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Naturally fermented Jijelian black olives: microbiological characteristics and isolation of lactic acid bacteria

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RESUMEN

Aceitunas negras Jijelia fermentadas por métodos naturales: caracterización microbiológica y aislamiento de bacterias ácido lácticas.

Un estudio sobre la microflora de aceitunas negras fermentada por métodos tradicionales en el Este de Argelia es presentado. Se realizo el siguiente recuento de grupos de microorganismos: bacterias mesófilas, enterobacterias, bacterias ácido lácticas (LAB), staphylococcus y levaduras. En una segunda fase, la identificación y evaluación de aspectos tecnológicos de LAB fue realizada. Setenta bacterias ácido lácticas fueron aisladas e identificadas. Estos aislados contenían principalmente dos géneros: *Lactobacillus* y *Leuconostoc*. Los resultados mostraron que *Lactobacillus plantarum* fue la especie predominante en este producto tradicional.

PALABRAS CLAVE: Aceitunas negras – Bacterias ácido lácticas – Microflora.

SUMMARY

Naturally fermented Jijelian black olives: microbiological characteristics and isolation of lactic acid bacteria.

A study of the microflora of traditionally fermented black olives in Eastern Algeria is presented. A count of the following microbial groups was carried out: mesophilic bacteria, enterobacteria, lactic acid bacteria (LAB), staphylococci and yeast. In a second phase, the identification and assessment of the technological traits of LAB was performed. Seventeen lactic acid bacteria were isolated and identified. These isolates were represented by two genera: *Lactobacillus* and *Leuconostoc*. The results showed that *Lactobacillus plantarum* was the predominant species in this traditional product.

KEY-WORDS: Black Olives – Microflora – Lactic Acid Bacteria.

1. INTRODUCTION

The production of fermented foods is one of the oldest food processing technologies known to man. Since the dawn of civilization, methods for the fermentation of milks, meats and vegetables have been described (Fox, 1993). There are 21 different commercial vegetable fermentations in Europe along with a large number of fermented vegetable juices and blends, the most economically relevant of these are the fermentations of olives, cucumbers, and cabbage (Buckenhuskes, 1997).

The unified qualitative standard applying to table olives in international trade defines table olives as "the sound fruit of specific varieties of the cultivated olive tree (*Olea europea sativa*, Hoffm, link), harvested at the proper stage of ripeness and processed as specified in this standard. Such processing may include the addition of various products or spices of good quality (Campaniello *et al*., 2005).

Table olive production has a deep-rooted tradition in all Mediterranean countries. The olive tree is one of the major agricultural trees in Jijel (East of Algeria), with an area of 14,000 hectars distributed essentially in mountainous areas (Anonymous, 2006). The majority of the olives grown are dedicated to the production of olive oil. In Jijel areas, the Chemlal, Hamra and Sigoise varieties of *Olea europea* are also used for table olive production, mainly by traditional methods. In the traditional process, starter cultures are not employed during fermentation of the product, so, black olives are placed in brine at a variable salt concentration where a native microbial population naturally ferments them and where an initially small population of lactic acid bacteria (LAB) becomes the predominating microbial flora. LAB are know to produce a heterogonous array of functional products, which include organic acids, carbon dioxide, flavor compounds and antimicrobial compounds, such as bacteriocins that regulate the microbial development (Vandenbergh, 1993; Muriana and Luchansky, 1993).

The aim of this study was to characterize the microbial population of traditionally fermented black olives produced in Jijel areas (Eastern Algeria) and then isolate LAB.

2. MATERIALS AND METHODS

2.1. Samples

Ten samples of traditionally fermented black olives were collected from different locations in East and South-East Jijel (East of Algeria). Samples were collected in December 2007. For each sampling, one Kg of fermented black olives was collected.

2.2. Microbiological analysis

Twenty g of fermented black olives of each sample was diluted in 180 ml sterile saline (0.9% NaCl), homogenized and the dilutions were plated in duplicate onto appropriate media.

The media and the conditions used for microbial numeration were the following (Campaniello *et al*., 2005): Plate count agar (PCA) incubated at 37°C for 48h for mesophilic bacteria; violet red bile glucose agar (VRBG), incubated at 37°C for 24h for enterobacteria; Baird-Parker agar base, incubated at 37°C for 48h for staphylococci; MRS agar, incubated at 32°C for 48h to 72h in anaerobiosis for lactic acid bacteria; Oxytetracyclin glucose agar (OGA), incubated at 25°C for 3-7 days for yeasts.

2.3. pH measurements

The pH measurements of brines and fermented black olives were obtained with a pH meter (HANNA), calibrated with two standard solutions buffered at pH 4.00 and pH 7.00.

2.4. Isolation and identification of lactic acid bacteria

Serial decimal dilutions were spread in duplicate onto MRS agar (de Man *et al*., 1960) and M17 agar (Terzaghi and Sandine, 1975). The plates were incubated at 32°C for 24 h to 48h in anaerobic conditions. After incubation, 17 colonies were picked from the MRS and M17 plates and sub-cultured in MRS and M17 broths.

LAB was identified as reported by Idoui and Karam (2008). The isolates were initially subjected to the Gram staining and the catalase test (3% H_2O_2). Only the Gram positive, catalase negative isolates were further identified. Growth at different temperatures was determined in MRS and M17 broths at 10°C, 15°C, 40°C and 45°C. NaCl tolerance (4% and 6.5%) was performed on MRS and M17 broths; reductase and hydrolysis of arginine were also recorded. The acetoin production was determinate using the Voges-Proskauer test.

The fermentative type was determined on agar (Gibson and Abdelmalek, 1945). The use of citrate was performed in Kempler and Mc Kay (1980) medium. The haemolysis type was determined in agar medium with horse blood added (Leveau *et al*., 1991).The ability of the isolated strains to produce acid from different carbohydrates was also determined.

2.5. Technological traits of some isolated strains

The technological traits of LAB were evaluated as reported by Idoui and Karam (2008). The acidifying property was performed on skim milk. Sterilized milk was inoculated with an active culture (1%) of each strain and incubated at 37°C for 3h, 6h, 9h and 12hours. The determination of total acidity (°D) was performed by titration with N/9 NaOH in the presence of phenolphtaleine (Accolas *et al.,* 1977).

The exopolysaccharide production from sucrose was recorded in MRS and M17 agar (20 $g.L^{-1}$ sucrose). After incubation for 16 to 24 h, mucoid colony formation on agar medium was related to the production of exopolysaccharides.

The proteolytic activity was evaluated in Yeast Milk Agar. The diameter of the proteolysis zone was determined after incubation at 30°C for 24 hours.

3. RESULTS AND DISCUSSION

3.1. Microbiological Analysis and pH measurements

The results for pH and the microbiological characteristics of traditionally fermented black olives samples are summarized in table 1. The pH value for all samples ranges between pH5.24 and pH5.80. The pH values of the analyzed samples are similar to those observed by Campaniello *et al*. (2005).

Table 1
pH and Counts of microbial population in fermented black olive samples

Sample	pH	Mesophilic bacteria (10^7 cfu/ g)	Enterobacteria (10^5cfu/ g)	Staphylococci (10^1 cfu/ g)	Lactic acid bacteria (10^7 cfu/ g)	Yeasts (10^6 cfu/ g)
01	5.40	6.40	4.00	0.00	3.24	ND
02	5.24	3.24	4.01	ND	3.82	6.80
03	5.76	9.76	2.96	0.00	4.40	ND
04	5.52	4.00	1.01	0.00	8.00	4.80
05	5.40	3.40	1.20	0.00	5.00	3.70
06	5.80	2.80	4.00	0.00	3.24	2.20
07	5.40	2.40	5.00	ND	5.20	5.00
08	5.48	4.00	1.01	0.00	1.20	1.25
09	ND	ND	1.02	0.00	6.00	1.30
10	ND	ND	1.40	0.00	5.20	8.00

ND: Not determined

The results indicate that the olive samples reveal a diversity of microflora. This diversity could be linked to environmental conditions, especially to spontaneous fermentation. The count of mesophilic bacteria is between 2.40 and 9.76 $\times$ 10^7 cfu/g. This result is in agreement with those reported on Sicily olives samples by Poiana and Romeo (2006). The enterobacteria counts ranged from a count of 1.01 to 5 $\times$ 10^5 cfu /g. Staphylococci were not detected in any of the samples. Also, counts of lactic acid bacteria and yeast were between 1.20 and 8.00 $\times$ 10^7 cfu/g and 1.25 and 8.00 $\times$ 10^6 cfu/g respectively. These results show the changes in the lactic acid bacteria and yeast populations which grew in all black olive samples. The counts of lactic acid bacteria were higher than yeasts. Poiana and Romeo (2006) reported that, in general, yeasts coexisted with lactic acid bacteria throughout the whole fermentation period. Their counts were lower than those of the lactic acid bacteria through the most active fermentation period and their presence was stable. These results are in agreement with those reported on Algerian table olives samples by Kacem and Karam (2006). In the same way, Fernandez-Diez (1983) reported that the fermentation of table olives involves a complex microflora of lactic acid bacteria, yeasts, Gram-positive and Gram-negative bacteria. In the study by Campaniello et *al.* (2005), microbiological analyses of LAB and yeasts in olives showed that their cell load was higher in the raw material and during the first fermentation phase and increased during the storage of products. The same authors found that staphylococci were undetectable in samples and Enterobacteriaceae remained constant in all samples of analyzed olives, throughout the 80 days of fermentation.

As reported in the literature, the predominant microorganisms in Spanish style table olives are lactic acid bacteria; yeasts, though, are the organisms responsible for the fermentation of olives in natural processing (Garrido-Fernandez *et al*., 1997). In Algerian table olives (Western Algeria), lactic acid bacteria along with yeasts are the predominant microorganisms (Kacem and Karam, 2006).

3.2. Isolation and Identification of Lactic Acid Bacteria

The results of the identification of LAB are summarized in Tables 2 and 3. A total of 17 microbial isolates which were Gram positive, catalase negative and rods/ or cocci were selected from MRS and M17 media. All isolates have the ability to grow at 6.5% NaCl and grow at 15°C but not at 45°C. These were classified as LAB. Microflora of LAB was mainly composed of homofermentative and heterofermentative strains, while 9 out of 17 isolates were homofermentative.

The standard physiological and biochemical tests led to the identification of the isolates as follows: six isolates of *Lactobacillus plantarum* (35.29 %), three isolates of *Lactobacillus brevis* (17.65 %), one isolate of *Lactobacillus veridesens* (5.88 %), one isolate of *Lactobacillus curvatus* (5.88 %); two isolates of *Lactobacillus casei* ssp *tolerens* (11.76 %) and four isolates of *Leuconostoc mesenteroides* (23.53%). From the results presented here, lactobacilli represent 76.47 % of the isolates. In the case of Spanish olive fermentation, *Lb.plantarum* was mainly isolated as representative of the group of LAB (Ruiz Barba *et al*., 1994; Campaniello *et al.,* 2005).

Table 2
Some Physiological and biochemical characteristics of *Lactobacillus* isolates

Isolates	**FO1**	**FO3**	**FO4**	**FO11**	**FO12**	**FO17**	**FO2**	**FO5**	**FO6**	**FO7**	**FO15**	**FO13**	**FO16**
Gram Strain	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell Shape				Rods				Rods		Rods	Rods	Rods	Rods
Catalase test	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth at:													
15°C	+	+	+	+	+	+	+	+	+	+	+	+	+
45°C	−	−	−	−	−	−	−	−	−	−	−	−	−
4 % NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
6.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	+	+	+	+/−	+	+	+	+	+	−	−	−	+/−
Fermentative type	h	h	h	h	h	h	het	het	het	Het	h	h	h
Fermentationof:													
Lactose	+	+	+	+	+	+	+	+	+	−	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+/−	+	−	−
Gluconate	+/−	+/−	+/−	+/−	+/−	+	+	+	+/−	−	+	−	+/−
Ribose	+/−	+/−	+	+/−	+	+/−	+	+	+	−	+	−	−
Xylose	−	−	−	−	−	+/−	+/−	+	+	−	+/−	−	−
Identified as	*Lactobacillus plantarum*						*Lactobacillus brevis*			*Lb. veri-desens*	*Lb. curva-tus*	*Lb. casei* ssp *tolerens*	

FO: Traditionally fermented black olives; h: homofermentative; het: heterofermentative

Table 3
Some Physiological and biochemical characteristics of *Leuconostoc* isolates

Isolates	FO8	FO9	FO10	FO14
Gram Strain	+	+	+	+
Cell Shape	Cocci	Cocci	Cocci	Cocci
Catalase test	−	−	−	−
Growth:				
10°C	+	+	+	+
40°C	−	−	−	−
45°C	−	−	−	−
4% NaCl	+	+	+	+
6.5% NaCl	+	+	+	+
Arginine hydrolysis	−	+/−	−	+
Fermentative type	het	het	het	Het
Citratase	+	+	+	−
Fermentation of				
Arabinose	−	−	−	−
Rhamnose	−	−	−	−
Galactose	−	−	−	−
Inulin	−	−	−	−
Mannitol	+	+	+	+
Sorbitol	−	−	−	−
Lactose	+/−	+	+	+
Saccharose	+/−	+/−	−	+
Maltose	−	−	−	+/−
Raffinose	−	−	−	+
Starch	+	+	+	+
Glycerol	−	−	−	−
Identified as	*Leuconostoc mesenteroides*			

FO: Traditional fermented black olives; het: heterofermentative

In our LAB collection, *Lb. plantarum* is a dominant strain with 35.29%. This result is in complete agreement with those reported by similar works. Vandenberg *et al*. (1993) reported that the natural microflora of Portuguese olives is represented essentially by *Lb. plantarum* and *Lactobacillus paracasei*. In another study, Borcakli *et al.* (1993) reported that the microbial flora of Turkish fermented olives is mainly composed of Gram negative bacteria and yeasts while, *Lb.plantarum* are detected at the end of the fermentation. In a previous study, Harmon *et al*. (1987) reported that the indigenous LAB change spontaneously during the fermentation of natural olives. At the end of the process, only *Lb. plantarum* is detected. Our result shows that *Ln. mesenteroides* was isolated from the spontaneous fermentation of black olives. This is in agreement with the results found by Oliveira *et al.* (2004). In the same way, Lavermicocca *et al*. (1998) isolated *Lb. plantarum* and *Ln. mesenteroides* ssp *mesenteroides* from olive phylloplane and table olive brines.

In our study, we isolated *Lb.casei* ssp *tolerens* from naturally fermented Jijelian black olives. The *Lb.casei* species were isolated by Randazzo *et al*. (2004) but from naturally fermented Sicillian green olives. Finally, these results are not in complete agreement with those reported by Kacem and Karam, (2006) who isolated 32 lactic acid bacteria from naturally fermented Algerian green olives represented essentially by strains of *Lactococcus lactis*, *Lb.plantarum* and *Enterococcus* sp.

3.3. Technological performance of some isolated strains

The production of lactic acid by some lactic acid bacteria isolates is summarized in Table 4. The results showed that *Lb. plantarum* FO11 and *Lb.casei* ssp *tolerens* FO13 acidify skim milk faster than the other strains of lactic acid bacteria tested for this property. The slowest acidification agent was the isolate *Lb. plantarum* FO11; it produced 86.50 ± 0.00°D after incubation for 12 hours. This result is not in accordance with those reported by Ayad *et al*. (2004) who indicated that most strains of *Lb. plantarum* isolated from different sources have shown a slow acidification rate.

For the proteolysis activity, results showed that the isolates were able to grow on YMA media but with no clear zones around the colonies. The same results were obtained with exopolysaccharides production while none of the strains produced exopolysaccharides.

4. CONCLUSION

To our knowledge, no information existed on the microflora of naturally fermented black olives produced in Algeria and especially in the East of Algeria. Our results showed the diversity of the native microflora in this product. Also, this study demonstrated that lactobacilli are the dominant LAB but *Leuconostoc* strains are present too.

Table 4
Production of lactic acid by some lactic acid bacteria isolates

Time of incubation (hours)	Acidity (°D)				
	0	3	6	9	12
Lb.plantarum FO1	18.00 ± 0.00	24.00 ± 0.00	31.00 ± 0.60	41.00 ± 1.00	62.00 ± 0.60
Lb. brevis FO5	18.00 ± 0.00	24.00 ± 0.01	24.50 ± 0.00	27.00 ± 0.80	48.00 ± 0.95
Lb .veridesens FO7	18.00 ± 0.00	21.00 ± 1.00	24.50 ± 0.50	29.50 ± 0.50	55.00 ± 0.81
Lb.plantarum FO11	18.00 ± 0.00	28.00 ± 0.10	33.80 ± 0.10	59.00 ± 0.50	86.50 ± 0.00
Lb.curvatus FO15	18.00 ± 0.00	25.00 ± 1.00	35.00 ± 0.00	47.00 ± 0.25	66.00 ± 0.20
Lb.casei ssp *tolerens* FO13	18.00 ± 0.00	26.00 ± 0.70	35.00 ± 0.80	49.00 ± 0.00	77.00 ± 0.23
Leuconostoc mesenteroides FO9	18.00 ± 0.00	24.00 ± 1.15	26.00 ± 0.80	27.50 ± 0.50	41.00 ± 1.46

For future applications, isolates will be analyzed for several other physiological properties related to the black olive fermentation process such as lipolytic activity, production of antimicrobial substances and hydrolysis of the bitter glucoside oleuropein.

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