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Lactic acid bacteria isolated from fermented green olives produced in Western Algeria

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عزل للبكتيريا اللبنية من الزيتون الأخضر المتخمر المنتج في الغرب الجزائري

تم عزل 23 عازلة للبكتيرية اللبنية من 10 عينات من زيتون المائدة المخمر في الغرب الجزائري. واعتمدت طريقة التشخيص والتعريف البكتيري على الفحص المجهري والخصائص البيوكميائية والقدرة على تخمير عدد من السكريات وتم التعرف من خلال ذلك على 11 عازلة من Lactobacillus plantarum خمسة عزلات من Lactococcus lactis ssp. lactis وسبعة عازلات Enterococcus faecium تهدف هذه الدراسة الأولية إلى تشكيل مجموعة بكتيرية محلية

الكلمات المفتاحية : البكتيرية اللبنية ـ Lactococcus ـ Lactobacillus ـ Enterococcus ـ زيتون

Bactéries lactiques isolées d'olives vertes fermentées produites dans l'Ouest algérien

Vingt trois isolats de bactéries lactiques ont été isolés à partir de 10 échantillons d'olives vertes fermentées dans l'ouest algérien. Elles ont été caractérisées et identifiées sur la base de l'observation microscopique, des propriétés biochimiques et la capacité de fermentation des sucres. Onze souches étaient identifiées à l'espèce *Lactobacillus plantarum*, 7 à l'espèce *Enterococcus faecium* et 5 à l'espèce *Lactococcus lactis* ssp. *lactis*. Cette étude préliminaire a pour objectif l'élaborationd'une collection locale de culture bactériènnes starters.

Mots clés: Bactéries lactiques - *Lactococcus* - *Lactobacillus* - *Enterococcus* - Olives - Identification

Lactic acid bacteria isolated from fermented green olives produced in Western Algeria

A total of 23-isolates of lactic acid bacteria were isolated from 10 samples of fermented green olives in Western Algeria. They were characterized and classified on the basis of microscopic analysis and phenotypic characteristics. Eleven isolates were identified as *Lactobacillus plantarum* which was followed by seven isolates of *Enterococcus faecium* and five isolates of *Lactococcus lactis* ssp. *lactis* This preliminary study was carried out to make a local collection of starter cultures.

Key words: Lactic acid bacteria - *Lactococcus* - *Lactobacillus* - *Enterococcus* - Olives - Identification

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INTRODUCTION

Olives are mainly produced in the mediterranean region. They are particularly popular in Algeria. However, the national production at an industrial scale is still limited to olive oil. When harvested, olives undergo rapid deteriorations, especially in the high moisture regions where the prevailing environmental conditions may accelerate the process of decomposition (Battcock & Azam-Ali, 1998).

Olive fermentation as a preserving method is a process involving starter cultures of lactic acid bacteria pickling (Fleming et *al.*, 1969; de Castro *et al.*, 2002). However this process is still performed at the household or domestic factories level in a majority of algerian countries. No starters are used and fermentation is obtained by allowing the fruit to ferment spentaneously for three to six weeks, depending on the ambient temperature.

Upgrading the production of fermented olives from the household to the industrial level with consistent quality, may requir in a first step, the isolation and selection of the microorganisms associated with the traditional fermentations. In recent years, a considerable number of studies have focused on isolation of lactic acid bacteria from fermented olives in order to use them as starter cultures in various fermentations (Lavermicocca *et al.*, 1998; Randazzo *et al.,* 2004). Fernández-Diéz (1983) and Van Den Berg *et al.* (1993) had reported that the natural microflora of Portuguese olives is represented essentially by *L. plantarum* and *L. paracasei* species.

In the case of Spanish olive fermentation *L. plantarum* was mainly isolated as the most representative species of lactic acid bacteria (Ruiz-Barba *et al.*, 1991; Ruiz Barba *et al.*, 1994). In a previous study, Borcakli *et al.* (1993) reported that the microbial flora of Turkish fermented olives are mainly composed of Gram- negative bacteria and yeasts while, *Lactobacillus plantarum* are detected in the end of the fermentation. In Italy, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc* sp., *Enterococcus faecium* and *Enterococcus* sp. were isolated from olive phylloplane and olive brines in Apulia. More recently, *Lactobacillus casei* species were isolated from naturally fermented Sicilian green olives (Randazzo *et al.,* 2004).

It is of value to isolate lactic acid bacteria from fermented olives for further uses as starter cultures for green olive fermentation.

The persent paper deals with the isolation of new strains of lactic acid bacteria from fermented olives collected in Western Algeria. It also includes characterization of the isolates based on phenotypic criteria and constitutes a preliminary study in order to elaborate a local starter culture collection.

MATERIAL & METHODS

1. Samples

Ten samples of traditional fermented green olive samples were obtained from domestic factories located in two regions (Sig and Remchi) of Western Algeria. After collection, samples were transported to laboratory in a thermoflasks containing ice.

2. Plate count and isolation of lactic acid bacteria

Olives (50 g) were cut into small pieces and homogenized by grinding with 10 ml of 1% peptone water. After homogenization, serial decimal dilutions (10^{-2} to 10^{-6}) were made in 1% peptone water. 0.1 ml volumes of each dilution were surface plated in duplicate.

Total lactic acid bacteria were enumerated on MRS agar (de Man *et al.*, 1960) after 3 days at 30°C. Lactobacilli were counted in MRS agar ajusted with acetate (pH 5.4) so that the growth of other organisms could be inhibited (Garcia *et al.*, 1987). Plates were incubated under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C. for 2 to 3 days until growth was evident. Lactococci were counted on M17 agar (Terzaghi & Sandine, 1975) after incubation for 2 days at 30°C.

After bacterial counts, 10 colonies belonging to different types were randomly picked from each 30-50 colony count plates of MRS or M17 agar and purified through three cycles of single colony cultures.

3. Morphological, physiological and biochemical analysis

Cell shape and arrangement, Gram-staining, catalase activity (3% H_2O_2), production of gas from

glucose (1% glucose and Durham tubes), temperature requirement (4, 8, 10, 15, 37, 40 and 45°C), NaCl tolerance (4, 6.5, 7 and 10% NaCl) and growth at pH 3.9 and 9.6 were stutied in M17 or MRS broth.

L-and D-lactic acid were analysed enzymatically by the kit method according to the instructions protocol by the kit manufactuurer (F-Kit L-lactic acid/D-lactic acid, Roche diagnostic, Mannheim, Germany).

3.1. Clonies from M17 agar

Homofermentative cocci which were capable of growing at 10 and 40°C but not at 45°C or at 4% salt or at pH 9.6 were considered as lactococci and were classified according to the method and criteria of Mundt (1986). Homofermentative cocci, grouped in pairs or short chains, which grew at 10, 37, 40 and 45°C, survived heating at 60°C after 30 mn, grew in a 6.5% salt and at a pH 9.6 were considered as enterococci (Devriese *et al*., 1987).

The following test were carried out on each isolate using Api 20 STREP (API-System, S.A., France) according to the manufacturer's instructions: acetoïn production; hyppurate, esculin and arginine hydrolysis; pyrrolidonyl-aralamydase, α-galactosidase, β-galactosidase, β-glucuronidase activity and utilization of ribose, arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, starch, glycogen and glycerol. Api streeps were incubated at 32°C and examined after 4, 24 and 48 hours

3.2. Colonies from MRS agar

Homofermentative lactobacilli isolates was characterized according to the method and criteria of Kandler & Weiss (1986). Carbohydrates fermentation test was performed with Api 50 CHL (API-System, S.A.,France) according to the manufacturer's instructions (Sneath et *al*., 1986).

Arginine hydrolysis was tested in MRS broth (without glucose) containing 3 g/l arginine and 2 g/l sodium citrate replacing ammonium citrate. Ammonia was detected using Nessler's reagent. Acetoïn production was determined (for lactobacilli) in MRS broth using the Voges-Proskauer test.

After determination of genera, lactococci, lactobacilli and enterococci isolates were identified by comparing the results of tests of each isolate to those reported by Teuber *et al*. (1992), Hammes *et al.* (1992) and Devriese *et al*. (1987) respectively. All isolates were stored at 4°C in sterile (120°C, 10 min) (10%) reconstituted skim milk or at -20°C in M17 or MRS broth supplemented with 20% glycerol.

RESULTS & DISCUSSION

1. Lactic acid bacteria counts

Table 1 shows the results of microbial counts from fermented green olive samples. In all samples, means of values were ranged approximately between 2.7×10^4 to 5.1×10^5 bacteria/ml (ml, *i. e*., ml of homogenate) in MRS agar without acetate, 1.1×10^2 to 2.1×10^4 bacteria/ml in M17 agar and 1.1×10^3 to 4.0×10^3 bacteria/ml in MRS agar with acetate.

Table 1. Lactic acid bacteria counts in traditional butter samples

Samples	.....Counts (CFU/ml).....		
	MRS-agar	MRS-agar with acetate	M17-agar
S 1	2.7×10^4	1.7×10^3	1.5×10^3
S 2	6.0×10^4	1.9×10^3	2.0×10^3
S 3	4.0×10^5	3.1×10^3	1.4×10^3
S 4	5.1×10^5	1.3×10^3	$1.5 . 10^3$
S 5	1.2×10^5	1.1×10^3	1.6×10^3
S 6	6.2×10^4	1.8×10^3	1.2×10^3
S 7	3.0×10^4	4.0×10^3	2.1×10^4
S 8	6.0×10^4	3.4×10^3	1.3×10^3
S 9	3.0×10^4	1.1×10^3	1.1×10^2
S 10	4.0×10^4	1.9×10^3	1.4×10^3

2 Characterization and identification of bacteria

A total of 23 isolates of lactic acid bacteria were isolated from ten samples of fermented green olives. For characterization of bacteria, we have attached much importance to a sharp distinction between cocci and rods, Gram staining, catalase activity, acetoïn and arginine dihydrolase reactions.

The result concerning the identification using the physiological, biochemical and morphological tests are summarized in table 2 for lactococci and enterococci.

Table 2. Physiological characteristicsand identification of the lactococci isolates*

Isolates	0L1	0L8	0L19	0L21	OL22	OL17	OL20	OL32	OL35	OL37	OL98	OL106
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+
Cell shape	c	c	c	c	c	c	c	c	c	c	c	c
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-
Growth at or in:												
10°C	+	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	+	+	+	+	+	+	+
60°C after 30 mn	n	n	n	n	n	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
6.5% NaCl	-	-	-	-	-	+	+	+	+	+	+	+
7% NaCl	-	-	-	-	-	+	+	+	+	+	+	+
10% NaCl	n	n	n	n	n	-	-	+	-	-	+	+
pH 9.6	-	-	-	-	-	+	+	+	+	+	+	+
Lactic acid isomer	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)
Fermentation type	h	h	h	h	h	h	h	h	h	h	h	h
Api 20 Strep Sys												
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	+	+	-	+	-	+	+	+	+	+	+	+
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	+	+	+	+	+	+	+
Identified as	*Lc. lactis* ssp. *lactis*	*En. faecium*										

Five isolates obtained from M17-agar were identified as *Lactococcus lactis* ssp. *lactis*. They produced L-lactic acid without gaz formation, grew in 4% NaCl but not in 6.5% NaCl and at pH 9.6.

All isolates grew at 10°C and 40°C but not at 45°C. They have the ability to hydrolyse arginine but they were unable to produce acetoïn. All isolates fermented ribose, lactose and trehalose.

Seven isolates obtained from M17 agar were identified as *Enterococcus faecium* . They did not produce gas from glucose fermentation, produced L-lactic acid, grew at 10, 37, 40 and 45°C, survived 60°C after 30 mn, grew in a 6.5%, 7% and three of them have the ability to grow in 10% salt (OL9, OL32 and OL106). All isolates fermented arabinose, ribose, trehalose and starch.

Table 3 shows the physiological characteristics of 11 isolates of lactobacilli picked from MRS with acetate.

These results together with the API 50 CHL pattern of carbohydrate fermentation (Table 4), and compared to the scheme for identifying species developed by Hammes *et al.* (1992). All isolates were identified as *L. plantarum*. They produced L-lactic acid without gaz formation, grew in 6.5% NaCl but not in 10% NaCl.

Table 3. Physiological and biochemical characteristics of lactobacilli *

Isolates	OL2	OL7	OL9	OL12	OL15	OL16	OL23	OL33	OL36	OL40	OL53
Gram stain						+					
Cell shape						Rods					
Catalase test	-										
Growth at or in:											
4°C	-	-	-	-	-	-	-	-	-	-	--
8°C	-	-	-	-	-	-	-	-	-	-	--
15°C	+	+	+	+	+	+	+	+	+	+	++
40°C	+	+	+	+	+	+	+	+	+	+	++
45°C	-	-	-	-	-	-	-	-	-	-	--
6.5% NaCl	+	+	+	+	+	+	+	+	+	+	++
7.0% NaCl	+	-	+	-	+	+	+	-	-	+	++
10% NaCl	-	-	-	-	-	-	-	-	-	-	--
pH 3.9	+	+	+	+	+	+	+	+	+	+	++
Lactic acid isomer	n	n	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)
Fermentation type	h	h	h	h	h	h	h	h	h	h	hh
Acetoïn	-	-	-	-	-	-	-	-	-	-	--
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	++

* +: positive; -: negative; h: homofermentation ; n: no performed

All isolates grew at 10°C, 15 and 40°C but not at 4, 8 and 45°C. They have no ability to hydrolyse arginine but they were able to produce acetoïn.

From the results presented here it is clear that *L. plantarum* were the main species of lactic acid bacteria isolated from fermented olive (11 isolates) samples, which is the most frequent species most often occurring in fermented olives (Ruiz-Barba *et al.*, 1991, 1994). Therefore, this species has been extensively studied with the aim of its use in starters (Delgado e*t al.*, 2001).

Seven isolates of *E. faesium* and five isolates of *L. lactis* ssp *lactis* were also isolated from the product. These isolates were capable of growing in olives and were able to reach counts of 10^3 bacteria/ml.

To our knowlege genera of lactococci are the most occurring lactic acid bacteria in dairy products but not in fermented olives (de Roissart & Luquet, 1994; Desmazeaud, 1996). The presence of Lc. *lactis* ssp *lactis* in fermented olives has not been reported before.

In this study all isolates of lactobacilli were obtained from MRS agar with acetate, which is generally described as selective media on which only typical colonies of lactobacilli are selected (Garcia *et al.*, 1987). In contrast, M17 agar showed a moderate selectivity for the isolation of lactococci from our samples. We found that isolates of *Lactococcus* as well as *Enterococcus* were able to grow either in M17 medium. Also, high colonies of yeasts were observed in both MRS and M17 agar probably which explain sush the high counts of microorganisms in our samples and the low number of lactic acid bacteria isolated from these samples. Asehraou *et al.* (2000) also, reported that fermented olives sampled from two factories in Morocco showed that only yeast colonies appeared and no growth of lactic acid bacteria was detected.

CONCLUSION

The characterization of isolates on the basis of microscopic analysis and phenotypic characteristics (especially biochemical properties and sugar fermentation abilities) is very useful and remains the most widely recognized approach but, in the future, it would be interesting to conduct a more detailed study on bacterial identification using molecular methods.

At present, isolates of *L. plantarum* are further investigated in order to elaborate an adequate starter culture which would permit the manufacturing on industrial scale of a uniform fermented green olives, and keep the quality characteristics of the artisanal product as much as possible.

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
Carbohydrates											
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	-	-	-	-	-	-	-	-	-	-	-

Identified as: *Lactobacillus plantarum*

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