

Isolation of Lactic Acid Bacteria from Naturally Fermented Algerian Olives

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Abstract. Twenty-two isolates of lactic acid bacteria isolated from the spontaneous fermentation of olives were identified on the basis of the phenotypic criteria. Eleven of them were identified as *Lactobacillus plantarum* which was followed by 4 *Lactococcus lactis* ssp. *lactis* and 7 *Enterococcus* spp. Cell free supernatant of *Lactobacillus plantarum* OL9 was active against *Lactobacillus*, *Enterococcus*, *Propionibacterium* and *Erwinia* (of spoilage significance). The antibacterial activity was attributed to bacteriocin like substances.

Keywords: Olives, Lactic acid bacteria, Identification, Antagonism.

Introduction

Lactic acid bacteria are naturally involved in various olive fermentations and are consequently used as starter cultures [1-7]. Various starter cultures of lactic acid bacteria produce several antimicrobial substances [8]. Therefore, application of starter cultures after eliminating the bitter substances are recommended to initiate the fermentation of olives and also to increase their availability [9].

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 factories level by simply allowing the fruit to ferment spontaneously. Moreover, fermented products retained their desirable quality even after conservation for long period of time at ambient temperature. No information exists on indigenous lactic acid bacteria microflora of Algerian fermented olives.

It seems to be interesting to isolate lactic acid bacteria from naturally fermented olives in order to offer their possible use as starter cultures for the fermentation of olives. This might preserve the fermented products from a abnormal flavours 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 aims of the present study was to isolate lactic acid bacteria from naturally fermented green olives produced in western Algeria. This study also included characterization of isolates based on phenotypic criteria and antagonistic activity production of the isolates.

Materials and Methods

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.

Isolation and identification of lactic acid bacteria

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

Lactobacilli were enumerated in MRS agar medium (Oxoid) under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 3 days. Lactococci were counted in M17 agar medium (Oxoid) after incubation for 2 days at 30°C. After bacterial counts, 8 colonies were randomly picked from both the MRS or M17 agar plates. Cell shape, cells arrangements, Gram-staining, catalase activity (3% H₂O₂), production of gas from glucose (in 1% glucose with Durham tubes), temperature requirement (15, 40 and 45°C), NaCl tolerance (4 and 6.5% NaCl) and growth at pH 3.9 and 9.6 were performed in M17 or MRS broth.

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 [10, p. 1244-1251]. 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 [11]. The following tests were 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 lactobacilli isolates were characterized according to the criteria of Kandler & Weiss [12, p. 1208-1234]. 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.

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.

Inhibition study

Isolates were examined for their antagonistic activity against organisms (Table 1) by the direct [13] or well diffusion methods [14]. Lactobacilli were tested in MRS agar under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 18 hours, Propionibacteria in YELA agar [15] at 32°C for 48 hours and *Erwinia* in Nutrient agar at 25°C for 3 days. The characterization and identification of the antibacterial factors was done in the Cell Free Supernatant (CFS) obtained from producer isolates.

Table 1. Bacterial species used for inhibition study

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

* CFS1: Cell free supernatant of *Lactobacillus plantarum* OL9.

**CFS2: Cell free supernatant of *Lactobacillus plantarum* OL9.

** : American Type Culture Collection.

*** : Centre National de la Recherche Zootechnique (Jouy-en-Josas, France).

Results

A total of 22 of lactic acid bacteria were selected from agar plates of MRS or M17 media (cocci or rods, Gram positives and catalase negatives). The results of the identification summarized in Table 2 for lactococci and enterococci. Of 21 isolates obtained from M17 agar, 4 were identified as *Lactococcus lactis* ssp. *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* (Tables 3 and 4). Among the isolates tested for inhibition, *Lactobacillus plantarum* OL2, OL9, and OL15 and *Enterococcus faecalis* OL20 exhibited the broadest activity spectrum because they inhibited 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 shown inhibition against the inhibitor 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* ssp. *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 1, 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 [16, 17]. The characterization of the antibacterial substances revealed that they were extremely heat stable (2 h at 100°C and 20 min at 121°C) and active at acidic pH values. Antagonistic activity CFS was inactivated by protease (Chymotrypsin, Trypsin, and Proeinase K), and greatly reduced when treated with SDS or Triton X-100. In accordance with the guidelines outlined by Tagg *et al.* [18], the antibacterial activity could be attributed to bacteriocin like substances.

Discussion

From the results presented here, it is clear that lactic acid bacteria represented by 11 of *Lactobacillus plantarum*, 7 of *Enterococcus* sp. and 4 isolates of *Lactococcus lactis* ssp. *lactis* 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.*, [19] who isolated only strains of *L. plantarum* from Spanish fermented olives. In similar works, Fernández-Diez [20, pp. 379-397] and Van Den Berg *et al.* [21] have shown that the natural microflora of Portuguese olives was represented essentially

Table 2. Characteristics of the lactococci and enterococci isolates^a

Isolates	OL1	OL8	OL19	OL21	OL17	OL20	OL32	OL35	OL37	OL98	OL106
Gram stain			+								
Cell Shape			Cocci								
Catalase test	-	-	-	-	-	-	-	-	-	-	-
Growth at or in:											
40°C	+	+	+	+	+	+	+	+	+	+	+
45°C	-	-	-	-	+	+	+	+	+	+	+
60°C after 30 mn	n	n	n	n	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+	+	+	+
6,5% NaCl	-	-	-	-	+	+	+	+	+	+	+
pH 9.6	-	-	-	-	+	+	+	+	+	+	+
Fermentation type	h	h	h	h	h	h	h	h	h	h	h
<i>Api 20 STREP</i>											
<i>System:</i>											
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Pyrrolidonyl arylamidase	+	+	+	-	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	-	-	-	-	-	-
β-Galactosidase	+	+	+	+	+	+	+	+	+	+	+
β-Glucuronidase	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Acetoïn	-	-	-	-	+	+	+	+	+	+	+
Fermentation of											
Ribose	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	+	+	+	+	+	+	+
Mannitol	+	-	-	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-
Starch	+	+	-	+	+	+	+	+	+	+	+
Glycerol	-	-	-	-	+	+	+	+	+	+	+
Identified as			<i>Lc. lactis ssp lactis</i>				<i>Enterococcus faecalis</i>				

Lc: Lactococcus, ^a+: positive; -: negative; h: homofermentation; n: not performed.

Table 3. Characteristics of lactobacilli isolates^a

Isolates	OL2	OL7	OL9	OL12	OL15	OL16	OL23	OL33	OL36	OL40	OL53
Gram stain											
Cell shape											
Catalase test											
Growth at or in:											
15°C	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	-	-	-	-	-	-
6.5%NaCl	+	+	+	+	+	+	+	+	+	+	+
pH 3.9	+	+	+	+	+	+	+	+	+	+	+
Fermentation type	h	h	h	h	h	h	h	h	h	h	h
Acetoïn	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+

^a+: positive; -: negative; h: homofermentation; n: not performed.

Table 4. Pattern of carbohydrate fermentation by lactobacilli isolates (API 50 CHL micro-identification systems). Readings were done under anaerobic conditions at 30°C for 48 hours

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

by *Lactobacillus plantarum* and *Lactobacillus paracasei* species. In a previous study, Fernández Gonzalez *et al.* [22] and Harris [23, pp. 45-72] reported that the indigenous lactic acid bacteria change spontaneously during natural olive fermentations. At the end of the process, only *Lactobacillus plantarum* is detected. 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 [24, 25].



Fig. 1. Direct method showing antibacterial activity in isolates. Indicator strain is *L. lactis ssp. lactis* OL8. Isolates to be tested for production of an antimicrobial compounds were spotted onto the surface of agar plates and incubated overnight. Isolates to be tested for sensitivity were inoculated into soft MRS agar and poured over the plate on which the producer had grown. Plates were then incubated overnight.

Our study shows that *Lactococcus lactis ssp. lactis* was isolated from fermented olive samples. To our knowledge, the presence of this bacterium in fermented olives has not been reported yet. These isolates are capable of growing in olives and are able to reach counts of 10^5 cfu/ml. Members of the *Enterococcus* genus were also isolated in this study. This bacterium has been also isolated as constituents of the dominant flora from a Spanish-style green olive fermentation and isolates have been identified and characterized [26].

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 liquid medium) an active agent when tested by well diffusion method. Similar results have been reported by Geis *et al.* [27] or Klaenhammer [28] who detected activity only in agar medium. Another study showed also that cell-free culture supernatants were inhibitory [29].

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 Jémenez-Díaz *et al.* [29] where 26 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 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 antimicrobial compounds has not been shown in any previous report.

Conclusion

This study demonstrated that *Lactobacillus plantarum* OL9 and OL15 isolated from naturally fermented olives produced in liquid medium factor that was inhibitory to some Gram-positive or Gram-negative bacteria. Therefore, it might be investigated in order to elaborate an adequate starter culture which would permit the manufacturing homogeneously fermented green olives, and preserve the quality characteristics of the artisanal product as much as possible. The nature of this (or these) inhibitory agent(s) are currently investigated.

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ملخص البحث. تم عزل مجموع ٢٢ عزلة لبكتيريا حمض اللبن من الزيتون الأخضر المتخمر في الغرب الجزائري، وتم التعرف من خلال ذلك على ١١ عزلة من *Lactobacillus plantarum*. وأربعة عزلات من *Lactococcus lactis* و سبعة عزلات *ssp. lactis* و سبعة عزلات *Enterococcus faecalis*. كما تم دراسة التضاد البكتيري لدى ١٨ عزلة وتبين من خلال النتائج المتحصل عليها. قدرة ثلاثة عزلات من *Lactobacillus plantarum* وعزلة واحدة من *Enterococcus faecalis* على تثبيط نمو خمسة عزلات أخرى من بكتيريا حمض اللبن. كما ثبت طافئ وسط غذاء العزلة *Lactobacillus plantarum* OL9 نمو بكتيريا أخرى موجبة لصبغة جرام *Lactobacillus*, *Enterococcus*, *Propionibacterium* بالإضافة لسلالة بكتيرية سالبة موجبة لصبغة جرام *Erwinia*.

