., 2008, Servili *et al*., 2006, Hurtado *et al*., 2012).

The tolerance and bio-degradation of oleuropein by certain strains of LAB was demonstrated (Ciafardini *et al*., 1994, Rozes and Peres, 1996, Marsilio *et al*., 1996, Marsilio and Lanza, 1998). In a previous work we have selected strains of *L. plantarum* and *L. pentosus* to show the in-vitro bio-degradation capacity of oleuropein (Ghabbour *et al*., 2011). Their application in un-debittered olive fermentation may improve the nutritional, organoleptic and functional properties of the end product by reducing the nutrient losses due to the de-bittering and washing of the olives, and producing fermented olives rich in LAB cells and their metabolism products. The main objective of this work was to control the natural fermentation process of un-debittered Moroccan Picholine green olives by LAB inoculation, using two lactobacilli strains previously selected for their oleuropein biodegradation capacity. The inoculation was performed in non-sterilized olives in order to evaluate the capacity of the lactobacilli strains to improve the natural fermentation process, widely practiced at the industrial level.

2. MATERIALS AND METHODS

2.1. Olive preparation

Green olives of the Moroccan Picholine variety were purchased at a market in the Oujda area (east of Morocco). The olives were sorted manually, and then 400g of olives were brined at 5% (w/v) of NaCl, in flasks with a volume of about 494 mL, and then adjusted to pH 6 with lactic acid (0.1 N).

2.2. Starter preparation and olive inoculation

The starters were prepared with two separate lactobacilli strains (*L. plantarum* S175 and *L. pentosus* S100) previously selected for their in-vitro oleuropein bio-degradation capacity (Ghabbour *et al*., 2011). The strains were cultivated twice in MRS broth containing 5% (w/v) sodium chloride and incubated overnight at 30 °C. 1.5mL of each MRS culture was diluted in a brine solution containing sodium chloride (5%, w/v) and then used to inoculate 100ml of brine to obtain a final concentration of about 10^7–10^8cfu·mL^{-1}. The inoculation of the olives was done 24 hours after their brining. The assays were carried out as follows: (assay 1): un-inoculated control (spontaneous fermentation), (assay 2): olives inoculated with *L. plantarum* S175 and (assay 3): olives inoculated with *L. pentosus* S100. The assays, made in duplicate, were incubated at 30 °C. The brines regularly and aseptically

sampled were subjected to physicochemical and microbiological analyses.

2.3. Microbiological analysis

The brines sampled were subject to successive decimal dilutions in sterile saline water. From the decimal dilutions, each microbial group was inoculated in its specific medium, using the pour-plate method. The mesophilic aerobic bacteria, coliforms, staphylococci, lactic acid bacteria, and yeasts and moulds were determined, respectively, on Trypticase Soya Agar pH 7.3±0.2 (Biokar, France), Deoxycholate lactose agar pH 7.3±0.2 (Biokar, France), chapman medium pH 7.4±0.2 (Biokar, France), de Man Rogosa & Sharpe agar pH 5.7±0.1 (Biokar, France) containing pimaricin at 0.02%, and potato dextrose agar (Biokar, France) acidified with lactic acid (0.1N) to pH 3.5. The mesophilic aerobic bacteria and staphylococci were counted after 2 days of incubation at 30 °C. The coliforms were counted after 2 days of incubation at 37 °C. The LAB were counted after 3 days of incubation at 30 °C. The yeasts and moulds were counted after 3 to 4 days of incubation at 25 °C.

2.4. Physico-chemical analysis

The physicochemical parameters analyzed in the brine samples were pH, free acidity, chloride and sugars. The pH was measured using a pH meter type Crison pH 2000 after calibration at pH 4 and 7. The free acidity was determined using NaOH (0.1 N) and phenolphthalein as indicator. The results were expressed as percent of lactic acid. The chloride content in brine was measured by titration with AgNO3 (0.1N) in the presence of potassium chromate (0.5%, w/v) as indicator, the content of chloride in the brine was determined based on a standard curve made with NaCl, and expressed as percent of NaCl. The soluble sugar contents expressed in g per 100 mL of brine, were determined using the Ashwell method (Ashwell, 1957), based on the measurement of the green color developed by the reaction of soluble sugars with anthrone in the presence of sulphuric acid. The green color developed was measured at 630 nm. The results obtained were expressed in grams of total sugars per 100 mL of brine.

2.5. Polyphenols analysis

The brines of all the inoculated assays weres ampled in triplicate ont the 1st, 5th, 10th, 15th, 23th and at the 38th day of the fermentation process; the brines of the control assay were sampled on the 1st and the 38th day of the fermentation process. The sampled brines were analyzed by HPLC-DAD for their oleuropein and hydrolysis product contents. The samples were centrifuged at 1000 rpm/10min, and phenolic compounds were extracted from the supernatant three times with ethyl acetate (8:2, v/v). After decanting, the organic phase was harvested and left in the dark for 30 minutes in the presence of disodium sulfate, and then evaporated to dryness at 50 °C. The residue obtained was dissolved in 1 mL of methanol and stored at −20 °C and then analyzed by an HP isocratic HPLC, equipped with an HP-UV detector at 280 nm and a C18 column (250 mm×4,6 cm, 5 µm) maintained at 40 °C. The mobile phase consisted of a milli-Q water acidified with acetic acid (97:3, v/v) (solution A) and acetonitryl-methanol (1:1, v/v) (solution B). The samples were filtered through a PVDF syringe filter (Sartorius, France), and then a volume of 20 µL was injected at a flow rate of 1 mL·min^{-1}. The solvent gradient changed according to that reported by (Kaltsa *et al*., 2015). The identification of polyphenolic compounds in olive brines was carried out by comparing the retention times of each peak with those of oleuropein and hydroxytyrosol standards. Phenolic compound quantification was achieved by measuring the absorbance at 280 nm recorded in the chromatograms relative to external standards.

2.6. Tentative examination of the sensory characteristics

At the end of the fermentation process, all the fruits were sorted manually and examined for their attack by olive spoilage. The fermented olive properties, including off-odor, bitterness, acid taste, hardness and crunchiness, were assessed by 20 panelists composed of teachers and doctorate degree students in our university, not by specialized panelists, and compared to a commercial sample of green olives fermented according to the Spanish process. The sensorial evaluation was indicated using a line scale ranging from 0 (no perception) to 10 (extreme) as described by (Meilgaard *et al*., 1991). From each sample, 4 olives were tasted for the evaluation. Between each tasting the panelists washed their mouths out with mineral water.

2.7. Statistical analysis

All the determinations were carried out in triplicate and the results were expressed as mean values and standard mean error. Data were analyzed by an analysis of variance (ANOVA). Means were compared using the One-way ANOVA with Tukey's post tests and the Two-way ANOVA with Bonfferoni's post tests, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. The significant level was set at 5% ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1. Microbiological analysis

During the first 2 weeks of the process all the assays showed a rapid development of the microbiota associated with olives, to achieve 7.6–9.8 10^8cfu/ml for LAB and mesophilic microbiota, and 3–5 10^6 cfu·mL^{-1} for yeasts and moulds, and after a reduction to stabilize, around 10^5 cfu·mL^{-1} until the end of the fermentation process (Figure 1).

Significant differences were observed when inoculated olives (with *L. plantarum* S175 or with *L. pentosus* S100) and un-inoculated olives (control) were compared. The interaction between inoculation and time effects significantly affected ($p<0.001$) the evolution of the LAB population. This population of inoculated olives showed a slight increase from 5.7–9 10^7 to about 10^9cfu·mL^{-1} during the first 15 days of the process, followed by a decrease to stabilize at around 2.10^5cfu·mL^{-1} at the end of the process (Figure 1). The natural LAB population of the un-inoculated olives (control) showed a rapid development from about 10^2cfu·mL^{-1} to achieve 1.2 10^6cfu·mL^{-1} during the first 20 days, followed by a slight decrease to stabilize at 1.8 10^4cfu·mL^{-1} at the end of the fermentation process. This decrease may be due to the elimination of the LAB species non-supporting high acidity values (Balatsouras, 1985) and polyphenols (Landete *et al*., 2008, Rodríguez *et al*., 2009) accumulating during the fermentation process. In accordance with (Panagou *et al*., 2003), the results obtained showed a better performance of selected lactobacilli strains (*L. plantarum* S175 and *L. pentosus* S100) in developing a good fermentation process by dominating the yeast biochemical activity and leading to the improvement of the hygienic quality of fermented olives. The final concentration of LAB cells (2 10^5cfu·mL^{-1}) obtained in fermented olives is of great importance for developing the functional properties of the end product since this microbiota is responsible for the flavor and the texture of the end products (Garrido-Fernández *et al*., 1997, Sánchez *et al*., 2000).

The yeast and mould populations of all the assays showed a rapid development during the first 15 days of the fermentation process to achieve a maximum of 3–5 10^6cfu·mL^{-1} (Figure 1). After this first phase, the yeast population showed a slight decrease to stabilize at around 10^5cfu·mL^{-1} until the end of the fermentation process. Slight differences in yeast and mould and mesophilic microbiota evolutions were observed in the inoculated olives compared to the un-inoculated olives (control). The inoculation of olives with *L. plantarum* showed significant differences in the evolution of the yeast and mould population during the first four weeks compared to un-inoculated olives (control), but no significant differences were observed towards the end of the process. However, the inoculation with *L. pentosus* S100 showed a significant effect on this population at some different times of the process in comparison with the olives inoculated with *L. plantarum* S175 and the un-inoculated olives (control).

The active development of the yeast and mould population during the first days of fermentation may be due to the accumulation of nutrients and low concentrations of polyphenols. This microbiota can play a double role as fermentative and spoilage microorganisms in olive fermentation (Arroyo-López *et al*., 2008). Their presence at the beginning of fermentation can improve the growth of LAB (Tsapatsaris and Kotzekidou, 2004, Hurtado *et al*., 2012). Their decrease during the second phase of the fermentation may lead to important organoleptic attributes, determining the quality and flavor of the end product, and may improve the dominance of the biochemical activity

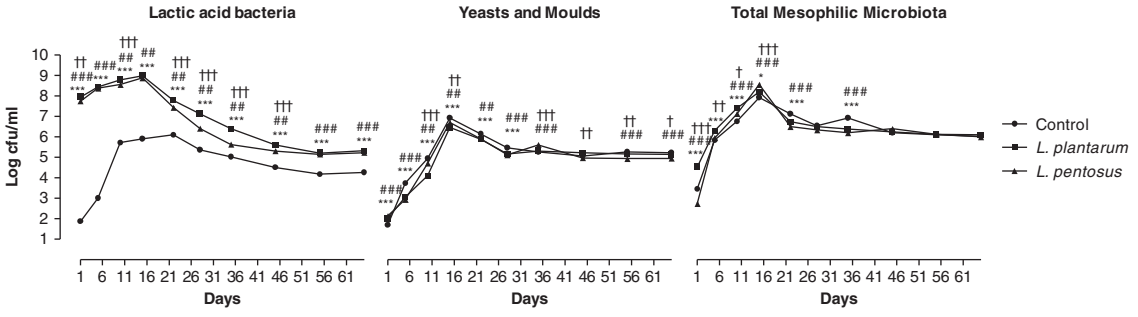

FIGURE 1. Evolution of lactic acid bacteria, yeasts and moulds, and total mesophilic microbiota in olive brines during fermentation at 30 °C. —●— Control: un-inoculated green olives (spontaneous fermentation), —■— *L. plantarum*: Green olives inoculated with *Lactobacillus plantarum* S175strain, —▲— L. pentosus: Green olive inoculated with *Lactobacillus pentosus* S100 strain. *Values (*L. plantarum* vs Control), $^{\#}$Values (*L. pentosus* vs Control), †Values (*L. plantarum* vs *L. pentosus*). $^{*, \#, \dagger}$Values differ significantly ($p<0.05$), $^{**, \#\#, \dagger\dagger}$Values differ significantly ($p<0.01$), $^{***, \#\#\#, \dagger\dagger\dagger}$Values differ significantly ($p<0.001$).

of LAB which is highly desired to assure the olive biotransformation.

The coliform population showed, in inoculated assays, a rapid development from about 10^2cfu·mL^{-1} to achieve a maximum of 3.6–710^3cfu·mL^{-1} during the first 5 days, followed by a drastic reduction and elimination at the end of the second week of fermentation (Figure 2). The un-inoculated olives (control) showed the same appearance, but with a delay of about 10 days in comparison with inoculated olives. The staphylococci population showed, in all the assays, a rapid development during the first 10 days of fermentation, followed by a drastic reduction and elimination at the third week of fermentation (Figure 2). Two-way ANOVA with Bonfferoni's post tests data revealed that the inoculation of olives (with *L. plantarum* S175 and *L. pentosus* S100) significantly affected the evolution of coliforms and staphylococci populations, while the LAB strains used as starter grew during the fermentation process. This finding is in agreement with the results obtained by (Benincasa *et al*., 2015), indicating the presence of *L. plantarum* starter throughout the fermentation process, while staphylococci and coliforms disappeared after 30 and 90 days of fermentation, respectively. The elimination of these populations leads to the improvement in the hygienic quality of fermented olives. It may be due to the antimicrobial compounds and to a progressive acidification of the fermenting brine produced by lactic acid bacteria (Klaenhammer *et al*., 1994, Marsilio *et al*., 2005) and to the oleuropein and its hydrolysis products (Nychas *et al*., 1990).The reduction in the mesophilic microbiota population downstream of the fermentation process should lead to the stabilization of the end product during the post fermentation phase.

3.2. Physicochemical analysis

The inoculated olives, with *L. plantarum* S175 or *L. pentosus* S100, showed a rapid drop in pH from 6 to 4.5 during the first 5 days of fermentation, followed by a slight reduction to stabilize at around pH 4 until the end of the fermentation process (Figure 3). The un-inoculated olives (control) showed the same trend with higher pH values than those obtained in inoculated olives. The free acidity showed a continuous increase in all assays to achieve, at the end of the fermentation process, 0.9% and 1% in olives inoculated respectively with *L. pentosus* S100 and *L. plantarum* S175, and 0.5% in the control (Figure 3). Throughout the fermentation process, the acidity values obtained in the inoculated olives were higher than those of the control.

Significant differences ($p<0.001$) in pH and acidity values were observed during the fermentation process when the inoculated and un-inoculated olives were compared. However, no significant differences were observed, during 45 days of the process, between assays inoculated with *L. plantarum* S175 and *L. pentosus* S100. These differences in pH and acidity changes between the controlled process (inoculated olives) and spontaneous process (un-inoculated olives), particularly the rapid acidification, indicate the rapid installation and acidification rates of the LAB strain over the microbiota naturally associated with the spontaneous process.

L. plantarum and *L. pentosus* were demonstrated to have a high acidification rate in the controlled fermentation of Portuguese Azeiteira and Arbequina green olive varieties (Hurtado *et al*., 2010, Peres *et al*., 2008).Other authors reported a faster acidification of inoculated Manzanilla green olives with

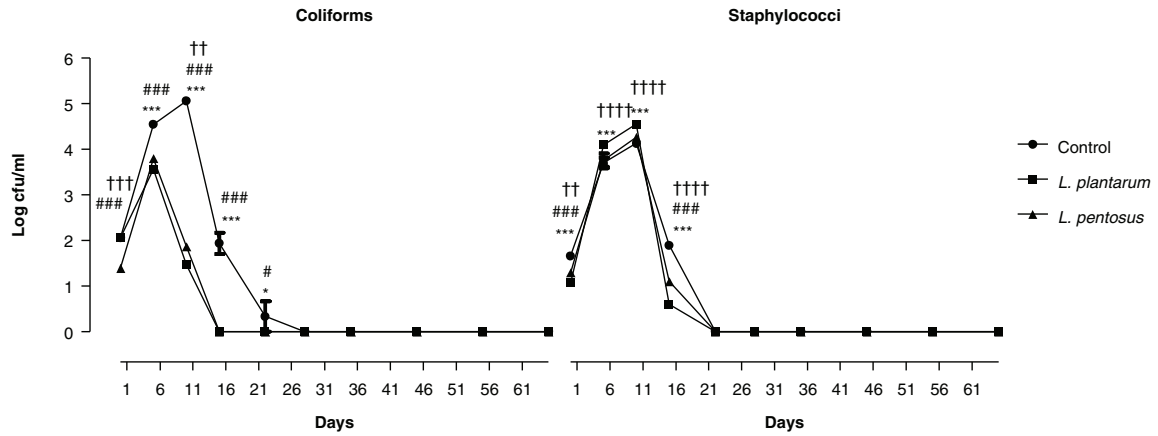

FIGURE 2. Evolution of coliforms and staphylococci in olive brines during fermentation at 30 °C.—●— Control: un-inoculated green olives (spontaneous fermentation), —■— *L. plantarum*: Green olives inoculated with *Lactobacillus plantarum* S175strain, —▲— *L. pentosus*: Green olives inoculated with *Lactobacillus pentosus* S100 strain. *Values (*L. plantarum* vs Control), $^{\#}$Values (*L. pentosus* vs Control), †Values (*L. plantarum* vs *L. pentosus*). $^{*,\#,\dagger}$Values differ significantly ($p<0.05$), $^{**,\#\#,\dagger\dagger}$Values differ significantly ($p<0.01$), $^{***,\#\#\#,\dagger\dagger\dagger}$Values differ significantly ($p<0.001$).

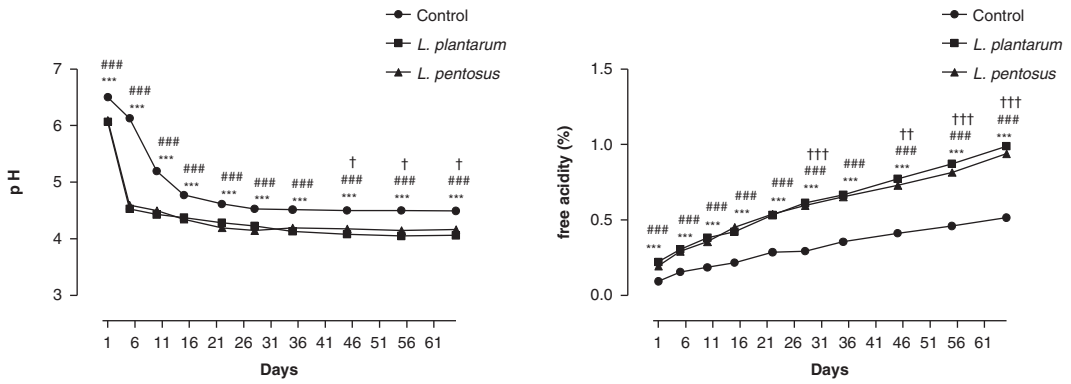

FIGURE 3. Evolution of pH and free acidity (% lactic acid) in olive brines incubated at 30 °C. —●— Control: un-inoculated green olives (spontaneous fermentation), —■— *L. plantarum*: Green olives inoculated with *Lactobacillus plantarum* S175strain, —▲— *L. pentosus*: Green olives inoculated with *Lactobacillus pentosus* S100 strain. *Values (*L. plantarum* vs Control), #Values (*L. pentosus* vs Control), †Values (*L. plantarum* vs *L. pentosus*). *, #, †Values differ significantly ($p<0.05$), **, ##, ††Values differ significantly ($p<0.01$), ***, ###, †††Values differ significantly ($p<0.001$).

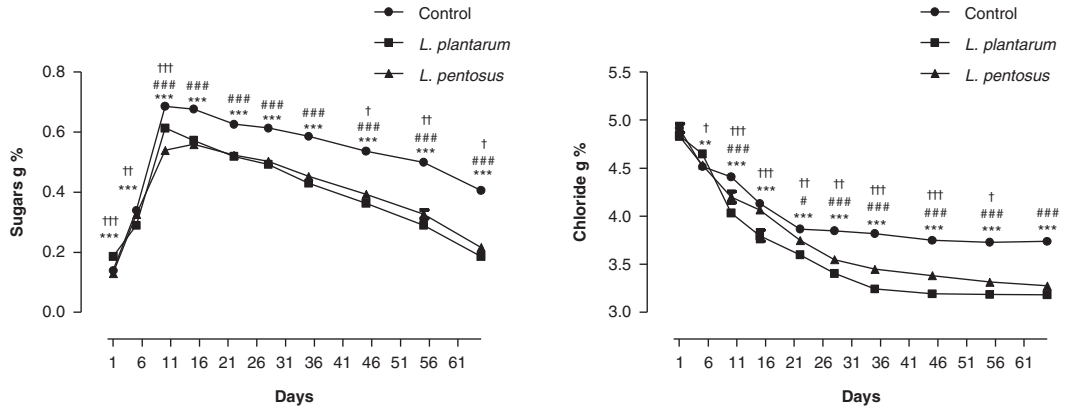

FIGURE 4. Evolution of sugars and chloride contents in olive brines incubated at 30 °C in olive brines incubated at 30 °C. —●— Control: un-inoculated green olives (spontaneous fermentation), —■— *L. plantarum*: Green olives inoculated with *Lactobacillus plantarum* S175strain, —▲— *L. pentosus*: Green olive inoculated with *Lactobacillus pentosus* S100strain. *Values (*L. plantarum* vs Control), #Values (*L. pentosus* vs Control), †Values (*L. plantarum* vs *L. pentosus*). *, #, †Values differ significantly ($p<0.05$), **, ##, ††Values differ significantly ($p<0.01$), ***, ###, †††Values differ significantly ($p<0.001$).

L. plantarum higher than the spontaneous process during the first 25 days of brining (Leal-Sánchez *et al*., 2003).

The increase in acidity may be due to the microbial activity of mesophilic acid producing microorganisms, mainly LAB, spore forming bacteria, coliforms, and fermentative yeasts (Balatsouras, 1985, Fernández-Diez *et al*., 1985). The biochemical activity of these microorganisms may be activated by the optimal temperature of incubation maintained at 30 °C, and high contents of sugars and other nutrients in brines, due to their rapid infiltration from olives to brine. The acidity values obtained are highly desired to improve the hygienic quality of the end product, since the pathogenic bacteria are sensible and rapidly eliminated in acidic conditions (Peres *et al*., 2008, Panagou *et al*., 2008). Indeed, the rapid acidification of the brines induced by the LAB starter may increase the prevalence of LAB in the epiphytic population and may reduce the risk of pathogen growth in intestinal tract of the consumer. These actions improve the safety and the pro-biotic properties of fermented olives.

The sugar contents showed, in all the assays, a rapid increase during the first 10 days of fermentation, to achieve a maximum of around 0.6%, and decrease continuously throughout the process to 0.3% in the un-inoculated olive (control) and 0.1% in the inoculated olives (Figure 4). The inoculation of olives with *L. plantarum* or with *L. pentosus* significantly affected the evolution of sugars during the fermentation process. High sugar contents were

detected in un-inoculated olives (control) in comparison with inoculated olives. Sugars are required for fermentation installation, and their diffusion from fruit to brine depends on various parameters like skin permeability, salt concentration, olive variety and temperature (Garrido-Fernández *et al*., 1997). There is a relative correlation between the consumption of sugars and the accumulation of lactic acid. This result may be due to the high microbial activity, mainly the acidification rate observed in inoculated olives.

The chloride contents showed a decrease in olive brines, in all the assays, during the fermentation process to stabilize at the end of the process at around 3.3% in inoculated olives and 3.8% in un-inoculated olives (control) (Figure 4). Significant differences in the final chloride contents were observed between inoculated olives and the control. This difference may be due to the osmosis exchange of polyphenols and sodium chloride between olive flesh and brine; these exchanges are more expressed in inoculated olives where the oleuropein released from fruits is subject to biodegradation by the starter. Significant differences were observed in some points of the fermentation process when the types of inoculation were compared. The decrease in chloride contents is due to their infiltration from brine into olive flesh until achieving the equilibrium, this infiltration of chloride into olive flesh is of great interest since it facilitates the release of oleuropein from olives to the brine (Ozdemir *et al*., 2011), which accelerates their de-bittering process. The salt content may influence the fermentation process by affecting the growth of lactic acid bacteria (Leal-Sánchez *et al*., 2003).The low salt content (5% of NaCl) we used in olive brining may allow a faster growth of the LAB starter without affecting biochemical activity, particularly the biodegradation of oleuropein (Romeo and Poiana, 2007).

3.3. Polyphenol analysis

To measure the degree of oleuropein disappearance, the oleuropein and hydroxytyrosol in brine were monitored. The results of the polyphenol analysis by HPLC-DAD are reported in (Table 1). The oleuropein and hydroxytyrosol contents were expressed in mg per 100 ml of olive brine. The oleuropein was the major phenolic compound found in the olive brines at the onset of the fermentation process. During the first 5 days of fermentation, the oleuropein content decreased from about 84.9 to 39.6 mg per 100 mL of brine and from 72.7 to 42.8 mg per 100 mL of brine, respectively in the inoculated olives with *L. plantarum* S175 and *L. pentosus* S100. This reduction of oleuropein was accompanied by a simultaneous increase in the hydroxytyrosol content from about 11.1 to 64.5 mg per 100 mL of brine and from 10.8 to 69.9 mg per 100 mL of brine, in inoculated olives with *L. plantarum* S175 *and L. pentosus* S100, respectively.

The oleuropeinolytic activity obtained with both lactobacilli strains is significantly higher than the control (un-inoculated olives). This result indicates the high involvement of the LAB starter in oleuropein degradation. During the lactic fermentation process the degradation of oleuropein may be attributed to the acidic condition and the β-glucosidase, related to the biochemical activity of the LAB starter (Brenes *et al*., 1993).

From the 5th day of the fermentation process, the accumulation of oleuropein in inoculated brines exceeds its degradation rate. This result may be explained by the higher and continuous diffusion of oleuropein by osmosis from olive flesh to the brine (Ozdemir *et al*., 2011), which is higher than its biodegradation by LAB strains, indicated by a continuous increase of hydroxytyrosol in the brine. Hence, the hydroxytyrosol contents increased

Table 1. Evolution of Oleuropein and Hydroxytyrosol contents in olive brines during the fermentation process of inoculated and un-inoculated olives. (nd : not determined)

Inoculated Green Olive	Days	1	5	10	15	23	38
		Concentrations of Oleuropein and Hydroxytyrosol (mg·100 mL^{-1} of olive brine) ±ESM					
L. plantarum 175	Oleuropein	$84.9^{***\#\#\#}$±2.2	39.6^{ns}±3.1	79.1^{***}±3.5	133.3^{***}±1.5	159.6^{***}±1.6	$169.3^{**\#\#\#}$±1.3
L. pentosus S100	Oleuropein	$72.7^{\dagger\dagger\dagger}$±0.7	42.8±0.1	57.9±0.6	106.1±0.6	149±0.3	$176.6^{\dagger\dagger\dagger}$±0.3
Un-inoculated olive	Oleuropein	94±0.4	nd	nd	nd	nd	193±0.2
L. plantarum 175	Hydroxytyrosol	11.1^{nsns}±0.9	64.5^{ns}±1.9	94.7^{***}±7	122.2^{ns}±3.5	132.9^{*}±3.1	$154.2^{ns\#\#\#}$±2.5
L. pentosus S100	Hydroxytyrosol	10.8^{ns}±0.1	69.9 ±0.6	118±0.7	128.6±0.8	142.7±1.3	$160.6^{\dagger\dagger\dagger}$±0.3
Un-inoculated olive	Hydroxytyrosol	3±0.2	nd	nd	nd	nd	42±0.4

*Values (*L. plantarum* 175 vs *L. pentosus* S100), $^{\#}$Values (*L. plantarum* 175 vs Control), †Values (*L. pentosus* S100 vs Control).
$^{*, \#, \dagger}$Values differ significantly ($p<0.05$).
$^{**, \#\#, \dagger\dagger}$Values differ significantly ($p<0.01$).
$^{***, \#\#\#, \dagger\dagger\dagger}$Values differ significantly ($p<0.001$).
^{ns}Values not significant at ($p<0.05$).

from 11.1–10.8 mg per 100 mL of brine initially to 154.2–160.6 mg per 100 ml of brine as final concentrations were obtained after 38 days of culture with *L. plantarum* S175 and *L. pentosus* S100, respectively; while the un-inoculated olives showed an increase of hydroxytyrosol from 3 mg per 100 mL of brine initially to 42 mg per 100 mL of brine after 38 days of fermentation. The oleuropein contents, obtained after 38 days of culture with *L. plantarum* S175 and *L. pentosus* S100, were respectively of 169.3 mg per 100 mL of brine and 176.6 mg per 100 ml of brine, while un-inoculated olives showed 193 mg of oleuropein per 100 ml of brine. The higher contents of hydroxytyrosol found in the inoculated olive brine with *L. plantarum* S175 and *L. pentosus* S100 which were respectively154.2 mg per 100 mL and160.6 mg per 100 ml of brine as final concentrations were obtained after 38 days of culture, compared to that obtained in the un-inoculated control (42 mg per 100 mL), confirms the oleuropeinolitic activity of the starter. These results indicate the possible use of these LAB strains, instead of chemicals, in the biological de-bittering of green olives.

Hydroxytyrosol was reported as the main product of oleuropein biodegradation by lactic acid bacteria (Ciafardini *et al*., 1994, Landete *et al*., 2008, Rodríguez *et al*., 2009, Zago *et al*., 2013, Benincasa *et al*., 2015). It is considered a marker to determine the oleuropein degradation and the diffusion of phenols from drupes to brine (Randazzo *et al*., 2011). Its accumulation in brine may be due to the oleuropeinolytic activity of the LAB starter and other microorganisms naturally associated with the fermentation process (Garrido-FernÁndez and Vaughn, 1978, Kaltsa *et al*., 2015). These microorganisms with ß-glucosidase and esterase activities are useful for table olives bio-processing by assuring the biological de-bittering and fermentation of olives, instead of a chemical process.

L. plantarum was reported as a potentially active species in the biological de-bittering and lactic fermentation process of olives (Landete *et al*., 2008, Rodríguez *et al*., 2009, Zago *et al*., 2013, Benincasa *et al*., 2015, Kaltsa *et al*., 2015). However, the LAB starter activity may depend on the LAB species and olive variety. *L. plantarum* was reported to be unsuitable for controlled fermentation of Arbequina green olives, while *L. penstosus* was suitable for Arbequina and Leccinocv olive processing (Servili *et al*., 2006, Hurtado *et al*., 2010). These authors demonstrated that *L. pentosus* improved the fermentation process by reducing entero-bacteria survival during the first stage of fermentation and modifying the sensorial quality of fermented olives. In our study, both LAB strains of *L. plantarum* S175 and *L. pentosus* S100, showed high adaptation and biochemical activities, with a slight difference in oleuropein biodegradation and the lactic fermentation installation of the non alkali-treated Moroccan Picholine green olive variety. This result indicates their possible use as autochthonous LAB starters for the industrial biological processing of this local variety.

3.4. Tentative examination of sensory characteristics

The main olive spoilage identified in fermented olives was the bloater, called "gas pocket". The bloater incidences obtained were 9% and 12% in inoculated olives with *L. plantarum* S175 and *L. pentosus* S100, respectively. However, higher bloater incidence (34%) was obtained in un-inoculated olives. Compared to the control (un-inoculated olives), the lower bloater incidences obtained in inoculated olives indicate the effectiveness of the LAB starter tested in controlling the natural fermentation process.

The bloater spoilage is due mainly to the gas producing microorganisms, including coliforms, fermentative yeasts and hetero-fermentative LAB (Asehraou *et al*., 2000).The low level of bloater incidence obtained in inoculated olives may be explained by the rapid elimination of coliforms, and the reduction in hetero-fermentative LAB and fermentative yeast activities, mainly due to the dominance of the biochemical activity of LAB starters (*L. plantarum* S175 and *L. pentosus* S100) over the other fermentative microorganisms. LAB are known for their anti-microbial production capacity (organic acids, bacteriocins and hydrogen peroxide) (Marsilio *et al*., 2005, Reis *et al*., 2012).

All the panelist members preferred the taste of the inoculated olives as good palability products than the un-inoculated, market olives. No off-odors were detected by panelists in any of the samples of fermented olives. However different levels of the other sensory attributes were revealed. Bitterness, acidic taste, hardness and crunchiness analyses were significantly inoculation-dependent. The one way ANOVA data with Tukey's post tests revealed significant differences between the control and inoculated olives with *L. plantarum* S175 and *L. pentosus* S100. Higher levels of acidic taste, hardness, and crunchiness than the control and the market sample (control 2) were obtained in fermented olives (Figure 5). Furthermore, the bitterness obtained in the inoculated olives was lower than the control and higher than the market sample (Figure 5). No significant differences in bitterness or crunchiness were obtained between the inoculated olives with *L. plantarum* S175 or *L. pentosus* S100, but significant differences ($p<0.05$) in acidic taste and hardness were observed. Significant differences ($p<0.001$) were observed between the inoculated (*L. plantarum* S175 or *L. pentosus* S100) and un-inoculated olives (control or control market). The acidic taste, crunchiness and hardness were significantly higher in the inoculated olives than in the controls. However, the inoculated olives showed a bitter taste significantly lower than un-inoculated olives (control)

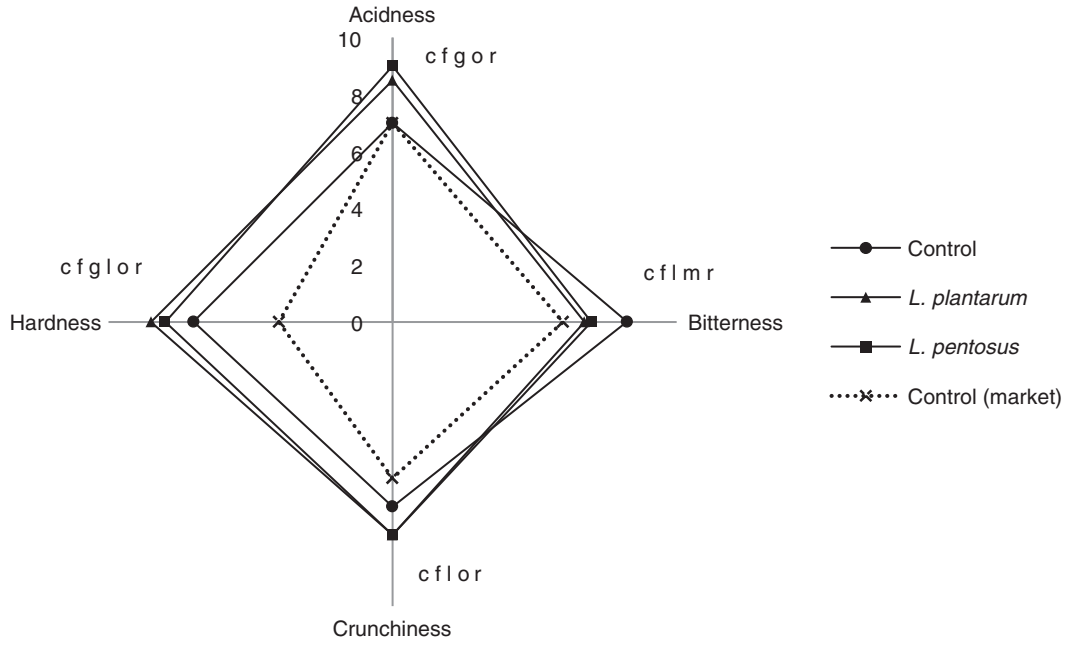

FIGURE 5. Evaluation of some sensory attributes of fermented green olives. —●— Control: uninoculated green olives (spontaneous fermentation), —■— *L. plantarum*: Green olives inoculated with *Lactobacillus plantarum* S175 strain, —▲— *L. pentosus*: Green olives inoculated with *Lactobacillus pentosus* S100 strain, …x… Control 2 (market): Green olive market sample (control 2) de-bittered chemically by sodium hydroxide.[a b c] Values (*L. plantarum* vs Control), [d e f] Values (*L. pentosus* vs Control), [g h i] Values (*L. plantarum* vs *L. pentosus*), [j k l] Values (Control vs Control (market)), [m n o] Values (*L. plantarum* vs Control (market)), [p q r] Values (*L. pentosus* vs Control (market)).[a d g j m p] Values differ significantly ($p<0.05$). [b e h k n q] Values differ significantly ($p<0.01$).[c f i l o r] Values differ significantly ($p<0.001$).

and higher than the market sample (control 2) which was de-bittered chemically by sodium hydroxide. The low bitter taste appreciated by the panelists in inoculated olives may be due to the high biological acidity covering the residual bitterness. This finding indicates the effectiveness of the oleuropeinolytic LAB strains tested in olive de-bittering, but at a lower rate than the chemical de-bittering process. Significant differences in hardness and crunchiness were observed between un-inoculated olives (control) and the market sample (control 2), due mainly to the low quality of the olives of the market sample caused by chemical de-bittering. No significant difference in acidic taste was obtained between the controls, which may be explained by the chemical acidification of the olives. This result is in accordance with that of (Tataridou and Kotzekidou, 2015), who recently reported the possible improvement in sensorial and nutritional characteristics of the final product by using oleuropeinolytic strains of the *L. plantarum* group as both de-bittering and fermentation agents.

4. CONCLUSIONS

The results obtained showed the better performance of LAB strains used in this experiment (*L. plantarum* S175 and *L. pentosus* S100) in controlling the natural fermentation process of Moroccan Picholine green olives, by their biological de-bittering and improving the hygienic quality and sensory attributes of the end product, and reducing the bloater spoilage incidence in fermented olives. These LAB strains showed high adaptation to the non alkali-treated Picholine green olive brine environment (at 30 °C and 5% NaCl), and high biochemical capacity to assure the biodegradation of oleuropein and the dominance of the lactic fermentation process over the other off-odor fermentations and spoilage microorganisms. The application of these selected LAB strains as autochthonous starters for the industrial biological processing of Moroccan Picholine green olives, including de-bittering and fermentation, should lead to economic benefits by improving the quality of fermented olives and reducing olive spoilage.

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