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Effects of *Lactiplantibacillus plantarum* 11 and Chloride Sodium on Biochemical and Microbiological Quality of Table Olives during Fermentation

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HIGHLIGHTS

- The hygienic quality of olives was improved during incubation at 30 °C.
- Pathogenic germs were inhibited at 4, 8, and 12% (w/v) of NaCl, in batches inoculated and incubated at 30 °C.
- *Lactiplantibacillus plantarum* 11 could be considered as a probiotic starter culture of table olives.

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Acronyms and abbreviations
CFU=Colony Forming Unit
LAB=Lactic Acid Bacteria

ABSTRACT

Background: Table olives are nutritionally a complete food and considered as one of the oldest fermented products. This study aimed to evaluate the effect of *Lactiplantibacillus plantarum* 11 as a starter culture on the fermentation of table olives at two incubation temperatures 22 and 30 °C and different salt concentrations (0, 4, 8, and 12% m/v) of sodium chloride (NaCl).

Methods: The fermentation of table olives was carried out according to the Spanish style. *L. plantarum* 11 was inoculated as a starter culture (10^6 Colony Forming Unit (CFU)/ml), and *Listeria monocytogenes* CECT 4032 was used as an indicator strain. Under the same experimental conditions, the fermentation of olives without the inoculation of starter culture was used as a control. Then, biochemical and microbiological quality of each experimental batch was tested.

Results: Unlike the incubation temperature of 22 °C, the pH values obtained in salted batches and incubated at 30 °C were all below the marketing limits for table olives. At the end of the process, the maximum load of yeasts and molds (>5 log CFU/ml) was recorded in the batches incubated at 22 °C. At 22 °C, *Listeria* was absent in inoculated fermenters at a concentration greater than or equal to 8% (w/v) of NaCl. However, at 30 °C, *Listeria* was not detected in treatment groups and in the control group with 12% NaCl.

Conclusion: *L. plantarum* 11 could be potentially considered as a probiotic starter culture during the fermentation of black table olives.

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Introduction

Table olives are nutritionally a complete food and considered as one of the oldest fermented products. According to the latest studies, olive is generally a source of natural fiber, rich in vitamin E and cholesterol-free (Lanza et al., 2014; Pasten et al., 2019). Several technological processes have been used in the development of black olives, the best known of which are the Greek style and the Californian style. The Greek method is milder

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and includes washing, natural fermentation in brine and oxidation in air for color enhancement, it is a method based on spontaneous fermentation which is more or less uncontrollable on an industrial scale. The Californian style includes washing, treatment with iron salt and air oxidation, canning, and finally heat treatment. The latter process involves final sterilization, so it is generally considered safer production (De Castro et al., 2019; Grounta et al., 2017).

To guarantee the quality and microbiological safety of the finished product, preparation of table olives is based on the use of high salt concentrations in fermentation brines, in order to reduce the load of undesirable and pathogenic microorganisms. In Morocco, black olives are produced using concentrations ranging from 6 to 12% (w/v) of sodium chloride (NaCl). On the other hand, black olives of Greek style are brined with 7 to 10% (w/v) of salt for 6 to 12 months of fermentation (Code des pratiques loyales pour les olives de table, 2018). This high concentration of sodium chloride is closely related to serious health problems such as high blood pressure cardiovascular and kidney diseases (He and MacGregor, 2018; Mozaffarian et al., 2014).

The alkaline treatment of table olives with sodium hydroxide (NaOH) is commonly used for the production of green olives (De Castro et al., 2019). According to the literature, this pre-treatment not only affects the fruit by eliminating the bitter taste resulting from the oleuropein, but it also affects the brine exhibiting the bacterial olives population as was shown by the study of Chammem et al. (2005) that treatment with soda during lactic fermentation of olives contributed to the elimination of coliform population.

Recently, studies have evaluated the processing of table olives while using reduced concentrations of NaCl (Mateus et al., 2016; Zinno et al., 2017). However, it has already been established that a low concentration of sodium chloride could be responsible for the multiplication of pathogens such as *Clostridium botulinum* (Maslanka et al., 2013). Other research based on comparative studies of olives obtained by spontaneous fermentation and olives produced using a starter culture has suggested that inoculation with appropriate Lactic Acid Bacteria (LAB) as starter culture helps improve and make olives fermentation process more predictable (Anagnostopoulos et al., 2020). The use of *Lactiplantibacillus plantarum* in combination or not with another probiotic strain as a starter culture has been widely used in the production of black olives. Pino et al. (2018) demonstrated the application of *L. plantarum* in the probiotic formulation of black olives as well as a reduction in the concentration of sodium chloride allowed to obtain a final product fulfilling microbiological criteria and exhibiting the most appreciated sensory characteristics.

In the light of the above, this work aimed to evaluate effects of *L. plantarum* 11 and chloride sodium on biochemical and microbiological quality of table olives during 30 days of fermentation.

Materials and methods

Olive processing and fermentation

Black olives of "Moroccan picholine" variety were harvested in the Tangier-Tetouan region of north Morocco in mid-December. The treatment was carried out in batches containing 500 g of fruit and 250 ml of mineral water. Olives were first debittered for 2 h using 2.3% (m/v) of NaOH solution, washed thoroughly with mineral water and subsequently subdivided into control and experimental batches, in which were brined with four different concentrations of sodium chloride, including T0, T4, T8, and T12 that represent 0, 4, 8, and 12% (m/v). Batches T were inoculated with an adequate concentration of *L. plantarum* 11 (10^6 Colony Forming Unit (CFU/ml). However, *Listeria monocytogenes* CECT 4032 was used as model organism and was inoculated in both types of batches.

Experiments were performed at two different temperatures, 22±2 and 30±2 °C, on day 0, 4, 10, 19, 24, and 30 of fermentation. The pH and acidity measurements, as well as microbiological analysis were carried out at specified times. Total phenolic content was determined at the end of fermentation process. All of the experimental tests were carried out in triplicate.

Starter culture preparation

L. plantarum 11 which possessed specific technological and safety characteristics was isolated from table olives in Morocco and identified in laboratory of Microbiology at Jaèn University in Spain (El Issaoui et al., 2021). The strain was cultured twice in MRS broth (Biokar Diagnostics, Beauvais, France) for 24 h at 30 ºC, second inoculation was carried out in MRS broth at 4.5% (w/v) of NaCl to allow isolate to adapt to saline environments (De Castro et al., 2019). The culture was centrifuged at 8 000 rpm for 15 min using a model centrifuge (Hettich universal 230), and washed with saline solution (0.9% (w/v) NaCl). Pellet was re-suspended in brine of the corresponding batches at a concentration of 10^6 CFU/ml.

Preparation of indicator strain

L. monocytogenes CECT 4032 was revivified twice in a BHI broth (Biokar Diagnostics, Beauvais, France). Second revivification was carried out on BHI broth at 4.5% (w/v) of NaCl. After incubation at 37 °C for 18 h, the culture was centrifuged at 8 000 rpm for 15 min, then

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washed with saline solution (0.9% (w/v) NaCl) and pellet obtained was re-suspended in brine of all samples at a final concentration of 10^4 CFU/ml.

Biochemical analysis

-pH and acidity

The pH values of brines were monitored by a pH meter (PHSJ-3F, China). Total free acidity was measured by titration of the brine using 0.1 N sodium hydroxide (NaOH).

-Total phenolic content

The quantification of total phenolic compounds contained in olive pulp was carried out by the method of Folin Ciocalteu (Singleton et al., 1999). Total phenolic content of the extracts was expressed as µg of Gallic Acid Equivalents (GAE) per mg of sample in dry weight (µg/mg). Three ml of the olive pulp were added to 3 ml of hexane (v/v). The delipidated pulp was collected after complete separation into two phases: hexane (supernatant) and the delipidated pulp (pellet) ready for liquid-liquid extraction. Three ml of ethyl acetate was added to 3 ml of delipidated pulp (v/v). After centrifugation, the organic phase rich in phenolic compounds undergoes evaporation under vacuum using a rotary evaporator at 75 °C. The dry residue was stored in 3 ml of methanol at -18 °C and this solution was used for the determination of the phenolic compounds (Gueboudji et al., 2021). The dosage of the total polyphenols was determined by spectrophotometry. In glass hemolysis tubes, a volume of 0.3 ml of each extract was added, with a mixture of 1.5 ml of 10-fold diluted Folin-Ciocalteu reagent and 1.2 ml of sodium carbonate (Na2CO3) at 7.5%. The tubes are shaken and stored for 30 min at 40 °C. The absorbance was read at 765 nm (Wabaidur et al., 2020).

Microbiological analysis

Microbiological analysis of olive brines was carried out by counting the total mesophilic bacteria, total and fecal coliforms, yeasts and molds, LAB, *Listeria* and *Staphylococcus* respectively on Plate Count Agar, Deoxycholate Lactose agar, Sabouraud agar, de Man Rogosa & Sharp agar, Palcam agar and Baird Parker agar respectively (all provided from Biokar Diagnostics, Beauvais, France). Incubation was carried out for 24 to 48 h (up to 96 h for *Listeria*), at 30 °C for LAB, at 44 °C for fecal coliforms and at 37 °C for the rest (Anagnostopoulos et al., 2020).

Statistical analysis

Statistical tests were performed using SPSS statistical software (IBM SPSS software version 16.0). Statistical analysis of the data was performed using a unidirectional analysis of variance (ANOVA) in order to test the differences between fermentation conditions (inoculation with starter culture, salt concentration and incubation temperature); significant differences were expressed at $p<0.05$. The post-hoc Tukey test was used to assess the overall differences between the samples.

Results

Biochemical changes

According to the results, the lowest pH values were revealed by brines incubated at 30 °C compared to 22 °C (Figures 1 and 2). For T0% and T4%, at 30 °C, rapid acidification of the medium was recorded in the batches test and controls, whose pH values dropped faster (during the first 2 days) to more than 3 units. Uninoculated brines added with a concentration greater than or equal to 4% (w/v) of sodium chloride and incubated at 30 °C, showed pH values close to 4.3. However, only the brine of T4% (30°C) which was the most acidified (4.28) as shown in Figure 2.

Unlike pH, high values of titratable acidity were recorded in batches incubated at 22 °C. The production of acids in experimental and control samples was relevant in the presence of a salt concentration less than or equal to 4% w/v (Figure 3), but this production was significantly higher ($p<0.05$) in uninoculated brines. Results of total phenol content were determined and expressed as µg/mg of GAE. The values recorded were between 17.64 and 41.2 µg/mg. The highest concentration was obtained at 22 °C in experimental batches brined with 4% (w/v) of NaCl. However, the lowest value of total phenol content was obtained at the same incubation temperature in the control samples brined with 12% (w/v) of salt (Table 1).

By comparing inoculated and uninoculated batches, it can be seen that values obtained in the assays inoculated with *L. plantarum* 11 were significantly higher ($p<0.05$) than those of the control, since three experimental batches (T4% at 22 °C, T0%, and T12% at 30 °C), showed a total phenol concentration greater than 30 µg/mg, compared to a single control batch (C12% at 30 °C) as illustrated in Figure 4. As clearly observed, total phenols content recorded in the raw material (untreated olives) was greater than 46 µg/mg, which was significantly higher ($p<0.05$) than the content after fermentation.

Microbiological changes

Microbial counts expressed in $\log_{10}$ CFU/ml of both treatment and control batches, added with different salt concentrations (0, 4, 8, and 12%) and incubated at 22 and 30 °C, are recorded in Tables 2 and 3. Highly significant

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differences were observed between inoculated and uninoculated assays (p<0.05). A high level of mesophilic population was detected in the control samples. At 22 °C and in the presence of a concentration less than or equal to 8% (w/v) of salt, a significant decrease was observed. Identical behavior was recorded at 30 °C for salt-free brine and brine added with 4% (w/v) of NaCl (Table 3).

Data from statistical analysis revealed that olives inoculation with *L. plantarum* 11 as starter culture, significantly affected the evolution of coliform population during fermentation (Table 2). At 22 °C, total and fecal coliforms load persisted throughout fermentation process excepted for T8%, the growth was completely inhibited after 24 days. The highest values were obtained in salt-free control assays, with 3.6 and 3.2 log CFU/ml respectively for total and faecal coliforms (Table 2).

At 30 °C, an almost constant evolution of yeasts and molds was detected in free-salt brine of experimental and control batches. However, brines with 0% and 4% (w/v) of NaCl, showed a slight increase. In fact, the concentrations of yeasts and molds have been generally increased in the presence of 8 and 12% (w/v) of salt. Incubation at 22 °C favored increase in yeast and mold concentrations in all tested assays, excepted for T8% and C8% which showed a reduction in the load of these microorganisms as a function of fermentation time. The lowest value (3.45 log CFU/ml) was recorded after 30 days of fermentation in T8%.

In case of LAB, the population was present in experimental and control samples throughout the fermentation. In uninoculated samples, the highest values were detected after 19 days of fermentation at 30 °C, except for C0%. However, in experimental samples, the highest values (> 6 log CFU/ml), was obtained after at least 10 days of fermentation. At the end of the process, T4% and T8% batches showed the highest concentrations.

At 22 °C, LAB multiplication reached its maximum at the end of fermentation process for all tested samples. Values ranging from 6.1 to 6.6 log CFU/ml were recorded in assays inoculated with starter culture, compared to 5.1 and 5.6 log CFU/ml in the case of uninoculated samples. Statistical analysis of lactic population showed no significant difference between experimental and control tests (p>0.05).

With regard to *Staphylococcus* and *Listeria*, highly significant differences (p<0.05) were observed between treatment and control batches. For *Staphylococcus*, no growth was detected in T0% and T4% assays incubated at 30 °C. On the other hand, 4.38 and 4.53 log CFU/ml were obtained in T8% and T12%. These values disappeared later after 10 (T8%) and 24 (T12%) days of fermentation. Regarding the controls, only C12% was devoid of this pathogen at the end of the process. At 22 °C, there was no remarkable difference between treatment and control batches, *Staphylococcus* was present throughout the fermentation period.

Listeria did not detect regularly during analysis periods. On the day 4 of fermentation at 30 °C, no colony was counted in the samples of T8% and T12%. Values were then positive (4.84 and 3.4 log CFU/ml, respectively for T8% and T12%) and disappeared after 30 days. For C12%, *Listeria* was detected only after 10 days of fermentation (p<0.013). Similar results have been obtained during fermentation at 22 °C, *Listeria* was not detected in T8% and T12% after 30 days of fermentation. However, the other samples showed different concentrations.

Table 1: Total phenol content expressed in inoculated and uninoculated brines

Incubation temperature (°C)	Extract	Phenol content (µg of GAE/mg of SDW)
-	Raw material	46.12±0.14 ^{i}
22±2°C	T0%	25.08±0.21 ^{d}
	T4%	41.20±0.30 ^{h}
	T8%	20.09±0.01 ^{e}
	T12%	20.44±0.05 ^{f}
	C0%	18.68±0.11 ^{c}
	C4%	25.32±0.02 ^{g}
	C8%	18.00±0.70 ^{b}
	C12%	17.64±0.10 ^{a}
30±2°C	T0%	32.92±0.13 ^{e}
	T4%	25.32±0.09 ^{c}
	T8%	29.36±0.08 ^{d}
	T12%	32.12±0.01 ^{e}
	C0%	18.6±0.14 ^{a}
	C4%	26.00±0.71 ^{c}
	C8%	23.68±0.39 ^{b}
	C12%	23.12±0.01 ^{e}

a, b, c, d, e, f, g, h and i, the means in the same column with a different exponent are significantly different (p<0.05); GAE: gallic acid equivalent; SDW: sample in dry weight

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Table 2: Microbial counts (log CFU/ml) of inoculated and uninoculated assays during fermentation at 22 °C

	Days of fermentation at 22 °C				
	4	10	19	24	30
Mesophilic bacteria					
T0%	4.75±0.24^{a}	4.64±0.06^{bc}	4.62±0.07^{d}	4.59±0.03^{c}	4.29±0.02^{f}
T4%	5.29±0.32^{c}	4.54±0.15^{b}	4.50±0.03^{c}	4.34±0.03^{bc}	4.17±0.10^{e}
T8%	5.67±0.07^{d}	5.21±0.04^{d}	3.77±0.22^{a}	3.70±0.18^{a}	3.12±0.22^{a}
T12%	5.39±0.42^{c}	4.29±0.05^{a}	4.24±0.28^{b}	3.90±0.07^{ab}	3.71±0.25^{b}
C0%	5.00±0.28^{b}	4.87±0.12^{c}	4.77±0.05^{e}	4.70±0.10^{c}	4.61±0.03^{g}
C4%	4.90±0.02^{b}	4.77±0.23^{ce}	4.55±0.72^{cd}	4.50±0.20^{bc}	3.87±0.07^{c}
C8%	5.99±0.19^{e}	5.30±0.05^{d}	4.64±0.14^{d}	4.50±0.14^{bc}	4.00±0.12^{d}
C12%	5.77±0.02^{d}	5.4±0.14^{e}	4.61±0.04^{d}	4.59±0.03^{c}	4.54±0.40^{g}
Total coliforms					
T0%	3.24±0.15^{c}	3.22±0.63^{c}	3.22±0.84^{c}	3.20±0.19^{d}	3±0.09^{c}
T4%	3.66±0.44^{d}	3.60±0.29^{d}	3.55±0.44^{d}	3.40±0.29^{d}	3.22±0.11^{c}
T8%	2.40±0.22^{a}	2.05±0.25^{a}	2.30±0.39^{a}	0^{a}	0^{a}
T12%	3.00±0.19^{b}	3.12±0.52^{bc}	3.01±0.34^{b}	2.70±0.33^{c}	2.49±0.23^{bc}
C0%	3.88±0.21^{e}	3.60±0.32^{d}	3.69±0.79^{d}	3.66±0.51^{e}	3.60±0.06^{c}
C4%	3.90±0.10^{e}	3.01±0.33^{b}	3.60±0.56^{d}	3.6±0.28^{e}	3.00±0.14^{c}
C8%	2.49±0.11^{a}	2.09±0.08^{a}	2.43±0.11^{a}	2.00±0.31^{b}	1.94±0.10^{b}
C12%	3.51±0.34^{d}	3.30±0.22^{c}	3.23±0.18^{c}	3.31±0.36^{d}	3.20±0.15^{c}
Fecal coliforms					
T0%	3.11±0.21^{c}	3.00±0.04^{c}	2.89±0.11^{b}	2.76±0.79^{d}	2.60±0.13^{d}
T4%	3.08±0.05^{c}	3.02±0.55^{c}	2.80±0.24^{b}	2.51±0.29^{c}	2.30±0.29^{c}
T8%	2.39±0.05^{b}	2.36±0.02^{b}	1.47±0.02^{a}	0^{a}	0^{a}
T12%	3.15±0.04^{c}	2.90±0.24^{c}	2.70±0.04^{b}	2.56±0.16^{d}	2.24±0.14^{c}
C0%	3.60±0.11^{e}	3.46±0.66^{d}	3.30±0.27^{c}	3.26±0.70^{e}	3.20±0.31^{e}
C4%	3.41±0.11^{d}	3.3±0.06^{d}	3.20±0.15^{c}	3.11±0.30^{e}	3.00±0.07^{e}
C8%	2.00±0.25^{a}	1.35±0.08^{a}	1.29±0.31^{a}	1.20±0.36^{b}	1.05±0.33^{b}
C12%	3.41±0.17^{d}	3.30±0.23^{d}	2.82±0.21^{b}	3.10±0.19^{e}	2.70±0.27^{d}
Yeasts and Molds					
T0%	3.58±0.33^{d}	4.01±0.42^{d}	5.02±0.30^{d}	5.39±0.11^{d}	5.47±0.12^{d}
T4%	3.08±0.61^{a}	3.82±0.38^{c}	5.00±0.19^{d}	5.11±0.40^{c}	5.26±0.19^{c}
T8%	4.30±0.13^{f}	4.27±0.35^{e}	3.77±0.29^{b}	3.69±0.16^{a}	3.45±0.20^{a}
T12%	3.19±0.39^{b}	3.40±0.40^{a}	3.63±0.29^{a}	5.12±0.18^{c}	5.25±0.20^{c}
C0%	3.92±0.36^{e}	4.03±1.15^{d}	5.79±0.11^{g}	5.73±0.25^{f}	5.87±0.09^{e}
C4%	3.41±0.94^{c}	3.57±1.13^{b}	5.68±0.36^{f}	5.84±0.32^{g}	5.96±0.19^{e}
C8%	4.53±0.35^{g}	4.59±0.63^{f}	4.39±0.33^{c}	4.18±0.32^{b}	4.21±0.23^{b}
C12%	3.92±0.18^{e}	4.30±0.48^{e}	5.18±0.50^{e}	5.44±0.22^{e}	5.50±0.39^{d}
Lactic acid bacteria					
T0%	5.20±0.06^{cd}	5.40±0.26^{bc}	5.90±0.1^{d}	5.95±0.26^{c}	6.10±0.60^{c}
T4%	5.10±0.43^{bc}	5.50±0.18^{c}	6.00±0.25^{d}	6.12±0.20^{d}	6.24±0.62^{c}
T8%	4.08±0.58^{a}	5.21±0.05^{b}	5.57±1.14^{c}	6.07±0.31c^{d}	6.31±0.35^{c}
T12%	4.91±0.19^{b}	5.45±0.10^{c}	6.52±0.31^{e}	6.55±0.10^{e}	6.60±0.32^{d}
C0%	5.00±0.15^{c}	5.00±0.25^{a}	5.10±0.10^{a}	5.30±0.05^{ab}	5.30±0.37^{a}
C4%	4.90±0.15^{b}	5.00±0.50^{a}	5.00±0.23^{a}	5.20±0.94^{a}	5.10±0.64^{a}
C8%	5.17±0.12^{cd}	5.21±0.22^{b}	5.31±0.40^{b}	5.46±0.39^{b}	5.60±0.07^{b}
C12%	5.31±0.23^{d}	5.32±0.13^{bc}	5.34±0.07^{b}	5.35±0.16^{b}	5.38±0.05^{ab}
Staphylococcus					
T0%	3.24±0.02^{c}	3.24±0.12^{b}	3.22±0.19^{e}	3.20±0.16^{c}	3.00±0.08^{c}
T4%	3.27±0.12^{d}	3.18±0.21^{b}	3.15±0.10^{d}	3.11±0.08^{c}	3.10±0.05^{c}
T8%	2.84±0.20^{a}	3.91±0.17^{c}	2.00±0.13^{a}	2.00±0.10^{a}	2.10±0.08^{a}
T12%	2.96±0.25^{b}	2.90±0.04^{a}	2.85±0.37^{b}	2.80±0.18^{b}	2.77±0.09^{b}
C0%	3.87±0.31^{e}	3.84±0.24^{c}	3.75±0.09^{f}	3.81±0.11^{d}	3.78±0.12^{e}
C4%	3.93±0.04^{f}	3.90±0.06^{c}	3.92±0.04^{g}	3.87±0.03^{d}	3.89±0.06^{e}
C8%	4.53±0.10^{g}	4.00±0.04^{c}	3.00±0.03^{c}	2.10±0.50^{a}	3.50±0.07^{d}
C12%	3.92±0.06^{f}	3.88±0.13^{c}	4.00±0.07^{h}	3.84±0.16^{d}	3.80±0.05^{e}
Listeria					
T0%	4.74±0.20^{c}	6.88±0.02^{g}	6.84±0.06^{g}	5.95±0.08^{g}	4.84±0.50^{e}
T4%	6.76±0.73^{f}	6.69±0.18^{f}	6.54±0.51^{f}	5.92±0.17^{g}	3.50±0.10^{g}
T8%	4.30±0.15^{a}	3.45±0.02^{b}	0^{a}	1.40±0.23^{b}	0^{a}
T12%	4.30±0.32^{a}	1.01±0.15^{a}	4.84±0.05^{d}	0^{a}	0^{a}
C0%	7.67±0.06^{g}	7.00±0.31^{h}	5.93±0.12^{e}	4.84±0.11^{f}	5.30±0.50^{f}
C4%	4.47±0.21^{b}	5.75±0.88^{e}	0^{a}	4.59±0.04^{e}	2.80±0.03^{b}
C8%	5.98±0.01^{e}	5.53±0.13^{d}	4.39±0.11^{c}	3.30±0.54^{d}	1.17±0.05^{d}
C12%	5.77±0.10^{d}	4.00±0.65^{c}	3.58±0.19^{b}	2.29±0.08^{c}	1.55±0.01^{c}

a, b, c, d, e, f, g and h, the means in the same column with a different exponent are significantly different (p<0.05).

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Table 3: Microbial counts (log CFU/ml) of inoculated and uninoculated assays during fermentation at 30 °C

	Days of fermentation at 30 °C				
	4	10	19	24	30
Mesophilic bacteria					
T0%	6.36±0.06^{g}	5.34±0.50^{f}	3.32±0.02^{a}	3.32±0.02^{a}	3.33±0.01^{a}
T4%	5.27±2.30^{e}	5.26±0.01^{d}	4.87±0.06^{e}	4.40±0.01^{d}	3.76±0.01^{b}
T8%	5.24±0.03^{d}	5.67±0.06^{g}	3.77±1.67^{b}	3.70±0.02^{b}	3.22±0.5^{a}
T12%	4.28±0.01^{b}	5.52±0.02^{e}	5.58±0.01^{f}	5.25±0.01^{f}	4.44±0.01^{d}
C0%	6.46±0.01^{h}	4.82±0.02^{b}	4.61±0.01^{d}	4.21±0.01^{c}	4.12±0.43^{c}
C4%	5.52±0.08^{f}	4.69±0.03^{a}	4.66±0.02^{d}	4.63±0.01^{e}	4.63±0.01^{e}
C8%	4.24±0.34^{a}	5.21±0.45^{c}	4.50±0.12^{c}	4.31±0.04^{d}	4.10±0.01^{c}
C12%	4.83±0.05^{c}	6.23±0.01^{h}	6.01±0.01^{g}	4.21±0.01^{c}	4.72±0.01^{e}
Total coliforms					
T0%	2.60±0.01^{e}	2.52±0.06^{e}	2.23±0.01^{e}	0^{a}	0^{a}
T4%	2.53±0.02^{d}	2.46±0.01^{cd}	2.11±0.01^{cd}	0^{a}	0^{a}
T8%	2.46±0.01^{c}	2.44±0.01^{cd}	2.05±0.40^{b}	0^{a}	0^{a}
T12%	2.24±0.01^{a}	2.16±0.05^{a}	1.98±0.03^{a}	0^{a}	0^{a}
C0%	2.58±0.01^{e}	2.46±0.01^{d}	2.13±0.01^{d}	1.40±0.06^{e}	0^{a}
C4%	2.51±0.03^{d}	2.44±0.01^{cd}	2.10±0.01^{c}	1.02±0.02^{d}	0^{a}
C8%	2.47±0.01^{c}	2.43±0.01^{c}	2.09±0.02^{c}	0.47±0.40^{b}	0^{a}
C12%	2.41±0.03^{b}	2.30±0.01^{b}	2.03±0.03^{b}	0.86±0.40^{c}	0^{a}
Fecal coliforms					
T0%	2.51±0.01^{f}	2.33±0.03^{e}	2.16±0.01^{b}	0^{a}	0^{a}
T4%	2.47±0.01^{e}	2.42±0.01^{de}	2.04±0.02^{b}	0^{a}	0^{a}
T8%	2.39±0.01^{d}	2.36±0.05^{cd}	0^{a}	0^{a}	0^{a}
T12%	2.14±0.01^{b}	2.00±0.01^{a}	0^{a}	0^{a}	0^{a}
C0%	0.43±0.01^{a}	2.43±0.01^{e}	2.12±0.01^{b}	1.00±0.01^{c}	0^{a}
C4%	2.45±0.01^{e}	2.34±0.01^{c}	2.10±0.01^{b}	0.72±0.12^{b}	0^{a}
C8%	2.41±0.01^{d}	2.35±0.05^{c}	2.09±0.02^{b}	0^{a}	0^{a}
C12%	2.30±0.01^{c}	2.25±0.02^{b}	0^{a}	0^{a}	0^{a}
Yeasts and Molds					
T0%	3.28±0.01^{b}	5.22±0.49^{f}	3.31±0.01^{a}	3.31±0.01^{a}	3.32±0,01^{c}
T4%	2.56±0.01^{a}	4.21±0.01^{b}	3.90±0.60^{c}	3.88±0.01^{c}	3.76±0.01^{d}
T8%	4.36±0.01^{e}	4.36±0.25^{c}	3.77±0.01^{b}	3.77±0.01^{b}	3.7±0.04^{d}
T12%	5.59±0.01^{h}	5.81±0.02^{g}	5.52±0.01^{f}	4.59±0.01^{e}	4.44±0.01^{e}
C0%	3.63±0.20^{c}	4.11±0.01^{a}	4.31±0.01^{d}	4.82±0.01^{g}	3.03±0.43^{b}
C4%	3.69±0.01^{d}	4.77±0.39^{e}	4.7±0.01^{e}	4.63±0.01^{f}	4.62±0.01^{f}
C8%	4.65±0.44^{f}	4.64±0.52^{d}	4.54±0.01^{e}	4.21±0.01^{d}	2.92±0.02^{a}
C12%	5.23±0.06^{g}	6.26±0.01^{h}	6.01±0.01^{g}	5.77±0.01^{h}	4.72±0.01^{f}
Lactic acid bacteria					
T0%	5.01±0.16^{c}	6.40±0.02^{a}	6.42±0.01^{f}	5.52±0.01^{c}	5.42±0.08^{b}
T4%	5.58±0.02^{f}	5.74±0.02^{a}	5.94±0.08^{d}	6.00±0.11^{d}	6.23±0.02^{e}
T8%	4.71±0.23^{b}	6.31±0.02^{a}	6.07±0.08^{e}	6.00±0.01^{d}	6.00±0.02^{d}
T12%	3.66±0.02^{a}	6.30±0.01^{a}	6.06±0.01^{e}	5.40±0.07^{bc}	4.11±0.01^{c}
C0%	5.90±0.01^{g}	5.61±0.01^{a}	5.61±0.01^{a}	5.33±0.01^{b}	5.33±0.01^{b}
C4%	5.52±0.10^{f}	5.64±0.03^{a}	5.70±0.06^{b}	5.37±0.21^{b}	5.30±0.03^{b}
C8%	5.20±0.17^{d}	5.48±0.37^{a}	5.84±0.01^{c}	5.31±0.05^{b}	5.15±0.11^{a}
C12%	5.37±0.07^{e}	5.83±0.01^{a}	5.91±0.01^{d}	5.12±0.01^{a}	5.10±0.01^{a}
Staphylococcus					
T0%	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
T4%	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
T8%	4.38±0.08^{d}	3.58±0.11^{c}	0^{a}	0^{a}	0^{a}
T12%	4.53±0.06^{e}	4.47±0.01^{e}	3.00±0.06^{b}	0.5±0.01^{b}	0^{a}
C0%	4.00±0.01^{b}	4.13±0.13^{d}	3.00±0.01^{b}	2.00±0.13^{c}	1.00±0.02^{c}
C4%	4.15±0.15^{c}	3.00±0.01^{b}	3.00±0.15^{b}	2.00±0.08^{c}	2.00±0.01^{d}
C8%	4.53±0.06^{e}	4.00±0.15^{d}	3.00±0.06^{b}	2.00±0.50^{c}	0.50±0.01^{b}
C12%	4.78±0.01^{f}	4.69±0.01^{f}	0^{a}	0^{a}	0^{a}
Listeria					
T0%	4.56±0.03^{c}	0^{a}	5.25±0.02^{d}	4.85±0.02^{d}	1±0.05^{b}
T4%	4.53±0.16^{c}	1.50±0.05^{e}	3.34±0.02^{c}	0.42±0.01^{b}	0^{a}
T8%	0^{a}	4.84±0.01^{d}	1.05±0.25^{b}	0^{a}	0^{a}
T12%	0^{a}	3.40±0.06^{b}	0^{a}	0^{a}	0^{a}
C0%	4.56±0.11^{c}	6.17±0.01^{f}	6.10±0.05^{f}	5.79±0.06^{e}	2.69±0.01^{d}
C4%	4.30±0.01^{b}	0^{a}	5.54±0.06^{e}	5.54±0.15^{e}	2.00±0.01^{c}
C8%	4.39±0.1^{b}	0^{a}	5.54±0.01^{e}	3.30±0.01^{c}	2.80±0.01^{d}
C12%	0^{a}	4.39±0.01^{c}	0^{a}	0^{a}	0^{a}

a, b, c, d, e, f, g and h, the means in the same column with a different exponent are significantly different (p<0.05).

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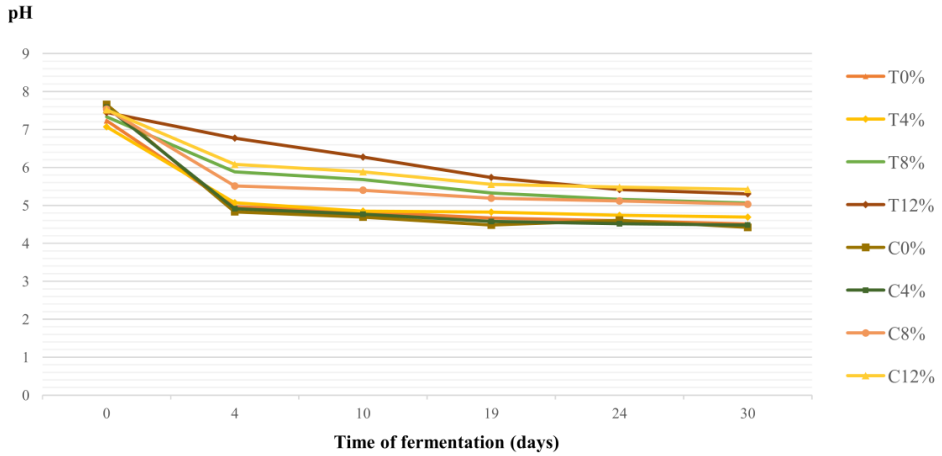


Figure 1: Changes in pH of inoculated and uninoculated brines during fermentation at 22 ℃

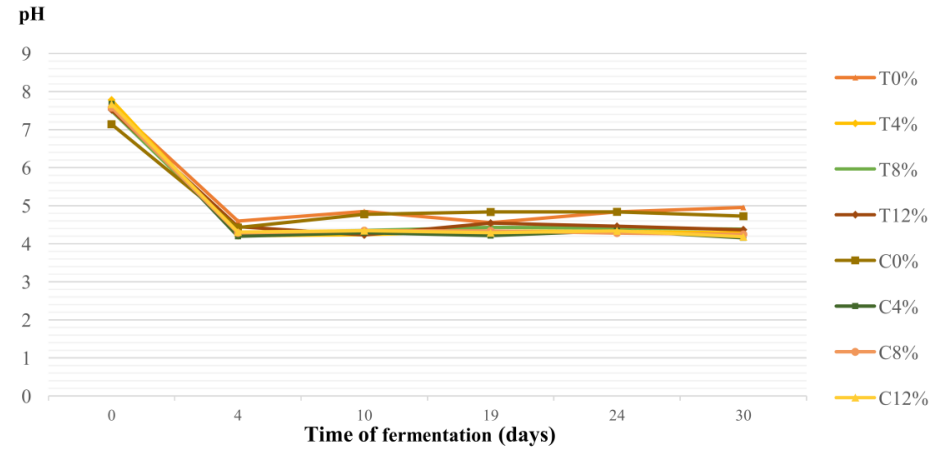


Figure 2: Changes in pH of inoculated and uninoculated brines during fermentation at 30 ℃

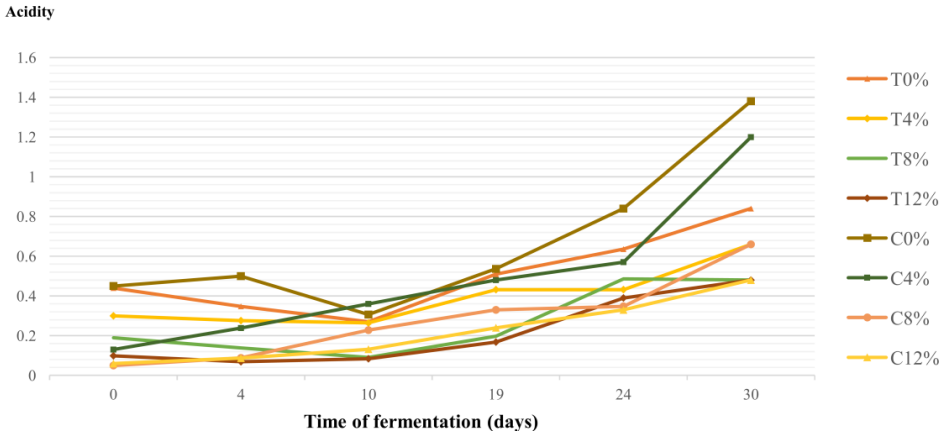


Figure 3: Changes in titratable acidity (g) of inoculated and uninoculated brines during fermentation at 22 ℃

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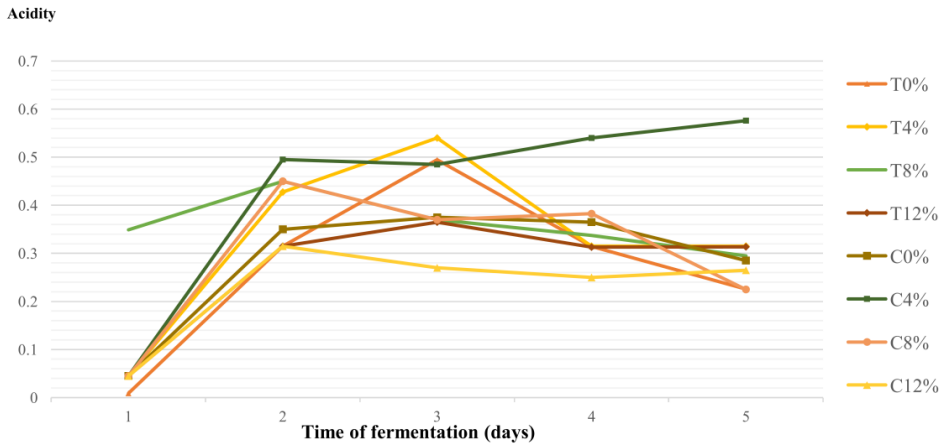


Figure 4: Changes in titratable acidity (g) of inoculated and uninoculated brines during fermentation at 30 °C

Discussion

In the recent years, considerable research has been undertaken on the elucidation of technological properties of LAB, which could transform a traditional fermented product into a functional food providing new perspectives for olive industry. In this study, the effect of *L. plantarum* 11 as starter culture in olives fermentation of the Moroccan picholine variety was tested. The subsequent selection of this strain was based on the ability to growth at different pH and salt concentrations, antibiotic resistance, capacity to produce antibacterial substances as well as acidifying and antioxidant properties (El Issaoui et al., 2021).

According to the results, fermentation at 30 °C showed a rapid decrease in pH (first 4 days), compared to 22 °C, which is an important preliminary step in olives fermentation and might be due to the formation of lactic acid, which is the main metabolic product of LAB. The pH value is a parameter which directly influences the success of fermentation, and is considered to be an important factor in terms of technology and safety of finished product. The pH obtained after 30 days of fermentation at 30 °C was below the international standard for table olives (around 4.30) (Trade standard applying to table olives, (2004), for all brines supplemented with a concentration higher than or equal to 4% (w/v) of NaCl. On the other hand, at 22 °C, the values were all higher than 4.4. Similar results have been reported by Tassou et al. (2002) who demonstrated that pH values of brines supplemented with 6 and 8% (w/v) of NaCl were lower than the pH of brines at 4%. In a recent study of Medina et al. (2020) on aerobic fermentation of black olives, the recorded pH was often higher than the recommendations. Differences in pH and acidity between inoculated and uninoculated assays have been detected. The decrease in pH was important when starter culture was not applied, and therefore acidity values were higher. This difference might be due to LAB with potential acidifying capacity spontaneously developed in control batches, compared to experimental batches which might be dominated by *L. plantarum* 11 with moderate acidifying capacity. These results were in agreement with a previous work undertaken by Blana et al. (2014) who noted that the drop in pH was significant in the processes inoculated with *L. pentosus* B281 and in the case of co-culture with *L. pentosus* B281 and *L. plantarum* B282, compared to high pH values (>6) recorded for assays inoculated only with *L. plantarum* B282.

The high acidity levels were obtained for samples salt-free or brined with 4% (w/v) of sodium chloride at 22 °C. Same observations have been reported by other studies which have shown that high acidity was obtained at low salt concentration during olives fermentation (Özay and Borcakh, 1995). In the study of Anagnostopoulos et al. (2020), the maximum values of acidity were obtained after 120 days of fermentation, for spontaneously fermented olives, inoculated with *L. plantarum* as starter culture, added with 7% of NaCl and 3.3% of citric acid. However, less significant acidity values (<0.5%) were obtained for olives fermented under the same conditions with 10% of NaCl.

Table olives are considered as rich in natural antioxidants and a source of phenolic compounds (Durante et al., 2018; García et al., 2018; Moreno-González et al., 2020). The total phenols content was considerably

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reduced in the various samples at the end of the process compared to raw material, which could be explained by alkaline treatment applied at the start of the process. Marsilio et al. (2005) compared olives treated and untreated with NaOH and found that the phenols content in untreated olives was higher compared to olives treated with lye.

Likewise, the decline in phenols content might be due to the degradation of these compounds by LAB and yeasts, or to their diffusion from fruits to the brine (Anagnostopoulos et al., 2020). Different studies (Romero et al., 2004; Sánchez-Rodríguez et al., 2018) show that phenol content depends as much on olive variety as on processing method, and also on the ripeness of olive. Similarly, experimental assays inoculated by starter culture showed high retention of these compounds, compared to the controls. A study undertaken by Romeo et al. (2018) indicated that the use of *L. plantarum* as a starter culture led to a high increase in total phenols content in three varieties of olives analyzed.

LAB population was predominant during the process in both inoculated and uninoculated assays. LAB growth kinetics were almost similar in the presence or absence of starter culture and no remarkable difference was observed in the values of Figures 3 and 4, which can be explained by the natural and spontaneous multiplication of LAB during fermentations. *L. plantarum* 11, used as a starter culture in this experiment, may be resistance to the microflora naturally presented in the different fermentation processes, as well as to the different salt concentrations applied, which can be justified by the number of LAB obtained in the inoculated (6 log CFU/ml) and uninoculated (5 log CFU/ml) processes incubated at 22 °C. However, only a dominance test using molecular assays can demonstrate this observation.

The presence of yeasts in olives can be advantageous from the point of view of flavor, by improving organoleptic characteristics of fruits (Arroyo-López et al., 2008) as well as by their probiotic activity (Porru et al., 2018). However, at high concentrations, yeasts are known to be responsible for an undesirable fermentation caused mainly by accumulation of CO_2 and acidity reduction in finished product (Anagnostopoulos et al., 2017). In this experiment, dominance of yeasts towards the end of fermentation, compared to other microbial populations, can be justified by their high salt and high acidity tolerance (Bautista-Gallego et al., 2011; Tofalo et al., 2013). At 22 °C, the load of these germs was reduced in experimental brines added with 4 and 8% (w/v) of NaCl, which can be explained by the occurrence of starter culture used in the maintenance of yeasts growth.

The elimination of coliforms indicates a good evolution of olive fermentation process towards lactic fermentation, instead of other fermentations, and therefore a natural improvement in the hygienic quality of fermented olives. The load of total and fecal coliforms was reduced in all samples. This decrease was more rapid and significant in experimental samples incubated at 30 °C. Elimination of coliforms was delayed for at least a week in samples uninoculated with starter culture. Ghabbour et al. (2016) studied the control of the spontaneous fermentation process of un-debittered Moroccan Picholine green olives using two strains of lactobacilli (*L. plantarum* S175 and *L. pentosus* S100) and indicated that elimination of coliforms in uninoculated trials was delayed by 10 days compared to the tests inoculated with a combination of *L. plantarum* and *L. pentosus*. In addition, several studies have demonstrated the intervention of starter culture in the rapid elimination of coliforms through olives processing (Anagnostopoulos et al., 2020; Chranioti et al., 2018).

Generally, the presence of *Listeria* and *Staphylococcus* in food is an indicator of poor hygiene. Even if the fermentation brine does not present favorable environment to this pathogens, several studies have reported their survival during different storage times (Bevilacqua et al., 2018; Medina et al., 2020). In this study, *Staphylococcus* and *Listeria* which was used as target microorganisms were not detected at the end of the process, in experimental trials brined with 4, 8 and 12% (w/v) of sodium chloride at 30 °C. According to Franzetti et al. (2011), the biomass and metabolic activity of LAB as well as a pH below 4.5 pose significant obstacles for *L. monocytogenes* and *Staphylococcus* growth.

Fermentation temperature must be close to 25 °C because at below the fermentation is blocked and above, other harmful fermentations expand (Rokni et al., 2015). In this study, microbiological quality of olives was more satisfactory at the optimal growth temperature of LAB (30 °C). However, the best biochemical characteristics (pH, acidity, and polyphenol content) were obtained at 22 °C. This can be explained by the fact that at 30 °C, the fermentation process is accelerated, and a prolonged fermentation period can lead to a decrease in nutritional quality. Pistarino et al. (2013) showed that total phenolic content was remarkably reduced for the three applied temperatures (22, 30, and 37 °C) after an extended fermentation time of black olives.

Conclusion

Data obtained by this experiment showed that starter culture (*L. plantarum* 11) was able to establish a controlled fermentation of black olives at 30 °C. The hygienic and nutritional quality of the olives, mainly in terms of the polyphenol content, was improved by inoculating *L. plantarum* 11 with an initial concentration of 10^6

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CFU/ml, and brining with a reduced concentration of sodium chloride (4%). These fermentation conditions guaranteed a healthier final product that met the recommended microbiological criteria. As a result, *L. plantarum* 11 could become a promising candidate for production of olives with technological potential provided these results can be applied on an industrial scale.

Author contributions

K.E., S.B., and E.O.K. designed the study; K.E. conducted the experimental work and wrote the manuscript; K.E., S.B., N.A., N.S.S., and J.A. analyzed the data. All the authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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