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Isolation of the Lactic Acid Bacteria from Sigoise and Chemlale Olive Varieties in Ain Defla (Algeria)

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Research Article

Abstract

Sigoise and Chemlal are two varieties of olives (*Olea europea)* that are popularly consummated in Algeria. Considering that lactic acid bacteria are an integral part of the microbial flora of fermentation and biopreservation importance in the olive tegument, the aims of this work are to isolate and characterise the lactic acid bacteria flora of shredded olive in salt samples, collected in the region Kodiat Zebbouj-Bourached- Ain Defla, Algeria. The isolated lactic acid bacteria strains were identified, using phenotypic, biochemical and physiologic characteristics including proteolytic, lipolytic and acidifing activities.

The twenty lactic acid bacterial (8 from Sigoise and 12 from Chemlal) were identified as belonging to, *Enterococcus*, *Lactococcus*, *Streptococcus thermophilus, Leuconostoc* and *Pediococcus* genera. All the isolated lactic acid bacteria were non-lipolytic but produced lactic acid *Streptococcus thermophilus* however, produced a very high proteolytic activity (diameter=25 mm). Thus the lactic acid bacteria strains isolated from Sigoise and Chemlal olives can be used in agro-alimentary industries to prepare many products benefit to human and animals.

Keywords: Olive; Lactic acid bacteria; Proteolytic activity; Lipolytic activity; Acidifing power.

1. Introduction

The history of the olive-tree (*Olea europea* L.) merges with that of the ages of the Mediterranean basin. Thus, the tree and its oil occupy a dominating place in the culture and the heritage of the great ancient civilisations. The remote origin of *O. europea* L. was always accompanied by innumerable legends because the different people of the Mediterranean allotted to their own gods, the creation of the olive-tree. Consequently, the olive-tree became a sacred tree par excellence [1].

O. europea L. account of many varieties having important phenotypical diversities, although the origins of these varieties remain vague. Various works suggested that the inter-fertility between the cultivated forms and/or the wild forms is at the origin of the diversification of the cultivated olive tree [2].

The Chemlal variety is regarded as the best producing one of oil of good quality [3,4]. The Sigoise variety, also called Zitoune Tlemcen, which is intended for consumption as olive of table occupies most (80 to 90%) of the olive-trees [3,4]. The green olives are fruits harvested during the ripening period, prior to colouring and when they have reached normal size, but black olives are fruits harvested when fully ripe or slightly before full ripeness is reached [5]. Due to peptic substances and other fruit components, the olives have a very diverse microflora and according to Borcakli et al. [6] and Kotzekidou [7], these flora are primarily made up of Gram negative bacteria, yeast and Lactobacillus, though the Lactic Bacteria (LAB) are more abundant than the yeasts and mainly selected according to their acidifying, proteolytic and aromatic properties [8,9]. Lactic acid bacteria, which were firsty definded by Jensen [10] are commonly involved in a large number of spontaneous fermentations of food products and mainly used as starters in fermented food products, leading to the development of certain characteristic organoleptic properties and increase in shelf-lives. Indeed, LAB produce many metabolites, including those having antimicrobial properties, such as, hydrogen peroxide, reuterin, diacetyl, bacteriocins and principally organic compounds, i.e., lactic acid [11,12]. The LAB is also responsible for the fermentation process of olive of table production [13]. The objectives of this study therefore, are to identify isolated LAB from shredded olive samples obtained from the area of Bourrached-Ain Defla and determine their metabolite producing properties.

2. Materials and Methods

2.1. Description of location site

The wilaya of Ain Defla occupied a central geographical position, as the territory of the Wilaya remains inserted between the mountainous massifs of the DAHRA-ZACCAR in the North and OUARSNIS in the south, with a plain in the center, in the form of basin crossing from east to west by Wadi Cheliff, an important national stream.

The Wilaya of Ain Defla is located 145 km south-west of the capital and extends over an area of 4544.28 km² (Figure 1) [14]. The wilaya of AIN-DEFLA presents a semi-arid Mediterranean climate with a difference of 20°C between the temperatures in January and August. The summer extends for about 5 to 6 months, with masses of hot air from the month of May. Rainfall remains variable and reaches 500 to 600 mm/year. A series of climatic stages that goes from the sub-arid to the bottom from the valley to the sub-humid on the reliefs. This situation is linked to the orography: more the altitude is high and the floor is wet. The same applies to snow cover the reliefs of more than 600 m of altitude (Figures 1 and 2) [14].

2.2. Sampling

One sample of each variety is collected, starting from the olives, green and ripe fruits (black) of the area of Kodiat Zebbouj-Bourached-Wilaya of Ain Defla. The olives are preserved in bottles containing of sterile saline, and left to ferment for 4 months (December-March).

Variety 1 corresponds to green olive (Sigoise): (V1/OG).

Variety 2 corresponds to black olive (Chemlal): (V2/OB).

Figure 1. Situation of Ain Defla in Algeria (Google Maps, 2017) [43].

Figure 2. Situation of Bourached in Ain Defla (Google Maps, 2017) [43].

The pH of the preserved olive was measured before the isolation of the lactic bacteria; which makes it possible to determine the acidifying power of the lactic acid bacteria flora [15].

2.3. Isolation of the lactic acid bacteria

About 5 g of each variety of olive was crushed in saline (45 ml), followed by homogenisation, then, decimal dilutions up to 10^{-6}, prepared in physiological saline (0.9% NaCl). Aliquots of 1 ml of 10^{-3} to 10^{-6} dilutions were separately platted on the surface of sterile MRS (lactobacilli), M17 (lactococci), Elliker (streptococci and lactobacilli) and Mayeux (*Leuconostoc*s) agars, and incubated at 30°-37°C for 48 h-72 h [16,17]. The purified LAB strains were preserved by two methods: Short-term preservation: the pure LAB strains were preserved at +4°C, for which resuscitation was done by sub-culturing of the stock batch every four weeks [18]. Long-term preservation was however, by freezing the purified isolates at -20°C, in the culture medium containing 70% skimmed milk (enriched with 0.05% of yeast extract) and 30% glycerol, after centrifugation at 3000 tr/min for 10 min. Acording to Samelis et al. [19], the culture can be preserved for several months, although if necessary, the cultures are sub-cultured twice before use, into skimmed milk enriched with yeast extract [18].

2.4. Identification of the lactic acid strains

The purified isolated LAB strains were phenotypically identified, based on their colonial and microscopic morphologies, as well biochemical and physiological characteristics [20]. The determined biochemical and physiological characteristics included, catalase, oxidase, growth under various physiological test conditions (temperatures: 10°C, 37°C and 45°C; pH: 4.2, 6.5 and 9.6; as well as, 2.3, 4 and 6.5% of NaCl concentrations). Thermoresistance was determined at 63.5°C for 30 min; while other biochemical and physiological tests determined were, fermentative tests, mannitol mobility; research of enzymes (LDC: Decarboxylase Lysine; ODC: Decarboxylase Ornithine; ADH: Dihydrolase Arginine); production of acetoine; utilisation, urea-indole, β-galactosidase, nitrate-reductase; litmus milk tests; starch hydrolysis, esculin hydrolysis, gelatine hydrolysis, respiratory type; biliary salt test; production of exopolysacharides; resistance to antibiotics; and haemolysis test [17,19-28].

2.5. Lactic acid production potentials

To determinate the acidifying power following 16 h, we used the Bradley et al. [29] method modified for lactic acid bacteria isolated from olives.

Acidity is determined by the formula:

Acidity (°D)=V NaOH × 10

V NaOH: Volume of NaOH used to titrate lactic acid contents in 10 ml of the broth of acid bacteria strains.

2.6. Proteolytic activity of the lactic acid bacterial strains

To determine the proteolytic activity of the lactic acid bacteria, the agar medium with milk (10% of skimmed milk) was run, solidified and dried then paper discs were deposited on the surface of the agar. Each disc receives a volume of 20 µl of a young culture.

After incubation at 37°C during 24 h, the proteolysis is revealed by clear zones around the discs [30]. The proteolytic activity is evaluated on solid medium by the measurement of the zone diameter of the clearance, expressed in mm [31].

2.7. Lipolytic activity

This activity is determined on sterile MRS medium plugged with pH 7 and added with 1% of Tween 80 (artificial lipidic sources) [32]. The extracellular lipolytic activity is determined by proportioning of free fatty-acids in the supernatant of culture [33].

2.8. Statistical analysis

A calculation of the mean and margins of errors in measuring the amount of lactic acid secrete during 16 h by the strains of lactic acid bacteria isolated from sampling of olive was done by excel 2007.

3. Results

From the shredded olive samples taken of the area of Ain-Defla, we isolated 8 strains of lactic bacteria from the V1/OG and 12 strains from the V2/OB.

3.1. Morphological characteristics

After incubation at 30°C and 37°C (Figures 3 and 4), only the small, creamy, circulars, smooth and regular colonies having a size of 1 mm to 2 mm in diameter and the small creamy, irregular colonies from 1 to 3 mm in diameter were retained among all the found colonies of the microbial flora of olive samples (yeasts, moulds, other bacteria).

According the morphological characteristics, the lactic acid bacteria strains were included in 5 genera. From V1/OG samples: *Lactococcus* (37%), *Streptococcus* (25%) *Leuconostoc* (13%) *Pediococcus* (13%) and *Enterococcus* (12%); and from V2/OB: *Streptococcus* (28%), *Pediococcus* (27%), *Leuconostoc* (18%), *Lactococcus* (9%) and *Enterococcus* (8%). The green olive contains much more *Lactococcus*, while the black olive contains *Streptococcus*.

3.2. Physiological and biochemical characteristics

According to the results of phenotypical, biochemical and physiological characteristics the following species were identified: from V1/OG: a strain of *Enterococcus faecium*, three of *Lactococcus lactis* subsp. *diacetylactis*, two of *Streptococcus thermophilus*, one of *Leuconostoc pseudomesenteroides* and one of *Pediococcus cerevisiae*. For V2/OB: a strain of

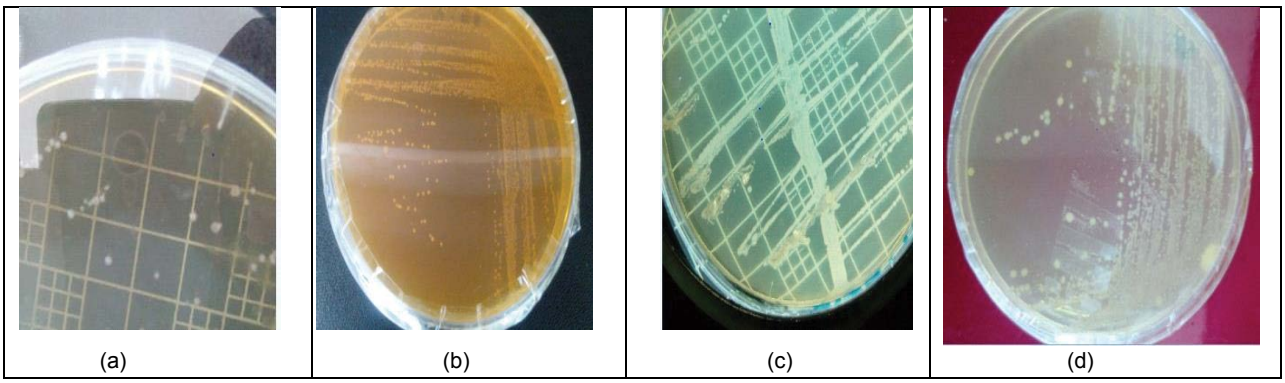

Figure 3. Colonies of lactic acid bacteria isolated from both olive varieties Sigoise (V1/OG) and Chemlal (V2/OB). Culture conditions: a: MRS medium (37°C); b: M17 medium (37°C); c: Elikker medium (30°C), and d: Mayeux medium (30°C)

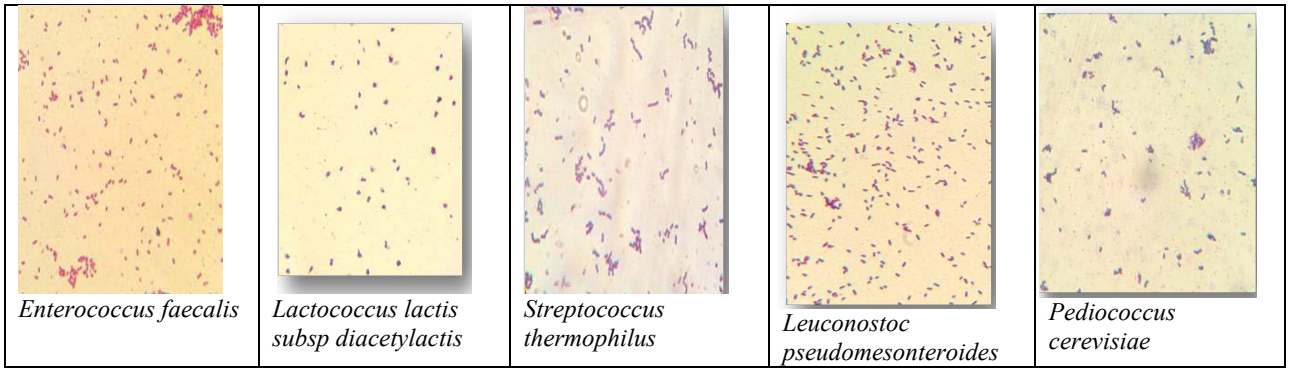

Figure 4. Microscopic appearance of lactic acid bacteria after Gram staining (GX100).

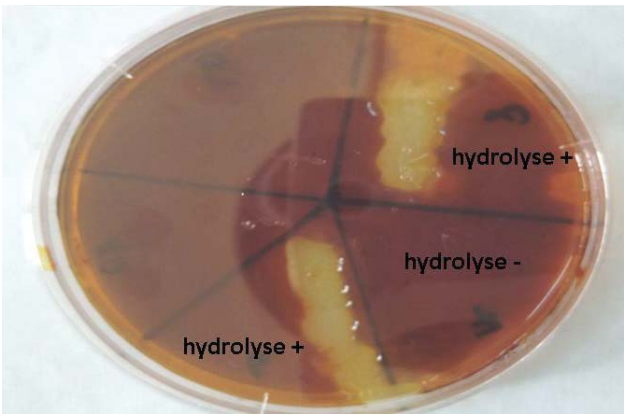

Figure 5. Hydrolysis of starch by lactic acid strains.

Enterococcus faecium, two of *Enterococcus faecalis*, one of *Lactococcus lactis* subsp. *lactis*, three of *Streptococcus thermophilus*, two of *Leuconostoc pseudomesenteroides*, one of *Pediococcus cerevisiae*, one of *Pediococcus acidilactis* and one of *Pediococcus parvulus*. These strains are not pathogenic because they have γ haemolysis (Figures 5 and 6 and Tables 1 and 2).

3.3. Technological characters

Proteolytic activity: After incubation at 37°C during 24 h, this activity expressed by appearance of halo around the discs contains the lactic acid bacteria isolated from V2/OB (Figure 4).

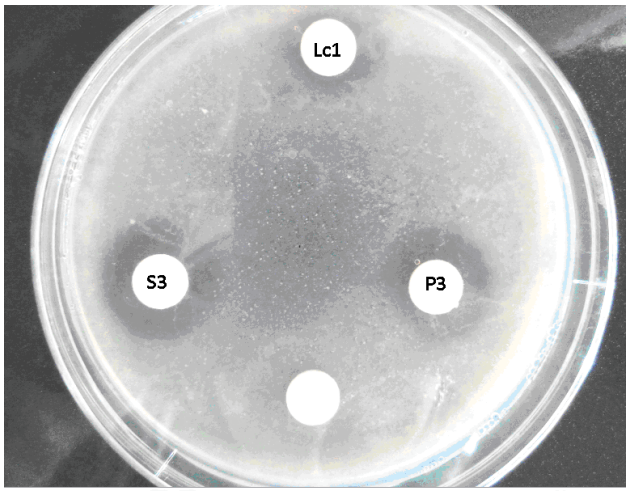

Figure 6. Proteolytic activity of lactic acid strains.

The best zone of proteolytic activity was found with *Streptococcus thermophilus* (diameter=25 mm), other zones were noted with the two strains of *Leuconostoc pseudomesonteroides* (diameter=15 mm) and one strain of *Pediococcus acidilactis* (diameter=23 mm). From the two varieties of olives, Chemlal (V2/OB) contains in this flora proteolytic strains of lactic acid bacteria.

3.4. Lipolytic activity

All the lactic acid strains isolated from green and

Character / Strains	Gra.	Cat.	Oxi.	T Growth °C			Th	pH			NaCl %			TF	DHA	DCL	DCO	Ac.	Cit	UI		ONPG	Nit. Red.		TRS	Ami.	Esc.	G	LM		BS		Dex.	Hem
				10	37	45		4.2	6.5	9.6	2.3	4	6.5							U	I.								Aer.	Anaer.	0.1	0.3		
E 1	+	-	-	V	+	+	+	+	+	+	+	+	+	H	+	+/-	+	-	+	-	+	+	-	+	RC	-	+	-	+	+	-	+	-	γ
E 2	+	-	-	+	+	+	+	+	+	+	+	+	+	H	+	-	-	-	-	-	+	+	-	+	RC	-	+	+	+	+	+	+	-	γ
E 3	+	-	-	+	+	-	+	+	+	+	+	+	+	H	+	+/-	+	+	+	+	+	+	+		RC	-	+	-	+	+	+	+	-	γ
E 4	+	-	-	+	+	-	+	+	+	+	+	+	+	H	+	-	-	+	-	-	+	+	-	+	RC	-	+	-	+	+	+	+	-	γ
L 1	+	-	-	+	+	+	-	+	+	+	+	+	+	H	+	+	-	+	-	-	+	+	-	+	RC	-	+	-	+	+	-	+	-	γ
L 2	+	-	-	+	+	+	-	+	+	+	+	+	+	H	+	-	-	+	-	-	+	+	+		RC	-	+	-	+	+	+	+	-	γ
L 3	+	-	-	+	+	+	+	+	+	+	+	+	+	H	+	-	-	+	-	-	+	+	+		RC	-	+	-	+	+	+	+	-	γ
L 4	+	-	-	+	+	-	+	+	+	+	+	+	+	H	-	-	-	+	-	-	+	+	+		RC	-	+	-	+	+	+	-	-	γ
S 1	+	-	-	-	+	+	+	+	+	+	+	+	+	H	-	+/-	-	-	-	-	+	+	-	-	RC	-	+	-	+	+	+	+	-	γ
S 2	+	-	-	-	+	+	+	+	+	+	+	+	+	H	-	+/-	-	-	-	-	+	+	-	-	RC	-	+	-	+	+	-	+	-	γ
S 3	+	-	-	-	+	+	+	+	+	+	+	+	+	H	-	+	-	-	-	-	+	+	+		RC	+	+	+	+	+	+	+	-	γ
S 4	+	-	-	-	+	+	+	+	+	+	+	+	+	H	-	+	-	-	-	-	+	+	-	+	RC	-	+	-	+	+	+	+	-	γ
S 5	+	-	-	-	+	+	+	+	+	+	+	+	+	H	-	-	-	-	-	-	+	+	-	+	RC	-	+	+	+	+	+	+	-	γ
Ln 1	+	-	-	-	+	+	+	+	+	+	+	+	+	Ht	-	+	+	-	+	-	+	+	+		RC	+	+	-	+	+	+	-	-	γ
Ln 2	+	-	-	-	+	+	+	+	+	+	+	+	+	Ht	-	+	-	-	+	-	+	+	+		RC	-	+	-	+	+	+	+	-	γ
L n 3	+	-	-	-	+	+	+	+	+	+	+	+	+	Ht	-	+	-	-	-	-	+	+	-	+	RC	-	+	-	+	+	+	+	-	γ
P 1	+	+	-	-	+	+	+	+	+	+	+	+	+	H	+	-	-	+	+	-	+	+	+		RC	+	+	-	+	+	+	-	-	γ
P 2	+	+	-	-	+	+	+	+	+	+	+	+	+	H	+	+	+/-	+	+	-	+	+	-	+	RC	-	+	-	+	+	+	+	-	γ
P 3	+	+	-	-	+	+	+	+	+	+	+	+	+	H	+	+	+	+	+	-	+	+	+		RC	-	+	-	+	+	+	+	-	γ
P 4	+	+	-	-	+	-	+	+	+	+	+	+	+	H	+	-	+	+	+	-	+	+	+		RC	+	+	-	+	+	-	+	-	γ

Temperature; Th: Thermoresistance; TF: Type Fermentation; H: Homofermentaire; Ht: Heterofermentaire; DHA: Dihydrolase Arginine; DCL: Decarboxylase Lysine, DCO: DECARBOXYLASE ornithine; Ac: Acetoïne, Cit: Citrate; UI: Uree-Indol; ONPG: Orthonitrophenyl-β-Galactoside; NitRed: Nitrate Reductase; TRS: Test Reduction Souche; RC: Reduction Coagulation; Ami: Amidon; Esc: Esculine; G: Gelatin; LM: Liver Meat; aer: aerobie; anaer: anaerobie; BS: Bile Salts; Dex: Dextrane; Heme: Hemolysis

(+): Growth positive, (-) negative growth, (+/-): unknown variable

Table 1. Characteristics of the lactic acid bacteria isolated.

Sugars / Strains	Ara. 20	Cel. 10	Fru.	Gala.	Glu.	Lac.	Mal.	Mann.	Sac.	Sorb.	Xyl.	Man.	Identification
E 1	+	+	+	+	+	+	+	+	+	+	+	+	*Enterococcus faecium*
E 2	+	+	+	+	+	+	+	+	+	+	+	+	*Enterococcus faecium*
E 3	-	+	+	+	+	+	+	+	+	+	-	+	*Enterococcus fecalis*
E 4	-	+	+	+	+	+	+	+	+	+	-	+	*Enterococcus fecalis*
L 1	+	+	+	+	+	+	+	+	+	+	+	+	*Lactococcus lactis* subsp *diacetylactis*
L 2	+	+	+	+	+	+	+	+	+	+	+	+	*Lactococcus lactis* subsp *diacetylactis*
L 3	+	+	+	+	+	+	+	+	+	+	+	+	*Lactococcus lactis* subsp *diacetylactis*
L 4	+	+	+	+	+	+	+	+	+	+	+	+	*Lactococcus lactis* subsp *lactic*
S 1	-	+	-	-	+	+	+	-	+	-	-	+	*Streptococcus thermoohilus*
S 2	-	+	-	-	+	+	+	-	+	-	-	+	*Streptococcus thermoohilus*
S 3	-	+	-	-	+	+	+	-	+	-	-	+	*Streptococcus thermoohilus*
S 4	-	+	-	-	+	+	+	-	+	-	-	+	*Streptococcus thermoohilus*
S 5	-	+	-	-	+	+	+	-	+	-	-	+	*Streptococcus thermoohilus*
Lc 1	+	+	+	+	+	+	+	+	+	+	+	+	*Leuconostoc pseudomesenteroïdes*
Lc 2	-	+	+	+	+	+	+	+	+	+	+	+	*Leuconostoc pseudomesenteroïdes*
Lc 3	+	+	+	+	+	+	+	+	+	+	+	+	*Leuconostoc pseudomesenteroïdes*
P 1	+	+	+	+	+	+	+	+	+	+	+	+	*Pediococcus cerevisiae*
P 2	+	+	+	+	+	+	+	+	+	+	+	+	*Pediococcus cerevisiae*
P 3	+	+	+	+	+	+	-	+	+	+	+	+	*Pediococcus acidilactic*
P 4	+	+	+	+	+	+	+	+	+	+	+	+	*Pediococcus parvulus*

(+): Growth positive, (-) negative growth, (+/-): unknown variable

Ara.20: Arabinose 20; Cel.10: Celubiose 10; Fru: Fructose; Gala: Galactose; Glu: Glucose; lac: lactose; Mal: Maltose; Mann: Mannose; Sac: Saccharose; Sorb: Sorbitol; Xyl: Xylose; Man: Manitol; Ident: Identification

Table 2: Fermentation of sugars by lactic acid bacteria.

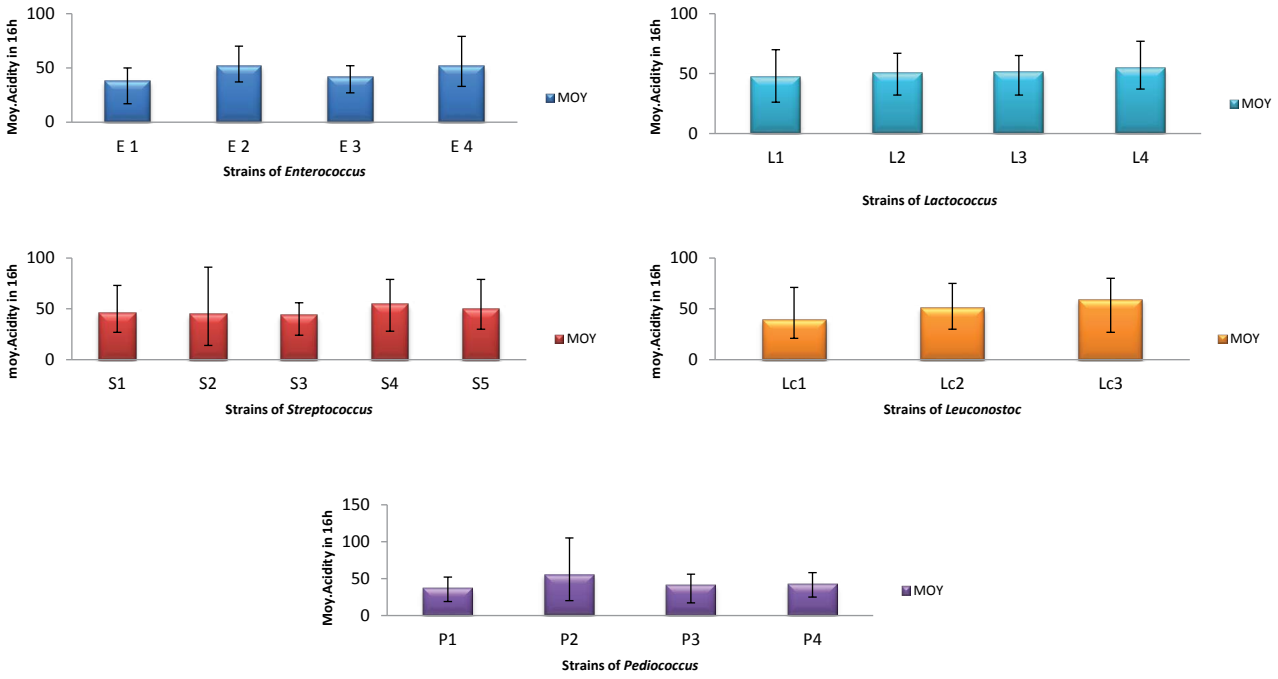

Figure 7. Dornic acidity as a function of lactic acid bacteria isolated from olives (*E: Enterococcus, L: Lactococcus, S: Streptococcus, Lc: Leuconostoc, P: Pediococcus*).

black olives have not a lipolytic activity (absence of clear halos around the discs).

3.5. Acidifying power

The totality of the identified lactic acid bacteria presented a progressive production of lactic acid (Figure 7). After 16 h of incubation, the pH varies between 4.01 and 6.00 for *Enterococcus*, 3.92 and 4.08 for *Lactococcus*, 3.94 and 6.10 for *Streptococcus* 3.90 and 6.00 for *Leuconostoc* and 4.57 and 6.00 for *Pediococcus*. Other quantities of lactic acid were recorded with the other species of the lactic acid bacteria. The olive green (Sigoise) (V1/OG) contains bacteria which have an important activity of acidification (production lactic acid) (Figure 7).

4. Discussion

We isolated 20 strains of lactic acid bacteria from the shredded olive. According to Marsilio et al. [34] Lactic Acid Bacteria (LAB) have long been employed in fermentation as a food preservation technique owing to their progressive acidification of the fermenting brine with a consequent pH decrease and the production of antimicrobial substances and bacteriocins. In addition, the use of LAB could standardize olive fermentation and reduce the use of highly polluting chemicals as NaOH (lye solution), contribute significantly to storage preventing microbial spoilage, and improve flavour [35].

The evolution of acidity and the variations of pH during the growth of the strains tested in skimmed milk show a differentiation between the kinds, the species and even between of the same strains species, these results are also reported by Luquet and Corrieu [36]. The increase in acidity is due to the fermentation of sugars into organic acids by lactic acid bacteria [37]. Of these results, we noted that the quantity of lactic acid produced changes according to the stage of life of the bacterium. This difference in production can be explained by deficiencies in the system of the transport of the fermentable substances of the medium towards the cellular cytoplasm [38].

It is known that the proteolytic system of the lactic acid bacteria degrades proteins and consequently, changes texture, the taste and the flavours of the fermented products [39].

The lactic bacteria isolated from two varieties do not present a lipolytic activity. These results are in agreements with those obtained by Crow et al. [40] which showed that the lactocoques ones have a weak lipolytic activity. Fernández et al. [41] noted that the esterase activity was necessary to the growth of the lactic acid bacteria, neither in a synthetic medium, nor in skimmed milk or entirety the resistance of the lactic acid bacteria isolated in this work was shown by the method of diffusion on agar. Several studies showed the natural resistance of an important range of lactic acid bacteria to antibiotics [42,43].

5. Conclusion

In the present work, from two varieties of olives Sigoise and Chemlal collected in Bourached region of Ain Defla, twenty strains of lactic acid bacteria are isolated in agar medium. Some classical techniques are used to identify some morphological, biochemical, physiological and technological characters. The genera of the isolated are *Enterococcus*, *Lactococcus*, *Streptococcus thermophilus*, *Leuconostoc* and *Pediococcus*.

This strains produce lactic acid measured in each two hours until 16 h. Lipolitic and proteolytic activities are searched in this work. These isolated strains are not lipolytic. *Streptococcus thermophilus* has a very high proteolytic activity (diameter=25 mm) observed in agar medium with milk. Theses strains of lactic acid bacteria witch is an integral part of olive flora have a benefit effect that can used in industries and to humans therapies.

6. Acknowledgement

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