Microorganisms associated with post-harvest green olives deteriorations in Morocco.

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RESUMEN

Microorganismos asociados con la deterioración de aceitunas verdes tras la recolección en Marruecos.

Se estudiaron los microorganismos envueltos en las alteraciones post-cosecha de aceitunas verdes alteradas recolectadas mediante el método de vareo y antes del proceso de fermentación. Las determinaciones incluyeron: recuento estandar en placa, bacterias gram-negativa (coliformes y pseudomonas), *bacillus* y levaduras. Los resultados mostraron que las alteraciones son debidas en primer lugar a las levaduras y a sus interaciones con pseudomonas y coliformes. Las levaduras más frecuentes estuvieron representadas por las especies: *Debaryomyces hansenii, Rodothorula glutinis, Pichia membranefaciens, P. anomala* y *Candida bacarum.* Las bacterias gram-negativa estuvieron representadas por las especies: *Erwinia carotovora, Hafnia alvei, Enterobacter agglomerans, E. aerogenes* y *Serratia marcescens, S. liquefaciens* y *Shigella flexneri.* Las bacterias oxidasa-positiva fueron las más abundantes, predominando las especies de *Pseudomonas, P. aeruginosa, P. alcaligenes* y *P. syringae.* La mayoría de los aislados de estos microorganismos fueron celulolíticos y lipolíticos.

Las especies *bacillus* fueron también aisladas e identificadas. Las especies principales fueron *Bacillus megaterium, B. pumilus, B. cereus* y *B. olei.*

Las especies de *bacillus* no parecieron estar relacionadas con la deterioración de las aceitunas.

PALABRAS-CLAVE: Aceituna verde — Alteración — Marruecos — Microorganismo — Recolección.

SUMMARY

Microorganisms associated with post-harvest green olives deteriorations in Morocco.

Altered green olive fruits harvested by the pole slender method were studied for the microorganisms involved in post-harvest alterations of the fruits before the fermentation process. The determinations included: standard plate count, Gram-negative bacteria (coliforms and pseudomonads), *Bacillus*, and yeasts. Results showed that the alterations are due first to yeasts and their interactions with pseudomonads and coliforms. The most frequent yeasts were represented by the species: *Debaryomyces hansenii, Rodothorula glutinis, Pichia membranefaciens, P. anomala* and *Candida bacarum.* Gram-negative fermenting bacteria were represented by the species: *Erwinia carotovora, Hafnia alvei, Enterobacter agglomerans, E. aerogenes* and *Serratia marcescens, S. liquefaciens* and *Shigella flexneri.* The oxidase-positive bacteria were most abundant and mainly dominated by *Pseudomonas* species including *P. aeruginosa, P. alcaligenes* and *P. syringae.* Most of the isolates of these microorganisms were cellulolytic and lipolytic. *Bacillus* especies were also isolated and identified. The main species were *Bacillus megaterium, B. pumilus, B. cereus* and *B. olei. Bacillus* species seem not involved in olive deteriorations.

KEY-WORDS: Alteration — Green olive — Harvesting — Microorganism — Morocco.

1. INTRODUCTION

The high production of olives in Morocco (6.9% of the world production), the traditional harvest method, and the long storage of the fruits at relatively high room temperatures (20-24 °C) before processing result in a high loss and poor quality of the fermented olives and/or the extracted oil.

Up to now, no work had been done in Morocco to identify the microorganisms responsible of olive fruits alterations. Various species would be involved in the olive attacks. These include bacteria, yeasts and moulds.

The natural microflora is very complex and may vary widely with the conditions of harvesting, transporting, and storing. Injured fruits during these operations are the most susceptible to alterations. Species with a cellulase and/or lipase may have an important concern in the deterioration of the fruits and also an incidence on the products obtained from altered fruits (Karbassi and Luh, 1979). The spot attacks on the fruits are the most frequent alterations of the fruits. This is followed by a severe softening and/or sloughing. The fruits reaching this deterioration stage become inedible even if they are fermented. Morever the lipases produced by some lipolytic microorganisms may induce high acid degree value in the extracted oils due to the high free fatty acids content. A late harvest and an extended storage of fruits may increase these deteriorations.

In the present work, the microflora responsible for the olive fruits alterations was studied and the most frequent species of various groups were identified and characterized.

2. MATERIALS AND METHODS

2.1. Sampling

Lots of 25 kg of green and red olives were purchased from the orchard of Marrakech. The altered fruits were manually separated in the laboratory to make 10 bulks to be sampled for the plate counts and isolation of the different microorganisms that would cause the alteration.

Ten grams of the attacked fruits removed with a sterilized knife were introduced in erlenmeyer flasks containing saline-water (0.85%) to make the initial dilution from which serial dilutions up to 10^{-5} were prepared in tubes containing sterile saline water (0.85%).

2.2. Microbiological

Isolation and identification. The standard plate count (SPC) were determined by plating appropriate

dilutions on Plate Count Agar (PCA Merck, Germany). The plates were incubated for 3 days at 30°C. Coliforms were evaluated by plating dilutions from 10^{-1} to 10^{-4} on Deoxycholate-Lactose-Agar (Merck, Germany) and incubated for 24 hours at 37°C. Red colonies were counted and some of them were streaked on the same medium for purification. The isolates were maintained on trypticase soya agar slants (Biocar, France) and kept at 4°C for further identification and characterization. Coliform isolates were checked for their shape, Gram and oxidase reactions. Rod shaped Gram-negative and oxidase negative were then submitted to the biochemical identification according to the method described by Larpent and Larpent-Gourgaud (1975).

The oxidative bacteria were plated directly on Agar F and agar P (Difco laboratories USA) and incubated for 10 days at 4°C. The pigmented colonies were counted and some of them were purified on the same media and streaked on trypticase-soya-agar slants for further studies. The isolates were also submitted to the same tests (Gram and oxidase). Oxidase-positive Gram-negative rod shaped isolates were then identified according to the method described by Larpent and Larpent-Gourgaud (1975).

For the determination of sporeforming bacteria, the initial dilution of the sample was heated 15 min at 70°C in a water bath to activate the spores and to destroy the vegetative cells of non-sporeforming bacteria. Appropriate dilutions (10^{-2}, 10^{-4}) were then realized and plated on trypticase soya agar (Biocar, France). The plates were incubated for 48 hours at 30°C under aerobic conditions. The grown colonies on the medium were counted and some of them were checked for Gram reaction and spore formation. Gram-positive rod shaped sporeforming isolates were identified according to the method described by Larpent and Larpent-Gourgaud (1975). 5 mL, 2 mL and 1 mL from the heated initial dilution were inoculated to tubes containing a solid medium (Reinforced *Clostridium* agar) and before it can solidify, 0.1 mL of a 5% sodium sulfite solution and 0.1 mL of a 5% ferric ammonium citrate solution were added. The medium was shaked, allowed to solidify and incubated at 30°C for 24 to 48 hours. Black colonies on the medium are considered sulfite-reducing *Clostridium*.

Yeasts counts were carried out by plating dilutions from 10^{-1} to 10^{-4} on potato-dextrose-agar (PDA) (Difco Laboratories, USA) acidified to 3.5 with lactic acid. The plates were incubated at 28°C for 3 to 4 days. The grown colonies were counted and some of different morphology were streaked on PDA slants for further identification and characterization. The simplified identification key described by Deak and Beuchat (1987) was used for the identification of the isolates.

Characterization. Isolates of most abundant microorganisms including Gram-negative bacteria and yeasts were characterized for their lipase, protease, and cellulase formation. Lipase and protease were determined respectively on the victoria blue B agar (Alford, 1975) and gelatin medium (Lee, 1976). Presence of cellulase in the isolates was determined by inoculating yeast nitrogen base (Difco Laboratories, USA) supplied with cellulose (Merck, Germany) for yeasts. For the Gram-negative isolates a mineral medium containing g/l: Yeast extract (Merck, Germany) 5, sodium chloride (Merck, Germany) 5, cellulose (Merck, Germany) 10 was inoculated. In both cases, growth on the medium was considered as positive reaction.

3. RESULTS AND DISCUSSION

3.1. Viable counts

Standard plate counts of the different samples ranged from $1 \cdot 10^4$ to $80 \cdot 10^4$ cfu/g (Colony Forming Unit). The

Table I. **Microbial counts of the green olive fruits harvested by the slender pole method**

N	SPC (10^4)	FC (10^3)	Ps (10^2)	Bac (10^2)	ClostYeasts (10^3)
1	1	<1	1	1	- 10
2	4	1	16	0	- 3
3	3	0.4	2	14	- 1
4	8	22	8	12	- 4
5	9	2	5	13	- 20
6	20	6	68	40	- 70
7	50	1	140	10	- 70
8	-	<1	260	<1	- 80
9	-	12	58	<1	- 240
10	80	100	120	3	- 680
11	-	28	1000	3	- 650

SPC: Standard Plate Count.
FC: Fecal Coliforms.
Ps: Pseudomonas.
Bac: Bacillus
Clost: Clostridium

microbial profiles of the olive fruits are high relatively to some other vegetables. This is most probably due to the intensive pollution of the fruits during harvesting and handling. Data reported in table I showed an abundance of the Gram-negative bacteria and yeasts. Coliform counts ranged from numbers $<10^3$ to 10^4 cfu/g. Numbers of oxidase-positive bacteria ranged from 10^2 to 10^5 cfu/g. This may tell about the microflora involved in the post harvest attacks of olives and may also tell about a heavy contamination of some samples. Yeast counts were the highest and ranged from $1 \cdot 10^3$ to $6.8 \cdot 10^5$ cfu/g. Not only yeasts were the most abundant microorganisms but they also showed a wide variation among the samples. *Bacillus* did not show high counts relatively to the other groups and seems to have a little role in olive alterations.

3.2. Identification and characterization

The biochemical and physiological characteristics of the Gram-negative isolates are reported in Table II. Among the studied isolates, *Erwinia carotovora, Hafnia alvei,* and *Serratia liquefaciens* were the most abundant species. Some others were also isolated from the altered fruits but they are of little significance because of the low proportions recovered relatively to the other species.

Pseudomonas species isolated from the altered fruits were *Pseudomonas sp, P. aeruginosa, P. alcaligenes and P. syringae* (Table II). All the isolates belonging to *P. aeruginosa* and *P. alcalifaciens* were lipolytic and cellulolytic while 50% the isolates of *P. syringae* were lipolytic and proteolytic. Lipolytic and cellulolytic strains of *E. carotovora* were also highly represented 92.9 and 74% respectively (Table III).

Table III. **Spoilage activities through lipase, protease and cellulase presence in the isolates of Gram-negative bacteria (figures in %)**

Number	Lipolytic	Proteolytic	Cellulolytic	Strains
29	92.9	0	74	*Erwinia carotovora*
4	100	25	100	*Pseudomonas aeruginosa*
3	100	0	100	*Pseudomonas alcaligenes*
2	100	0	50	*Enterobacter agglomerans*
2	100	0	100	*Serratia marcescens*
5	100	0	80	*Serratia liquefaciens*
2	50	0	50	*Pseudomonas syringae*
1	100	0	100	*Tatumella sp.*
5	100	0	100	*Shigella flexineri*
5	75	0	75	*Pseudomonas sp.*
2	100	0	100	*Shigella boydii*
2	50	-	100	*Proteus alcalifaciens*
6	100	17	67	*Hafnia alvei*
2	100	0	100	*Erwinia aerogenes*
1	0	0	0	*Proteus sp.*
1	100	0	100	*Pseudomonas inconstans*
1	100	0	100	*Serratia rubideae*

The mechanism by which microorganisms act in olive spoilage is still not well understood. The interactions of some species with each other and with yeast species may help elucidating the phenomena and especially the potentially active microorganisms in the alteration during the fermentation and/or during the storage of post process of fermented olives.

Table II. **Physiological and biochemical characteristics of Gram-negative bacteria isolated from green olive fruits**

Nb	Oxy	Ur	MR	Act	Ind	Gaz	H_2S	Pig	Glu	Lac	Suc	Gal	Mal	Xyl	Ara	Fru	Cel	Ado	Ino	Sor	Strains
29	-	-	+	-	-	+	-	-	-	+	+	+	+	+	+	+	+	-	+	+	*Erwinia carotovora*
4	+	±	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	*Pseudomonas aeruginosa*
3	+	±	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*Pseudomonas alcaligenes*
2	-	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	*Enterobacter agglomerans*
2	-	-	-	+	-	-	-	-	+	-	-	-	+	+	+	+	+	-	+	+	*Serratia marcescens*
5	-	-	+	-	-	+	-	+	-	-	+	+	+	+	+	+	-	-	+	+	*Serratia liquefaciens*
2	-	-	-	+	-	+	-	+	+	-	+	+	+	+	+	-	+	-	+	+	*Pseudomonas syringae*
1	-	-	-	+	-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	+	*Tatumella sp.*
5	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	-	-	*Shigella flexineri*
5	-	+	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	-	+	*Pseudomonas sp.*
2	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	*Shigella boydii*
2	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	*Proteus alcalifaciens*
6	-	-	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	-	*Hafnia alvei*
2	-	-	-	+	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	*Erwinia aerogenes*
1	-	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	*Proteus sp.*
1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	*Pseudomonas inconstans*
1	-	-	+	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*Serratia rubideae*

Oxy: oxydase; Ur: urea; MR: methyle red; Act: acetoin; Ind: indol; Gaz: gaz formation on glucose; H_2S: H_2S formation; Pig: pigments formation; Glu: glucose fermentation; Lac: lactose; Suc: sucrose; Gal: galactose; Mal: maltose; Xyl: xylose; Ara: arabinose; Fru: fructose; Cel: cellobiose; Ado: adonit; Ino: inositol; Sor: sorbitol

Most of the isolates belonging to the Gram-negative bacteria showed a cellulolytic activity in a minimum medium. This may tell about the potential hydrolysing systems of the fruits. Cellulase formation may lead to physical deteriorations of olives during fermentation. The destruction of cellulotic matrix by cellulase may result in sloughing of the fruits and consequently the produce is not edible.

Almost the same pattern was observed for the lipase formation by the isolates on Victoria Blue B agar. High proportions of the isolates showed a lipolytic activity. This may have a direct incidence on the quality of the obtained products either fermented olives or olive oil. A high acid degree value due to the occurrence of lipolysis by lipases freed by the lipolytic microbiota in the fruits prior to the processing, may lead to a low quality of oil and/or to a non edible fermented olives.

Low proportions of the isolates showed a proteolytic activity in the Gram-negative isolates. The presence of protease was determined by gelatin liquefaction by the strains. Some proteolytic species may not develop a gelatinase activity.

All the sporeforming isolates were aerobic and catalase positive. These isolates were identified as *Bacillus* species and were grouped in the species classified by Nourris et al, 1977. *Bacillus* were reported to have an effect on the pectic matrix of olive fruits (Karbassi et Luh, 1979). However in our case *Bacillus* species seem not involved in the deterioration of the olive fruits during the post-harvest storage because of the low counts recovered from some spoilt samples.

Gram-negative bacteria including coliforms and oxydase-positive bacteria of the genera *Pseudomonas* are known by their large distribution in the nature (soil, vegetables, water, food, etc...) and by their proteolytic/lipolytic activity leading to some food alterations through the destruction of the protein/lipid matrix. Morever, these microorganims can tolerate unfavourable conditions and grow on some foods such as vegetables.

The taxonomic criteria reported in Table IV show the physico-chemical and the biochemical properties of the yeast strains isolated from altered olives. One can figure out that some criteria are of great significance for the microbiology of fermented olives. That is some species are very common in the olive fermentations as it was reported by some authors (Vaughn et al, 1969, Vaughn et al, 1972) and may have an effect on the technology of the fermented green olives. Among the species widely distributed, *Debaryomyces* and *Pichia* are the most abundant.

As it can be seen the strains belonging to *Debaryomyces* had some interesting properties including the growth in presence of high sodium chloride concentrations up to 12-15%, growth at 37°C, use of nitrate and urea as sole source of nitrogen, cellulose and lipids hydrolysis and growth on various carbohydrates. The other species belonging to the genera *Pichia* are also highly represented. 94.73% of the isolates belonging to *D. hansenii* showed a positive lipolytic reaction and 89.48 % of the isolates were cellulolytic (Table V). *P. anomala* isolates were lipolytic (50%) and cellulolytic (50%) while all the isolates belonging to *C. bacarum* were lipolytic (100%) and proteolytic (100%).

The contamination of olive fruits by yeast strains in high numbers on one hand, and the presence of active strains (presence of hydrolases) on the other hand may involve these microorganisms in the olive fruits spoilage. The presence of yeasts in high numbers in the fermentor during the fermentation process is seemingly harmful to the organoleptic quality of the fermented olives. The eating quality rely on the organoleptic characteristics including: physical state of the fermented fruits, color and

Table IV. **Physiological and biochemical characteristics of yeast strains isolated from green olive fruits**

Nb	Sp	myc	37°C	NO_3	Ur	NaCl (%)				Glc		Mal		Treh		Lev		Lac		Ara		Cel		Gal		Suc		Strains
						5	10	12	15	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	
1	4	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-	*Pichia media*
19	2	-	-	-	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Debaryomyces hansenii*
3	-	-	-	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Rhodotorula glutinis*
1	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	-	-	-	-	*Candida lipolitica*
4	-	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*C. bacarum*
4	4	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	*P. membranefaciens*
2	-	-	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Cryptococcus terreus*
8	4	-	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*P. anomala*
1	-	-	+	-	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	*Cryptococcus flavus*
2	-	-	+	-	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Rh. mucilaginosa*
1	-	-	+	+	-	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*C. fragarium*
1	4	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*P. etchelsii*
1	-	-	+	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	-	-	+	-	+	-	+	+	*C. versatilis*

Sp: spore formation; myc: mycelium formation; 37°C: growth at 37°C; NO_3: NO_3 utilisation; Ur: urea utilisation; Na Cl: growth on NaCl concentrations; Glc: glucose; Mal: maltose; Treh: treahalose; Lev: levulose; Lac: lactose; Ara: arabinose; Cel: cellobiose; Gal: galactose; Suc: sucrose; G: growth on; F: fermentation.

Table V. **Spoilage activities through lipase, protease and cellulase presence in yeast isolates (figures in %)**

Number	Lipolytic	Proteolytic	Cellulolytic	Strains
1	100	100	100	*P. media*
29	94.73	5.26	89.48	*D. hansenii*
3	33.33	0	66.6	*R. glutinis*
1	100	0	0	*C. lipolytica*
4	100	0	75	*C. bacarum*
4	50	25	50	*P. membranefaciens*
2	100	0	100	*Cry. terreus*
8	50	12.5	50	*P. anomala*
1	100	0	100	*Cry. flavus*
2	100	0	100	*R. mucilaginosa*
1	100	0	100	*C. fragarum*
1	0	0	100	*P. etchelsii*
1	100	0	0	*C. versatilis*

texture, taste and flavour. Brown spots on the fermented fruits, sloughing, softening, and discoloring are the main relevant problems that can occur during the fermentation. Some of these are due to a poor quality of the raw material because of the alteration problems that can occur during the harvest and storage before the processing.

Yeasts are the most active microorganisms in the decaying process of vegetables and the high counts of yeasts found in the samples of olive fruits are reported in Table I. The level reached by yeasts in the fruits may give the evidence that these microorganisms are seemingly active in the olive fruits alterations. This activity may be encouraged by the presence of other microorganisms (*Pseudomonas* species) which can grow in interactions with yeasts. *Debaryomyces* species may survive high salt concentration (Walker and Ayrges, 1970) and induce some deteriorations during the fermentation process or during storage.

Lipase formation is more dangerous in the crude fruits because they may induce some changes in the properties of the products during the oil extraction process and may lower the quality of oil by increasing the free fatty acid content in the fruits before extraction and during the storage of the extracted oil. Attacked fruits are usually used in oil processing because they are susceptible to alterations during the fermentation and can not be accepted by the consumer.

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