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Selection of *Lactobacillus plantarum* strains for their use as starter cultures in Algerian olive fermentations

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RESUMEN

Selección de cepas de *Lactobacillus plantarum* para su uso como cultivos iniciadores en la fermentación de aceitunas de Argelia.

El objetivo de esta investigación fue evaluar algunos aspectos tecnológicos de cepas de *L. plantarum* previamente aisladas de aceitunas fermentadas. Para este propósito, 11 cepas fueron usadas para estudiar su susceptibilidad a antibióticos *in vitro*, resistencia a valores de pH bajos, actividad acidificante, proteolítica, y hemolítica, producción de ácido láctico y exopolisacáridos, y resistencia a la liofilización. En general, las cepas fueron susceptibles a la mayoría de los antibióticos ensayados y mostraron supervivencia a pH 2. La mayoría de las cepas mostraron una actividad de acidificación alta (1.035 ± 0.29 a 0.912 ± 0.21 mmol/l de ácido láctico) o media (0.556 ± 0.29 a 0.692 ± 0.18 mmol/l) con una buena actividad proteolítica (1.49 ± 0.25 a 5.25 ± 0.11 mg l^{-1} tirosina). Ninguna de las cepas produjo exopolisacáridos o hemolisis en sangre de oveja.

PALABRAS CLAVE: Aceitunas fermentadas – Antibióticos – Cultivos iniciadores – Lactobacillus plantarum.

SUMMARY

Selection of *Lactobacillus plantarum* strains for their use as starter cultures in Algerian olive fermentations.

The aim of this research was to evaluate some technological traits of *L. plantarum* strains previously isolated from fermented olives. For this purpose, 11 strains were tested for their *in vitro* antibiotic susceptibility, resistance to low pH values, acidifying activity, proteolytic activity, haemolytic activity, lactic acid and exopolysaccharide production and resistance to freeze-drying .Collectively, the strains were susceptible to most of the antibiotics tested and showed survival at pH 2. Most strains showed high (1.035 ± 0.29 to 0.912 ± 0.21 mmol/l ± sd of lactic acid) or medium (0.556 ± 0.29 to 0.692 ± 0.18 mmol/l±sd) acidification activity with good proteolytic activity (1.49 ± 0.25 to 5.25 ± 0.11 mg l^{-1} tyrosine). None of the strains produced exopolysaccharides or haemolysis in sheep's blood.

KEY-WORDS: Antibiotics – Fermented olives – Lactobacillus plantarum *– Starter cultures.*

1. INTRODUCTION

Olives are the fruit of the olive tree (*Olea europaea*), which is cultivated in all the countries of the Mediterranean region, especially in the central and southern regions of Spain and Italy, Greece, Turkey, Tunisia, Algeria and Morocco. In western Algeria (Sig), the green olives are directly brined and preserved by natural fermentation. Starter cultures are not employed during fermentation. This process is still performed by simply allowing the fruit to ferment spontaneously. No means whatsoever are taken to control fermentation, which in most cases is incomplete or affected by microbial spoilage. For these reasons great attention must be paid to the quality of fermenting olives.

Lactobacillus isolated from fermented olives (Ruiz-Barba *et al.,* 1991 Lavermicocca *et al.,* 2002. Kacem *et al.,* 2004; Kacem, and Karam, 2006) has been extensively studied with the aim of its use in starter cultures for olives or other vegetable fermentations (Costilow and Fabian, 1953 and Leal-Sánchez *et al.,* 2002, Lavermicocca *et al.,* 2005, Kacem, 2007). However, little is known about its antibiotic resistance.

Antibiotic resistance has been reported for lactic acid bacteria (Salminen *et al.*, 1998). This is of interest because of its possible use to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis. On the other hand, the transmission of antibiotic resistant genes to unrelated pathogenic or potentially pathogenic bacteria in the gut is a major health concern (Danielsen and Wind, 2003).

In a previous study (Kacem *et al.* 2004), a total of 11 *L. plantarum* strains were isolated from the fermented green olives produced in western Algeria (region of Sig). Of these, *L. plantarum* OL15 and OL9 strains produced bacteriocins towards Gram-positive and negative bacteria (Kacem *et al.*, 2005, 2006).

In this work, we aimed to evaluate the resistance of these strains to a range of antibiotics, and to screen them for some criteria (carbohydrate

fermentation, lactic acid and exopolysaccharide production, tolerance to acidic pH values, proteolytic and haemolytic activities and resistance to freeze-drying) in order to select starter culture strains.

2. MATERIALS & METHODS

2.1. Bacterial strains and media

The strains of *L. plantarum* (OL2, OL7, OL9, OL12, OL15, OL16, OL23, OL33, OL36, OL40 and OL53) used in this study were isolated from fermented green olives and were described previously by Kacem *et al.* (2004). They were kept as a frozen stock at –20°C in distilled water plus 20 % (v/v) glycerol and propagated twice in Man Rogosa Sharpe (MRS) broth (Oxoid Ltd., UK) (de Man *et al.*, 1960) at 30°C before use.

2.2. Testing for resistance to antibiotics

Bacterial antibiotic resistance was determined on solid MRS medium by the use of 11 different antibiotic discs (bioMérieux, Marcy-l'Etoile, France) (Table 3). The results (average of 3 reading) were expressed as sensitive (S) or resistant (R) thanks to the standard disc diffusion method (National Committee for Clinical Laboratory Standards, 1999). Two strains with known antibiotic resistances (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) were used as the control strains (Kacem and Kaid-Harche, 2008).

2.3. Fermentation of carbohydrates

The ability of the different *L. plantarum* strains to ferment different carbohydrates was determined using API 50 CHL test kits (bio Mérieux SA). The API test strips were prepared as recommended by the kit supplier and scored after incubation for 24 and 48 hours at 37 or 30°C. The interpretation of the fermentation profiles was facilitated by the use of the computer-aided database "API-LAB plus" (API bioMe´rieux).

2.4. Lactic acid production

Production of lactic acid was measured by the precipitate method according to Pryce (1969) and expressed in mmol/l. Results are the average of three replicates.

2.5. Acidifying activity

Acidification was measured by the change in pH (ΔpH) over time according to the methods of Lombardi *et al.* (2002) and Ayad et al. (2004) . Fifty millilitres of MRS (Oxoid) were inoculated with 2% culture (in order to standardize the assay, the inoculums were approximately 10^6 cfu/ml) and incubated at 37°C. The pH was measured at 0, 2, 4 and 6 h using a pH-meter (Micro pH 2002, Crison, Barcelona, Spain). The acidification values were expressed as pH decrease, calculated as the difference between the value immediately after inoculation and values at 0, 2, 4 and 6 h ($\Delta pH = pH_{at\ time} - pH_{zero\ time}$). The cultures were considered fast, medium or slow acidifying when a ΔpH of 0.4 U (pH units) was achieved after 3, 3-5 and > 5 h, respectively (Kacem and Kaid-Harche, 2008).

2.6. Tolerance to acidic pH values

Strains were grown in MRS broth (Oxoid) at 30°C overnight, then subcultured into fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 5000 × g for 10 min. at 4°C. The pellets were washed in sterile phosphate-buffered saline (PBS) (Oxoid), pH 7 and resuspended in PBS. Each strain was diluted 1/100 in PBS at pH 1, 2 and 3. Incubation times were 2, 4 and 6 h. Bacteria were then transferred to MRS broth (Oxoid) and incubated at 37°C overnight (El-Naggar, 2004). Counts of surviving cells were determined as described by Kacem and Kaid-Harche (2008). Bacterial growth was expressed in colony forming units per milliliter (cfu/ml) and the survival percentage (% ± sd) of strains to different pH values was then calculated. The experiment was repeated twice and each reading represents the means of three observations.

2.7. Proteolytic activity

The proteolytic activity of strains was determined in a skimmed-milk medium (reconstituted skimmed milk powder 10% w/v) using the tyrosine method (Hull, 1947), in accordance with the International Dairy Federation (IDF) standard 149A (1997). Milk was inoculated at 0.2% with each strain precultured in MRS broth (Oxoid) at 37°C for 18 h, to obtain approximately 10^6 cfu / ml and then incubated at 30°C for 24 h. The IDF method is based on the reaction of the amino acids tyrosine and tryptophan released from the milk substrate at 72 h with a phenol reagent, yielding a blue color which is measured at 650 nm. The results were calculated from a calibration curve obtained from dilutions of tyrosine in distilled water and expressed as mg tyrosine l^{-1} of milk (Kacem and Kaid-Harche, 2008).

2.8. Exopolysaccharide production

Exopolysaccharide production was evaluated as reported by Kacem and Kaid-Harche (2008). Overnight cultures were streaked on the surface of plates containing ruthenium red milk (10% w/v, skim milk powder, 1% w/v, sucrose and 0.08 g/l ruthenium red, 1.5% w/v agar) (Oxoid). After incubation at 37°C for 24 h, non-ropy strains gave red colonies due to the staining of the bacterial cell wall, while ropy strains appeared as white colonies.

2.9. Haemolytic activities

Blood haemolysis was evaluated on Columbia agar plates (Oxoid) supplemented with 5% sheep's blood which were incubated at 37°C for 24 h (Lombardi et al., 2004; Kacem and Kaid-Harche, 2008).

2.10. Influence of freeze-drying

Resistance of strains to freeze-drying was determined according to the Font de Valdez *et al.* (1985) method modified by Kacem and Kaid-Harche, 2008). Strains were grown in MRS broth (Oxoid) for 18 h at 37°C, centrifuged (10000 g at 4°C for 10 min), and then suspended to the initial volume in a cryoprotective solution containing 100 g/l non-fat milk solids, 100 g/l sucrose, and 4 g/l ascorbic acid. The mixture was frozen at –80°C for 24 h and freeze-drying was then carried out at –50°C for 48 h under 0.01 mbar vacuums (Model Lyph Lock 4.5, Labconco Corporation, Kansas City, MO, USA). Freeze-dried cultures were immediately rehydrated to their initial volume in MRS broth (Oxoid) for 10 min at 37°C, and homogenized by high speed vortexing. Cell survival was then determined by the pour plate method.

3. RESULTS AND DISCUSSION

In this study, a total of 11 *Lactobacillus plantarum* strains were screened for their performance regarding several growth characteristics. This characterization could be used as selection criteria for novel starter cultures in olive fermentations.

The results of carbohydrate fermentation by the 11 strains of lactobacilli tested are shown in Table 1. All strains fermented D and L-arabinose, galactose, D-glucose, inositol, salicin, lactose, cellobiose and inulin. Additionally, Table 2 shows the best two final identifications for each strain on API gallery. Bacteria were identified as *L. plantarum* which confirms our previous results (Kacem *et al.,* 2004).

Table 3 shows the results obtained for the antibiotic susceptibility of the 11 strains tested. Results indicate that our strains were susceptible to most of the antibiotics tested and low multiple

Table 1
Pattern of carbohydrate fermentation by lactobacilli isolates (API 50 CHL micro-identification systems)

Isolates	OL2	OL7	OL9	OL12	OL15	OL16	OL23	OL33	OL36	OL40	OL53
Carbohydrates											
Control	–	–	–	–	–	–	–	–	–	–	–
D-Arabinose	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+
β-Methyl-Xyloside	+	+	–	+	+	+	+	+	+	–	–
Galactose	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	–	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	–	–	+	+	+	+	+	+
Sorbitol	–	+	+	+	+	+	+	+	+	–	–
α-Methyl-D-glucoside	+	–	–	+	+	+	–	+	+	+	–
N-Acetyl-glucosamine	+	+	+	+	+	+	+	+	–	+	+
Amygdalin	+	+	+	–	+	+	+	+	+	+	+
Arbutin	+	+	–	+	+	+	–	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	–	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	–	–
Inulin	+	+	+	+	+	+	+	+	+	+	+
Melezitose	–	+	–	+	–	+	+	+	–	+	–
D-Raffinose	+	+	+	+	+	+	+	+	+	+	+
Starch	+	–	–	–	+	–	–	–	–	+	–
Xylitol	+	+	+	+	–	+	+	+	+	+	+
CD-Lyxose	+	–	–	–	+	–	+	–	–	–	+
Identified as:	*Lactobacillus plantarum*										

The Carbohydrates (glycerol, erythritol, ribose, d-xylose, l-xylose, adonitol, rhamnose, l-sorbose, d-mannose, dulcitol α-methyl-d-mannoside, melibiose, trehalose, glycogen, gentiobiose, d-turanose, d-tagatose, d-fucose d-arabitol, l-arabitol, gluconate,2-ceto-gluconate and 5-ceto-gluconate) are not fermented by strains.

Table 2
Results of the biochemical tests for the identification of the isolated strains using API gallery

Isolates	Identification	% ID	T- index	Type*
L. plantarum OL2	Very good identification	99.6	0.89	S
L. plantarum OL7	Very good identification	99.6	0.89	S
L. plantarum OL9	Excellent identification	99.9	0.94	S
L. plantarum OL12	Very good identification	99.6	0.89	S
L. plantarum OL15	Excellent identification	99.9	1	S
L. plantarum OL16	Very good identification	99.6	0.89	S
L. plantarum OL23	Very good identification	99.2	0.86	S
L. plantarum OL33	Excellent identification	99.9	1	S
L. plantarum OL36	Very good identification	99.6	0.89	S
L. plantarum OL40	Very good identification	99.2	0.86	S
L. plantarum OL53	Very good identification	99.9	0.88	S

(*) S = Significant.

Table 3
Antibiotic susceptibility of lactic acid bacteria isolates

Antibiotic	OL2	OL7	OL9	OL12	OL15	OL16	OL23	OL33	OL36	OL40	OL53
Penicillin G (10µg)	S	S	S	S	S	S	S	S	S	S	S
Ampicillin (10 µg)	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin (30 µg)	S	S	S	**R**	S	S	S	S	S	**R**	S
Oxacillin (1 µg)	S	S	S	**R**	**R**	S	S	S	S	**R**	S
Vancomycin (30 µg)	S	S	S	S	S	S	S	S	S	S	S
Cloramphenicol (30 µg)	S	S	S	S	S	S	S	S	S	S	S
Clindamycin (2 µg)	S	S	S	S	S	S	S	S	S	S	S
Rifampicin (5 µg)	S	S	S	S	S	S	S	S	S	S	S
Tetracyclin (30 µg)	**R**	**R**	**R**	S	**R**	S	S	S	S	S	S
Kanamycin (30 µg)	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
Ciprofloxacin (5 µg)	S	S	S	S	S	S	S	S	S	S	S

(R): Resistant. (S): Sensitive.

resistance was observed. This is not in accordance with various reports indicating that lactic acid bacteria are normally resistant to the principal antibiotics, such as penicillin G, ampicillin, vancomycin, cloramphenicol or ciprofloxacin (Halami *et al.,* 2000 and Coppola *et al*., 2005). In a similar study conducted by Herreros *et al*. (2005) most of the tested *L. plantarum* strains from different sources were resistant to the antibiotics used. In addition, our results show that 4 strains were tetracycline resistant which is in accordance with other reported studies (Danielsen, 2002; Herreros *et al.* 2005). It is well known that vancomycin is an antibiotic belonging to glycopeptide antibiotics and inhibits peptidoglycan synthesis which is an important structural component of the bacterial cell wall. Therefore, Gram-positive bacteria, including lactic acid bacteria are especially vulnerable to vancomycin treatment (Reynolds, 1989). In our case, all strains tested were sensitive to vancomycin. This result does not confirm the finding of Salminen *et al*. (1998), who reported that vancomycin resistance is an intrinsic property of lactobacilli.

Generally, antibiotic sensivity or resistance has been studied in strains of lactobacilli isolated from dairy products, animals or humans (Torriani *et al.,* 1988 and Salminen *et al*. 1998). All these works have reported that several isolates showed multiple resistance to most antibiotics tested. However, our strains of *L. plantarum* tested for sensivity or resistance to antibiotics were isolated from vegetable material (fermented green olives) (Kacem *et al.,* 2004) where this type of treatment is not practiced. This is probably one of the reasons, among others, that can explain our findings.

As shown in Table 4, the values of proteolytic activity of the 11 strains studied ranged from 1.49 ± 0.25 and 5.25 ± 0.11 mg l^{-1} tyrosine at 72 h. Our results are high in comparison with those reported by other authors for lactobacilli isolated from milk (Schmidt *et al*., 1994 and Badis *et al*., 2004).

Lactic acid production is reported in Table 4. These results are not in accordance with those reported by Ayad *et al.* (2004) who indicated that most strains of *L. plantarum* isolated from different sources show a slow acidification rate.

Resistance to freeze-drying is described in Table 4. The results indicate that the strains readily grew in MRS agar after freeze-drying and show high survival percentages (up to 60 ± sd %) (Table 4). This characteristic is critical for the storage of frozen

Table 4
Proteolytic activity, lactic acid production and freeze-drying resistance of the 11 *L. plantarum* strains studied

Strains	Proteolytic activity (mg l^{-1} ± sd tyrosine/72h)	Lactic acid production (mmol/l ± sd)	Freeze-drying resistance (%)
L. plantarum OL2	4.20 ± 0.22	0.997 ± 0.21 (F)*	66 ± 22
L. plantarum OL7	3.56 ± 0.35	0.692 ± 0.18 (M)*	52 ± 23
L. plantarum OL9	3.13 ± 0.41	1.035 ± 0.29 (F)	56 ± 11
L. plantarum OL12	5.25 ± 0.11	0.999 ± 0.11 (F)	68 ± 23
L. plantarum OL15	4.49 ± 0.18	0.998 ± 0.32 (F)	46 ± 20
L. plantarum OL16	3.44 ± 0.15	0.589 ± 0.29 (M)	53 ± 12
L. plantarum OL23	1.49 ± 0.25	0.456 ± 0.23 (S)*	58 ± 17
L. plantarum OL33	3.28 ± 0.15	0.912 ± 0.21 (F)	55 ± 15
L. plantarum OL36	2.12 ± 0.33	0.399 ± 0.29 (S)	42 ± 27
L. plantarum OL40	2.55 ± 0.22	0.556 ± 0.29 (M)	38 ± 14
L. plantarum OL53	4.56 ± 0.14	0.923 ± 0.29 (F)	44 ± 33

*: The cultures were considered as fast, medium or slow acidifying when a ΔpH of 0.4 U was achieved after 3, 3-5 and > 5 h, respectively.
sd: standard deviatio.n

Table 5
Survival percentage of *L. plantarum* strains after their incubation in phosphate buffered saline atvarious pH values

	Survival percentage (% ± sd) after incubation						
	pH 1.0	pH 2.0			pH 3.0		
	2 h	2 h	4 h	6 h	2 h	4 h	6 h
Strain							
L. plantarum OL2	0 ± 0.0	40 ± 2.0	30 ± 2.1	15 ± 0.1	62 ± 2.3	52 ± 0.3	47 ± 3.3
L. plantarum OL7	0 ± 0.0	44 ± 3.1	39 ± 0.1	20 ± 2.1	65 ± 0.2	61 ± 3.2	60 ± 0.2
L. plantarum OL9	0 ± 0.0	49 ± 0.5	40 ± 0.2	19 ± 2.2	76 ± 0.3	72 ± 3.3	65 ± 2.3
L. plantarum OL12	0 ± 0.0	55 ± 2.7	44 ± 0.7	26 ± 1.7	84 ± 0.4	74 ± 1.4	73 ± 1.2
L. plantarum OL15	0 ± 0.0	65 ± 0.3	53 ± 2.3	28 ± 1.4	73 ± 2.1	70 ± 3.1	52 ± 2.1
L. plantarum OL16	0 ± 0.0	54 ± 3.0	24 ± 2.3	11 ± 3.3	64 ± 1.3	56 ± 3.3	51 ± 0.3
L. plantarum OL23	0 ± 0.0	33 ± 2.0	18 ± 2.6	12 ± 2.6	58 ± 2.3	50 ± 0.3	42 ± 1.4
L. plantarum OL33	0 ± 0.0	57 ± 0.6	36 ± 1.6	21 ± 1.0	76 ± 0.6	68 ± 0.4	56 ± 2.4
L. plantarum OL36	0 ± 0.0	52 ± 3.1	24 ± 2.1	11 ± 2.1	71 ± 1.1	55 ± 1.7	51 ± 1.1
L. plantarum OL40	0 ± 0.0	52 ± 0.3	44 ± 0.6	24 ± 0.4	68 ± 0.3	58 ± 2.3	44 ± 1.3
L. plantarum OL53	0 ± 0.0	43 ± 1.2	29 ± 0.2	16 ± 2.2	65 ± 0.4	53 ± 0.1	47 ± 2.1

sd: standard deviatio.n

stock cultures used in industrial production. Finally, none of the strains produced exopolysaccharides or haemolysin in sheep's blood.

Table 5 shows the results of acid tolerance (survival percentage of *L. plantarum* strains at various pH values). All tested strains survived incubation periods of 2 h to 6 h at pH 2.0 and pH 3.0 with a decrease in survival percentage when exposure time progresses for the strains. Generally, *L. plantarum* OL12, OL15, OL16 and OL33 strains survived acidic conditions better than the rest of strains. At pH 2.0, *L. plantarum* OL15 strain showed the highest survival percentage (65 ± 0.3%, 53 ± 2% and 28 ± 1.4%) after 2, 4 and 6 h incubation periods, respectively. No growth occurred after incubation at pH 1 for 2 h. Similar results were reported by Draser *et al.* (1969); Dunne *et al*. (2001) and El-Naggar (2004).

In addition, among these interesting strains, it has been reported in previous studies (Kacem *et al.,* 2005, 2006; Kacem, 2007) that *L. plantarum* OL15 and OL9 strains produced bacteriocins (proteinaceous antimicrobial substances compound) with inhibitory activity against olive spoilage and phytopathogenic bacteria organisms, including, *Pseudomonas* genera that can cause severe damage in olive groves and occasionally on the fruits (Holtsmark and al., 2008). This suggests that these strains are favorable for use as starter cultures for olives production.

4. CONCLUSION

The results obtained in the present study show the survival ability of *L. plantarum* strains tested under the conditions of low pH values. Most strains were fast lactic acid producing and have good proteolytic activity. The strains were susceptible to most of the antibiotics tested, which belong to the major classes of antibiotics used in human clinical therapy. The absence of antibiotic resistance can be considered a positive trait for bacteria used in starter cultures. We expect that the present study will become an increasingly important component of the scientific foundation underpinning the biological control of production and fermentation of olives in Algeria. Further studies are necessary in order to establish if the presence of *L. plantarum* OL15 and OL9 may affect the flavor or rheological characteristics of olives.

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