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# TECHNOLOGICAL PROPERTIES OF POTENTIAL PROBIOTIC *LACTOBACILLUS* STRAINS ISOLATED FROM TRADITIONAL FERMENTING GREEN OLIVE

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**ABSTRACT**

The aim of the present study was to evaluate some technological properties and potential probiotic of 14 *Lactobacillus* strains isolated from brines of natural fermenting Moroccan Picholine green olive. The brine samples, collected from industrial environments, were analyzed for their Physico-chemical and microbiological properties. The *Lactobacillus* strains were characterized for their technological and physiological properties. The results obtained showed that the olive brines have an average pH of 4.32, an acidity of 0.61% and chloride contents of 6.42%. LAB and yeasts are the most dominant microorganisms in olive brine samples. The selected *Lactobacillus* (14 strains) showed low resistance to pH 2 and high resistance to bile salts (up to 2%), with values ranges from 1.98%-4.70% and 63.12%-86.48%, respectively. All the *Lactobacillus* strains displayed high levels of acidification and can produce β-glucosidase, protease, and cellulase in large amounts. The diacetyl production is detected in five *Lactobacillus* strains.

**Keywords:** Lactic acid bacteria, *Lactobacillus*, Probiotic, Fermentation, Olives

## INTRODUCTION

Table olive is a major fermented product in Mediterranean countries. The International Olive Oil Council estimates the world production of table olive to about 2 829 500 tones during the 2016/2017 season (**IOOC, 2017**). The main industrial processes of table olives production include the Spanish style for green olives, the Greek style for black olives, and the Californian style for oxidized black olives **(Fernandez *et al.*, 1997).**
In Morocco, industrial table green olive processing is mainly based on the Spanish style, obtained with alkali-treatment with sodium hydroxide of olives in a lye solution to eliminate the oleuropein, the main polyphenol responsible for the bitterness of olive fruit **(Kailis and Harris, 2007).** After debittering, the olives were subject to successive washes to remove residual sodium hydroxide and then brined at 10-12% of NaCl, where they undergo a natural lactic fermentation **(Fernandez *et al.*, 1997, Kailis and Harris, 2007).** This spontaneous fermentation process is mainly carried out by lactic acid bacteria, the most dominant microorganisms in olive brine **(Fernandez-Diez *et al.*, 1985).**
The fermentation environment is characterized by alkaline pH (due to lye treatment of the olives), high initial salt concentration (10-12%) and polyphenol contents (i.e. oleuropein and its by-products). Lactic acid bacteria (LAB) should support these stress factors and compete with other microorganisms to succeed in the natural lactic fermentation process of the olives. The biochemical activity of LAB should overcome those of undesirable microorganisms (mainly enterobacteria, *Staphylococcus*, *Bacillus*, *Propionibacterium*, *Clostridium*, yeasts, and molds) to avoid olive spoilage and foodborne diseases **(Fernandez-Diez *et al.*, 1985).** LAB autochthonous to this environment is a good candidate in starter selection for olive fermentation.
LAB to be used as a starter must be easily adapted to the fermentation environment and have non-pathogenic, probiotic and technological characteristics. LAB are known for their capacity of producing antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins, or useful enzymes, or for their probiotic properties **(Bonatsou *et al.*, 2017)**. The technological characteristics of LAB in table olive have to be survival in brine, production of high amounts of lactic acid during fermentation, tolerance to high pH and NaCl values, the production of volatile compounds and specific enzymes, improving the sensorial properties of fermented olives **(Bautista-Gallego *et al.*, 2013, Bonatsou *et al.*, 2017).** The good evidence of microbial and/or specific enzymes to develop novel biotechnological methods in place of chemical treatment of the green table olives **(De Leonardis *et al.*, 2015).** The LAB to be used in this biotechnological process must meet technological properties, health promotion, and disease prevention. Previous studies showed that the microbiota of fermented olives exhibited specific probiotic properties **(Martins *et al.*, 2013),** thus natural fermented green olives are good matrices to develop autochthonous starters for controlled fermentation of green olives with probiotic properties.
The main objective of this work is the characterization of the technological properties of potential probiotic *Lactobacillus* strains isolated from natural fermenting green brines.

## MATERIAL AND METHODS

### Samples Collection

Seven brine samples of natural fermenting non-alkali-treated green olive collected aseptically from the industrial environment from enterprise TRIFFA, were transported at 4°C for their Physico-chemical and microbiological analyses.

### Physico-chemical Analyses

The pH of brine samples was measured, using a pH meter (VWR Symphony SB70P). The titratable acidity of brine samples was determined by titrating 10 mL of brine with sodium hydroxide (0.1N) in the presence of phenolphthalein. The results of titratable acidity were expressed in percent of lactic acid (g/100 mL). Chlorides contents in brine were determined as follows: to 10 ml of brine samples we added two drops of potassium chromate (0.1N), then this solution was titrated with (0.1N) silver nitrate solution (AgNO3) until the appearance of the persistent brick red color. The chlorides were expressed as a percentage of NaCl (g/100 mL of brine). All the Physico-chemical analyses were performed in triplicate.

**Microbiological Analyzes**

The microbiological analyses of olive brine, made in duplicate, included an enumeration of total aerobic mesophilic flora (FMAT), *enterobacteriaceae*, spore-forming Bacteria, yeasts and molds, and lactic acid bacteria (LAB). Brine samples were serially diluted in sterile saline solutions (9g/L of NaCl) and then 0.1mL of each dilution was plated on a specific medium of each microorganism. The FMAT were enumerated on the Plate Count Agar (PCA) (BIOKAR, FRANCE) after 48 h of incubation at 37°C. The spores of *Bacillus* were enumerated, in brine samples heated at 80°C/10 min, using PCA and incubated at 37°C 48 h. The *enterobacteriaceae* were enumerated on the Mac Conkey agar (BIOKAR, FRANCE) after 48 h of incubation at 37°C. Yeasts and molds were enumerated on Potato-Dextrose-Agar (PDA) (BIOKAR, FRANCE) after 48-72 h of incubation at 25°C. Lactic acid bacteria were enumerated on de Man Rogosa and Sharpe agar (MRS) (BIOKAR, FRANCE), supplemented with cycloheximide (0.01%), after 48 to 72 h of incubation at 37°C.

***Lactobacillus* Strains and Growth Conditions**

14 LAB strains used in this work were isolated from the brine samples of natural fermenting green olives, and selected for their high antifungal activity, and characterized for their probiotic and safety properties **(Abouloifa *et al.*, 2019)**. They were identified by the 16S rRNA sequencing method using the universal primers 27F and 1492R (data not shown), and they included *Lactobacillus brevis* (5 strains), *Lactobacillus pentosus* (2 strains) and *Lactobacillus plantarum* (7 strains), and the accession numbers for their 16S rRNA gene sequences are NR_044704.2, NR_029133.1, and NR_104573.1, respectively. These strains were maintained in 20% glycerol (v/v) at −80ºC, they were routinely reactivated in de Man Rogosa and Sharpe (MRS) broth (BIOKAR, FRANCE) at 37ºC for 18 h before use.

**Physiological Characterization of *Lactobacillus* Strains**

The 14 *Lactobacillus* strains were characterized physiologically by testing their growth capacity at different temperatures (15, 30, 37 and 45°C), at different pHs (4, 6, 7 and 9) and different concentrations of NaCl (2, 4, 6, 8, 10 and 12%). The growth tests (temperature, pH and NaCl) were performed in MRS broth inoculated with overnight cultures and incubated at 37°C for 24 h for pH and NaCl. The presence of biomass growth in MRS broth indicated the tolerance to the parameter studied.

**Resistance to Bile Salts**

The 14 *Lactobacillus* strains were studied for their resistance to higher concentrations of bile salts. 0.1mL of the overnight culture was inoculated in 10 mL of MRS broth containing bile salts at different concentrations (0.3, 0.5, 1 and 2 %, w/v). After incubation at 37°C for 48 h, the biomass of the strains was evaluated by measuring the absorbance (OD) at 600 nm. The MRS without bile salts was used as a control in this test. All the tests were performed in triplicate. The resistance (%) to bile salts was calculated as follows:

$$\%\ \text{Resistance} = \frac{\text{OD in MRS broth with bile salts (0.3, 0.5, 1 and 2\%)}}{\text{OD in MRS broth without bile salts}} \times 100$$

**Resistance to Acid pH**

The resistance to the acid pH was studied by inoculating 1% of overnight cultures, of the 14 *Lactobacillus* strains, in 10 mL of MRS broth adjusted with HCl (4M) to different pH values (2, 2.5 and 3). After incubation at 37°C for 48 h, the biomass of the strains was obtained by measuring the absorbance at 600 nm. The normal MRS (pH=6.5) was used as a control in this test. All the experiments were performed in triplicate. The resistance (%) to the pH was calculated as follows:

$$\%\ \text{Resistance} = \frac{\text{OD in MRS broth with pH (2, 2.5 and 3)}}{\text{OD in MRS broth normal (pH = 6.5)}} \times 100$$

**Acidification Activity**

The acidification activity of the *Lactobacillus* strains was evaluated by measuring the pH change of the medium during their culture. The culture was performed in MRS broth (50 ml), inoculated with 1% of an overnight culture of the *Lactobacillus* strains, and incubated at 37°C. The pH was measured at 0, 12, 24, 36, 48, and 72 h of incubation using a calibrated pH meter type VWR Symphony SB70P. The pH was measured in triplicate.

**Diacetyl Production**

The diacetyl production capacity of the *Lactobacillus* strains was determined according to the method of **King (1948).** Overnight cultures of *Lactobacillus* strains were inoculated (1%) in 10 mL of UHT milk and incubated at 37°C for 24 h. One milliliter of each *Lactobacillus* culture was combined with 0.5 mL of α-naphthol (1% w/v) (Sigma-Aldrich, USA) and KOH (16% w/v) (Sigma-Aldrich, USA) and then incubated at 37°C for 10 min. Diacetyl production is indicated by the formation of a red ring at the top of the culture in the tube. All the assays were performed in triplicate.

**Enzymatic Characterization of *Lactobacillus* Strains**

***β-glucosidase Activity***

The β-glucosidase activity was determined according to **Ciafardini *et al.* (1994).** Briefly, a volume of 0.2 ml of 0.3% (w/v, dissolved in N, N-dimethylformamide) of 5-bromo-3-indolyl-β-D-glucoside (X-Gluc) (Sigma-Aldrich, USA) was spread onto MRS agar, and then drop-inoculated by dropping 5 µl of overnight cultures of *Lactobacillus* strains. The assays were incubated at 37°C for 7 days. The positive strains producing β-glucosidase were identified as blue-colored colonies.

***Proteolytic Activity***

The proteolytic activity of the *Lactobacillus* strains was evaluated according to **Phyu *et al.*, (2015)**, on skim milk agar containing: Casein (5g/L), yeast extract (2.5g/L), dextrose (1g/L), skim milk powder (28g/L) and agar (20/L). Overnight cultures of the *Lactobacillus* strains were spot-inoculated on the medium and incubated for 48 h at 37°C. Proteolytic strains displayed clear zones around colonies**.**

***Lipolytic Activity***

The lipolytic activity of the *Lactobacillus* strains was determined on a culture medium composed of: peptone (10g/L), CaCl 2, 2H2O (0.1g/L), Tween 80 (1mL/L) and agar (20g/L), according to the method described by **Tanasupawat *et al.* (2015).** Overnight cultures of the *Lactobacillus* strains were spot-inoculated on the medium and incubated at 37°C for 48 h. Lipolytic activity causes opaque zones around the colonies.

***Amylolytic Activity***

The amylolytic activity of the *Lactobacillus* strains was evaluated by measuring their ability to hydrolyze starch in the agar medium. Overnight cultures of the *Lactobacillus* strains were spot-inoculated onto MRS agar supplemented with 1% (w/v) of potato starch and incubated at 37°C for 48 h. After incubation, the cultures were flooded with iodine solution (4%, v/v). A clear zone around the colonies indicates the production of the amylase enzyme.

***Cellulolytic Activity***

The cellulolytic activity of the *Lactobacillus* strains was evaluated according to the method of **Yang *et al.*(2014).** Overnight cultures of the *Lactobacillus* strains were inoculated on MRS agar supplemented with 1% (w/v) Carboxy Methyl Cellulose (CMC). The MRS agar was incubated at 37°C for 48 h. After incubation, the cultures (MRS-CMC plates) were flooded with 1% (w/v) Congo red for 15-20 min, then bleached with 1M NaCl for 15-20 min and allowed to stand for 15 minutes at room temperature. Clear zones obtained around colonies, indicated cellulolytic activity.

**Statistical Analysis**

The Student-Newman-Keuls (S-N-K) comparison test was used to identify the group of means by the one-way analysis of variance (ANOVA), followed by means comparisons. A p-value <0.05 was considered statistically significant. The results are represented by means of values ± standard deviation.

**RESULTS AND DISCUSSION**

**Physico-chemical and Microbiological Analyses of the Olive Brine**

Table 1 shows the results of the physicà-chemical analyses of the natural fermenting olive brine samples. The results showed value ranges of 4.3- 4.4 for pH, 0.5-0.67% (w/v of Lactic acid) for free acidity, and 6.14-6.72% (w/v) for chloride contents. These parameters values fall within the ranges tolerable by lactic acid bacteria (LAB).

The microbiological properties of the seven samples were evaluated, and the results are reported in Table 2. The microbial load ranged between 8.07 and 8.52 log CFU/mL for FMAT, 6.48 and 8.06 log CFU/mL for yeasts and molds, and 8.04 and 8.96 log CFU/mL for LAB. On the other hand, enterobacteria, staphylococci as well as aerobic spores forming bacteria (*Bacillus*) were not detected in all the brine samples, indicating their good hygienic quality. The microbiological analyses allowed us to isolate 104 isolates of LAB from the seven olive brine samples analyzed.

**Table 1** Physico-chemical properties of the fermenting green olive brine samples

| Brine Samples | pH | Titratable acidity % | Chloride % |
|---|---|---|---|
| E1 | 4.4[a] ±0.00 | 0.55[c]±0.01 | 6.16[c]±0.02 |
| E2 | 4.3[b]±0.01 | 0.67[a]±0.01 | 6.16[c]±0.02 |
| E3 | 4.4[a]±0.00 | 0.64[b]±0.01 | 6.45[b]±0.06 |
| E4 | 4.3[b]±0.01 | 0.63[b]±0.00 | 6.45[b]±0.06 |
| E5 | 4.3[b]±0.00 | 0.63[b]±0.00 | 6.45[b]±0.06 |
| E6 | 4.4[a]±0.01 | 0.64[b]±0.01 | 6.69[a]±0.04 |
| E7 | 4.3[b]±0.01 | 0.64[b]±0.01 | 6.69[a]±0.04 |

Values are mean ± standard error of triplicates.
[a-c]Means in same column of each parameter with different lower case letters differed significantly ($p < 0.05$).

The LAB dominated in all samples of olive brines. LAB is the main microorganisms responsible for the brine acidification by the production of lactic acid from fermentable substrates **(Bonatsou *et al.*, 2017)**. The low pH values obtained (pH<4.5), may provide microbiological stability of fermented olives. However, these conditions may encourage the growth of yeasts and molds, leading to the development of olive spoilage, mainly gas-pockets **(Asehraou *et al.*, 2000)**. The chloride contents can strongly inhibit some microorganisms involved in olive spoilage, especially *enterobacteria*, without affecting LAB **(Rokni *et al.*, 2015)**. These results are similar to those obtained on Spanish-style green table olives **(Lucena-Padros *et al.*, 2014)**.
The absence of these microorganisms is probably due to a low pH, high acidity and chloride values obtained in brine samples. It may be due also to the antimicrobial compounds commonly produced by LAB, such as organic acids, bacteriocins, peptides, H2O2, etc (**Henning et al. 2015)**. These antimicrobial compounds are known for their inhibitory effect against pathogens, such as *enterobacteriaceae* **(Hurtado *et al.*, 2008)**. In addition to the improvement of the hygienic quality of the olives, LAB is highly desired in olive brines to improve the organoleptic attributes **(Asehraou *et al.*, 1993)**, and to reduce the bloater spoilage incidence in fermented olives **(Ghabbour *et al.*, 2016)**. LAB and yeasts are the most dominant microflora in brine samples. These results confirm those obtained by other authors **(Fernandez *et al.*, 1997, Pereira *et al.*, 2008)**. One of the roles of yeasts in olive fermentation is to synthesize nutrients essential to improve the growth of LAB **(Arroyo-López *et al.*, 2008)**.

**Table 2** Microbiological properties of the fermenting green olive brine samples

| Brine Samples | Log CFU/mL | | | |
|---|---|---|---|---|
| | FMAT | *Enterob.* | Staph. | *Bacillus* |
| E1 | 8.36[bc]±0.09 | ND | ND | ND |
| E2 | 8.52[a]±0.06 | ND | ND | ND |
| E3 | 8.41[b]±0.03 | ND | ND | ND |
| E4 | 8.32[c]±0.02 | ND | ND | ND |
| E5 | 8.50[a]±0.04 | ND | ND | ND |
| E6 | 8.07[e]±0.04 | ND | ND | ND |
| E7 | 8.21[d]±0.05 | ND | ND | ND |

**Legends**: FMAT: total aerobic mesophilic flora, Enterob.: *Enterobacteriaceae*, Staph: *Staphylococcus*, Y&M : Yeasts and Molds, LAB: lactic acid bacteria, ND: not detected
Values are mean ± standard error of triplicates.
[a-e]Means in same column of each parameter with different lower case letters differed significantly ($p < 0.05$)

**Physiological Characterization of *Lactobacillus* Strains**

All the *Lactobacillus* strains showed a growth capacity at 30°C, 37°C and 45°C, which may be due to their natural selection during olive fermentation in the temperate region, with annual temperatures between 15°C and 44°C (east of Morocco). All the strains showed a wide pH range of growth, which may be due to their isolation from green olives treated with alkaline solutions. All the strains showed their tolerance to up to 12% of NaCl, which may be due to their selection during the first days of brining, characterized by high NaCl contents (10-12%, w/v), widely used in olive industry to avoid spoilage.

**Resistance of *Lactobacillus* Strains to Acid pH and Bile Salts**

The resistance of 14 *Lactobacillus* strains to acid conditions close to those of the stomach was carried out by culture in MRS adjusted to different initial pHs and higher bile salts concentrations for 48 h at 37°C. The results obtained are shown in Table 3. All strains of *Lactobacillus* could grow at pH acid and bile salt (acid pH and in presence of high bile salts contents).
The resistance values of the *Lactobacillus* strain ranges of 2.97%-7.61%, 2.63%-6.74% and 1.98%-4.70% to pH 3, pH 2.5 and pH 2, respectively. The resistance of all *Lactobacillus* strains to bile salts concentrations ranged between 79.36%-97.26%, 78.83%-95.28%, 74.50%-92.44% and 63.12%-86.48% for 0.3, 5, 1 and 2% of bile salts, respectively. All the *Lactobacillus* strains showed low resistance to pH 2. With respect to the bile salts, all strains showed high resistance up to 2% of bile salts. The resistance values, to different pH and bile salt concentrations, obtained for all the *Lactobacillus* strains, are mostly not significantly ($p<0.05$) different between the three species (*L. brevis, L. pentosus and L. plantarum*).
The *L. brevis* S82, *L. pentosus* S42, and *L. plantarum* S72 demonstrated a good resistance to acid pH; while *L. brevis* S63, *L. pentosus* S75, and *L. plantarum* S71 showed high resistance to bile salt. This finding indicates that the resistance to pH and bile salts concentrations seems to be strain-dependent.
The resistance to acid pH and bile salts are now considered the basic criteria of screening for potentially probiotic strains **(Dunnel *et al.*, 1999)**. Resistance to 0.3% of bile salts is considered as the main criterion in the selection of probiotics **(Gilliland *et al.*, 1984)**. Probiotics must be able to survive in the human gastrointestinal tract and exercise their biochemical and physiological activities. LAB strains, isolated from naturally fermenting Portuguese and Italian-green olive varieties, were found to tolerate bile salts (0.3%) and low pH **(Bevilacqua *et al.*, 2010, Peres *et al.*, 2014)**. It should be emphasized that the *Lactobacillus* strains tolerated higher concentrations (2 %) of bile salts, known for its antimicrobial activity mainly against Gram-positive bacteria **(Hofmann and Eckmann, 2006)**. The high tolerance of these strains to bile salts may be due to their production of bio-protecting and/or hydrolyzing agents against this detergent **(Horackova *et al.*, 2018)**. The acidity (pH 2.5 to 3.5) of gastric secretions and bile salts destroyed the majority of bacteria that enter the gastrointestinal tract **(Holzapfel *et al.*, 1998)**. All of these *Lactobacillus* strains are resistant to high bile concentration and low pH, indicating their ability to survive in the gastrointestinal tract. These findings allow us to select these *Lactobacillus* strains as potential candidates in deep studies for probiotic applications.

**Acidification Activity**

The results of the acidification activity of the *Lactobacillus* strains obtained after 12, 24, 36, 48 and 72 h of culture in MRS broth at 37°C are reported in table 4. After 12h of incubation, all of the *Lactobacillus* strains showed a low-reduction of pH from 6.35 to 5.2-5.8. However, a drastic reduction of pH to 3.8 was obtained after 36 h of incubation, remaining stable for 72h, with no significant differences ($p<0.05$) between *Lactobacillus* species and strains. While the pH of the non-inoculated MRS (control) was stable at 6.35 after 72h of incubation.
All of the *Lactobacillus* strains showed a good acidification capacity, which may prevent the growth of pathogenic and spoilage microorganisms. The results of acidification activity are similar to those obtained on LAB strains isolated from Spanish fermenting green olives **(Abriouel *et al.*, 2012)**.

**Diacetyl Production**

The results obtained in this work indicate that 5 *Lactobacillus* strains (*L. brevis* S14 and S18, *L. pentosus* S75, and *L. plantarum* S49 and S61), out of the 14 *Lactobacillus* strains, showed their capacity to produce diacetyl. The Diacetyl (2,3-butanedione) is a volatile compound generated from citrate metabolism by certain LAB strains and contributes directly to the flavor formation **(Rincon-Delgadillo *et al.*, 2012)**. Diacetyl is known for its antimicrobial action towards pathogens and spoilage microorganisms **(Jay, 1982)**. Diacetyl is produced by the main genera of LAB (*Streptococcus, Leuconostoc, Lactobacillus,* and *Pediococcus*) **(Jay, 1982)**. Some *Lactobacillus* strains, of *L. plantarum, L. pentosus, L. brevis* and *L. casei* species, were confirmed for their diacetyl production capacity **(Chuang and Collins, 1968, Hickey *et al.*, 1983, Phalakornkule and Tanasupawat, 2007, Pan *et al.*, 2014)**. Diacetyl is highly involved in flavor formation and antimicrobial activity of LAB **(Jay, 1982)**.

**Enzymatic Characterization of *Lactobacillus* Strains**

The enzymatic characterization of *Lactobacillus* strains was evaluated by the ability to produce different enzymes. The results are reported in table 5. All of *Lactobacillus* strains showed a blue color after 7 days of incubation at 37°C, indicating their capacity for the production of β-glucosidase. All the strains showed high levels of producing β-glucosidase activity. These results may be due to the natural selection of these *Lactobacillus* strains in olive brine rich of oleuropein, the major phenolic glucoside of olives. LAB strains producing β-glucosidase have been isolated from natural fermenting green olives brines of Moroccan, Portuguese, Spanish and Italian olive varieties **(Ghabbour *et al.*, 2011, Abriouel *et al.*, 2012, Peres *et al.*, 2014, Tofalo *et al.*, 2014)**.

**Table 3** Resistance (%) of *Lactobacillus* strains to acid pH and bile salts obtained after 48 h of incubation at 37°C

| LAB strains | pH | | | Bile salts (%. w/v) | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 2.5 | 3 | 0.3 | 0.5 | 1 | 2 |
| *L.brevis* S14 | $3.40^{d}$±0.20 | $3.60^{h}$±0.22 | $4.60^{g}$±0.28 | $92.74^{bcd}$±3.55 | $91.36^{abc}$±3.50 | $86.63^{bc}$±3.31 | $76.79^{e}$±1.47 |
| *L.brevis* S18 | $3.55^{d}$±0.16 | $5.44^{d}$±0.25 | $5.56^{f}$±0.26 | $85.74^{e}$±3.94 | $80.42^{ef}$±3.69 | $74.50^{h}$±3.42 | $68.59^{fgh}$±1.58 |
| *L. brevis* S27 | $2.65^{g}$±0.04 | $3.02^{i}$±0.04 | $3.10^{j}$±0.04 | $93.74^{abcd}$±1.30 | $81.64^{ef}$±1.13 | $79.37^{fg}$±1.10 | $63.12^{i}$±0.44 |
| *L. brevis* S63 | $2.92^{f}$±0.03 | $6.19^{b}$±0.07 | $6.78^{d}$±0.08 | $95.71^{abc}$±1.07 | $89.34^{c}$±1.00 | $87.65^{bc}$±0.98 | $86.48^{a}$±0.48 |
| *L. brevis* S82 | $3.69^{c}$±0.02 | $5.78^{c}$±0.04 | $7.09^{c}$±0.05 | $96.25^{ab}$±0.65 | $90.88^{bc}$±0.61 | $83.43^{de}$±0.56 | $82.24^{bc}$±0.28 |
| *L.pentosus* S42 | $2.42^{h}$±0.22 | $3.22^{i}$±0.29 | $3.62^{hi}$±0.32 | $96.77^{ab}$±2.05 | $83.46^{de}$±2.84 | $77.32^{gh}$±1.63 | $64.40^{1ij}$±2.89 |
| *L. pentosus* S75 | $2.39^{h}$±0.03 | $2.98^{i}$±0.06 | $3.46^{i}$±0.07 | $96.65^{ab}$±2.00 | $94.32^{ab}$±1.95 | $90.98^{a}$±1.88 | $80.23^{cde}$±0.83 |
| *L. plantarum* S23 | $2.39^{h}$±0.04 | $2.63^{i}$±0.04 | $3.59^{i}$±0.06 | $92.50^{bcd}$±1.45 | $92.14^{abc}$±1.44 | $82.57^{de}$±2.92 | $76.59^{e}$±0.60 |
| *L. plantarum* S46 | $1.98^{i}$±0.07 | $2.72^{i}$±0.10 | $2.97^{j}$±0.11 | $95.65^{abc}$±3.47 | $94.59^{ab}$±3.43 | $88.41^{b}$±3.21 | $82.23^{bc}$±1.49 |
| *L. plantarum* S49 | $4.08^{b}$±0.07 | $6.36^{b}$±0.11 | $7.23^{bc}$±0.13 | $90.13^{d}$±1.60 | $84.99^{d}$±1.51 | $80.50^{ef}$±1.43 | $77.94^{de}$±0.69 |
| *L. plantarum* S61 | $3.49^{d}$±0.04 | $6.29^{b}$±0.07 | $7.16^{c}$±0.08 | $86.15^{e}$±0.92 | $79.17^{fg}$±0.85 | $68.69^{i}$±0.74 | $65.78^{hij}$±0.35 |
| *L. plantarum* S62 | $3.44^{d}$±0.10 | $5.93^{c}$±0.18 | $6.10^{e}$±0.18 | $79.36^{f}$±2.39 | $78.83^{fg}$±2.38 | $75.86^{h}$±2.29 | $69.94^{fg}$±1.05 |
| *L. plantarum* S71 | $3.14^{e}$±0.33 | $4.78^{e}$±0.51 | $6.02^{e}$±0.64 | $94.52^{abc}$±0.89 | $93.51^{ab}$±1.44 | $91.68^{a}$±1.24 | $85.32^{ab}$±1.27 |
| *L. plantarum* S72 | $4.70^{a}$±0.14 | $6.74^{a}$±0.21 | $7.61^{a}$±0.23 | $97.26^{a}$±2.98 | $95.28^{a}$±2.92 | $92.44^{a}$±2.83 | $80.80^{cd}$±1.24 |

*Values are mean ± standard error of triplicates.*
*[a-l]Means in same column of each parameter with different lower case letters differed significantly ($p < 0.05$).*

**Table 4** Acidification activity of *Lactobacillus* strains obtained in MRS broth at 37°C

| LAB strains | pH | | | | | |
|---|---|---|---|---|---|---|
| | 0h | 12h | 24h | 36h | 48h | 72h |
| *L.brevis* S14 | $6.35^{a}$±0.07 | $5.65^{bcd}$±0.07 | $4.00^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L.brevis* S18 | $6.35^{a}$±0.07 | $5.60^{bcde}$±0.00 | $3.95^{b}$±0.07 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. brevis* S27 | $6.35^{a}$±0.07 | $5.30^{g}$±0.00 | $3.95^{b}$±0.07 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. brevis* S63 | $6.35^{a}$±0.07 | $5.45^{efg}$±0.07 | $3.95^{b}$±0.07 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. brevis* S82 | $6.35^{a}$±0.07 | $5.75^{b}$±0.07 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L.pentosus* S42 | $6.35^{a}$±0.07 | $5.60^{bcde}$±0.14 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. pentosus* S75 | $6.35^{a}$±0.07 | $5.40^{fg}$±0.14 | $3.95^{b}$±0.07 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S23 | $6.35^{a}$±0.07 | $5.60^{bcde}$±0.14 | $3.95^{b}$±0.07 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S46 | $6.35^{a}$±0.07 | $5.50^{def}$±0.00 | $3.95^{b}$±0.07 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S49 | $6.35^{a}$±0.07 | $5.30^{g}$±0.00 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S61 | $6.35^{a}$±0.07 | $5.55^{cdef}$±0.07 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S62 | $6.35^{a}$±0.07 | $5.50^{def}$±0.00 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S71 | $6.35^{a}$±0.07 | $5.65^{bcd}$±0.07 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| *L. plantarum* S72 | $6.35^{a}$±0.07 | $5.65^{bcd}$±0.07 | $3.90^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 | $3.80^{b}$±0.00 |
| Control (MRS broth) | $6.35^{a}$±0.07 | $6.35^{a}$±0.07 | $6.35^{a}$±0.07 | $6.35^{a}$±0.07 | $6.35^{a}$±0.07 | $6.35^{a}$±0.07 |

Values are mean ± standard error of triplicates.
[a-g]Means in same column of each parameter with different lower case letters differed significantly ($p < 0.05$).

The β-glucosidase activity of LAB strains is associated with the production of hydroxytyrosol **(Ghabbour *et al.*, 2011)**, the stable antioxidant compound highly desired in foods.

The proteolytic activity of *Lactobacillus* strains was determined, on skim milk agar, by observing the presence of a clear zone of proteolysis around colonies. Most of the strains showed proteolytic activity, but with variable importance. Out of 14 strains, 3 *L. plantarum* strains (S23, S46, and S49) and 3 *L. brevis* (S14, S18 and S27) and *L. pentosus* S42 displayed high proteolytic activity, while *L. brevis* S82 showed no proteolytic activity. The results of proteolytic activity are in agreement with those obtained by other authors **(Peres *et al.*, 2014, Phyu *et al.*, 2015)**. **Abriouel *et al.* (2012)** reported that the proteolytic activity was not detected in any of the LAB isolates from Spanish green table olives. While, *Lactobacillus* strains obtained from fermented dairy products exhibited high proteolytic activity **(Beganovic *et al.*, 2013, Phyu *et al.*, 2015)**. The proteolytic activity is due to various protease and peptidase enzymes, produced by LAB strains to procure their nutrients. These enzymes may lead to the production of bioactive peptides, improving the functional properties of fermented products.

The absence of an opaque halo around colonies was obtained for all the *Lactobacillus* strains, indicating that no lipolytic activity was detected for these strains. The absence of lipolytic activity was also obtained in *Lactobacillus* strains isolated from fruit processing and semi-hard artisanal goat cheeses **(De Albuquerque *et al.*, 2017, Meng *et al.*, 2018)**. Generally, *Lactobacillus sp* are considered to possess no or weak lipolytic activity **(Thakkar *et al.*, 2015)**.

The amylolytic activity of the *Lactobacillus* strains was evaluated on the starch agar medium. The absence of a clear zone of amylolysis around colonies indicated the incapacity of all the *Lactobacillus* strains to degrade starch. A similar result of amylolytic activity was obtained in LAB strains originating from Spanish-green olive brines **(Abrioue*l et al.*, 2012)**. Amylolytic LAB strains have been isolated from different beverages and tropical amylaceous fermented foods, prepared mainly from cassava and cereals (e.g., maize and sorghum) rich in starch **(Reddy *et al.*, 2008)**. *Lactobacillus* strains did not exhibit hydrolysis of starch, this may be due to their isolation environment (olive brine) characterized by the absence of starch contents **(Preedy and Watson, 2010).**

The cellulolytic activity of the *Lactobacillus* strains was evaluated on MRS agar containing carboxymethylcellulose (CMC). The presence of a clear zone around colonies, after revelations by Congo red and NaCl, indicated the capacity of *Lactobacillus* strains to degrade CMC by the production of cellulases. All of the *Lactobacillus* strains demonstrated high cellulolytic activity. The strains producing cellulases are good candidates in probiotic selection. Cellulases can improve the enzymatic digestion of fruits and vegetables in the gastrointestinal tract **(Juturu and Wu, 2014)**.

**Table 5** Enzymatic characterization of *Lactobacillus* strains

| LAB strains | Proteolysis[a] | Lipolysis[a] | Amylolysis[a] | Cellulolysis[a] | β-glucosidase[a] |
|---|---|---|---|---|---|
| *L.brevis* S14 | +++ | - | - | ++ | +++ |
| *L.brevis* S18 | +++ | - | - | ++ | +++ |
| *L. brevis* S27 | +++ | - | - | +++ | +++ |
| *L. brevis* S63 | + | - | - | ++ | +++ |
| *L. brevis* S82 | - | - | - | ++ | +++ |
| *L.pentosus* S42 | +++ | - | - | +++ | +++ |
| *L. pentosus* S75 | ++ | - | - | +++ | +++ |
| *L. plantarum* S23 | +++ | - | - | ++ | +++ |
| *L. plantarum* S46 | +++ | - | - | +++ | +++ |
| *L. plantarum* S49 | ++ | - | - | ++ | +++ |
| *L. plantarum* S61 | + | - | - | ++ | +++ |
| *L. plantarum* S62 | ++ | - | - | ++ | +++ |
| *L. plantarum* S71 | ++ | - | - | +++ | +++ |
| *L. plantarum* S72 | + | - | - | +++ | +++ |

a: (-) no activity; (+) low activity; (++) medium activity and (+++) high activity

## CONCLUSION

Lactic acid bacteria are the dominant microorganisms in fermenting green olives. Some *Lactobacillus* strains, isolated from the olive brine samples, showed the high acidification and the production of flavor compounds (Diacetyl. The production of specific enzymes (i.e. β-glucosidase) showed the ability to use the *Lactobacillus* in production of biological table olive. The Resistance of all *Lactobacillus* strains to acid pH and Bile salt indicated their ability to survive in the gastrointestinal tract. Based the profile of technological and probiotic properties, some *Lactobacillus* strains can be used as the probiotic starters in different food fermentations.

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