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Isolation of lactic acid bacteria for its possible use in the fermentation of green algerian olives

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RESUMEN

Aislamiento de bacterias del ácido láctico para su posible uso en la fermentación de aceitunas verdes argelinas.

Este estudio se emprendió con el objetivo de obtener bacterias del ácido láctico con capacidad para utilizarse como cultivo iniciador en la fermentación de aceitunas. Por esta razón, 32 cepas de bacterias del ácido láctico procedentes de fermentaciones espontáneas de aceitunas verdes se caracterizaron e identificaron en función de criterios morfológicos y bioquímicos. Catorce cepas se identificaron como *Lactococcus lactis*, 11 cepas como *Lactobacillus plantarum* y 7 cepas como *Enterococcus* sp. De las 18 cepas que se examinaron para detectar actividades antagónicas, se encontró que 3 cepas de *Lactobacillus plantarum* y una de *Enterococcus* sp. mostraban zonas de inhibición contra 5 cepas indicadoras de bacterias del ácido láctico aisladas en este estudio. El sobrenadante libre de células *Lactobacillus plantarum* OL9 fue activo contra diversas bacterias Gram-positivas (*Lactobacillus, Enterococcus y Propionibacterium*) y contra una cepa de bacteria Gram-negativa relacionada con alteraciones (*Erwinia*).

PALABRAS-CLAVE: Aceitunas - Antagonismo - Bacterias del ácido láctico - Identificación.

SUMMARY

Isolation of lactic acid bacteria for its possible use in the fermentation of green algerian olives.

This study was undertaken with the aim of obtaining lactic acid bacteria with the ability to ferment olives for possible use as starter cultures. For this reason, 32 isolates of lactic acid bacteria isolated from the spontaneous fermentation of green olives were characterized and identified on the basis of morphological and biochemical criteria. 14 of them were identified as *Lactococcus lactis*, 11 isolates as *Lactobacillus plantarum* and 7 isolates as *Enterococcus* sp. Of the 18 isolates examined for antagonistic activity, 3 isolates of *Lactobacillus plantarum* and one isolate of *Enterococcus* sp. were able to give distinct zones of inhibition against 5 indicator strains of lactic acid bacteria isolated in this study. Cell free supernatant of *Lactobacillus plantarum* OL9 was active against Gram-positive bacteria (*Lactobacillus, Enterococcus and Propionibacterium*) and also against one Gram-negative bacteria strain of spoilage significance (*Erwinia*).

KEY-WORDS: Antagonism - Identification - Lactic acid bacteria - Olives.

1. INTRODUCTION

Lactic fermentation permits the maintenance of high organoleptic, along with the nutritional and quality characteristics of several vegetables such as cucumbers, carrots, and olives and increases their availability (Andersson *et al.*, 1990 and Vescovo *et al.*, 1995).

Lactic acid bacteria are naturally involved in various olive fermentations and are consequently used as starter cultures (Etchells *et al.*, 1966; Ruiz-Barba *et al.*, 1991; Leal-Sánchez *et al.*, 1998). Various starter cultures of lactic acid bacteria produce several antimicrobial substances, including organic acid, hydrogen peroxide, diacetyl and bacteriocins (Vandenbergh, 1993). Therefore, the application of starter cultures after eliminating the bitter substances are recommended to initiate the fermentation of olives and also to increase their availability (Borcakli *et al.*, 1993b).

Many studies have focussed on the isolation of lactic acid bacteria from traditionally fermented olives. Vandenberg *et al.* (1993) reported that the natural microflora of Portuguese olives is represented essentially by *Lactobacillus plantarum* and *Lactobacillus paracasei*.

In the case of Spanish olive fermentation, *Lactobacillus plantarum* was mainly isolated as representative of the group of lactic acid bacteria (Ruiz-Barba *et al.*, 1991; Ruiz Barba *et al.*, 1994). In a previous study, Borcakli *et al.* (1993a) reported that the microbial flora of Turkish fermented olives are mainly composed of Gram- negative bacteria and yeasts while, *Lactobacillus plantarum* are detected at the end of the fermentation.

In Italy, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc* spp., *Enterococcus faecium* and *Enterococcus* spp. were isolated from olive phylloplane and olive brines in Apulia. The isolates were analyzed for several physiological properties related to the olive fermentation process such as acidification properties, lipolytic activity, synthesis of exopolysaccharides, production of antimicrobial substances, and hydrolysis of the bitter glucoside oleuropein (Lavermicocca *et al.*, 1998, 2002). More recently, *Lactobacillus casei* species were isolated from

naturally fermented Sicilian green olives (Randazzo *et al.,* 2004).

Olives are one of the major agricultural products of Algeria. However, starter cultures are not employed during fermentation of this product. This process is still performed at the household or domestic factory level by simply allowing the fruit to ferment spontaneously. Moreover, fermented products retained their desirable quality even after conservation for long periods of time at room temperature. No information exists on the indigenous lactic acid bacteria microflora of Algerian fermented olives.

The isolation of lactic acid bacteria from naturally fermented olives seems to be interesting in order to offer their possible use as starter cultures for the fermentation of olives. This might preserve the fermented products from the introduction of abnormal flavors and / or textures. To achieve this goal, it will be necessary to characterize the lactic acid microflora and select the appropriate strains of lactic acid bacteria.

The aim of the present study was to isolate lactic acid bacteria from naturally fermented green olives produced in western Algeria. This study also includes the characterization of isolates based on phenotypic criteria and antagonistic activity production of the isolates.

2. MATERIAL AND METHODS

2.1. Samples

In the present study, 11 samples of traditionally fermented green olives were obtained from domestic factories located in western Algeria. After collection, samples were transported to the laboratory in ice-containing thermoflasks and analyzed on arrival.

2.2. Isolation and counting of bacteria

Fifty grams of olives were homogenized by grinding with 10 ml of a 1% (w/v) sterile peptone solution. After homogenization, serial decimal dilutions (10^{-2} to 10^{-5}) were made in a 1% peptone solution and spread on plates in duplicate.

Lactobacilli were numbered in MRS agar medium (g/L, peptone 10.0, meat extract 8.0, yeast extract 4.0, D(+) glucose 20.0, di-potassium hydrogen phosphate 2.0, tween-80 1.0, di-ammonium hydrogen citrate 2.0, sodium acetate 5.0, magnesium sulphate 0.2, manganese sulphate 0.04 supplemented with 14.0 or 7.0g agar-agar for solid or soft MRS, respectively,) (de Man *et al.*, 1960) under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 3 days.

Lactococci were counted in M17 agar medium (Difco) (Terzaghi and Sandine, 1975) after incubation for 2 days at 30°C.

After bacterial counts, 8 colonies were randomly picked from both the MRS and M17 agar plates.

2.3. Morphological, physiological and biochemical examination.

Cell shape, cell arrangement, Gram-staining, catalase activity (3% H_2O_2), production of gas from glucose (in 1% glucose with Durham tubes), temperature requirement (15, 40 and 45°C), NaCl tolerance (4, 6.5, 8 and 10% NaCl) and growth at pH 3.9 and 9.6 were performed in M17 or MRS broth.

L-and D-lactic acids were analyzed enzymatically by the kit (F-Kit L-Lactic acid/D-Lactic acid, Roche diagnostic, Mannheim, Germany) according to the manufacturer's instructions.

Cocci, Gram-positive and catalase-negative from M17 agar:

Homofermentative cocci, which were capable of growing at 15 and 40°C but not at 45°C or at pH 9.6, were considered as lactococci according to the methods and criteria of Mundt (1986). The following test was carried out on each isolate using the Api 20 STREP (API-System, S.A., La Balme Les Grottes, Montalieu-Vercieu, France) according to the manufacturer's instructions. Incubation was at 30°C for 48 hours

Homofermentative cocci, grouped in pairs or short chains, which grew at 15, 40 and 45°C, survived after heating at 60°C after 30 min, and grew at pH 9.6 were considered as enterococci (Devriese *et al*., 1987). L-arabinose, arbutin, melizitose, melibiose, sorbitol, lactose, starch, ribose and sucrose fermentation were determined by adding the test substances after autoclaving to the basal medium (M17 broth without glucose but with 40 mg/l bromocresol purple). Tubes were inoculated and incubated at 30°C for 3 days.

Rods, Gram-positive and catalase-negative from MRS agar:

Homofermentative lactobacilli isolates were characterized according to the criteria of Kandler and Weiss (1986) and Schillinger and Lüke (1989). Arginine hydrolysis was tested in MRS broth containing 3 g/l arginine and 2 g/l sodium citrate replacing ammonium citrate. Ammonia was detected using Nessler's reagent.

Acetoin production was determined in MRS broth using the Voges-Proskauer test.

Identification of lactobacilli was performed with the API 50 CHL micro-identification systems (API-System, S.A., La Balme Les Grottes, Montalieu-Vercieu, France) at 30°C for 48 hours.

Table I

Bacterial species used for inhibition study. (CFS1 and CFS2 are Cell Free Supernatants obtained from *L. plantarum* OL9 and *L. plantarum* OL15 respectively). The producer isolate was in MRS broth, then centrifuged to remove cells. The supernatant was tested against *Lactococcus lactis* BO8 (indicator strain) as well as 19 other bacterial species by well diffusion method. The antibacterial activity was evaluated by measuring the diameter of inhibition zones

Bacterial species	Diameter of inhibition (mm)		Source
	CFS1	CFS2	
Lactococcus lactis BO8	13	11	Isolated in this study
Lactococcus lactis TF18	12	11	
Lactococcus lactis NS63	13	11	
Lactococcus lactis LB88	10	11	
Lactobacillus salivarius B23	12	0	
Lactococcus lactis CHT1	9.5	0	
Lactococcus lactis CHT5	13	0	
Lactococcus lactis CHT7	13	0	Our collection of strains
Lactococcus lactis CHT9	13	0	
Lactococcus lactis CHT14	14.3	0	
Lactococcus lactis CHT16	9.5	0	
Lactococcus lactis CHT27	10	0	
Enterococcus faecalis CHT15	10.5	10	
Enterococcus faecalis CHT28	12	0	
P. freudenriechii shermanii 9615	10	0	
P. freudenriechii shermanii 9619	14	0	
P. freudenriechii shermanii 1367	15	0	
P. freudenriechii shermanii 8262	13	0	
Erwinia chrysanthemi 1254	11	0	
			ATCC
Lc. lactis subsp. *lactis* ML3	11	0	CNRZ

CNRZ Centre National de la Recherche Zootechnique (Jouy-en-Josas, France)
ATCC: American Type Culture Collection.

All Isolates were stored at 4°C in sterile (120°C, 10 min) (10%) reconstituted skim milk or at -20°C in MRS broth supplemented with 20% glycerol.

2.4. Bacterial interaction

Isolates were examined for their antagonistic activity against organisms by the direct (Fleming *et al.*, 1975) or well diffusion (Barefoot and Klaenhammer, 1983) method.

Direct method

Isolates to be tested for production of antimicrobial compounds were spotted (5 %l of 10^6 cfu / ml from 18-hour culture in MRS broth) onto the surface of agar plates (MRS with 1.5% agar) and incubated overnight at 30°C.

Isolates to be tested for sensitivity were inoculated (0.2 ml of 10^4-10^5 cfu / ml from 12-hour cultures in MRS broth) into soft MRS agar (7 ml, 0.7% agar) and poured over the plates on which the putative producer had grown. Inhibitory activity due to the action of H_2O_2 was excluded by the addition of catalase (1mg/ml) to the overlaying agar (Geis *et al.*, 1983).

In order to minimize the effect of pH, modified MRS agar was prepared in a 0.2 M potassium phosphate buffer (pH 7.0) and the lactose level was reduced to 0.25% (Charmaigne and Shaw, 1990).

The antibacterial activity was evaluated after 18 hours of incubation at 30°C by measuring the inhibition zones. Only zones wider than 10 mm in diameter were scored positive. Bacteria, which showed inhibition by this direct method, were examined afterwards by the well diffusion method.

Well diffusion method

Prepoured MRS agar plates were overlaid with 7 ml MRS soft agar containing 0.2 ml of indicator cultures. Wells sized 5 mm in diameter were cut into off the agar plate using a sterile Durham tube and 100 µl of the culture supernatant fluid was placed

Table II

Characteristics and identification of the lactococci isolates[a]

Isolates	B01	B08	B019	B021	B05	B018	B025	B027	B029	B040	B045	B063	B064	B097
Gram stain							+							
Cell Shape							Cocci							
Catalase test							-							
Growth at or in:														
15°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6,5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH 9.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactic acid isomer							L(+)							
Fermentation type							h							
Api 20 STREP Syst:														
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyrrolidonyl arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	+	-	-	-	-	+	-	-	-
β-Galactosidase							+							
β-Glucuronidase							-							
Arginine hydrolysis							+							
Acetoin							-							
Fermentation of														
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	+	-	-	-	+	+	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Identified as	*Lactococcus lactis*													

[a] +: positive; -: negative; h: homofermentation

into each well. After overnight incubation at 30°C, the plates were examined for zones of inhibition around the wells.

Preparation of cell free culture supernatants

Producer isolates were grown in 5 ml of MRS broth for 18 hours at 30°C, then centrifuged at 7000 g for 15 minutes at 4°C to remove cells. The supernatant was adjusted to pH 7.0 with a 3M NaOH solution and then filter-sterilized (0.22-μm pore size filter, Gelman Acrodisc 13, Pall Corp., Ann Arbor, USA). The solution thus obtained, designated as Cell Free Supernatant (CFS), was stored at 4°C and –20°C.

2.5. Search for antimicrobial spectrum

Inhibitory activity of CFSs was tested against many lactococci, lactobacilli and other non-lactic acid bacteria by the well diffusion method (Table I). Lactobacilli were tested in MRS agar under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 18 hours, Propionibacteria in YELA agar (Malik *et al.*, 1968) at 32°C for 48 hours and *Erwinia* in Nutrient agar at 25°C for 3 days.

3. RESULTS

In all olive samples, mean values of microbial counts ranged from 3.0×10^4 to 6.0×10^6 cfu / ml in MRS agar and 1.1×10^4 to 2.0×10^5 cfu / ml in M17 agar.

A total of 32 lactic acid bacteria (Gram positive, catalase negative and. cocci or rods) were selected from agar plates of MRS or M17 media for identification as described in Materials and Methods. Yeasts were detected in all samples and were discarded from this study.

The results of the identification are summarized in Tables II and III for lactococci and enterococci respectively.

Of 21 isolates obtained from M17 agar, 14 were identified as *Lactococcus lactis* and 7 as *Enterococcus* sp.

Eleven isolates of lactobacilli picked from MRS agar grew at 15°C but not at 45°C or at 10% NaCl. They produce L-Lactic acid with no gas production from glucose and have the ability to hydrolyze arginine. All of these characteristics, together with the API 50 CHL pattern of carbohydrate fermentation, identified the 11 isolates as *Lactobacillus plantarum* (Table IV).

A total of 18 isolates represented by 10 isolates of *Lactobacillus plantarum*, 5 isolates of *Enterococcus*

Table III
Characteristics of the enterococci isolates[a]

Isolates	0L17	0L20	0L32	0L35	0L37	0L98	0L106
Gram stain				+			
Cell shape				cocci			
Arrangement of cells				chains or pairs			
Catalase test				-			
Growth at or in:							
15°C	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+
60°C after 30 min	+	+	-	+	+	+	+
6.5%NaCl	+	+	+	+	+	+	+
8.0% NaCl	+		+	+	+	+	+
10% NaCl	-	-	-	+	+	-	-
pH 9.6	+	+	+	+	+	+	+
Lactic acid isomer	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)
Fermentation type	h	h	h	h	h	h	h
Acetoin	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	-	+	+	+
D-arabinose	+	+	+	+	+	+	+
arbutin	-	-	-	+	+	-	-
lactose	+	+	-	-	+	+	-
ribose	+	+	+	+	+	+	+
sorbitol	+	-	+	-	+	+	+
starch	+	+	+	+	+	+	+
sucrose	+	+	+	-	-	+	+
melibiose	-	+	+	+	-	-	-
melezitose	-	+	-	-	-	-	-
Identified as:				*Enterococcus* sp.			

[a] +: positive; -: negative; h: homofermentation

sp. and 3 isolates of *Lactococcus lactis* were screened for their antagonistic activity against 5 indicator strains which were isolated in this study (*Lactococcus lactis* BO8; *Enterococcus faecalis* OL37 and OL98; *Lactobacillus plantarum* OL16 and OL23).

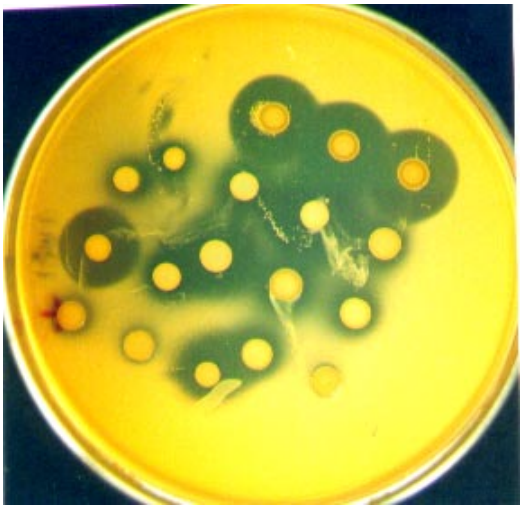

Figure 1
Demonstration of the bactericidal mode of action of lactic acid bacteria as evidenced by the inhibition zones around the spot of the lactococcal strains on a preincubated lawn of *Lactococcus lactis* BO8.

A demonstration of antagonism exhibited by the isolates is shown in Figure 1. Among the isolates tested for inhibition, *Lactobacillus plantarum* OL2, OL9, and OL15 and *Enterococcus faecalis* OL20 exhibited the broadest activity spectrum because they could inhibit all indicator isolates. The other *Lactobacillus plantarum* or *Enterococcus* sp. isolates exhibited antagonistic effects against 1 to 3 indicators. This inhibition was not prevented by the addition of catalase to the agar plates. None of the lactococci showed inhibition against the indicator strains used in this study.

Using the well diffusion method, only cell free supernatants of *Lactobacillus plantarum* OL9 (CFS1) and *Lactobacillus plantarum* OL15 (CFS2) were inhibitory against the indicator strain BO8 of *Lactococcus lactis* (data not shown).

The inhibitory action of filter-sterilized supernatants was also tested against 19 Gram-positive and Gram-negative bacteria.

As shown in Table I, all tested strains were inhibited by CFS1, while only 3 *Lactococcus lactis* and one *Enterococcus faecalis* were inhibited by CFS2.

Notably, the supernatant from *Lactobacillus plantarum* OL9 (CFS1) proved its ability to inhibit *Erwinia chrysanthemi* ATCC 1254, a Gram-negative bacteria mainly associated with vegetable spoilage (Brenner *et al.*, 1973 and Kwon *et al.*, 1997).

Table IV

Pattern of carbohydrate fermentation by lactobacilli isolates (API 50 CHL micro-identification systems). Readings were done under anaerobic conditions after 48 hours at 30°C

Isolates	OL2	OL7	OL9	OL12	OL15	OL16	OL23	OL33	OL36	OL40	OL53
Carbohydrates											
Control	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+
Ribose	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-
ßMethyl-Xyloside	+	+	-	+	+	+	+	+	+	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	-	-	-	-	-	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-
Inositol	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	-	-	+	+	+	+	+	+
Sorbitol	-	+	+	+	+	+	+	+	+	-	-
aMethyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-
aMethyl-D-glucoside	+	-	-	+	+	+	-	+	+	+	-
N-Acetyl-glucosamine	+	+	+	+	+	+	+	+	-	+	+
Amygdalin	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	-	-	-	-	-	+	+	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-
Inulin	+	+	+	+	+	+	+	+	+	+	+
Melezitose	-	+	-	+	-	-	+	+	-	-	-
D-Raffinose	+	+	+	+	+	+	+	+	+	+	+
Starch	+	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-
Xylitol	+	+	+	+	-	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	+	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-
2-Ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-
5-Ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-
Identified as:	*Lactobacillus plantarum*										

4. DISCUSSION

It is well documented that *Lactobacillus plantarum* is one of the species of lactic acid bacteria most often found in fermented olives. Therefore, this species has been extensively studied with the aim of its use in starter cultures (Delgado *et al*., 2001 and Leal-Sánchez *et al*., 2002).

From the results presented here, it is clear that lactic acid bacteria represented by 14 isolates of *Lactococcus lactis,* 7 of *Enterococcus* sp. and 11 of *Lactobacillus plantarum* were isolated from the spontaneous fermentation of green olives produced in western Algeria. This result is not in complete agreement with those reported by Ruiz-Barba *et al.* (1991, 1994) who isolated only strains of *Lactobacillus plantarum* from Spanish fermented olives. In similar works, Fernández-Díez (1983) and Vandenberg *et al.* (1993) have shown that the natural microflora of Portuguese olives was represented essentially by *Lactobacillus plantarum* and *Lactobacillus paracasei* species. In a previous study, Fernández González *et al.* (1993) and Harris (1998) reported that the indigenous lactic acid bacteria change spontaneously during the fermentation of natural olives. At the end of the process only *Lactobacillus plantarum* is detected. Our study shows that, *Lactococcus lactis* was isolated from fermented olive samples. To our knowledge, the presence of this bacterium in fermented olives has not yet been reported. These isolates are capable of growing in olives and are able to reach counts of 10^5 cfu /ml. Also, it was found that, all isolates of *Lactococcus lactis* isolated and identified in this study could grow in up to 8% salt concentration and at 15^{o}C temperature. So it is necessary to search suitability of isolates for olive fermentation.

We have likewise isolated members of the *Enterococcus* genus in this study. This bacterium has also been isolated as constituents of the dominant flora from a Spanish-style green olive fermentation and isolates have been identified and characterized (Floriano *et al*., 1998). In a previous study, Lavermicocca *et al.* (2002) reported that, *Lactobacillus plantarum, Enterococcus faecium* and *Enterococcus* spp. were isolated from olive brines in Apulia. Fermentations were carried out with the above strains (single or mixed cultures) on green and black table olives using brines containing 3% NaCl and 1% saccharose. In mixed cultures, *Lactobacillus plantarum* developed and persisted until the end of fermentation, while *Enterococcus faecium* survived for about 20 days.

Of the 18 isolates screened, 4 exhibited a broad activity spectrum (in solid medium) against indicator cultures. Of these isolates, only two (*Lactobacillus plantarum* OL9 and OL15) were found to produce (in a liquid medium) an active agent when tested by the well diffusion method. Similar results have been reported by Geis *et al*. (1983) or Klaenhammer (1988) who detected activity only in an agar medium. Other authors have also found that cell-free culture supernatants were inhibitory (Schillinger and Lüke, 1989; Stiles, 1997).

The fact that certain isolates showed no antimicrobial activity against the lactic acid bacteria that we isolated in this study, may not indicate the actual number of inhibitory strains, because factors other than pH, growth medium and indicator strain used are involved (Parente and Ricciardi, 1999).

Cell free supernatant of *Lactobacillus plantarum* OL9 exhibited the broadest activity spectrum against other *Lc. lactis, Lactobacillus* and *Enterococcus* strains and also against *Propionibacterium* and *Erwinia*. This type of result was also described by Jiménez-Díaz *et al*. (1993) where twenty-six strains of *Lactobacillus plantarum* isolated from green olive fermentations were tested for cross-antagonistic activities in an agar drop diffusion test. Only cell-free supernatants from four of these strains were shown to inhibit the growth of at least one of the *Lactobacillus plantarum* indicator strains. The inhibitory compound from this strain was active against propionibacteria as well as natural competitors of *L. plantarum* in olive fermentation brines. However in our study, we noticed that the cell-free supernatant of *Lactobacillus plantarum* OL9 was shown to inhibit the growth of *Propionibacterium* and also *Erwinia chrysanthemi* ATCC 1254. To our knowledge the inhibition of *Erwinia* by lactic acid bacteria as well as by their anti-microbial compounds has not been shown in any previous report. This opened a good perspective for *Lactobacillus plantarum* OL9 to be used as a starter culture for fermentation and preservation of olives as well as other fermented vegetable products

6. CONCLUSION

The identification of our isolates on the basis of physiological and morphological characteristics and sugar fermentation is not definite. In the future, it would be interesting to conduct a more detailed study on bacterial identification using molecular methods.

Also, this study demonstrated that *Lactobacillus plantarum* OL9 isolated from the spontaneous fermentation of green olives produced in a liquid medium a factor that was inhibitory to some Gram-positive and Gram-negative bacteria. Therefore, this might be investigated in order to elaborate an adequate starter culture, which would permit the uniform manufacturing of fermented green olives, and preserve the quality characteristics of the traditional product as much as possible. We are currently investigating the nature of this (or these) inhibitory agent(s).

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