RESEARCH ARTICLE

Probiotic potential of *Lactobacillus* strains isolated from Iranian traditional pickled garlic and using them in fermented olive

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Article info:
Received: 26 July 2020
Accepted: 30 October 2020

ABSTRACT
In this study, the dominant lactic acid bacteria (LAB) in pickled garlic were identified by biochemical tests and a PCR technique. The LAB isolates were used as the starter culture in the fermented olive, in order to produce the fermented olive with probiotic potential, longer shelf life, and faster fermentation process. To determine the probiotic potential of the isolates, confirmatory tests were performed. Based on the BLAST results, five isolates of *Lactobacillus plantarum*, two isolates of *Pediococcus ethanolidurans* and one isolate of *Lactobacillus brevis* were identified. The LAB strains isolated in this study have had the survival ability at pH 2.5, antimicrobial activity against some pathogens, growing in the medium containing 0.3% bile salt, sensitivity to antibiotics, and without hemolytic activity, and therefore recognized as probiotics. The treatments with the starter culture of *L. plantarum* have had the lowest pH and the highest lactic acid content. In the sensory evaluation, these treatments had higher desirability and were significantly different from the control.

Key words: pickled garlic; fermented olive; lactic acid bacteria; probiotic

Introduction

In the past, bacteria are identified based on different properties such as cell and colony morphology, plate conditions, motility, spore production ability, and carbohydrate fermentation. Recently, it is clear that the identification of bacteria based on the phenotypical and biochemical properties is not so accurate. As well as, it is reported that the bacteria have been classed in a group according to the phenotypic and biochemical properties, but they were different based on genotypic property in several cases (Fessard and Remize, 2019). On the other side, the use of molecular methods to identify microorganisms has now enabled us to understand the ecology of food fermentation. Today, many different genetic techniques are used as an approach to identify and distinguish between lactic acid bacteria (LAB) isolates (Mohania et al., 2008).

The LAB family is composed of a diverse group of gram-positive, low-GC content, non-spore, often lack catalase, rod-shaped or spherical bacteria, which have a key role in food fermentation, and some of them, are known as "Probiotic" (Mathur and Singh, 2005). The probiotic term, meaning "for life", was used for the first time by Lilly and Stillwell (1965). They introduced probiotics as substances developed by protozoa for motivation of growth of other organisms. Nowadays, probiotics are known as live bacteria and/or yeasts that are good for the health of the host, especially the digestive system. *Lactobacillus, Bifidobacterium, Streptoccocus, and Bacillus (B. coagulans)* are the main species of probiotics (Argyri et al., 2013).

The health-promotion activities of probiotics have recently become an important subject of LAB. This activity of probiotics is including immune system modulation (Yahfoufi et al., 2018), antimicrobial activity (AA) (Marianelli et al., 2010), anti-inflammatory activity (Oh et al., 2018), modulation of allergic responses (Kalliomäki et al., 2010), anticancer activity (Singh et al., 2018), and reduction of lactose intolerance disease (Oak et al., 2019).

Garlic (*Allium sativum*) belongs to the *Alliacae* family and is native to central Asia. Garlic is used widely as a flavoring and medicinal agent in the world. The history of garlic dates back to 7000 years ago to make the desirable taste (Bayan et al., 2014).

Olive is one of the main agricultural harvests of Iran, which has high consumers. Iran is the 16th largest producer of olives in the world with annually harvesting of 47,000 tons. A remarkable amount of this product is used in the food

fermentation industries to improve organoleptic and functional properties (Azizpour et al., 2017). However, starter cultures are not used most of the time during the fermentation of this product. This fact is still done at the domestic factories or household level by spontaneous fermentation of the fruit. At this condition, it is very difficult to achieve a stable organoleptic property by a proper fermentation period (as short as possible), due to it may the natural microbial flora of fruits are different. In addition, the fermentation process by the natural microbial flora of fruits is too long, which can cause several problems in the fermented product such as microbial spoilage, due to a slow decrease in pH. Moreover, fermented products retained their desirable quality even after conservation for long period at ambient temperature (Nuraida, 2015).

There is an opinion that isolation of LAB from naturally traditionally fermented foods in order to offer their use as starter culture in the fermentation of olives can achieve a fermented olive product with good functional and sensorial properties. This might present an abnormal texture and/or flavor in the fermented foods. To achieve this target, the selection of an appropriate strain of LAB is necessary, which the task requires characterization of LAB (Bautista-Gallego et al., 2013).

Fermented olives and their products are rich in LAB that produces beneficial compounds in olive processing. LAB, especially *Lactobacillus*, carry out the natural fermentation process of olives, decomposition of oleuropein (de-bittering) (Lavermicocca et al., 2002). LAB is the main microorganisms used to produce traditional and novel fermented food products; they are responsible for the protection and make the sensory characteristics of the product. *Lactobacillus plantarum* has a long history of safe and natural use in a variety of food products. It plays an important role in the fermentation and processing of olive. *L. plantarum* species do not have the ability to cause inflammation and are resistant to gastric acid and bile salts, so they have the potential probiotic activity.

According to various studies on the isolation of probiotics, LAB has different properties in products depending on their sources of isolation. Therefore, the isolation of LAB and potential probiotics from new sources and using them in fermented foods is useful. Hence, the aims of this paper were isolation and identification of dominant LAB of traditional Iranian pickled garlic, characterization of its potential probiotic activity, and it's using in fermented olive to improve organoleptic and functional properties.

Materials and Methods

Material

De-bittered olives and Iranian traditional pickled garlic (7-year-old) were prepared from Armaghane chashni Toos Company and the local market of Quchan, respectively. All chemicals and mediums used in this study were purchased from Sigma-Aldrich (USA) and Merck (Germany) Companies.

Acidity

The acidity was measured according to the standard titration method (AOAC, 1990).

LAB isolation

10 g of the sample was put in a stomacher bag at sterile conditions and 90 ml of sterile physiological saline was added to it. Each bag was mixed at 250 rpm for 5 min and diluted to 10^{-2}-10^{-4}, then plated on MRS broth at anaerobic conditions (in a jar and along with gas pack) and incubated at 37 °C for 24 h. Then, streak culture was prepared from grown broth culture, then gram stain and catalase tests were performed (Bautista-Gallego et al., 2013).

DNA extraction

DNA of the LAB isolates was extracted from 1 ml of overnight culture of each isolate by GeneAll Hybrid isolation reagent (Korea) according to the Company instructions and the method described by Andrigetto et al. (2001), and supplemented with the addition of lysozyme (50 mg ml^{-1}, Sigma).

PCR reaction

The reaction volume was considered 30 µL, which its components are including Master Mix (11.25 µL), pattern DNA (3 µL), forward primer (2.25 µL), reverse primer (2.25 µL), and sterilized double-distilled water (11.25 µL). 16S rRNA was used as an identification criterion and forward and reverse primers (27F: GAGAGTTTGATCCTGGCTCAG and 1492R: GAAAGGAGGTGATCCAGCCG) were used. The thermocycler program used in this PCR reaction is shown in Table 1 (Van Hoorde et al., 2008).

Electrophoresis

After the PCR reaction, 3 µL of each PCR product was loaded along with 1 µL of loading dye and 2 µL of twice

Table 1: *Thermocycler program used for PCR.*

Program	Step	Temperature (˚C)	Time	
1	Initial denaturation	95	5 min	
2	Denaturing	94	30 s	33 cycles
	Annealing	54	30 s	
	Extending	72	2 s	
3	Final extending	72	10 min	

distilled water in each electrophoresis well. The electrophoresis was done in agarose gel (1.5%) containing 1 µL of safe stain and TBE 1X buffer at 90 volts for 45 min. The bands formed were observed under UV irradiation in gel doc (Zanirati et al., 2015).

Sequencing

The sequences were sent to Macrogen Co (Korea) for one-way read sequencing from the 27F primer. The most similar strain to the isolate was determined by comparing the obtained sequences with those given in the GenBank DNA database using the Basic Local Alignment Search Tool (BLAST). The similarity over 97% was considered as a significant similarity (Zanirati et al., 2015).

Inoculation of selected isolates into fermented olives

In order to accustom and resistance to high concentrations of salt in olive brine, the isolates were cultured in MRS broth containing 4.5% NaCl and incubated at 37 ˚C. The grown bacteria were harvested by centrifuging at 6000 rpm and 4˚C for 10 min and washed with phosphate buffer. The LAB isolates were added to de-bittered olive in brine (6-8% NaCl) as individual and mixed form at 0.5 McFarland standard (10^6-10^7 cfu/mL). Each treatment was including de-bittered olive (250 g), 1% (v/v) inoculum, and brine (300 ml) as packed in glass (Sánchez et al., 2001).

Chemical properties

The acidity and pH of the treatments were evaluated during fermentation time (0, 4, 7, 14, 21, 30, 45, 60, 75, 90, 105, 120, 135 and 150 days of fermentation) (AOAC, 1990).

LAB population

One cc of each treatment was cultured in MRS agar as pour plate on 0, 4, 7, 14, 21, 30, 45, 60, 75, 90, 105, 120, 135, and 150 days of fermentation, and incubated at anaerobic condition (in an anaerobic jar along with GasPak kit), 37˚C for 24 h (Panagou et al., 2008).

Potential probiotic activity

Resistance to simulated gastric and acidic conditions

3.2 g/l pepsin and 2% NaCl were dissolved in MRS broth and adjusted pH by HCl to 1.5, 2, 2.5, and 3. 1 ml of overnight culture (MRS broth) of each isolate was added to 19 ml of simulated stomach environment with different pH, and incubated at 37˚C for 0.5, 1, 1.5 and 2 h, then the survival of the isolates was evaluated by plating on MRS agar (Millette et al., 2008).

In the evaluation of resistance to acidic conditions, the pH of the MRS broth culture containing the isolates was adjusted to 2.5 and exposure by 2 h at 37˚C. After incubation of the culture at 37 ˚C for 24 h, the grown colony was counted (Manini et al., 2016). In both tests, the control sample was considered at similar conditions.

Bile tolerance

MRS broth containing 0.3% oxgall was inoculated with the LAB strains in 1% v/v and followed by incubation at 37 °C for 24 h. The inoculated strains were then plated on MRS agar and incubated at 30°C for 48 h, the survival was then studied (Manini et al., 2016).

Antibacterial activity

Agar disk diffusion method

AA of microbial suspension and their supernatant were studied. After turbidity of the isolates and pathogenic bacteria reached 10^7, 40 µ of suspension of pathogenic bacteria were surface cultured on Mueller-Hinton agar (MHA). Discs (6 mm in diameter) were placed on the plate and fresh microbial suspension and its supernatant (40 µL, neutralized with NaOH 0.1%) were poured on the disks and followed by incubated at 37˚C for 24 h. Tetracycline and gentamicin antibiotics were also used as positive controls (Flórez et al., 2005).

Spotting and microtitre plate

40 µL of pathogenic bacteria from overnight culture (Mueller-Hinton broth, MHB) was plated on MHA. 5 µL of supernatant of the LAB isolates (prepared through centrifugation at 12,000 rpm and 4˚C for 15 min) was spotted on the plate and incubated at 37˚C for 24 h, and then the inhibition zone diameter was measured as mm.

The microplate was used to find minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), so different concentrations of the supernatants were diluted with MHB to 200 µL and poured on 96-well microplate, along with 20 µL pathogenic bacteria (10^7 ml/cfu) to each well. The absorbance was read before and after incubation of the microplate at 37˚C for 24 h at 630 nm. In order to define MBC, one loop was transferred from each not-grown well to nutrient agar plate, and then incubated at 37 ˚C for 24 h.

Antibiotic resistance

100 µL microbial suspension of LAB containing 1×10^8 CFU/mL was surface cultured on MRS agar. Antibiotic discs were then placed on the culture under sterile conditions. The plates were incubated at 30°C for 24 h and followed by an evaluation of the inhibition zone (Angmo et al., 2016). The antibiotic discs used in the study were penicillin G (10 units), clindamycin (2 mcg), co-trimoxazole (25mcg), erythromycin (15 mcg), vanomycin (30 mcg), and ampicillin (10/10 mcg).

Haemolytic activity

LAB isolates were streaked on the surface of Columbia blood agar plates (Merck, Germany) complemented with 5% sterile sheep blood. The plates were incubated at 30°C for 48 h and evaluated haemolytic activity of isolates. This evaluation based on the formation of the zone around colonies so that clear zones, green-hued zones, and no zones around colonies represent β-haemolysis, α-haemolysis, and γ-haemolysis, respectively (Angmo et al., 2016).

Statistical analyses

SPSS software (SPSS 16 software) and a completely randomized method were used for the statistical analysis of observed data. Duncan's test ($p<0.05$) was used to compare the means. Diagrams prepared and analyzed using Excel software.

Results and Discussion

Identification of dominant LAB isolates

Grown colonies with different appearances were selected and the catalase-negative and Gram-positive strains were isolated. Based on the above tests, 8 strains were isolated and

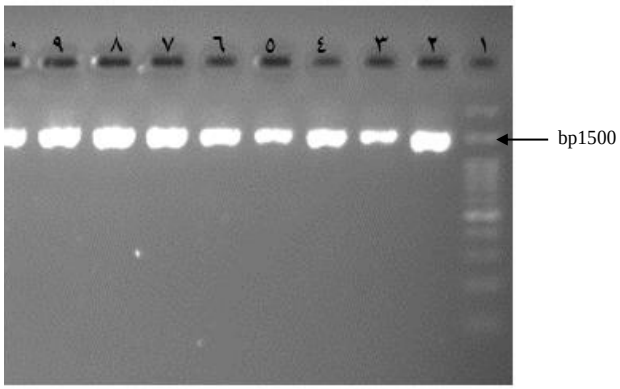

Figure 1. *Bonds of PCR products on gel. The first well: ladder (1-2 kbp).*

selected for molecular identification. The gel electrophoresis of PCR products to specific detection of dominant LAB isolated from pickled garlic is shown in Figure 1. The sequencing result of PCR products was verified by the BLAST procedure to confirm the identity of the amplicon. According to the results, *Lactobacillus plantarum*, *Pediococcus ethanolidurans*, and *Lactobacillus brevis* were identified as dominant LAB isolates. Since *Lactobacillus* strains have high competitiveness and adaptation potentials to starvation conditions and acidic environments, these strains are the most dominant than the other LAB species such as *Weissella*, *Leuconostoc*, and *Pediococcus*; while, *streptococci*, *enterococci*, and *lactococci* are rarely found (Sadeghi, 2016).

Table 2. *The treatment of fermented olive inoculated with the LAB isolates.*

Treatment (starter)	Code	Treatment	Code
L. plantarum PGNM2	A	*L. plantarum* PGNM8	B
P.ethanolidurans PGNM6	C	*L. brevis* PGNM1	D
L. plantarum PGNM2/ *L. plantarum* PGNM8	AB	*P.ethanolidurans* PGNM6/ *L. brevis* PGNM1	CD
L. plantarum PGNM2/ *L. plantarum* PGNM8/ *P.ethanolidurans* PGNM6	ABC	*L. plantarum* PGNM2/ *L. plantarum* PGNM8/ *L. brevis* PGNM1	ABD
L. plantarum PGNM2/ *L. plantarum* PGNM8/ *P.ethanolidurans* PGNM6/ *L. brevis* PGNM1	ABCD	Control	N

In this study, *L. plantarum* PGNM2, *L. plantarum* PGNM8, *P. ethanolidurans* PGNM6, and *L. brevis* PGNM1 were used to study the probiotic potential and as a starter in the fermentation of olives.

Inoculation of isolates to olive

The identified LAB isolates (four isolates) were used as a starter in fermented olive as individual and mixed forms at 6 and 8 % NaCl (Table 2).

Probiotic potential

Resistance to simulated gastric and acidic conditions

The survival of bacteria in the digestive system is one of the most important factors in the selection of probiotic strains. Acid and bile resistance are two essential features to predict the ability of bacteria to pass through the digestive tract (Zanirati et al., 2015).

All isolates were viable at pH 1.5, 2, and 3 for 0.5, 1, and 1.5 h, and except for *L. brevis* PGNM1 and *P. ethanolidurans* PGNM6 isolates, the other isolates were able to survive at pH 1.5 for 2 h (Table 3). The results showed that *L. plantarum* PGNM2 and *L. plantarum* PGNM8 had a population decline approximately equivalent to one logarithmic cycle. This decrease was observed three and four logarithmic cycles for *L. brevis* PGNM1 and *P.ethanolidurans* PGNM6, respectively. Overall, all isolates were able to survive in acidic conditions, but the most resistant isolate was *L. plantarum* PGNM2 (Table 4).

Delgado et al. (2007) studied subtractive screening for probiotic properties of *Lactobacillus* species from the human gastrointestinal tract in the search for new probiotics. Their result showed that acidic conditions (pH 2.5) are destructive

Table 3. *Resistance of the LAB to simulated gastric condition.*

LAB isolate	Time (h)	Pepsin at pH 3	Pepsin at pH 2.5	Pepsin at pH 2	Pepsin at pH 1.5
L. plantarum PGNM2	0.5	+	+	+	+
	1	+	+	+	+
	1.5	+	+	+	+
	2	+	+	+	+
L. plantarum PGNM8	0.5	+	+	+	+
	1	+	+	+	+
	1.5	+	+	+	+
	2	+	+	+	+
L. brevis PGNM1	0.5	+	+	+	+
	1	+	+	+	+
	1.5	+	+	+	+
	2	+	+	+	–
P. ethanolidurans PGNM6	0.5	+	+	+	+
	1	+	+	+	+
	1.5	+	+	+	+
	2	+	+	+	–

for bacteria, but LAB species must be able to survive in the conditions in which the isolates were resistant to these conditions, and *P. ethanolidurans* PGNM6 showed the least resistance to the acidic conditions.

Table 4. *Resistance of the LAB to acidic condition.*

LAB isolate	pH 2.5	
	Primary log cfu mL^{-1}	log cfu mL^{-1} after 2 h
L. plantarum PGNM2	7.2±0.7	6.4±0.5
L. plantarum PGNM8	7.9±0.5	6.5±0.5
L. brevis PGNM1	7.4±0.1	5.2±0.5
P. ethanolidurans PGNM6	8.2±0.1	4.7±0.5

Resistance to bile salt

The bile plays an essential role in the specific and nonspecific defense mechanisms of the intestine and its inhibitory effect is mainly determined by the concentration of bile salts. It is believed that 0.3 w/v is the average bile salt concentration in the human gastrointestinal tract (Blana et al., 2014).

All isolates were not only resistant to the medium containing 0.3% bile salt but also capable of growth approximately equal to the control sample (the medium without bile salt). The LAB isolates were able to grow at a 3% bile salt concentration. *L. plantarum* PGNM2 was capable of hydrolysis of bile salt (6%) and growth on the medium containing it. However, *P. ethanolidurans* PGNM6 strain was not able to grow in the medium containing 4% bile salt (Table 5).

Table 5. *The growth potential of the LAB isolates at different concentrations of bile salt.*

LAB isolate	1%	2%	3%	4%	5%	6%
L. plantarum PGNM2	+	+	+	+	+	+
L. plantarum PGNM8	+	+	+	+	+	–
L. brevis PGNM1	+	+	+	+	+	–
P. ethanolidurans PGNM6	+	+	+	–	–	–

According to Fuller et al. (1992) bile salt, even at low concentrations, can inhibit the growth and survival of microorganisms. Gilliland et al. (1984) reported that 0.3% of bile salt is the critical concentration for isolation of resistant LAB. The study on-resistance of the isolates to bile salt in MRS agar showed that *L. plantarum* PGNM2 was more able to survive and grow than the other three isolates and this isolate was able to grow in a medium containing 6% bile salt. However, *P. Ethanolidurans* PGNM6 cannot grow and survive in the medium containing bile salt more than 4%. Abriouel et al. (2012) studied the diversity of microbial populations in brines during fermentation of naturally-fermented *Aloreña* green table olives. They reported that some *L. pentosus* and *L. pseudomesenteroides* strains were able to grow in the medium containing 3% bile salt, but none of them was able to grow at higher concentrations. In addition, in agreement with the results of this study, they reported that both strains are resistant to acidic environments (pH 2, 2.5, and 3 for 30 min exposure), but only a few strains of *L. pentosus* and *L. pseudomesenteroides* were able to survive at pH 1.5.

Antibiotic resistance

Although LAB is generally known to be GRAS, it has been reported that antibiotic resistance genes can be transmitted between different types of bacteria, and thus they

Table 6. *The sensitivity of the LAB isolates to different antibiotics.*

LAB isolate	*L. plantarum* PGNM2	*L. plantarum* PGNM8	*L. brevis* PGNM1	*P. ethanolidurans* PGNM6
K	++	++	++	++
GM	+	+	+	+
AM	+	+	+	+
E	+	+	+	+
TE	+	+	+	+
S	++	+	++	++
P	+	+	+	+
C	+	+	+	+
V	+++	+++	+++	+++

(K) kanamycin, (GM)gentamycin, (AM) ampicillin, (E) erythromycin, (TE) tetracycline, (S) streptomycin, (P) penicillin, (C) chloramphenicol, (V) vancomycin

may make food-borne bacteria resistant to antibiotics. The LAB isolates identified in this study were semi-sensitive to the streptomycin and kanamycin antibiotics and sensitive to other antibiotics. In addition, all four isolates were resistant to vancomycin antibiotics (Table 6). This resistance could be attributed to the absence of di-alanine-D-lactate in the cell wall of these isolates (Wang et al., 2019).

Haemolytic activity

Hemolytic activity is one of the characteristics of pathogenic bacteria so it should be investigated in the selection of probiotic bacteria. All isolates were identified in this study without hemolytic activity.

To date, several pieces of researche have been done about the study on probiotic activities and to find the best probiotic strains. For example, Manini et al. (2016) investigated the probiotic properties of LAB isolated from wheat bran sourdough (two *L. brevis* strains, four *L. plantarum* strains, and two P. pentosaceus strains). They reported that the isolates were sensitive to the most of antibiotics studied. Also, three *L. plantarum* strains were susceptible to acidic conditions and disappeared after 2 h at pH 2.5, but all *L. brevis* and *P. pentosaceus* strains and one *L. plantarum* strain had the ability to survive in these conditions. All isolates except *P. pentosaceus* were viable and growing under 0.3% bile salt. In our study, all four lactic isolates were able to survive in the above conditions.

Antibacterial activity

Agar disk diffusion method

As shown in Table 7, the highest AA against *B. cereus*, *E. coli*, *L. monocytogenes*, and *S. enteritidis* were observed for *L.brevis* PGNM1, *L. plantarum* PGNM8, *L. brevis* PGNM1, and *P. ethanolidurans* PGNM6, respectively. According to Table 7, there is no significant difference between AA of all isolates against a pathogenic bacterium. There is a significant difference between AA of the LAB isolates and the antibiotic disks used in this study ($p \leq 0.005$).

According to the results, gram-positive bacteria showed higher sensitivity to the LAB isolates compared to gram-negative types. This is probably due to the presence of lipopolysaccharides layer in the cell wall of gram-negative bacteria. The layer inhibits the penetration of antimicrobial agents of LAB (Rostami et al., 2016).

Spotting and microtitre plate method

According to Figure 2, *L. brevis* PGNM1 had the highest AA on all the pathogens; on the other hand, *L. plantarum* PGNM8 and *P. ethanolidurans* PGNM6 had the lowest AA on *S. enteritidis* and the other pathogens. Figure 3 shows AA of supernatant of the LAB isolates from pickled garlic on some pathogenic bacteria. As shown in the figure *L. brevis* PGNM1 had the highest AA.

After 18 h incubation, the lowest concentration of supernatant of the LAB isolates that had equal absorption with the initial absorption was considered as MIC. After spotting from each well of the microplate, the lowest concentration of supernatant that caused the death of pathogenic bacteria was considered as MBC. As shown in Table 8, all four LAB isolates were able to inhibit the growth of pathogenic bacteria at high concentrations (100-200 mg/ml).

At a concentration of 12.5 mg/mL of supernatant of each LAB isolate, none of the treatments was able to inhibit the growth of pathogenic bacteria; however, supernatant of *L. brevis* PGNM1 and *L. plantarum* PGNM8 isolates at a concentration of 25 mg/mL had the ability to inhibit the growth of *L. monocytogenes*, and this concentration was

Table 7. *The AA of the LAB isolates on some pathogenic bacteria.*

LAB isolate	*S. enteritidis* (ATCC 13076)	*E. coli* (ATCC 25922)	*L. monocytogenes* (ATCC 19111)	*B. cereus* (ATCC 14579)
L.brevis PGNM1	$8/5\pm0/50^{Ba}$	$11/3\pm0/57^{Ab}$	$11/8\pm0/76^{Aa}$	$12/0\pm0/86^{Ab}$
L. plantarum PGNM8	$8/0\pm0/50^{Ba}$	$8/8\pm0/76^{Ba}$	$11/1\pm0/57^{Aa}$	$11/5\pm0/50^{Ab}$
P. ethanolidurans PGNM6	$7/7\pm0/76^{Aa}$	$10/0\pm0/50^{Bab}$	$8/2\pm0/76^{Ab}$	$10/3\pm0/57^{Ba}$
L. plantarum PGNM2	$8/0\pm1/00^{Aa}$	$9/5\pm0/50^{ABab}$	$11/2\pm0/76^{Ba}$	$9/5\pm1/32^{Aba}$
GM	$17/8\pm0/76^{Ab}$	$18/5\pm2/18^{Ac}$	$20/5\pm0/86^{Bb}$	$18/7\pm0/76^{Ac}$
TE	$14/5\pm0/5^{Bc}$	$30/7\pm0/76^{Cd}$	$20/1\pm0/44^{Ab}$	$20/7\pm0/28^{Ad}$

Table 8. *MIC and MBC of supernatant of LAB isolates on some pathogenic bacteria.*

Supernatant of isolate	MBC (mg/ml)	MIC (mg/ml)	Pathogenic bacteria
L. brevis PGNM1	100	50	*E. coli*
	100	25	*L. monocytogenes*
	-	50	*S. enteritidis*
	50	50	*B. cereus*
L. plantarum PGNM8	100	100	*E. coli*
	100	25	*L. monocytogenes*
	-	100	*S. enteritidis*
	100	100	*B. cereus*
P. ethanolidurans PGNM6	50	50	*E. coli*
	-	50	*L. monocytogenes*
	-	100	*S. enteritidis*
	50	25	*B. cereus*
L. plantarum	50	50	*E. coli*
	50	50	*L. monocytogenes*
	100	50	*S. enteritidis*
	100	100	*B. cereus*

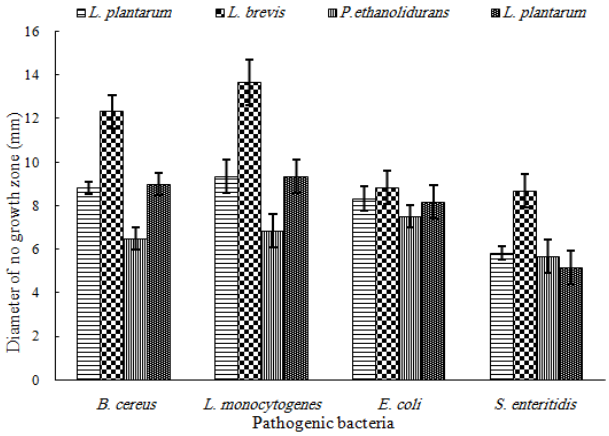

Figure 2. *The AA of supernatant of LAB isolates on pathogenic bacteria by spotting method.*

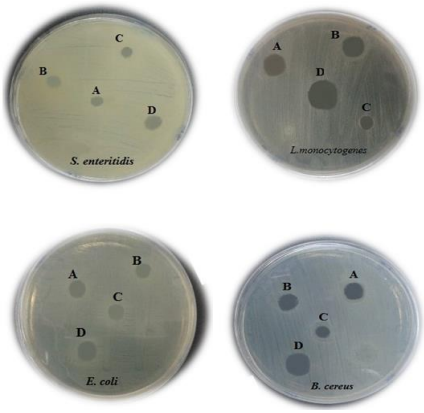

Figure 3. *AA of supernatant of the LAB isolated from pickled garlic on some pathogenic bacteria (L. plantarum PGNM2, (B) L. plantarum PGNM8, (C) P.ethanolidurans PGNM6, (D) L. brevis PGNM1).*

considered as MIC. Also, the MIC of supernatant of *P. ethanolidurans* on *B. cereus* was 25 mg/mL.

Only *L. plantarum* PGNM2 has had a bactericidal effect on the pathogenic bacteria (50 and 100 mg/ml). Among the pathogens examined in this study, *B. cereus* had the lowest survival capacity against supernatant of LAB isolates, so that the MBC was observed on it by all the isolates. *S. enteritidis* showed MBC for the only supernatant of *L. plantarum* PGNM2; but for Other LAB isolates, this was not observed.

Abriouel et al. (2012) observed a AA for *L. pentosus* and *L. pseudomesenteroides* isolated from fermented olive against *Staphylococcus aureus*, *L. monocytogenes*, *Streptococcus mutans*, *B. cereus*, *Enterococcus faecalis*, and *Salmonella enteric*. However, they reported that this LAB has no AA against *Pseudomonas fluoresce*, *E. coli*, *Candida albicans*. Manini et al. (2016) studied AA of lactic acid bacteria isolated from wheat bran sourdough by spotting method and reported that several *L. plantarum* spp. has AA against several species of *Listeria*, however, this has been not observed for *L. brevis*.

Lactic acid production

Lactic acid is the most important organic acid produced by LAB. Although the measurable acidity was low (about 0.1 %) in both salt percentages, but increased during fermentation time. As shown in Figure 4(a), the fermented olive prepared at 6% salt, the highest (1.08%) and lowest (0.80%) lactic acid contents were observed for treatment B (including *L. plantarum* KLDS PGNM8) and control sample, respectively. In the fermented olive prepared at 8% salt, AB treatment (inoculumed with two strains of *L. plantarum*) had the highest lactic acid content (1.13%), as shown in Figure 4(b).

The difference in the lactic acid content produced by LAB, especially at the beginning of fermentation, is due to the type of bacteria and the salt concentration (Blana et al., 2014). The use of LAB in the fermentation of olive rapidly decreases pH and this lead to the prevention of spoilage and shortening fermentation time, and improve organoleptic properties (Shah et al., 2018).

LAB population

After 60 days of fermentation at 6% NaCl, the highest (about 10^7 ml/cfu) and lowest (about 10^5 ml/cfu) population of LAB were observed for B and ABD treatments (Figure 5). In the next days, the population of all treatments was decreased, so that the treatment of N had the lowest population among treatments (Figure 5). This decrease in the population is due to low pH and microbial competition between strains (Shah et al., 2018). As shown in Figure 6, the AB and CD treatments had the highest (about 10^7 ml/cfu) and

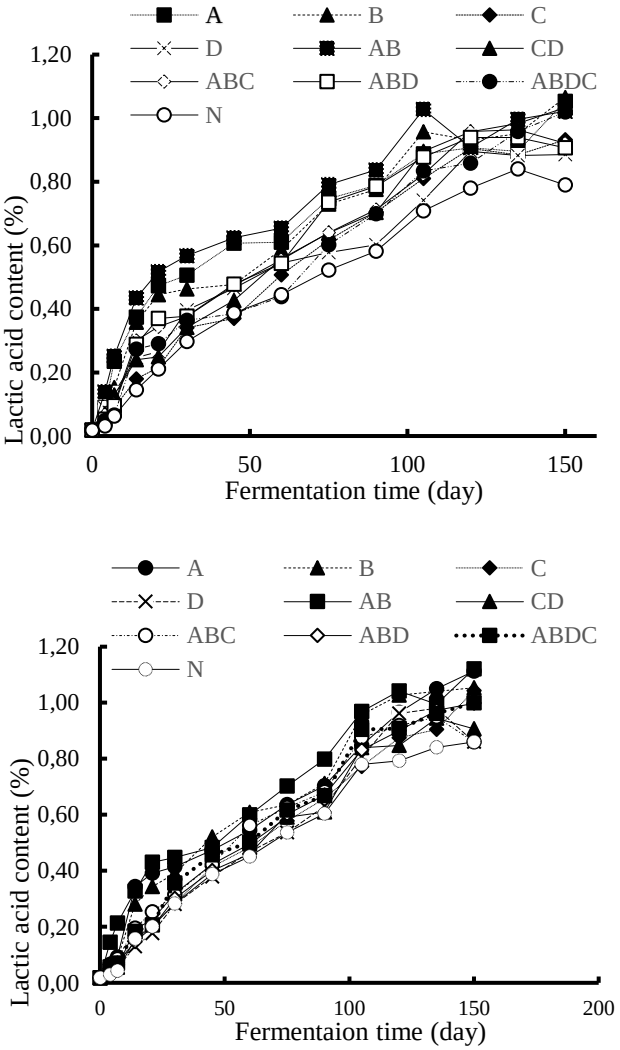

Figure 4. *Lactic acid content produced by LAB in fermented olive at 6% (a) and 8% NaCl (b) during fermentation time (A: L. plantarum PGNM2, B: L. plantarum PGNM8, C: P. ethanolidurans PGNM6, D: L. brevis PGNM1, AB: L. plantarum PGNM2/L. plantarum PGNM8, CD: P. ethanolidurans PGNM6/ L. brevis PGNM1, ABC: L. plantarum PGNM2/L. plantarum PGNM8/P. ethanolidurans PGNM6, ABD: L. plantarum PGNM2/L. plantarum PGNM8/L. brevis PGNM1, ABCD: L. plantarum PGNM2/L. plantarum PGNM8/ P. ethanolidurans PGNM6/ L. brevis PGNM1, N: control sample).*

lowest (about 10^5 ml/cfu) population of LAB, after 60 days of fermentation at 8% NaCl (Figure 6). In the treatments in which the population of LAB isolates was 10^6-10^7 cfu/ml, it can be stated that the product has the delivery ability of a sufficient number of LAB to the body.

Therefore, they can be introduced as a probiotic product (Jackson et al., 2019).

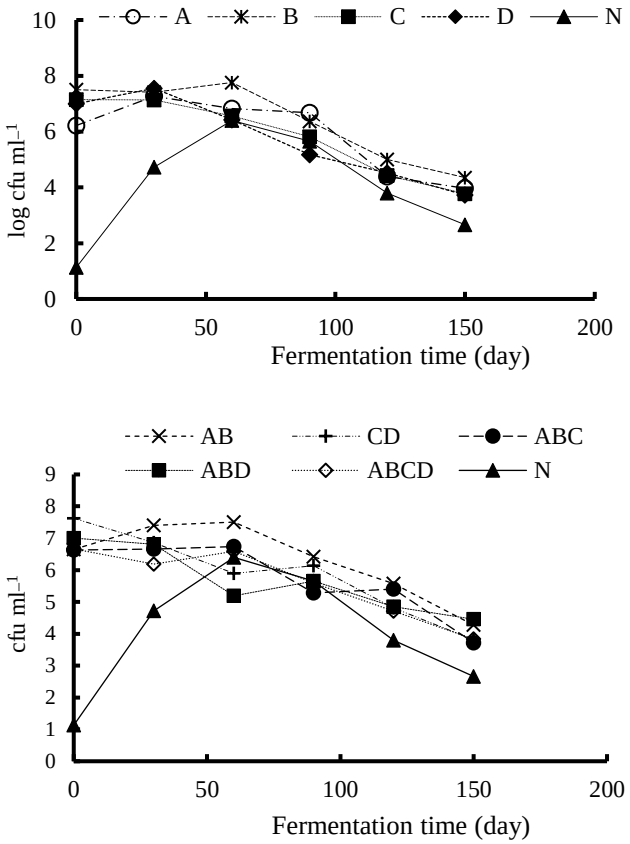

Figure 5. *LAB population in fermented olive inoculumed with individual form of the LAB isolates (a) and mixed of them (b) at 6% NaCl during fermentation time (A: L. plantarum PGNM2, B: L. plantarum PGNM8, C: P. ethanolidurans PGNM6, D: L. brevis PGNM1, AB: L. plantarum PGNM2/L. plantarum PGNM8, CD: P. ethanolidurans PGNM6/ L. brevis PGNM1, ABC: L. plantarum PGNM2/L. plantarum PGNM8/P. ethanolidurans PGNM6, ABD: L. plantarum PGNM2/L. plantarum PGNM8/L. brevis PGNM1, ABCD: L. plantarum PGNM2/L. plantarum PGNM8/ P. ethanolidurans PGNM6/ L. brevis PGNM1, N: control sample).*

Organoleptic evaluation

All organoleptic properties evaluated for the control treatment in this study had the lowest average acceptance, except for the brittleness. The highest flavor and bitterness acceptances were observed for treatment of A, which was significantly different from the control sample. The highest average acceptance of odor, color, and brittleness was observed for the treatment of AB. However, the treatment of ABCD and CD had the highest average acceptance of salinity and shell thickness, respectively. Eventually, OA of AB and A (the samples inoculated with *L. plantarum*) was the highest and significantly higher than the control sample (Table 9).

Sabatini et al. (2008) studied volatile compounds in uninoculated and inoculated table olives with *Lactobacillus*

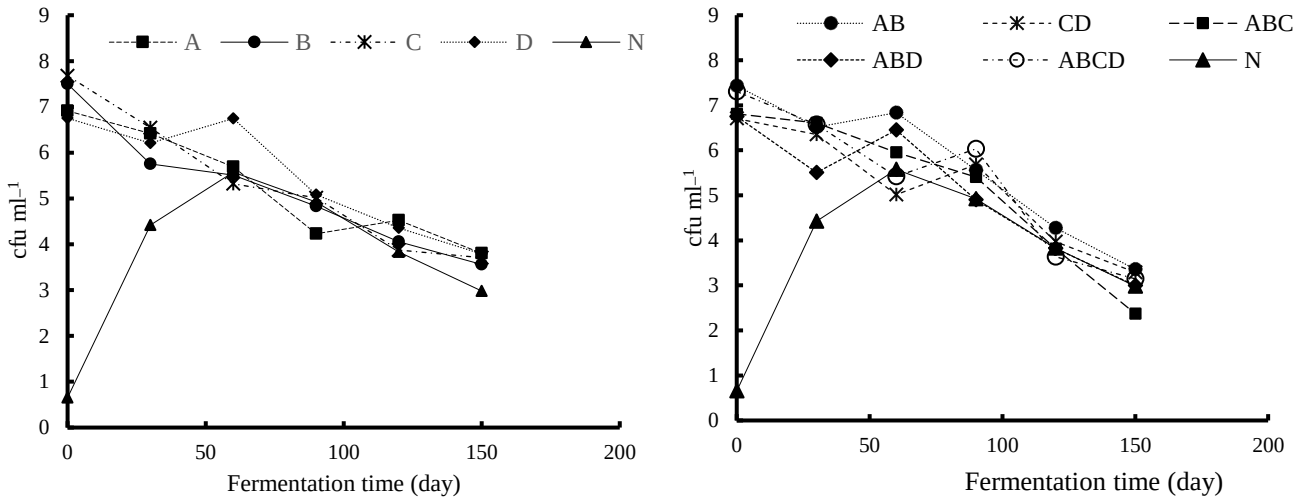

Figure 6. *LAB population in fermented olive inoculumed with individual form of the LAB isolates (a) and mixed of them (b) at 8% NaCl during fermentation time (A: L. plantarum PGNM2, B: L. plantarum PGNM8, C: P. ethanolidurans PGNM6, D: L. brevis PGNM1, AB: L. plantarum PGNM2/L. plantarum PGNM8, CD: P. ethanolidurans PGNM6/ L. brevis PGNM1, ABC: L. plantarum PGNM2/L. plantarum PGNM8/P. ethanolidurans PGNM6, ABD: L. plantarum PGNM2/L. plantarum PGNM8/L. brevis PGNM1, ABCD: L. plantarum PGNM2/L. plantarum PGNM8/ P. ethanolidurans PGNM6/ L. brevis PGNM1, N: control sample).*

Table 9. *Organoleptic properties of fermented olive prepared by the LAB isolates.*

Treatment*	Odor	Brittleness	Color	Salinity	Flavor	Bitterness	Shell thickness	Overall acceptance
A	4/00±0/86 ab	3/80±0/77 ab	4/25±0/64 ab	3/30±1/03 ab	4/25±0/71 a	4/10±0/79 a	4/05±0/67 a	4/10±0/79 ab
B	3/90±0/72 abc	3/90±0/79 ab	4/05±0/67 abc	3/15±1/18 ab	3/95±0/83 abc	3/80±0/89 abc	3/85±0/81 ab	3/90±0/72 abc
C	3/65±0/81 bc	3/30±0/86 b	3/45±0/61 de	2/80±1/05 b	3/20±0/83 ce	3/45±0/94 abc	3/40±1/09 bc	3/35±0/59 ce
D	3/80±0/76 abc	3/55±0/99 ab	4/25±0/64 ab	3/00±0/79 ab	3/40±0/88 cd	3/40±0/82 bc	3/30±0/80 c	3/50±0/83 cd
AB	4/25±0/64 a	4/05±0/89 b	4/50±0/69 a	3/40±0/75 ab	4/10±0/72 ab	3/95±0/99 ab	4/10±0/64 a	4/20±0/77 a
CD	3/95±0/76 abc	3/90±0/96 ab	4/15±0/74 ab	3/05±0/94 ab	3/60±0/75 bcd	3/70±1/12 abc	4/15±0/59 a	3/65±0/67 bcd
ABC	3/65±0/81 bc	3/85±0/81 ab	3/90±0/78 bcd	3/50±1/05 a	3/90±0/72 bc	3/85±0/81 abc	3/95±0/60 a	3/55±0/76 cd
ABD	3/75±0/85 abc	3/75±0/78 ab	3/95±0/83 bcd	3/15±0/81 ab	3/70±0/57 bcd	3/70±0/80 abc	3/75±0/77 abc	3/55±0/83 cd
ABCD	3/95±0/76 abc	3/55±0/88 ab	3/60±0/99 cd	3/65±0/87 a	3/65±0/99 bcd	3/70±0/86 abc	3/85±0/67 ab	3/85±0/81 abcd
N	3/40±0/82 c	3/45±0/68 ab	3/10±0/72 d	3/00±0/85 ab	2/80±0/83 e	3/20±1/05 c	3/30±0/80 c	2/95±0/83 e

*(A) *L. plantarum* PGNM2, (B) *L. plantarum* PGNM8, (C) *P.ethanolidurans* PGNM6, (D) *L. brevis* PGNM1, (AB) *L. plantarum* PGNM2/*L. plantarum* PGNM8, (CD) *P.ethanolidurans* PGNM6/ *L. brevis* PGNM1, (ABC) *L. plantarum* PGNM2/*L. plantarum* PGNM8/*P.ethanolidurans* PGNM6, (ABD) *L. plantarum* PGNM2/*L. plantarum* PGNM8/*L. brevis* PGNM1, (ABCD) *L. plantarum* PGNM2/*L. plantarum* PGNM8/ *P.ethanolidurans* PGNM6/ *L. brevis* PGNM1, N: control sample.

**a, b, c and d show the significant difference between samples in each column ($p \leq 0.05$).

plantarum, and reported that the inoculation improves the flavor and odor of table olives. Blana et al. (2014) reported that the inoculation of *Lactobacillus pentosus* and *Lactobacillus plantarum* to green olives improves the organoleptic properties of the product.

Conclusion

P. ethanolidurans, L. plantarum and *L. brevis* were identified as the dominant LAB in traditional Iranian pickled garlic. The using *L. plantarum* in fermented olive as a starter acidifies the environment faster and reduces the fermentation period and the possibility of spoilage. In addition, *L. plantarum* as a starter improved remarkably organoleptic properties of fermented olive. As well as, this microorganism had suitable survival in fermented olive containing NaCl (6%) after 75 days (10^6 cfu/ml). According to the results of probiotic tests, *L. plantarum* can be used as a starter with probiotic potential in fermented olive. *L. plantarum* and *P. ethanolidurans* had the highest AA against *E. coli* and *B. cereus*, respectively. The gram-positive indicator bacteria had more sensitivity to the LAB isolate compared to gram-negative types.

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