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Microbiological study of naturally fermented Algerian green olives: isolation and identification of lactic acid bacteria and yeasts along with the effects of brine solutions obtained at the end of olive fermentation on *Lactobacillus plantarum* growth

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RESUMEN

Estudios microbiológicos de aceitunas verdes argelinas fermentadas naturalmente: aislamiento e identificación de bacterias lácticas y levaduras, y efecto de las soluciones de salmuera obtenidas al final de fermentación sobre el crecimiento de *Lactobacillus plantarum*

La microflora de las aceitunas verdes fermentadas naturalmente elaboradas en Argelia Occidental fue estudiada en períodos de fermentación de 15, 60 y 90 días. Diferentes microorganismos (bacterias aeróbicas, coliformes, estafilococos, bacterias del ácido láctico, lactobacilos, enterococos, levaduras, psicotrofos y bacterias lipolíticas) fueron detectados a los 15 y 60 días de fermentación. Después de 90 días de fermentación (pH 4.40), la población de bacterias lácticas se hizo dominante y persistió junto con las levaduras a lo largo de todo el proceso. Las bacterias lácticas aisladas (343) fueron identificadas como *L. casei*, *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. lactis* subsp. *lactis*, *E. faecalis*, *E. faecium* y *E. durans*. La especie dominante fue *L. plantarum*. Las levaduras aisladas (32) de todas las muestras fueron identificadas como *Saccharomyces cerevisiae* o *Candida parapsilosis*. También se recoge en este estudio que las soluciones de salmuera obtenidas al final de fermentación de aceitunas verdes fueron capaces de estimular el crecimiento de varias cepas de *L. plantarum*.

PALABRAS-CLAVE: Aceitunas fermentadas - Bacterias lácticas - Lactobacillus plantarum- - Levaduras - Microbiología.

SUMMARY

Microbiological study of naturally fermented Algerian green olives: isolation and identification of lactic acid bacteria and yeasts along with the effects of brine solutions obtained at the end of olive fermentation on *Lactobacillus plantarum* growth

The microflora of naturally fermented green olives produced in Western Algeria was studied over 15, 60 and 90 day fermentation periods. Different microorganisms (aerobic bacteria, coliforms, staphylococci, lactic acid bacteria, lactobacilli, enterococci, yeasts, psychrotrophs and lipolytic bacteria) were recorded at 15 and 60 days of fermentation. After 90 days (pH 4.40) of fermentation, the lactic acid bacteria population became dominant and persisted together with yeasts throughout the fermentation period.

The lactic acid bacteria isolated (343 isolates) were identified as *L. casei*, *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. lactis* subsp. *lactis*, *E. faecalis*, *E. faecium* and *E. durans*. The dominant species was *L. plantarum.* Yeasts were isolated from all samples (32 isolates) and were identified as *Saccharomyces cerevisiae* or *Candida parapsilosis.* Also, in this study we reported that brine solutions obtained at the end of olive fermentation were able to stimulate the growth of several *L. plantarum* strains.

KEY-WORDS: Fermented olives - Lactic acid bacteria - Lactobacillus plantarum - Microbiology - Yeasts.

1. INTRODUCTION

Olives (*zaitun*) (*aceituna* in Spanish) are one of the major agricultural products in Algeria. With an area of 2,400,000 km^2, olive groves occupy a total area of 165,800 hectares distributed essentially in mountainous areas (Anonymous, 2000). More than 48% of the Segoise variety of olives are harvested in their green state and processed according to the traditional technique for fermenting green olives. For example, in many regions of Western Algeria (Sig, Remchi, Tlemcen, etc.), olives are fermented as follows: green olives are placed in 1 to 2% (w/v) sodium hydroxide (lye) solution until the lye penetrates the flesh. Cold water is then added to the solution to remove the lye. Olives are then placed in barrels with a 4 to 6% brine solution and allowed to undergo a spontaneous fermentation under ambient temperature (22 to 24 °C). The fermentation period usually takes between two and three months.

This product is consumed traditionally as a base for soup, eaten as pickles or mixed with bread and spices according to local tastes and traditions.

No measures whatsoever are taken to control fermentation, which in most cases is incomplete or

affected by microbial spoilage. Therefore, it is not always easy or feasible to control undesirable pathogens or micro organisms causing spoilage in the olives that can cause disease in the Algerian mountaineer population who live in primitive conditions with poor sanitation. In addition, olive fermentation is carried out without starter cultures and therefore the quality of the final product is frequently inconsistent. For these reasons greater attention must be given to quality control in the fermenting of olives.

The availability of information on the microbiological characteristics of natural microflora present in olives is limited to few reports. Microbiological studies show that several species of lactic acid bacteria and yeasts are present and some of them were prevalent throughout the fermentation period with a particular metabolic capability (Ruiz-Barba and Jiménez-Díaz, 1994; Asehraou *et al*., 2000; Catulo *et al*., 2002).

Lactic acid bacteria and yeasts developed their activity during the last fermentation phases (Fernández-Díez, 1983; Ruiz-Barba and Jiménez-Díaz, 1995, Oliveira *et al*., 2004). Borcakli *et al.* (1993a) reported that the microbial flora of fermented olives are mainly composed of Gram-negative bacteria and yeasts, while *L. plantarum* and *Pediococcus* sp. are detected only after 76 days of fermentation.

As far as we know, lactic acid bacteria has long been described as being auxotrophic for many vitamins (Rogosa *et al.,* 1961; Kandler and Weiss, 1986), and a number of wild-type *L. plantarum* strains isolated from the fermentation of green olives have been shown to require vitamins as growth factors (Ruiz-Barba and Jimenez-Diaz, 1994). Lacking some of these factors can dramatically affect the growth rate or even the survival of *L. plantarum* strains. Yeasts present in vegetable fermentation play a beneficial role, which is important in terms of *L. plantarum* nutritional requirements.

During the last decades, progressive attention has being given to the isolation and identification of lactic acid bacteria from fermented olives, due the beneficial impact of starter cultures used in olive fermentation. The main species of lactic acid bacteria isolated from different fermented olives produced in many Mediterranean regions can be observed in Table 1.

Recent work concerning lactic acid bacteria isolated from the spontaneous fermentation of green olives has been carried out by Kacem *et al.* (2004a) in Algeria. In this study, *Lactococcus lactis, L. plantarum and E. faecalis* species were isolated and identified according to morphological and biochemical criteria.

Until now, no analytical study has taken place on naturally fermented Algerian olives to evaluate their

Table 1
Lactic acid bacteria isolated from olives

Authors	Products	Lactic acid bacteria
Floriano *et al.* (1998)	Spanish-style green olive	*L. plantarum* *Enterococcus sp.*
Maldonado *et al*. (2002, 2003)	Fermented green olive of South Spain	*L. plantarum*
Ruiz-Barba *et al.* (1991); Ruiz-Barba and Jiménez-Díaz (1994, 1995)	Spanish fermented olives	*L. plantarum*
Fernández-Díez (1983); Van Den Berg *et al.* (1993), Oliveira *et al.* (1993)	Portuguese fermented olives	*L. plantarum* *L. paracasei* *L. pentosus* *Ln. pentosaceus*
Asehraou *et al*. (2002)	Moroccan fermented olives	*L. plantarum*
Borcakli *et al*. (1993)	Turkish fermented olives	*L. plantarum*
Kacem *et al.* (2004a)	Algerian fermented olives	*L. lactis* *L. plantarum* *E. faecalis*
Korukluoglu *et al.* (2002)	Turkish fresh olives	*L. plantarum* *L. brevis* *L. lactis* *Ln. mesenteroides* *P. damnosus*
Randazzo *et al.* (2004)	Sicilian (Italy) fermented green olives	*L. casei* *L. plantarum* *L. brevis* *E. faecium*

microbiological profile. The knowledge of bacterial flora present in olive fermentation is very important for predicting and determining the quality of the final fermented olive.

This study describes the microbiological changes occurring during the fermentation of green olives produced in Western Algeria with special reference to the changes in lactic acid bacteria and yeasts. In this study we also report the ability of brine solutions obtained at the end of olive fermentation to stimulate the growth of *L. plantarum.*

2. MATERIAL AND METHODS

2.1. Olive samples

Traditionally fermented green olives (Sigoise variety) were obtained from a domestic factory located in Sig (Western Algeria). Four lots (barrels of 60 Kg of olives in 40 L of brine, NaCl 6% w/v) were analyzed from December to March (2003) after 15, 60 and 90 days of fermentation. In addition to brine solution samples obtained after 90 days from barrels, 14 other brine solution samples were obtained from different olive fermentations in the same region. These brine solutions were used for a stimulation study.

2.2. Microbiological and physicochemical analyses

In this study, the olives were used for microbiological analyses and brines for pH measurement. Fifty grams of olives were removed from each barrel (after 15, 60 and 90 days of fermentation), pitted with a hand-operated pitting machine, homogenized with 10 ml of 1% (w/v) sterile peptone solution and then diluted using the dilution pour-plate method. Total aerobic counts were made on plate count agar (Oxoid Ltd., UK) after incubation at 30 °C for 72h, and coliform counts on violet red bile agar (Oxoid) after incubation at 30 °C for 24h. Staphylococci were numbered on Baird-Parker medium (Oxoid) after incubation at 37 °C for 48h, yeasts on potato dextrose agar (Oxoid) after incubation at 22 °C for 5 days, psychrotrophs on plate count agar incubated at 7 °C for 10 days, lipolytic bacteria on tributyrin agar (Oxoid) after incubation at 30 °C for 72h. Lactic acid bacteria were numbered on MRS agar (Oxoid) (de Man *et al*., 1960) adjusted to pH 5 so that the growth of other microorganisms could be suppressed (Garcia *et al.,* 1987) and lactobacilli on acetate agar (Rogosa *et al*., 1951) after incubation at 30 °C for 5 days under anaerobic conditions (Gas Pak System, Becton Dickinson). Enterococci were numbered on citrate acid agar after incubation at 37 °C for 72h and lactococci were grown on M17 agar (Merck Mikrobiologie) (Terzaghi and Sandine, 1975) incubated at 30 °C for 48h.

The pH of the brine solution was determined electrometrically with a pH meter (Micro pH 2002, Crison, Barcelona, Spain).

Significance of differences between means was assessed by the Student-Newman-Keul's multiple range test (Steel and Torrie, 1980) after log transformation for bacterial counts.

2.3. Isolation and identification of isolates

2.3.1. Lactic acid bacteria

Colonies (25-40 per sample) displaying the general characteristics of lactic acid bacteria were picked at random from MRS agar, acetate agar, and citrate acid agar. All the isolates were initially subjected to Gram straining, and the catalase test. Colonies and cell morphology characteristics on MRS and M17 agar were examined afterwards and then separated into phenotypic groups.

The identification of lactic acid bacteria was performed using the following tests: growth at 15 °C, 37 °C and 45 °C in MRS broth for 5 days and at 4 °C and 10 °C for 12 days; NaCl (4%, 6.5% and 8%) and pH (3.9 and 9.6) tolerance was performed on MRS broth; Sherman test and survival after heating at 60 °C for 30 min were also studied (Samelis *et al*., 1994); gas production from glucose, determined in MRS broth containing inverted Durham tubes; hydrolysis of arginine, tested on MRS with bromocresol purple (Thomas, 1973); citrate utilization in the presence of carbohydrates, according to Kempler and Mc Kay (1980) and production of acetoin from glucose, determined using the Vogs-Proskauer test (Zourari *et al*., 1991). The identification profile adopted also took into account API 50 CHS/L galleries (BioMerieux, S.A., France).

Isolates of lactococci, enterococci and lactobacilli were identified according to the criteria of Mundt (1986), Devriese *et al*. (1987) and Kandler and Weiss (1986) respectively.

2.3.2. Yeasts

The yeast isolates were distinguished separately according to their different morphological appearance upon growth in a potato dextrose agar. After being isolated as pure cultures, the yeast isolates were identified based on the morphological and physiological criteria described by Kreger-van Rij (1984) and Barnett *et al.* (1990). The other biochemical tests were done with an API 20C AUX system (BioMerieux, S.A.), and readings were made at 24, 48 and 72 h of incubation at 30 °C.

Microorganisms were maintained in sterile (120 °C, 10 min) reconstituted skim milk (10% w/v) at 4 °C or at -20 °C in MRS broth supplemented with 20% w/v glycerol. Working cultures were also kept on MRS agar, M17 agar or potato dextrose agar slant at 4 °C and streaked every four weeks.

2.4. Stimulation study

Besides *L. plantarum* strains isolated in this study, other *L. plantarum* strains from our collection

(Laboratory of Biology of Microorganisms and Biotechnology, University of Oran, Algeria) were used as indicator strains.

The brine solutions (5 ml) obtained at the end of olive fermentations was centrifuged at 12000 *g* for 15 minutes at 4 °C to remove cells. The supernatant was then filter-sterilized (0.22-µm pore size filter, Gelman Acrodisc 13, Pall Corp., Ann Arbor, USA). The solution thus obtained was designated as Cell Free Brine (CFB) and stored at 4 °C and -20 °C.

Pre-poured MRS agar plates were overlaid with 7ml MRS soft agar containing 0.2 ml of indicator (*L. plantarum*) culture. In order to standardize the assay, the inoculum was approximately 10^5 indicator cfu ml^{-1}. Wells of 5 mm in diameter were cut onto the agar plate using a cork borer, and aliquots of 50 µl from each brine solution sample (CFB) were placed into the wells. The plates were incubated for 5 days at 30 °C, and examined for the presence of turbid zones (bacterial growth) around the wells. Each assay was performed in duplicate.

3. RESULTS

The mean values of the pH of brine solutions were 5.90, 4.53 and 4.40 after 15, 60 and 90 days of fermentation respectively.

Table 2 shows the counts of aerobic bacteria, coliforms, staphylococci, lactic acid bacteria, lactobacilli, enterococci, yeasts, psychrotrophs and lipolytic bacteria recorded throughout the three stages (after 15, 60 and 90 days) of naturally green olive fermentation.

High counts of aerobic bacteria and lactic acid bacteria were recorded at 60 and 90 days of fermentation. However low incidences of coliforms as well as staphylococci were detected. In contrast to lactic acid bacteria, lactobacilli and enterococci, the incidence of yeasts was lower (mean log count 1.88) after 15 days of fermentation. After 60 and 90 days of fermentation high counts of yeasts were detected (mean log counts 3.84 and 5.98, respectively). At the end of fermentation (90 days), the levels of total lactic acid bacteria, lactobacilli, enterococci and yeasts increased to log counts 6.96, 5.66, 5.65 and 5.98 respectively. In this stage of fermentation, the levels of coliforms staphylococci, psychrotrophs and lipolytic bacteria decreased to log counts 1.96, 1.35, 1.53 and 2.42 respectively.

Table 2
Mean log plate counts of different microbial groups during naturally green olive fermentation

Microbial group	Fermentation time (days)		
	15	60	90
Total aerobic count	4.52[a]	7.76[b]	6.88[b]
Coliforms	2.12	2.33	1.96
Staphylococci	2.03	2.52	1.35
Lactic acid bacteria	3.8	6.55	6.96
Lactobacilli	3.6[a]	5.55[b]	5.66[b]
Enterococci	3.6	4.33	5.65
Yeasts	1.88[a]	3.84[b]	5.98[b]
Psychrotrophs	4.22	3.88	1.53
Lipolytic	3.1	3.56	2.42

Values are the means of lots of naturally green olives.
[a,b] Means of the same organism with different superscripts differ significantly (P<0.05).

Mean log count of aerobic bacteria was 4.52 after the first stage (15 days) and reached its peak growth to mean log counts 7.76 and 6.88 after 60 and 90 days respectively. Notably, aerobic and lactic acid bacteria were the predominant microbial group throughout the fermenting stages of the olives (Table 2).

Out of a total of 343 isolates of lactic acid bacteria obtained from fermented olives, 90 were isolated after the first stage of fermentation (15 days), 130 were isolated after the second stage (60 days) and 123 were isolated after the third stage (90 days) (Table 3).

The morphological, physiological and biochemical analyses of the 343 isolates revealed diversity and change in species of lactic acid bacteria during the fermentation of green olives. Bacteria were subdivided into 9 groups.

Seven groups were identified at the species level: *L. casei* (25 strains, 7.2% of the lactic acid bacteria isolated), *L. rhamnosus* (3 strains, 0.8%), *L. paracasei* (27 strains, 7.8%), *L. plantarum* (75 strains, 21.7%), *L. confusus* (7 strains, 2%), *L. cellobiosus* (9 strains, 2.6%), *L. lactis* subsp. *lactis* (27 strains, 7.8%), *E. faecalis* (27 strains, 7.8%), *E. faecium* (40 strains, 11.6%), *E. durans* (26 strains, 7.5%). Their biochemical and physiological characteristics are shown in Table IV.

Two groups were identified only at the genus level: *Lactococcus* sp. (41 strains, 11.8%) and *Lactobacillus* sp. (36 strains, 10.4%). All isolates grew at 15 °C but not at 45 °C or at 6.5% NaCl, and had the ability to hydrolyze arginine. They produced L-Lactic acid with no gas evolution in the presence of glucose, indicating their homofermentative metabolism.

L. plantarum, L. paracasei E. faecium, E. faecalis, E. durans, L. lactis subsp. *lactis* species, and other *Lactococcus* and *Lactobacillus* genera were isolated in greater amounts in fermented olives. The dominant strain was *L. plantarum* species. *L. plantarum* and *E. faecium* predominated in all stages of fermentation (more frequently at 90 days), *L. lactis* subsp. *lactis, E. faecalis* or *E. durans* were found more frequently than *E. faecium* after 60 days; however, their proportion diminished rapidly with ageing. At the end of the fermentation period, the main species was *L. plantarum* (31.7%) and no species of *L. rhamnosus, L. confusus* or *L. cellobiosus* was found. *L. casei*, *L. paracasei* and some other unidentified lactobacilli were also detected at the end of the third month of fermentation and represented 12.2%, 10.5% and 9.7% respectively.

Table 3
Changes in species of lactic acid bacteria during naturally green olive fermentation

	Fermentation time (days)					
	15		**60**		**90**	
pH	5.90		4.53		4.40	
Species	**No. strains**	**(%)**	**No. strains**	**(%)**	**No. strains**	**(%)**
L. casei	3	3.3	7	5.4	15	12.2
L. rhamnosus	2	2.2	1	0.7	-	-
L. paracasei	5	5.5	9	7	13	10.5
L. plantarum	11	12.2	25	19.2	39	31.7
L. confusus	7	7.7	-	-	-	-
L. cellobiosus	9	10	-	-	-	-
L. lactis subsp. *lactis*	4	4.4	18	13.8	5	4
E. faecalis	6	6.6	13	10	8	6.5
E. faecium	14	15.5	9	7	17	13.8
E. durans	9	10	11	8.4	6	4.8
Unidentified lactococci	12	13.8	21	16.3	8	6.5
Unidentified lactobacilli	8	8.8	16	12.3	12	9.7
Total	**90**		**130**		**123**	

Table 4
Physiological and biochemical characteristics of lactic acid bacteria isolates[a]

	Tests						
Growth at or in:	**1**	**2**	**3**	**4**	**5**	**6**	**7**
10 °C	-	+	-	+	+	+	+
15 °C	+	+	+	+	+	+	+
37 °C	v	+	-	+	+	+	+
45 °C	v	-	-	+	-	+	+
4% NaCl	+	+	+	+	+	-	+
6.5% NaCl	+	-	-	+	-	+	+
8% NaCl	-	-	-	+	-	+	+
pH 9.6	-	-	-	-	-	+	+
pH 3.9	+	-	-	-	-	-	-
Lactic acid isomer	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)
Citrate	-	+	-	+	+	-	-
Acetoin	-	+	-	+	-	-	-
Arginine hydrolysis	-	-	-	-	+	-	-
Survival at 60 °C for 30 min	-	-	+	-	-	+	+
CO2 from glucose	-	-	-	-	-	-	-
API 50 CH-Syst.							
Esculin	+	+	-	+	-	+	+
Lactose	+	+	-	+	-	+	+
Galactose	+	+	+	+	+	+	+
Fructose	+	+	+	N	+	+	+
Sorbitol	+	+	+	+	-	-	-
Mannose	+	+	+	+	+	+	+
Melibiose	+	+	+	N	+	-	-
Cellobiose	-	+	+	-	+		-
Raffinose	+	+	+	+	+	-	-
Xylose	+	+	+	+	+	-	-
Sucrose	+	-	-	+	-	+	+
Arabinose	-	-	-	+	+	-	+
Melezitose	+	+	+	+	-	-	-
Number of strains	41	3	27	75	27	27	66

[a]: All strains examined were gram-positive, motility-negative and catalase negative.
+: positive result; -: negative result
1. *L. casei,* 2. *L. rhamnosus,* 3. *L. paracasei,* 4. *L. plantarum* ,5. *L. lactis* subsp. *lactis*, 6. *E. faecalis*, 7. *E. faecium.*

Yeasts were isolated from all samples on potato dextrose agar plates. Of the 32 isolates, 22 were identified *Saccharomyces* genus because they reproduced by multilateral budding, formed pseudohyphae and asci containing one to four globose ascospores, fermented glucose, galactose and maltose; they did not assimilate lactose or nitrate and their cells were globose to subglobose or ellipsoidal to cylindrical. The other ten isolates seemed to belong to the genus *Candida*; they reproduced by multilateral budding, they did not form ascospores or carotenoid pigment, and their cells were ovoid or ellipsoidal to cylindrical.

All of these characteristics, together with the API 20C AUX system (Bio Merieux, S.A.) pattern of biochemical tests, identified the 22 isolates as *Saccharomyces cerevisiae* and the 10 other isolates as *Candida parapsilosis.* The strains of these two species of yeasts were isolated in all stages of olive fermentation.

Of the 18 CFB tested by the well diffusion method, 9 showed an ability to stimulate the growth of 17 strains of *L. plantarum* among the 70 isolated and tested in this study. A demonstration of the stimulation exhibited by CFB towards *L. plantarum* S33 is shown in Figure 1. On the other hand, all the CFB tested were not active with *L. casei, L. rhamnosus, L. paracasei, L. confusus, L. cellobiosus, L. lactis* subsp. *lactis, E. faecalis, E. faecium, E. durans* strains isolated in this study. This is a new finding since the brine solutions from olive fermentation to stimulate *L. plantarum* growth have not been reported before.

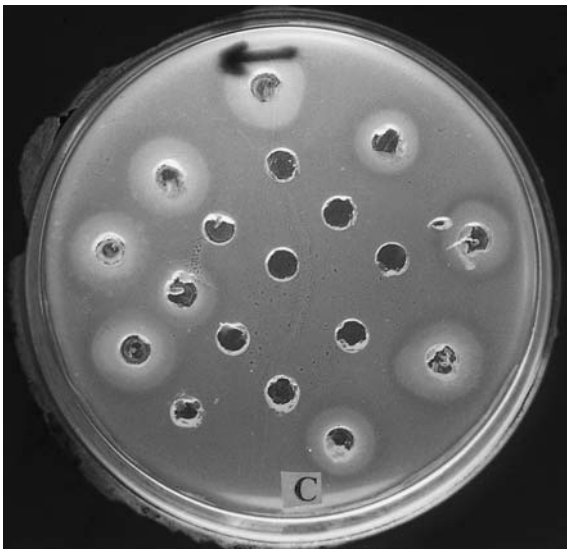

Figure 1
Agar well diffusion method showing the action of brine solutions (CFB) on *L. plantarum* S33. Brine solution samples (CFB) were placed into the wells. The plates were incubated for 5 days at 22 °C, and examined for turbid zones around the wells indicating the stimulation of *L. plantarum* S33 growth.

4. DISCUSSION

Results indicate that the naturally fermented Algerian olives reveal a diversity of microflora. This diversity could be linked to the pH variations throughout the different stages of fermentation. The significant decrease in pH during the second (pH 4.53) and the third (pH 4.40) stages of fermentation may be considered consequence of acid production by the increasing microbial populations.

Levels of most lactic acid bacteria, and yeasts increased during the 60 and 90 days of fermentation, when coliforms, staphylococci and lipolytic bacteria decrease their growth, probably due to the combined inhibitory effect of pH and brine concentration (Litopoulou-Tazanetaki, 1977).

In this study, the first stage of fermentation (15 days) was characterized by the growth of rather heterogeneous microflora, including aerobic bacteria, coliforms, staphylococci, lactic acid bacteria, lactobacilli, enterococci, psychrotrophs, lipolytic bacteria and yeasts. At the second stage (60 days) lactic acid bacteria and yeast population outnumbered the initial heterogeneous bacterial flora. Finally, at the third stage (90 days), *L. plantarum* population became dominant (31.7%) and persisted throughout the fermentation together with yeast *populations* (*Saccharomyces cerevisiae* and *Candida parapsilosis*), so that *L. plantarum* can be considered the dominant species in naturally fermented Algerian olives. In similar works, de la Borbolla y Alcalá (1971), Fernández Gonzalez *et al.* (1993), Ruiz-Barba and Jiménez-Díaz (1995) and Harris (1998) reported that the indigenous lactic acid bacteria change spontaneously during natural olive fermentation. At the end of the process only *L. plantarum* and yeasts were detected. Asehraou *et al.* (2002) described similar results in fermenting green olives that had been inoculated with *L. plantarum.* In contrast, Randazzo *et al.* (2004) found that about 50% of the isolated strains from naturally fermented Sicilian green olives belonged to the *L. casei* species.

Two species of yeasts (*Saccharomyces cerevisiae* and *Candida parapsilosis*) were isolated from the three stages of fermented olives. This result is not in complete agreement with those reported by Ruiz-Barba and Jimenez-Diaz (1995) who detected strains of *Saccharomyces cerevisiae, Candida parapsilosis* and other species of yeasts during the first stage of the fermentation. The same authors reported that *Saccharomyces cerevisiae* and *Candida parapsilosis* appeared very early in the olive brine and seemed to be well adapted to the first stage of fermentation.

An interesting question concerns the presence and persistence of *L. plantarum* and other lactic acid bacteria together with yeasts at the third stage of fermentation. However, the initial heterogeneous bacterial flora decreased during the fermentation process. In our study we demonstrated that brine solutions obtained at the end of fermentation have the capacity to stimulate several L. plantarum

strains. We have not established, however, the nature of this stimulatory factor (s). Tests to determine the nature of this stimulatory agent (s) will follow its purification.

Several authors described an increase in the vitamin content of products that had undergone a lactic acid bacteria fermentation (Akinrele, 1970; McFeeters, 1988). In most cases, this production of vitamins has been attributed to the activity of different microorganisms growing together with e lactic acid bacteria during fermentation (Costilow and Fabian, 1953a, 1953b; Rosen and Fabian, 1953). Ruiz-Barba and Jimenez-Diaz (1995) reported that several yeast strains were able to produce large amounts of different vitamins in a laboratory medium. These authors found some indications supporting the idea that these vitamins are produced by yeasts during fermentation.

At present, several brine solutions are being further investigated in our laboratory to characterize and identify the stimulating factors present in these solutions. More recently, we have reported a new proteinaceous bioactive substance, which stimulates the growth of several *L. plantarum* strains previously isolated from naturally fermented green olives (Kacem *et al*., 2004a). This factor named "Stimulin" (Kacem *et al*., 2004b) is produced by strains of lactococci isolated from *Lben*, a naturally fermented milk produced in Algeria.

On the other hand, our study indicated that low incidences of coliforms, staphylococci, psychrotrophs or lipolytic bacteria were detected at the end of fermentation. Probably, the growth of these bacteria must be restricted by brine acidification (the pH of the brine solution was 4.40 at the end of fermentation) and a bacteriocin produced by lactic acid bacteria (in particular, *L. plantarum* strains): as we know, some strains of *L. plantarum* isolated from fermented olives produce inhibitory substances, such as bacteriocins (Jiménez-Díaz *et al.,* 1993; Delgado *et al.,* 2001 and Maldonado *et al.,* 2003, Kacem *et al.,* 2005).

From the results presented here, it is clear that lactic acid bacteria represented by *L. casei*, *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. confusus*, *L. cellobiosus*, *L. lactis* subsp. *lactis*, *E. faecalis*, *E. faecium*, *E. durans* were isolated at different stages of the spontaneous fermentation of green olives produced in western Algeria. *L. plantarum* was the major species isolated in this study. Similar results have been reported in the literature (Fernández-Díez, 1983; Ruiz-Barba *et al*., 1994; Van Den Berg *et al.* 1993; Lavermicocca *et al*., 1998, 2002; Randazzo *et al.,* 2004, Kacem *et al.,* 2004a).

E. faecalis and *L. lactis* subsp. *lactis* species were also isolated in this study. *Enterococcus* genus, a frequent contaminant in olives has been isolated from green olive fermentations (Floriano *et al*., 1998). Isolates have been identified, characterized and utilized with *L. pentosus* as starter cultures for Spanish-style green olive fermentation (de Castro *et al*., 2002).

Concerning *L. lactis* subsp. *lactis,* this bacterium is occasionally isolated from fresh olives (Korukluoglu *et al*., 2002), olive oil (Kacem *et al.,* 2003) or fermented olives (Kacem *et al.,* 2004a) but has not ever before been reported as being related to olive fermentation.

5. CONCLUSION

The aim of this work was to gain a comprehensive view of the microbiological characteristics of naturally fermented green olives produced in Western Algeria during fermentation.

The results from this work show a significant presence of different microbial groups after 15 and 60 days of fermentation. At the end of fermentation, lactic acid bacteria (essentially *L. plantarum*) and yeasts (*Saccharomyces cerevisiae* and *Candida parapsilosis*) predominate over the other microbial groups.

Different species of lactic acid bacteria and yeasts present in this product were isolated and identified on the basis of morphological and biochemical criteria. The characterization of isolates based on a microscopic analysis and phenotypic characteristics (especially biochemical properties and sugar fermentation abilities) is very useful and remains the most widely recognized approach but it would be interesting to conduct a more detailed study on bacterial identification using molecular methods. Experiments concerning molecular identification of these bacteria are in progress.

At the same time, current research in our laboratory is aimed at studying the interactions (inhibition or stimulation) between *L. plantarum* strains and yeasts in laboratory media as well as in brined olive juice. This would allow us to select the best combinations of yeasts and *L. plantarum* strains to use them as starters in the fermentation of Algerian fermented olives with the flavor and sensorial qualities desired by consumers.

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