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Controlled fermentation of heat-shocked, unsalted and inoculated Moroccan Picholine green olives

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SUMMARY: The present work reports the controlled fermentation of heat-shocked, unsalted and inoculated green olives. The effects of heat-shock (60, 70 and 80 °C three times for 5 min), inoculation with the oleuropeinolytic strain of *L. plantarum* FSO175 (*L.p*-FSO175) and the addition of Cell-Free Supernatant of *C. pelliculosa* L18 (CFS of *C.p*-L18) on the fermentation process of unsalted green olives were examined. The results showed a drastic reduction in the initial indigenous *Enterobacteria*, and an improvement in the acidification of heat-shocked olives at 70 and 80 °C, when compared to 60 °C. The inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 enhanced the fermentation and preservation of unsalted green olives, indicated by a significant decrease in pH, increase in free acidity and total disappearance of *Enterobacteria*. The heat-shock treatment at high temperature (80 °C), inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 led to the best reduction in bitterness, and favorable color changes (L, a, and b) in fermented olives. This sequential method led to more appreciated sensory characteristics (mainly bitterness and color) of fermented olives, lower spoilage incidence in olives, and reduced fermentation time to 50 days, and therefore may be suitable to control the fermentation of unsalted green olives of the Moroccan picholine variety.

KEYWORDS: *C. pelliculosa; Fermentation; Heat-shock; L. plantarum; Olives; Un-salted.*

RESUMEN: ***Fermentación controlada de aceitunas verdes picholine marroquíes sometidas a choque térmico e inoculadas sin sal.*** El presente trabajo reporta la fermentación controlada de aceitunas verdes sometidas a choque térmico, sin salar e inoculadas. Se estudian los efectos del choque térmico (60 °C, 70 °C y 80 °C tres veces durante 5 min), la inoculación con cepa oleuropeinolítica de *L. plantarum* FSO175 (*L.p*-FSO175) y la adición de sobrenadante libre de células de *C. pelliculosa* L18 (CFS de *C.p*-L18), sobre el proceso de fermentación de aceitunas verdes sin salar. Los resultados mostraron la drástica reducción de las enterobacterias autóctonas iniciales, y la mejora de la acidificación de las aceitunas sometidas a choque térmico de 70 °C y 80 °C, en comparación con 60 °C. La inoculación con *L.p*-FSO175 y la adición de CFS de *C.p*-L18 mejoró la fermentación y conservación de las aceitunas verdes sin salar, indicada por una disminución significativa del pH, aumento de la acidez libre y desaparición total de enterobacterias. El choque térmico a alta temperatura (80 °C), la inoculación con *L.p*-FSO175 y la adición de CFS de *C.p*-L18 condujeron a una mejor reducción del amargor y cambios de color favorables (L, a y b) en aceitunas fermentadas. Este método secuencial, que permitió apreciar las características sensoriales (principalmente amargor y color) de las aceitunas fermentadas, y una menor incidencia de deterioro en las aceitunas, y redujo el tiempo de fermentación a 50 días, puede ser adecuado para controlar la fermentación de aceitunas verdes sin salar de Marruecos, variedad picholine.

PALABRAS CLAVE: *Aceitunas; C. Pelliculosa; Choque térmico; Fermentación; L. Plantarum; Sin sal.*

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1. INTRODUCTION

Fermented green olives are produced according to traditional and industrial processes. The traditional process is based on the natural lactic fermentation of directly brined green olive fruits (Bautista-Gallego *et al.,* 2010). The industrial process is based on the chemical debittering of olives with sodium hydroxide to eliminate the bitterness due to oleuropein (OLP), followed by washing with tap water and brining at 10-12% NaCl, where a natural fermentation process takes place (Garrido-Fernández *et al.,* 1997; Gómez *et al.,* 2006).

These processes have many drawbacks. The traditional process is characterized by a long (6-8 months) and non-controlled fermentation process (Ciafardini and Zullo, 2019), normally leading to fermented olives (end product) of variable quality and associated with high olive spoilage incidence (Fernández-Diez *et al.,* 1985). The industrial process, based on alkali treatment of olives, is characterized by the production of large amounts of wastewater which is rich in phenolic compounds and sodium hydroxide, constituting a serious environmental problem (Arroyo-López *et al.,* 2008). The development of a controlled bioprocess may overcome, or at least reduce, the impact of these drawbacks.

Lactic acid bacteria (LAB) with oleuropeinolytic activity are frequent in natural fermented olive brines (Ghabbour *et al.,* 2011). In a previous work, *L.p*-FSO175, used as an autochthonous oleuropeinolytic starter, allowed the control of olive fermentation in brine with low salt content (5%), but led to fermented olives with some bitterness (Ghabbour *et al.,* 2016). Recently, other authors demonstrated the strong oleuropeinolytic activity of *L. plantarum* starter in low-salt green olive brine (Pino *et al.,* 2019), which may reduce the bitterness in fermented olives. *L.p*-FSO175 was used in this work and demonstrated its high oleuropeinolytic activity in the absence of sodium chloride (Ghabbour *et al.,* 2020), indicating its possible use in biological debittering and fermentation of unsalted green olives.

Candida yeast species (i.e. *Candida pelliculosa*) are dominant in naturally fermented olives, and can lead to various olive spoilages (Arroyo-López *et al.,* 2008; Arroyo-López *et al.,* 2012a). Therefore, their use as starter culture in olive fermentation is not suitable; however, their cell-free supernatant (CFS) can be used as a source of nutrients for LAB (Arroyo-López *et al.,* 2008; Arroyo-López *et al.,* 2012b; Sidari *et al.,* 2019) and enzymes, particularly beta-glucosidase and esterase, highly valued in biological debittering of olives, due to their involvement in the biodegradation of oleuropein (Rodriguez-Gómez *et al.,* 2012, Anagnostopoulos *et al.,* 2017).

Several studies reported the benefits of heat-shock to olives prior to brining in order to improve their fermentation process, via the eradication of undesirable microbiota (Chorianopoulos *et al.,* 2005), and the increase in permeability of fruit (Balatsouras *et al.,* 1983). On the other hand, fermented olives are naturally processed in brine which is rich in sodium chloride (5-10%) (Garrido-Fernández *et al.,* 1997; Gómez *et al.,* 2006). A high consumption of sodium chloride is associated with risk of diseases for consumers (i.e. blood pressure, heart and kidney diseases). That is why the World Health Organization (WHO) set a global goal of reducing salt intake by 30% before 2025, and recommends to reduce salt intake for adults to less than 5g per day (World Health Organization, 2013). Several studies reported the replacement of NaCl with KCl and $CaCl_2$ (Mateus *et al.,* 2016; Zinno *et al.,* 2017), and other authors studied table olive processing at reduced NaCl concentrations (Pino *et al.,* 2018; Pino *et al.,* 2019). The production of unsalted fermented green olives is of great interest to overcome the drawbacks of chemicals (NaCl and NaOH) for human health and the environment.

The objective of this work is to control the fermentation process of unsalted and non-alkali treated green olives, based their prior heat-shock (60, 70 and 80 °C tree times for 5min), followed by the addition of oleuropeinolytic agents (strain of *L.p*-FSO175 and CFS of *C.p*-L18).

2. MATERIALS AND METHODS

2.1. Inoculum preparation

The strain used in this work (*L.p*-FSO175) was isolated from natural fermented green olives and selected for its oleuropein biodegradation capacity (Ghabbour *et al.,* 2011). The *L.p*-FSO175 strain was reactivated twice in de Man Rogosa and Shar-

pe (MRS) broth (BIOKAR, FRANCE) at 30 ºC for 18 hours before use, then transferred twice to modified MRS broth containing 1% oleuropein (OLP) as a sole carbon source and incubated at 30 °C for 18 hours. The culture in the presence of OLP was made to induce the production of enzymes involved in the biodegradation of oleuropein (i.e. β-glucosidase and esterase). An aliquot of this overnight culture was centrifuged at 12.000g/12min at 4 °C (Hermle Labnet Z216MK), and the pellet obtained was washed twice with sterile physiological saline solution and re-suspended in the same solution at 8 Log cfu/mL concentration.

2.2. Preparation of cell-free supernatant

The *C. pelliculosa* L18 strain used in this work was isolated from naturally-fermented green olives and selected for its positive oleuropeinolytic activity (Rokni *et al.,* 2021). The *C. pelliculosa* L18 strain was subcultured twice in modified Yeast Extract Glucose (YEG) broth, containing 1 % oleuropein as a sole carbon source for 4 days of incubation at 25 °C. The culture obtained was centrifuged twice at 12000g/12min at 4 °C (Hermle Labnet Z216MK). The CFS of *C.p*-L18 was sterilized using sterile 0.22 µm filters (ISOLAB, Germany) before use.

2.3. Preparation and inoculation of the olives

Fresh green olive fruits of the Moroccan Picholine variety were purchased from the Oujda market area (East of Morocco). The olives were sorted manually to obtain fruits with uniform color and maturity degree. The olives were separated into 3 lots of 7 Kg, and each one was subdivided into 4 sub-lots designated a, b, c and d. The olives were then placed in 2-L flasks at a ratio of 800 g olives to 650 mL tap water. The trials of the first lot (designated a-6, b-6, c-6 and d-6) were heat-shocked at 60 °C three times for 5min. The trials of the second lot (designated a-7, b-7, c-7 and d-7) were heat-shocked at 70 °C three times for 5min. The trials of the third lot (designated a-8, b-8, c-8 and d-8) were heat-shocked at 80 °C three times for 5min. After each heat-shock, the water was changed, except for the third treatment which was kept for olive fermentation.

The olives were cooled at room temperature and then inoculated (2%, v/v) with an overnight culture of *L.p*-FSO175 and/or added with (2%, v/v) sterile CFS *C.p*-L18. The assays were carried out as follows: trials (a-6, a-7 and a-8) were not inoculated, they were used as controls; trials (b-6, b-7 and b-8) were inoculated with *L.p*-FSO175; trials (c-6, c-7 and c-8) were added with CFS *C.p*-L18, and the trials (d-6, d-7 and d-8) were inoculated with *L.p*-FSO175 and added with CFS *C.p*-L18. All the assays, made in duplicate, were incubated at 30 °C. Water samples were taken aseptically during the fermentation process, and subjected to physico-chemical and microbiological analyses.

2.4. Physico-chemical analysis

The physico-chemical parameters analyzed in de-bittering waters were pH, free acidity, total sugars and total polyphenols. 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 (0.1N) and phenolphthalein as indicators. The results were expressed as percent of lactic acid. The soluble sugar contents were determined using the method of (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 olive water. The total polyphenols were determined as described by Marigo (1973) based on the measurement of the blue color developed during the reaction of the phenolic fraction with the Folin Ciocalteu reagent after neutralization with 20% sodium carbonate. The blue color developed was measured at 760 nm after incubation at 40 °C/20 min. The polyphenol contents in the olive water was determined by reference to a standard range prepared with gallic acid under the same conditions as those of the samples. The results were expressed in mM and they were the average of 3 measurements.

2.5. Microbiological analysis

The samples taken from the trials at the 1st, 4, 8, 15, 22, 29, 36, 43 and 50th day of the fermentation process were subjected to successive decimal dilutions in a sterile saline solution. From the decimal dilutions, each microbial group was inoculated in its specific medium, using the pour-plate method. The

lactic acid bacteria (LAB) were determined on de Man Rogosa & Sharpe Agar (MRS) pH 5.7 ± 0.1 (Biokar, France) containing cycloheximide (0.01%), after 48 hours of incubation at 30 °C. The yeasts and moulds were enumerated on Potato-Dextrose-Agar (PDA) (Biokar, France) acidified with lactic acid (0.1N) to pH 3.5, after 48-72 hours of incubation at 25 °C. The *Enterobacteria* were enumerated on Deoxycholate Lactose Agar (DCL) pH 7.3 ± 0.2 (Biokar, France), after 48 h incubation at 37 °C. The results were the average of 3 measurements.

2.6. Color evaluation of the olives

In order to evaluate the effects of heat-shock and inoculation on color changes in fermented olives, olive samples were carefully taken on the 1st, 22nd and 50th day of fermentation. The fruit samples were subjected to color measurements using BYK-Gardner Model 9000 Color View Spectrophotometer (Silver Spring, MD, USA) and expressed in terms of the CIE L*, a*, b* parameters. The results were the average of 3 measurements.

2.7. Examination of sensory characteristics and spoilage of fermented olives

At the end of the fermentation process, the organoleptic properties of fermented olives were assessed for color, flavor, crunchiness, bitterness and acidic by 20 trained panellists composed of teachers and doctorate degree students in our university. The sensorial evaluation was recorded using a line scale ranging from 0 (no perception) to 10 (extreme) as described by (Meilgaard *et al.,* 1991). The fermented olives were also evaluated for the presence of spoilage indicators such as white spots, gas pockets, softening and off-odor formation, and the results were expressed in %.

2.8. Statistical analysis

The statistical analysis performed using the STATGRAPHICS Centurion XVII package (Stat point Technologies, Inc., Virginia, USA) was used for all calculations. The Two-Way ANOVA were carried out for heat-shock and inoculation. The least significant difference (LSD) values were calculated at the 5% probability level to determine which levels of the factors influenced the dependent variables analyzed.

3. RESULTS

3.1. Physicochemical analysis

3.1.1. pH and free acidity

The pH and free acidity changes during the fermentation process of unsalted green olives are presented in (Figures 1, 2), and their mean values are presented in Table 1. Whatever the heat-shock applied, the pH showed a slight decrease from 5.5 to 4.4 in un-inoculated olives (control) (Figure 1a); While, in olives inoculated with *L.p*-FSO175, with or without the addition of CFS of *C.p*-L18, the pH decreased progressively from 4.5 to stabilize at the end of fermentation at around 3.8-3.9 and 3.7, respectively (Figure 1b, Figure 2d).

The free-acidity values obtained showed a continuous increase during the fermentation of all assays (Figures 1 and 2). At the end of fermentation, the highest free-acidity value (0.94%) was obtained for

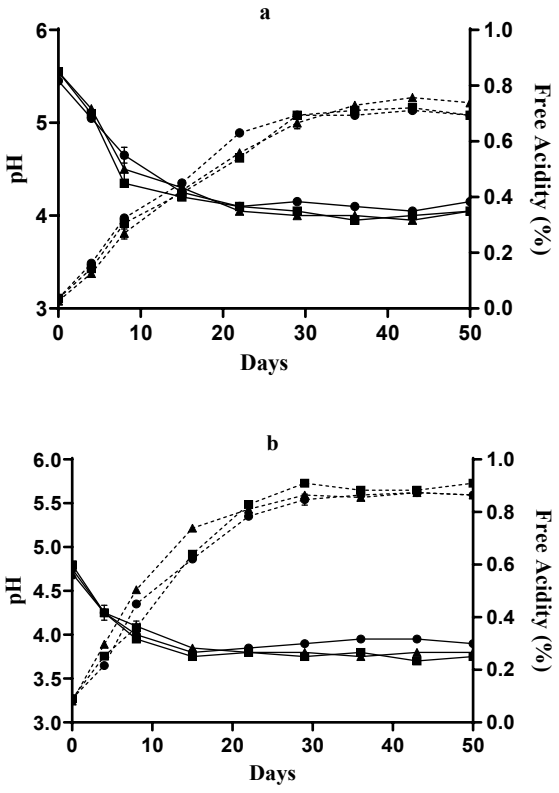

Figure 1. Evolution of pH (—) and free acidity (%) (----), during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (a): un-inoculated olives (control), (b): inoculated olives with *L.p*-FSO175. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

Table 1. Mean values and mean squares from the analyses of variance of pH, Free Acidity (% of lactic acid), Sugars and polyphenols (mM), LAB, Yeast and *Enterobacteria* biomass (Log CFU/mL)), during the controlled fermentation of heat-shocked (60 °C, 70 °C, 80 °C), un-salted and inoculated Moroccan green olives. a: un-inoculated olives (control), b: inoculated olives with *L.p*-FSO175, c: olives added with CFS of *C.p*-L18, d: olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18).

Factors			pH	Free acidity	Sugars	Polyphenols	LAB	Yeasts	Entero
Heat-shock	60 °C		4.06 a	0.60 ab	4.70 c	32.33 b	7.87 b	7.11 a	0.35 a
	70 °C		4.01 c	0.61 a	5.03 b	38.91 a	8.28 a	6.86 b	0.29 b
	80 °C		4.02 b	0.59 b	5.68 a	38.86 a	8.25 a	6.72 c	0.28 b
Inoculation	a		4.40 a	0.48 d	6.90 a	38.84 a	6.71 d	6.26 d	0.41 a
	b		3.99 b	0.64 b	4.44 c	35.81 c	9.25 a	6.74 c	0.29 b
	c		3.87 c	0.57 c	5.23 b	37.22 b	7.38 c	7.19 b	0.30 b
	d		3.85 d	0.71 a	3.98 d	34.93 d	9.19 b	7.39 a	0.26 c
Source of variation		Df							
Heat-shock		2	0.094	0.002	27.004***	1548.690***	5.809*	4.114	0.163
Inoculation		3	5.291***	0.778***	133.467***	237.420***	133.745***	20.301***	0.322
Heat-shock * Inoculation		6	0.018	0.012	0.565	9.482	0.670	0.293	0.009
Repetition		2	0.001	0.000	0.016	0.016	0.037	0.017	0.120
Residual		310	0.143	0.081	2.694	26.313	1.531	1.646	0.251

Means values in each column followed by the same letter are not significantly different according to LSD test at $p < 0.05$.

Df: Degrees of freedom, *Significant at 0.05 probability level, **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

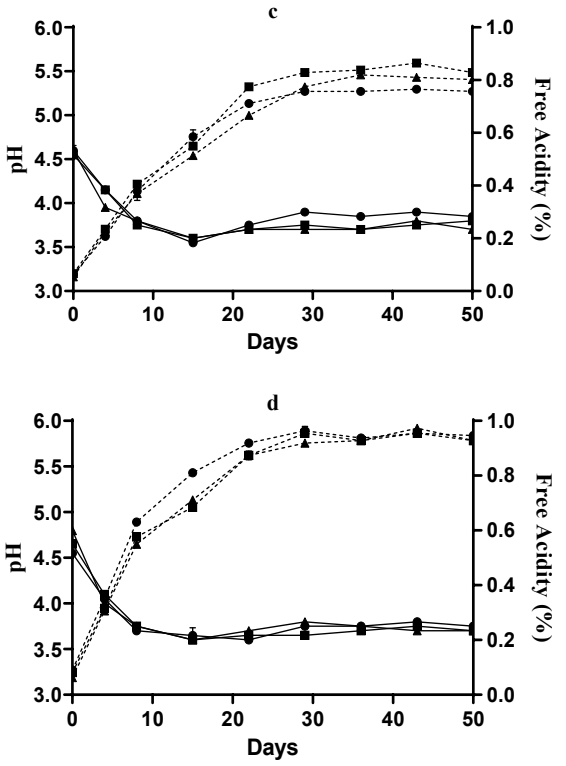

Figure 2. Evolution of pH (—) and free acidity (%) (----), during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (c): olives added with CFS of *C.p*-L18, (d): inoculated olives with *L.p*-FSO175 and added with CFS of *C.* L18. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18 (assay d), followed by that obtained (0.9%) with inoculation with *L.p*-FSO175 (assay b), then that (0.8%) obtained with the addition of CFS of *C.p*-L18 (assay c). The lowest acidity value (0.7%) was observed for un-inoculated olives (control).

Significant differences ($p < 0.05$) were obtained for pH and free acidity values between the assays, due to the effects of heat-shock and inoculation (Table 1). A high mean value for free acidity (0.64%) and low mean value for pH (3.99) were obtained in olives inoculated with *L.p*-FSO175 (b), compared to un-inoculated olives (control, a), with free acidity and pH values of 0.48% and 4.40, respectively. The inoculation with *L.p*-FSO175 and CFS of *C.p*-L18 addition (assay d) led to a substantial mean value for free acidity (0.71%) and lower mean value for pH (3.85). Thus, the highest mean value for free acidity (0.84%) and the lowest mean value for pH (3.81) were obtained, respectively, on the 43rd and the 15th days of processing. From these results, it can be observed that the increase in heat-shock led to a decrease in pH and increase in free acidity. Indeed, the inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 led to the obtention of the lowest mean value for pH (3.85) and highest mean value for free acidity (0.71%) (Table 1).

The results of the combined ANOVA (Table 1) showed the predominant effects of inoculation (more than 95%), respectively, on the variance in pH and free acidity. The influence of heat-shock was less than 1%.

3.1.2. Total sugars

The results of total sugar contents obtained for the olives are reported in (Figures 3 and 4). The sugar contents showed a progressive increase during the 20th day of the fermentation to achieve values ranging from 6-10 mM in all assays. They then decreased until the end of the process to reach 5-6 mM in un-inoculated olives (assay a) and less than 4mM in inoculated olives (assays b, c, and d).

The results of the combined ANOVA (Table 1) showed that the predominant effects of inoculation were of about 80% on the variance in sugars. The influence of heat-shock was less than 16%. According to the LSD test at $p < 0.05$, the accumulation of sugars in olive water was significantly dependent on heat-shock and inoculation (Table 1). The heat-shock at (70 and 80 °C) increased the mean value of total sugars significantly (5-5.6mM), when compared to that of 60 °C (4.7mM). At the same time, the olives inoculated with *L.p*-FSO175 and CFS of *C.p*-L18 (assay d) showed a decrease in sugars to obtain the lowest mean value of (3.97 mM), followed by that (4.44 mM) obtained in presence of *L.p*-FSO175 (assay b). The un-inoculated olives (assay a) and the olives added with CFS of *C.p*-L18 (assay c) showed high mean values for total sugars, which were 6.91 mM and 5.23 mM, respectively.

During the first 3 weeks of fermentation, the increase in temperature of heat-shock of olives led to significant increase in total sugars, and the highest mean value for sugars was obtained with heat-shock at 80 °C (Table 1).

3.1.3. Polyphenols

The results of polyphenols contents are reported in Figures 3 and 4. The results showed the same trend as sugars but with higher values in the un-inoculated olives, when compared to inoculated olives. They increased rapidly during the first week of fermentation to reach 44 mM in olives inoculated with *L.p*-FSO175 (assay b), and those added with CFS of *C.p*-L18 (assay c); 41 mM were

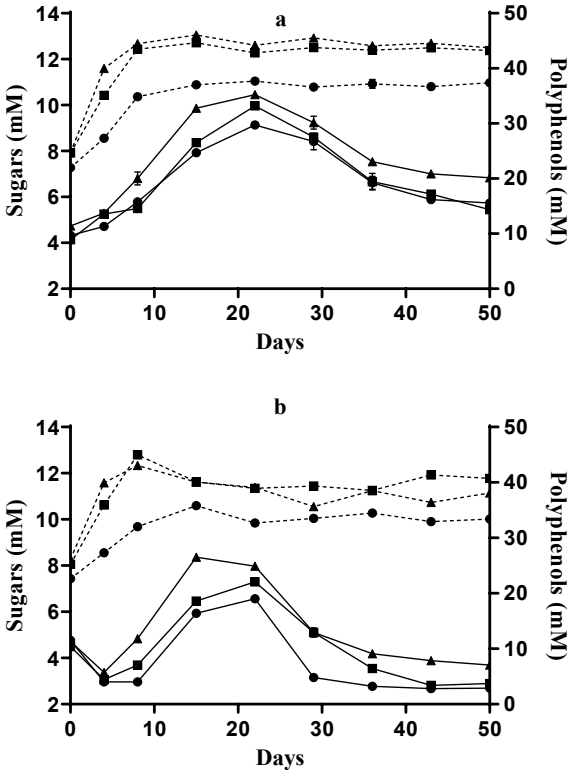

Figure 3. Total polyphenol (----) and total sugar (—) contents (mM) during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (a): un-inoculated olives (control), (b): inoculated olives with *L.p*-FSO175. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

obtained in the presence of *L.p*-FSO175 and CFS of *C.p*-L18 (assay d), and 45 mM was obtained in un-inoculated olives (assay a) (Figures 3a and 3b, (Figures 4c and 4d). After their maximum values, the polyphenol contents showed a slight decrease to stabilise at the end of fermentation, at around of 40 mM and 43 mM, respectively in assays (b, c, d) and (a). The heat-shock (70 and 80 °C) increased significantly ($p < 0.05$) the total polyphenols (38.9 mM), when compared to (32.33 mM) at 60 °C (Table 1). However, the inoculation with *L.p*-FSO175 and CFS of *C.p*-L18 (assay d) decreased the polyphenol content to the lowest mean value (34.93 mM), followed by that (35.81 mM) obtained for olives inoculated with *L.p*-FSO175 (assay b). Higher mean values for polyphenols (38.85 mM and 37.22 mM) were observed, respectively, for un-inoculated olives (assay a) and olives added with CFS of *C.p*-L18 (assay c).

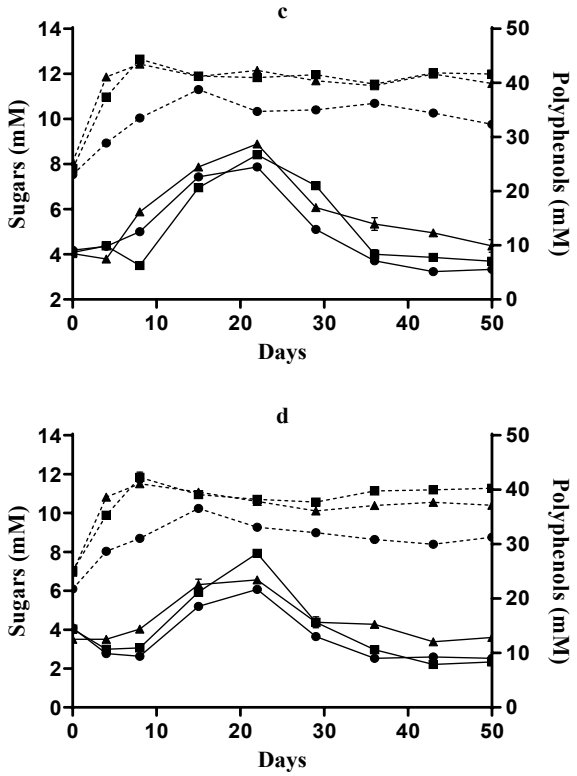

Figure 4. Total polyphenol (….) and total sugar (—) contents (mM) during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (c): olives added with CFS of *C.* L18, (d): inoculated olives with *L.p*-FSO175 and added with CFS of *C.p*-L18. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

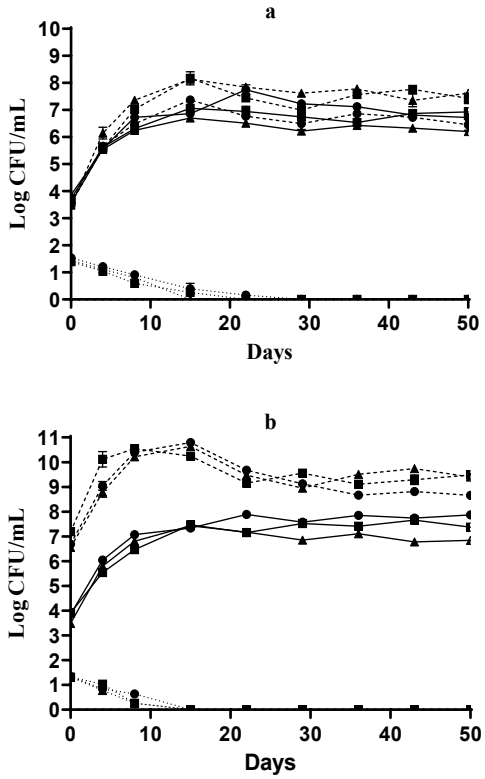

Figure 5. Evolution of LAB (----), Yeasts (—) and *Enterobacteria* (……) populations, during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (a): un-inoculated olives (control), (b): inoculated olives with *L.p*-FSO175. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown on the bars.

During the first 8 days of fermentation, the increase in temperature of heat-shock led to a significant increase in polyphenol content, and the highest polyphenol content was observed with heat-shock at 80 °C. The results of the combined ANOVA (Table 1) showed the predominant effects of Heat-shock of about 86% on the variance in polyphenols. The influence of inoculation explained less than 13%.

3.2. Microbiological analysis

3.2.1. Lactic acid bacteria (LAB)

The results of the microbial population of LAB in olive assays are reported in (Figures 5 and 6), and their mean values are presented in Table 1. In the olives inoculated with *L.p*-FSO175 (Figures 5b and 6d), the LAB population showed significant growth during the first 15 days of fermentation, from 7 Log CFU/mL to about 10 Log CFU/mL, followed by a very slight decrease to 9-10 Log CFU/mL at the end of the process. In the olives added with CFS of *C.p*-L18 (Figure 6c), LAB population showed an important growth from 3-4 Log CFU/mL to reach 8 Log CFU/mL and stabilize at this level until the 50th day of the process. However, in the un-inoculated olives (Figure 5a), the natural LAB population showed an increase from 3-4 Log CFU/mL to 7 Log CFU/mL during the first 15 days, followed by a slight decrease to stabilize at the end of the fermentation process between 6 and 7 Log CFU/mL, respectively, in assays treated at 60 °C and (70, 80 °C).

Significant differences ($p < 0.05$), were observed in LAB biomass due to the effect of heat-shock and inoculation (Table 1). Both heat-shock temperatures (70 and 80 °C) increased the LAB biomass significantly ($p < 0.05$) when compared to that obtained with 60 °C. A high increase in LAB growth was observed during the first 20 days of fermentation.

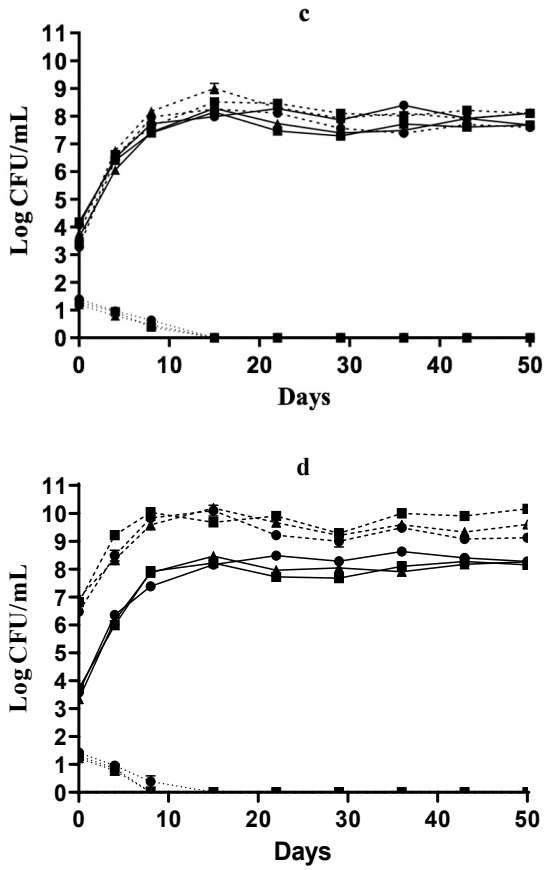

Figure 6. Evolution of LAB (----), Yeast (—) and *Enterobacteria* (……) populations, during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (c): olives added with CFS of *C.* L18, (d): inoculated olives with *L.p*-FSO175 and added with CFS of *C.p*-L18. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown on the bars.

The highest mean value for LAB count (9.25 Log CFU/mL) was obtained in olives inoculated with *L.p*-FSO175 (assay b), followed by that (9.2 Log CFU/mL) obtained in the presence of inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (assay d), and finally by that obtained (7.38 Log CFU/mL) in the presence of CFS of *C.p*-L18. The lowest mean value for LAB count was obtained in un-inoculated olives (assay a) (6.71 Log CFU/mL). The results of the combined analyses (Table 1) revealed that LAB biomass was highly influenced by the inoculation effect, which explained about 95% of the total variance observed. However, the impact of heat-shock on LAB biomass was lower than about 4%.

3.2.2. Yeasts and molds

The results from the microbial population of yeasts and molds in olives assays are reported in Figures 5 and 6, and their mean values are presented in Table 1. This population increased progressively during the first 15 and 22 days of the fermentation process, respectively, in the assays treated at (70 and 80 °C) and at 60 °C. In assays supplemented with CFS of *C.p*-L18, with or without inoculation with *L.p*-FSO175 (Figures 6, c and d), the yeasts and molds population showed significant growth during the first 15 days of fermentation, from 4 Log CFU/mL to about 8 Log CFU/mL, followed by a slight decrease to stabilize at 7 Log CFU/mL. In olives inoculated with *L.p*-FSO175, the natural yeast and mold population showed a progressive growth to reach the maximum of 7-8 Log CFU/mL (Figure 5b), which remains lower than that obtained in olives added with CFS of *C.p*-L18 (Figure 6c), and clearly higher than that (6-6.5 Log CFU/mL) observed in un-inoculated assays (Figure 5a).

The results of the combined ANOVA (Table 1) showed that the variance in yeast biomass was predominantly affected by inoculation (82%). However, even the effect of heat-shock was lower and explains less than 16%, the increase in temperature of heat-shock olives significantly decreased ($p < 0.05$) the mean value of the yeast and mold biomass (Table 1). The lowest mean value for the yeast and mold count (6.72 Log CFU/mL) was obtained with heat-shock at 80 °C; while the highest one (7.11 Log CFU/mL) was observed with heat-shock at 60 °C. However, with regard to inoculation, the assays (b, c and d) (table 1), showed the highest and significant mean values for yeasts and molds compared to that found in the un-inoculated olives (assays a) (6.27 Log CFU/mL). The inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (assay d) showed the highest mean value for yeasts and molds (7.39 Log CFU/mL), followed by that obtained in the presence of CFS of *C.p*-L18 (assay c) (7.2 Log CFU/mL) which was higher than that obtained with inoculation with *L.p*-FSO175 (b) (assay 6.75 Log CFU/mL).

3.2.3. Enterobacteria

The *Enterobacteria* population presented in (Figures 5 and 6) was initially low and in all olive, assays did not exceed 1.4 Log CFU/mL at the beginning of the fermentation. This population decreased rapid-

ly during the first week and disappeared completely on the 8th day of fermentation in heat-shocked olives at (70 and 80 °C) and inoculated with *L.p*-FSO175 and supplemented with CFS of *C.p*-L18 (assay d) (Figure 3d). With other treatments (*L.p*-FSO175 (assay b) or the addition of CFS of *C.p*-L18 (assay c)), the disappearance of this population was observed on the 15th day of the process. In the un-inoculated and heat-shocked (at 60 °C) olives, this population showed a delay to disappear and were totally eliminated on the 22nd and 30th day of the fermentation process, respectively (Figure 5a).

The results of the combined ANOVA (Table 1) showed that the variance in *Enterobacteria* was affected with the inoculation of about 36%, while the effects of heat-shock explained less than 18%. A significant difference ($p < 0.05$) in mean value for *Enterobacteria* counts was found between heat-shocked olives at 60 °C and heat-shocked at (70 and 80 °C). However, no significant difference was found between *Enterobacteria* counts obtained at 70 or 80 °C (Table 1).

In the presence of inoculation with *L.p*-FSO175, significantly ($p < 0.05$) lower mean values for *Enterobacteria* counts were found in olives (assays b, c and d) when compared to that of un-inoculated olives (assay a) (0.41 Log CFU/mL). The inoculation with *L.p*-FSO175 and the addition of CFS of *C.p*-L18 (assay d) allowed the reduction of *Enterobacteria* counts to reach the lowest mean value (0.26 Log CFU/mL); while no significant difference was observed in the mean value for *Enterobacteria* counts between olives inoculated with *L.p*-FSO175 (assay b) (0.29 Log CFU/mL) and olives added with CFS of *C.p*-L18 (c) (0.3 Log CFU/mL).

3.3. Sensorial and organoleptic analysis

3.3.1. Olives color changes

The mean values for the CIE *l *a *b parameters of olive color are represented in (Table 2). The olives from all the assays showed high lightness (*l) in the range 62-74, which is generally associated with better color. The increase in temperature of heat-shock olives increased the lightness (*l), yellowness (*b), but decreased the greenness (*a). Heat-shock at 80 °C led to a significant increase in the mean values for (*l = 69.61), (*b= 51.19) and decrease in greenness (*a =

Table 2. Mean values and mean squares from the analyses of variance for olives color basing CIE*l*a*b parameters (*l =lightness, *a = greenness, *b = yellowness) during the controlled fermentation of heat-shocked (60 °C, 70 °C, 80 °C), un-salted and inoculated Moroccan green olives. a: un-inoculated olives (control), b: inoculated olives with *L.p*-FSO175, c: olives added with CFS of *C.p*-L18, d: olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18).

Factors			CIE *l*a*b parameters		
			*l	*a	*b
Heat-shock	60 °C		67.64 b	1.97 a	48.91 c
	70 °C		68.52 b	1.36 b	49.45 b
	80 °C		69.61 a	1.11 b	51.11 a
Inoculation	a		65.82 c	0.98 c	48.17 d
	b		71.41 a	2.45 a	49.43 b
	c		68.37 b	1.01 c	48.75 c
	d		68.77 b	1.47 b	52.94 a
Source of variation		Df			
Heat-shock		2	35,205	7,081	47,328
Inoculation		3	141,252	12,740	124,049
Heat-shock * Inoculation		6	235,947	9,067	132,858
Repetition		2	6,228	0,062	6,766
Residual		94	141,512	10,390	119,117

Means values in each column followed by the same letter are not significantly different according to LSD test at $p < 0.05$. Df: Degrees of freedom, *Significant at 0.05 probability level, **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

1.11). Meanwhile, heat-shock at 60 °C led to the lowest mean values for the CIE *l *b parameters and the highest mean value for greenness (*a) (Table 2).

With regards to inoculation, a significant ($p < 0.05$) improvement in all CIE *l *a *b parameters was observed. The best improvement in color was observed in olives inoculated with *L.p*-FSO175 (assay b) (*l=71.71, *a=2.45 and *b=49.43) and in olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18 (assay d) (*l=68.77, *a=1.47 and *b=52.94) (Table 2). The best olive color was obtained at the end of the fermentation process (50th day), indicated by the best values for lightness (*l=74.08), greenness (*a=3.76) and yellowness (*b=57.54).

The combined analysis of variance (Table 2) showed the dominant effect of inoculation compared to that of heat-shock on all parameters of CIE*l*a*b, presenting about 32% of the observed variance for greenness (*a), 28% for yellowness (*b) and about 25% for lightness (*l). The influence of heat-shock was of about 6, 18 and 11%, respectively, on the variance for lightness (*l), for greenness (*a) and for yellowness (*b). An important effect due to the interaction (heat-shock * inoculation) was observed on the variance for lightness (*l) (42%), for greenness (*a) (23%) and for yellowness (*b) (30.9%).

3.3.2. Sensory characteristics of fermented olives

The mean values measured for the sensory characteristics of un-salted fermented green olives are reported in Table 3. Significant acceptability and palatability were determined by the panellist for all sensory characteristics due to the effects of inoculation and heat-shock. All the sensory characters showed significant differences ($p < 0.05$), due to the increase in heat-shock temperature (Table 3). Lowest mean values for all sensory characters were obtained with heat-shock at 60 °C, while, an acceptable sensory characteristic was found by the panellists for heat-shocked olives at 70 and 80 °C. Heat-shock at 80 °C led to a significant ($p < 0.05$) increase in the scores for acidic (7.1), bitterness (6.68) and color (6.8). A significant difference ($p < 0.05$) was obtained for bitterness between olives heat-shocked at (70 and 80 °C) and those treated at (60 °C). However, no significant difference was observed between heat-shocked olives at 70 and 80 °C.

Table 3. Mean values and mean squares from the analyses of variance of Sensory Characteristics (Color, Flavor, Crunchiness, Bitterness and Acidness) of un-salted fermented olives, under various effects of Heat shock (60 °C, 70 °C, 80 °C) and Inoculations. a: un-inoculated olives (control), b: inoculated olives with *L.p*-FSO175, c: olives added with CFS of *C.p*-L18, d: olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18).

Factors			**Color**	**Flavor**	**Crunchiness**	**Bitterness**	**Acidness**
Heat-shock	60 °C		4.58 c	4.86 c	5.80 c	5.30 b	6.17 c
	70 °C		6.46 b	6.61 a	7.26 a	6.57 a	6.42 b
	80 °C		6.80 a	6.17 b	6.92 b	6.68 a	7.10 a
Inoculation	a		4.90 c	4.58 c	5.70 c	5.18 d	5.28 d
	b		6.25 ab	6.36 a	7.26 a	6.95 a	7.50 a
	c		6.23 b	6.52 a	6.90 b	6.12 c	6.62 c
	d		6.42 a	6.07 b	6.78 b	6.50 b	6.85 b
Source of variation		Df					
Heat-shock		2	113.662***	66.354***	46.912***	47.512***	18.462***
Inoculation		3	29.811***	47.166***	27.248***	33.848***	52.015***
Heat-shock * Inoculation		6	33.206***	27.387***	27.856***	19.223***	18.106***
Repetition		19	0.319	0.311	0.267	0.226	0.437
Residual		209	0.245	0.221	0.238	0.279	0.292

Means values in each column followed by the same letter are not significantly different according to LSD test at $p < 0.05$.

Df: Degrees of freedom, *Significant at 0.05 probability level, **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

The un-inoculated olives (assay a) showed the lowest values for all characteristics, compared to inoculated ones (assays b, c and d). According to the LSD test significant differences were observed at $p < 0.05$ for all the characteristics analyzed due to the effect of inoculation. The lowest mean values for all scores were observed in the un-inoculated olives. However, the highest scores for all sensory characterteristics were obtained in the presence of inoculation with *L.p*-FSO175 (assay b), followed by inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (d). The inoculation with CFS of *C.p*-L18 (c) showed the highest score for flavor.

The results of the combined analysis of variance (Table 3) showed the predominant effect of heat-shock, which presented about 46% for crunchiness, flavor, and bitterness and 64% for color; while the acidic variance was affected by the effect of inoculation (58%). The inoculation effect presented about 33% of the variance for flavor and bitterness, and only slightly influenced the variance in color (16.8%). Indeed, the interaction between heat-shock and inoculation showed significant effects at $p < 0.001$ on the variance in all characteristics analysed and explained about 19% for color, flavor, bitterness acidic and 27% for crunchiness.

3.3.3. Examination of olive spoilage

The results of the assessment of fermented olive spoilage showed that the main olive spoilages recorded are gas pocket, softening, off-odors and lactic spots. The un-inoculated, heat-shocked olives at 60 °C (assay a) showed the highest gas pocket incidence at about 19%, along with the presence of softening and off-odors. However, the lowest rate of spoiled olives was observed in olives inoculated with *L.p*-FSO175, added or not with CFS of *C.p*-L18), and a low level of gas pocket spoilage (5% and 3.34%) was obtained in olives inoculated with L.p-FSO175 and treated at 70 and 80 °C, respectively. No off-odor was detected by panellists for heat-shocked olives at 70 or 80 °C, inoculated with *L.p*-FSO175, and/or supplemented with CFS of *C.p*-L18.

4. DISCUSSION

In a previous work, the controlled fermentation of Moroccan Picholine variety green olives, using the oleuorpeinolytic strain of *L.p*-FSO175, showed some bitterness in fermented olives (Ghabbour *et al.,* 2016). To overcome this problem and in order to improve both the bio-debittering and fermentation processes, the green olives were subjected to heat-shock (60, 70, 80 °C) tree times for 5 min, followed by inoculation with *L.p*-FSO175 and/or addition of CFS of *C.p*-L18.

The results showed that the highest mean values for sugars and polyphenols (5.6 mM and 38.9 mM, respectively), obtained in the olives heat-shocked at 80 °C, could be attributed to the increase in permeability of olive pulp due to heat-shock (Balatsouras *et al.,* 1983), facilitating the release of nutrients into the olive water. These results indicate the beneficial double technological roles of heat-shock: firstly, by providing sugars for the microorganisms, including LAB starter (Argyri *et al.,* 2014), and secondly by eliminating high amount of polyphenols (mainly oleuropein) by osmosis, thus accelerating the debittering process (Valenčić *et al.,* 2010).

It should be emphasized that the high temperatures of heat-shock (70 and 80 °C) significantly increased the LAB growth (to 8-10 Log CFU/mL) on the 15th day of fermentation. In fact, the heat-shock permitted the dominance of LAB during the first days of the fermentation process, through providing high amounts of nutrients, mainly sugars, polyphenols, and vitamins. In addition, when the inoculation was added, more significant ($p < 0.05$) differences in LAB were obtained compared to un-inoculated olives. The highest mean values for LAB were obtained, respectively, in olives inoculated with *L.p*-FSO175 (9.25 Log CFU/mL) and with *L.p*-FSO175 and added with CFS of *C.p*-L18 (9.2 Log CFU/mL). This result is similar to that reported by (Saravanos *et al.,* 2008).

In the presence of *L.p*-FSO175 and CFS of *C.p*-L18, lower mean values for sugars and polyphenols were observed in the olive water (3.98 mM and 34.94 mM, respectively), and in the presence of *L.p*-FSO175, the mean values obtained were (4.44 mM and 35.81 mM, respectively); while, the highest mean values for sugars and polyphenols were found in un-inoculated olives (6.9 mM and 38.85 mM, respectively). These results mean that high sugar contents were metabolized by the *L.p*-FSO175 starter culture and as a result, the lowest values for pH (3.7-3.8) and highest values for free acidity (of about 1%) were found. The latter is related to the accumulation

of organic acids produced by inoculum through the consumption of the sugars present in olives (Chorianopoulos *et al.,* 2005; Panagou *et al.,* 2008). Similar results for brine acidity (1.0-1.2%) and low pH values (3.8-3.9) were reported in heat-shocked olives which were fermented by *L. plantarum* (Etchells *et al.,* 1966).

The increase in temperature of heat-shock significantly decreased ($p < 0.05$) the yeast and mold population, and the lowest mean value (6.72 Log CFU/mL) was obtained for heat-shocked olives at 80 °C. Similar results were reported by Chorianopoulos *et al.,* (2005), who attributed the decrease in yeasts and molds to heat-shock. However, in the presence of *L.p*-FSO175 and CFS of *C.p* L18 (assay d), a significant ($p < 0.05$) increase in yeasts and molds was observed, allowing the highest mean value for yeasts and molds (7.39 Log CFU/mL), followed by that (7.2 Log CFU/mL) obtained in the presence of CFS of *C.p*-L18 (assay c). The presence of this population in all heat-shocked olives is due to the autochthonous yeasts and molds coming from the epidermis of the fruits themselves (Arroyo-Lopez *et al.,* 2012b; Pereira *et al.,* 2015). The olives added with CFS of *C.p*-L18 (assay c) showed significant ($p < 0.05$) differences in yeast and mold growth compared to un-inoculated olives. This result could be related to the wealth of nutrients in CFS of *C.p*-L18 (amino acids, fatty acids, vitamins and salts) which are necessary for yeasts and molds to grow. The persistence of the yeast and mold population during this process is of double technological importance. Firstly, they promote LAB growth (starter and/or autochthonous), providing them with the essential nutrients for their growth (Arroyo-López *et al.,* 2008; Arroyo-Lopez *et al.,* 2012b; Sidari *et al.,* 2019). Secondly, they improve the biological debittering process of fruits, based on their esterase and β-glucosidase activities (Rodriguez-Gomez *et al.,* 2012; Anagnostopoulos *et al.,* 2017).

The results obtained in this work showed that the increase in temperature of heat-shock led to a significant ($p < 0.05$) decrease in *Enterobacteria*, and a low count of this population was observed at the beginning of fermentation. Compared to 70 and 60 °C, heat-shock at 80 °C resulted in the lowest mean value for *Enterobacteria*. This finding indicates the beneficial effect of heat-shock on olives to give another advantage to the starter culture to dominate the fermentation process by eliminating most of the competitive and interfering microbiota (Etchells *et al.,* 1966; Balatsouras *et al.,* 1983; Chorianopoulos *et al.,* 2005).

In addition, inoculation with *L.p*-FSO175 and the addition of CFS of *C.p*-L18 significantly decreased the *Enterobacteria* count compared to un-inoculated olives, leading to the lowest mean value for this population (0.26 Log UFC/mL) (assay d). The earlier disappearance of *Enterobacteria* on the 8th day of fermentation was obtained in heat-shocked olives at (70 and 80 °C) and inoculated with *L.p*-FSO175 and added CFS of *C.p*-L18 (assay d). However, the un-inoculated and heat-shocked (at 60 °C) olives showed the highest mean value for *Enterobacteria* (0.41 Log UFC/mL), and their disappearance was obtained, with a delay, on the 22nd and 30th day of the fermentation process. The reduction in *Enterobacteria* can be attributed to antibacterial and antifungal compounds produced by *L.p*-FSO175, such as organic acids, hydrogen peroxide and bacteriocins (Abouloifa *et al.,* 2021). Furthermore, their total disappearance, during the first week of process, can also be related to the inhibitory effect of phenolic compounds (Landete *et al.,* 2008; Segovia-Bravo *et al.,* 2009). The safety of the final product can be ensured by the combination of two hurdles, namely acidity (pH) and antimicrobial compounds. However, a heat sterilization of fermented olives, packed in hermetically sealed containers, is mandatory to ensure their safety during storage.

With regard to color, the results showed that as the temperature of heat-shock increased, better color was obtained. It was demonstrated that the increase in olives storage temperature improved olive color (Rodríguez-Gómez *et al.,* 2014). The heat-shock at 80 °C showed a significant ($p < 0.05$) increase in mean values for lightness (*l = 69.61), yellowness (*b= 51.19) and decrease in greenness (*a = 1.11). This finding may be explained by the thermal inactivation of polyphenol oxidase (PPO) after heat-shock at 80 °C, thus preventing the browning of olive fruits (Whitaker and Lee, 1995). However, in olives heat-shocked at 60 °C, lowest color scales for CIE *L *a *b parameters were observed. This result may be explained by the increase in oxidation of polyphenols caused by the persistence of PPO activity in the raw material, favored by the pH of the olive water (4.5), which falls in the optimum pH range (pH 4-7) of PPO (Ben-Shalom *et al.,* 1977). Heat-shock at 60

°C seems to be insufficient to deactivate the PPO. This result is in agreement with previous works, reporting that short-term treatments (few minutes) at 70 or 90 °C, are generally sufficient to destroy all of the PPO activity in plants (Yemenicioğlu and Cemeroğlu, 2003).

The best color improvement (*l=71.71, *a=2.45 and *b=49.43) was observed in olives inoculated with *L.p*-FSO175 (assays b), followed by (*l=68.77, *a=1.47 and *b=52.94) inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18 (assay d). These results can be attributed to the favorable effect of lower pH and high acidity on the color parameter, through the diffusion of organic acids in fermented olives (Garrido Fernandez *et al.,* 1997b). Moreover, the inoculation with *L.p*-FSO175 and/or addition of CFS of *C.p*-L18 combined with heat-shock at 60 °C allowed significant improvement in color, meaning that the polyphenol oxidation observed in un-inoculated heat-shocked olives at 60 °C was overcome by the high acidification assured by the inoculum. The PPO involved in polyphenol oxidation was reported to be inhibited at pH lower than 4 in heat-shocked olives at 60° (Nicolas *et al.,* 1994). Thus, inoculation with *L.p*-FSO175 and heat-shock at 60 °C can be sufficient to avoid the enzymatic browning of fermented olives.

The results of sensory attributes of un-salted fermented green olives, indicate that the heat-shock at 80 °C permitted significant ($p<0.05$) improvement of acidness (7.1), bitterness (6.68) and colour (6.8). Significant ($p<0.05$) differences in values of bitterness were obtained between olives heat-shocked at (70 °C and 80 °C) and that treated at 60 °C, which may be linked with the effect of heat-shock in increasing release of polyphenols from olives observed in this work. These results indicate the beneficial effects of heat-shock at (70 °C and 80 °C) to promote the debittering of un-salted green olives, by increasing the permeability of olives pulp (Balatsouras *et al.,* 1983), and facilitating the diffusion of polyphenols and other nutrients from olives by osmosis, and consequently increasing debittering process (Valenčić *et al.,* 2010). On the other hand, the best appreciations of colorness by the panellists due to heat-shock of olives by high temperatures (70 °C and 80 °C), were confirmed by CIE*L*a*b parameters.

The inoculation of olives with *L.p*-FSO175 led to significant ($p<0.05$) appreciation of all sensory attributes. Previous works reported the improvement of sensory attributes of fermented olives by using an oleuropeinolytic strain of *L. plantarum* as debittering and fermenting agent (Tataridou and Kotzekidou, 2015, Ghabbour *et al.,* 2016). Thus, the reduction of bitterness in olives inoculated with *L.p*-FSO175 can be attributed to the high oleuropeinolytic activity of this strain, particularly in the absence of salt (Ghabbour *et al.,* 2020). The reduction of bitterness observed in olives added with the CFS of *C.p*-L18 can be related to the β-glucosidase enzyme induced in *C. pelliculosa* L18 by oleuropein (Rokni *et al.,* 2021). The effectiveness of some *Candida* species starters in bio-debittering was reported (Ciafardini and Zullo, 2019) to have led to lower concentrations of polar phenolic compounds in olive brines. The oleuropeinolytic activity of *L.p*-FSO175 and the CFS of *C.p*-L18, is of great interest because it contributes to the high accumulation of hydroxytyrosol in fermented olives (Chytiri *et al.,* 2020), which is highly desired to improve their nutritional value (Bertelli *et al.,* 2020).

The results of the analysis of spoilage in fermented olives showed that the increase in temperature due to the heat-shock (70 °C and 80 °C) of olives led to a low incidence of gas pockets (less than 5%) in fermented olives, which can be due to gas-producing microorganisms (Asehraou *et al.,* 2000). In the presence of inoculation with *L.p*-FSO175 with or without the addition of CFS of *C.p*-L18, the lowest rate of gas pockets was observed and no off-odors were detected by panellists. These results should be explained by an adequate balance in the olives' microbiota (LAB > yeasts) to reduce tissue damage (Golomb *et al.,* 2013). The benefits of inoculation with *Lactobacillus* starters in improving the hygienic quality of fermented olives was reported (Peres *et al.,* 2008, Hurtado *et al.,* 2010, Aponte *et al.,* 2012, Ghabbour *et al.,* 2016).

From all the obtained results, compared to 60 and 70 °C, the heat-shock of olives at 80 °C showed its advantage in improving the physicochemical, microbiological and the sensory properties of un-salted fermented olives. In addition, the inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (assay d) showed the best improvement in the fermentation profile of un-salted olives, compared to the olives inoculated with *L.p*-FSO175 (b) or added with CFS of *C.p*-L18 (assay c). These results indicate that the heat-shock of olives at 80 °C, followed by

inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18, could be a suitable process for the controlled fermentation of un-salted green olives. This process is characterized by a high decrease in pH (< 3.8) and increase in acidity (> 0.8%), and by an early disappearance of *Enterobacteria* (first week), and a reduced fermentation time (50 days). Furthermore, this process allows the production of un-salted green olives with considerable antioxidants (Bertelli *et al.,* 2020), and probiotic and organoleptic properties (Abouloifa *et al.,* 2019; Abouloifa *et al.,* 2020).

5. CONCLUSIONS

The present study reports the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan Picholine green olives for the first time. The results showed that heat-shock at 80 °C improved the fermentation profile of un-salted olives by increasing the release of nutrients (i.e. sugars and polyphenols), reducing the bitterness and improving the color of fermented olives. The inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 enhanced the fermentation process, indicated by a high decrease in pH, high increase in free acidity, and rapid disappearance of *Enterobacteria* obtained on the 8th day of fermentation. This process produced a substantial improvement in sensory attributes (bitterness, color and acidity) and reduced the spoilage incidence in fermented olives. Based on these results, the heat-shock of olives at 80 °C, followed by their inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18, can be appropriate for the biological debittering and fermentation of un-salted Moroccan Picholine green olive variety. Furthermore, this process presents promising prospects not only for consumers by providing salt-free olives, but also by protecting environment, by releasing chemical-free wastewater (NaCl and NaOH).

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