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LACTIC ACID BACTERIA OF WESTERN ALGERIA. I: CHARACTERISTICS OF STRAINS ISOLATED FROM RAW MILK AND OLIVE OIL

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Abstract

A total of 154 strains of *Lactococcus, Lactobacillus, Enterococcus and Streptococcus* were isolated from olive oil samples or from cow's, goat's and sheep's raw milk samples collected in West of Algeria. The lactic acid bacteria strains were phenotypically identified and characterized using microbiological and biochemical tests. Of the 119 isolates of *Lactococcus*, 50 strains were identified as *Lc. lactis* subsp. *lactis*, 34 strains were identified as *Lc. lactis* subsp. biovar. *diacetylactis* and 35 strains were identified as *Lc. lactis* subsp. *cremoris,* but with difference in carbohydrate fermentation patterns. 21 strains were identified as *Lactobacillus* sp., 11 strains were identified as *Enterococcus faecium* and 03 strains were identified as *Streptococcus thermophilus.*

***Keywords**: Lactic acid bacteria, Lactococcus, Lactobacillus, Milk, Olive oil, Identification.*

Résumé

154 souches de *Lactococcus*, *Lactobacillus*, *Enterococcus* ou *Streptococcus* étaient isolées à partir d'échantillons d'huile d'olive ou d'échantillons de laits crus de vache, de chèvre et de brebis collectés dans l'Ouest algérien. Les tests de caractérisation microbiologique et biochimique ont permis d'identifier 50 souches de *Lc. lactis* subsp. *lactis*, 34 souches de *Lc. lactis* subsp. biovar. *diacetylactis* et 35 souches de *Lc. lactis* subsp. *cremoris* avec des variations dans leurs profils fermentaires. 21 souches étaient identifiées à *Lactobacillus* sp., 11 souches à *Enterococcus faecium* et 03 souches à *Streptococcus thermophilus.*

***Mots clés**: Bactéries lactiques, Lactococcus, Lactobacillus, Lait, Huile d'olive, Identification.*

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ملخص

قمنا بعزل 78 سلالة من بكتيرية اللبن (*Lactococcus*) وذاك من عينات مختلفة لحليب (البقرة، النعجة والعنزة) وزيت الزيتون جمعت كلها من الجهة الغربية للجزائر. ولقد أدت الاختبارات الى تشخيص و تحديد 35 سلالة من نوع (Lc. lactis subsp. lactis) و 43 سلالة من نوع (Lc. lactis subsp. biovar. diacetylactis)، ثلاثة منها تخمر الغليسيرول وخمسة اخرى لها قدرة النمو فى وسط غذائى به 6.5 % من NaCl.

***الكلمات المفتاحية:** بكتيرية اللبن،الحليب،زيت الزيتون، التشخيص البكتيري.*

The lactic acid bacteria represented by lactococci, lactobacilli, pediococci and streptococci are of great significance for their contribution to food fermentation and preservation [1-2]. All these micro-organisms are associated with human's, animal's, dairy products, fermented beverages and plant material [3-4-5].

Generally, dairy products material constitute a major sources for isolation and screening of lactic acid bacteria [6-7-8]. Raw milk, for example, has always been the most powerful means for obtaining useful cultures for food and feed industry, because it is rich in very important nutrients ; it has a favourable pH; it contains air (aerobic condition) ; it's salt concentration and osmotic pression are low. All this make milk an extremely suitable medium for a large number of micro-organisms and the number of species growing in milk is indeed considerable. There are many detailed studies of lactic acid bacteria isolated from raw milk in many countries, and several vegetable products were used on the last few years for isolation and selection of new lactic acid bacteria strains. An example of those products is the traditional fermented olive in Mediterranean regions. Traditional olive fermentation occurs at ambient temperature, *i.e.* near or below 25°C in those regions, regard to the nature of the microflora, the fermentation conditions and olive itself. Diez [9] and Van Den Berg *et al.* [7] had reported that the natural microflora of Portuguese olives is represented essentially by *Lb. plantarum* and *Lb. paracasei* species. In the case of Spanish green olive fermentation mainly *Lb. plantarum* was mainly isolated as representative of the group of lactic acid bacteria [10-11] but in our knowledge, the presence of *Lactococcus* sp. genus in both fermented olive and olive oil has not yet been reported.

In Algeria there are a few works with lactic acid bacteria obtained from cow , goat and sheep [12] and recently from camel's raw milk [13] but not from olive or olive oil. Several regions of West Algeria are well known for traditionally fermented olive and olive oil. It seems to be feasible to isolate lactic acid bacteria from fermented olive oil where they have grown to high concentration and afterwards use them as starter cultures in the same kind of green olive and olive oil fermentation, with the purpose of preserving products without introducing a new flavour or texture. Obviously it will be necessary to carry out some degree of selection based on a characterization of the strains, but one evident advantage in using the bacterial strains will be their known ability to grow well in this environment.

This paper deals with the isolation of new strains of lactic acid bacteria from various raw milks from cow, goat and sheep and or from a traditional fermented olive oil collected in several regions of West Algeria. This study included characterization and identification of strains based on phenotypic criteria.

1- MATERIALS AND METHODS

As shown in table 1, different cow's, goat's and sheep's raw milk samples (50 ml) were collected in four regions of West Algeria and traditional fermented olive oil samples (50 ml) were obtained from small manufactories located in two regions (Remchi and Tlemcen).

Samples were kept between 4 and 10°C for 36 hours before analysis in order to prevent bacterial development during transportation.

Samples	*Regions*	**Number of samples**
Olive oil	Remchi	03
	Tlemcen	01
Sheep's raw milk	Remchi	02
	El Amria	02
Goat's raw milk	Zenata	03
	El Amria	01
Cow's raw milk	El Amria	07
	El Amria	01
	Oued Tlelate	02

Table 1: Origin and number of samples collected in five regions of West Algeria.

Isolation and counting of bacteria

A 100 µl portion of milk or oil were suspended in 10 ml of sterile 0.9% NaCl solution and vigorously mixed. Suitable decimal dilutions were spread according to standard method using M17 agar [14]. Cultures were incubated under aerobic conditions for 2 days at 32°C before the counting of total viable cocci.

In the same way MRS agar with acetate (pH 5.4) [15] were used to count total lactobacilli. Plates were inoculated and incubated under anaerobic conditions (Gas Pak System, Becton Dickinson) for 3 days at 32°C.

The bacterial numbers were expressed as cfu/ml. After bacterial counts, 8 to 15 colonies per plate) were picked up at random from agar medium and purified through 3 cycles of single colony cultures.

Identification and storage of bacteria

Morphological and cultural properties of lactic acid bacteria were examined according to standard methods of bacteriology: cell shape, cells arrangements, catalase activity and Gram-staining were checked for cells grown in M17 or MRS broth at 32°C for 18 hours. Biochemical and physiological tests were done with API 20 STREP and API 50 CH systems (API-System, S.A., La Balme Les Grottes, Montalieu-Vercieu, France) according to the manufacturer's instructions. Samples were incubated at 32°C and readings were done at 4, 24 and 48 hours. Temperature requirement (10°C, 15°C and 45°C), NaCl tolerance (4% and 6.5%) and production of gas from glucose fermentation were studied on M17 broth. The citrate utilization test was performed on the media of Nickels and Leesment [16] or Kempler and McKay [17].

The criteria of identification we used were in accordance with the system of Schleifer *et al.* [18] and Schleifer [19]. Bacterial stains were stored at 4°C in reconstituted skim milk (10%) or at -20°C in M17 broth supplemented with 20% glycerol.

2- RESULTS AND DISCUSSION

Counting of milk and oil microflora

Both in M17 or MRS medium and at 32°C, the counts of viable cells of bacteria were comprised between 10^4 – 1.3×10^5 cfu/ml, 1.2×10^4-1.1×10^5 cfu/ml and 1.8×10^4 - 4×10^4 cfu/ml in all of cow's, goat's and sheep's milk samples, respectively. Within the fermented olive oil samples, the average of number of bacteria was estimated to 10^6 cfu/ml either with M17 or MRS broth.

Isolation, characterization and identification of bacteria

Bacterial strains were designed by letters and numbers corresponding to the product and region from which they were isolated and order of isolation as listed in table 2.

Strains of lactococci, lactobacilli, streptococci and enterococci were detected in agar plates. Morphological, physiological and biochemical characteristics of *Lactococcus* strains are given in full in table 3 (a and b). Based on the results and the schemes for identifying species developed by Schleifer *et al.* [18] and Schleifer [19], 50 strains of the 154 isolates were identified as *Lactococcus lactis* subsp *lactis.* They did not produce gas from glucose fermentation, grew at 10°C, but not at 45°C. Five of them grew in 6.5% NaCl but tests performed with API 50 CH confirm that these strains have a *Lactococcus lactis* subsp. *lactis* traits. 34 isolates were assigned to *Lactococcus lactis* subsp. biovar *diacetylactis* because they produced acetoïn and have ability to utilize citrate and to hydrolyse arginin. 35 strains were identified as *Lactococcus lactis* subsp. *cremoris* but with difference in carbohydrate fermentation patterns (Tab. 4).

Samples	Regions	Number of strains	Designation of strains
Olive oil	Remchi	17	HOR1, HOR2, .. to HOR17
	Tlemcen	03	HOT1, HOT2 and HOT3
Sheep's raw milk	Remchi	07	LBR1, LBR2,...to LBR7
	El Amria	50	BA1,BA2,. .. to BA50
Goat's raw milk	Zenata	19	LCH1, LCH2, .. to LCH19
	El Amria	02	CA1 and CA2
Cow's raw milk	El Amria	35	LVA1, LVA2, .. to LVA35
	El Amria	20	VA1, VA2, .. to VA20
	Oued Tlelate	01	VT1

Table 2: Designation and origin of the 154 isolates of bacteria.

Strains	LVA	LCH	LBR	HOR	LVA	LCH	LBR	HOR
Number of strains	11	10	06	07	20	09	01	10
Cell shape	spherical to ovoid				ovoid			
Arrangement of cells	chains of pairs				pairs or short chains			
Growth at or in:								
10°C	+	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	-	-	-
4%NaCl	+	+	+	+	+	+	+	+
6,5%NaCl	(-)*	-	-	-	-	-	-	-
Fermentation type:	homo	homo	homo	homo	homo	homo	homo	homo
Esculin hydrolysis	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	-	-	-
β-Galactosidase	+	+	+	+	+	+	+	+
β-Glucuronidase	-		-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	+	+	+	+
Acetoin	+	+	+	+	-	-	-	-
Fermentation of								
Ribose	+	+	+	+	+	+	+	+
Arabinose	+	-	+	+	+	+	+	+
Mannitol	+	-	-	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+
Glycogen	-	-	-	-	-	-	-	-
Glycerol	-	-	(-)**	(-)**	-	(-)**	-	-
Identified as:	*Lc. lactis* subsp *lactis* biovar *diacetylactis*				*Lc. lactis* subsp. *lactis*			

Table 3a: Identification of lactococci isolated from raw milk and olive oil.
homo: homofermemtation.
Lc : *Lactococcus.*
All strains examined were Gram-positive, and catalase negative.
(*): Five strains of *Lc. lactis* subsp *lactis* biovar *diacetylactis* (LVA8, LVA9, LVA10, LVA24 and LVA27) grew at 6.5%NaCl.
(**): Two strains of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* (LBR3 and HOR17) and one strain of *Lc. lactis* subsp. *lactis* (LCH10) have ability to utilize glycerol.

All strains of *Lactococcus* fermented ribose, starch and trehalose. Three strains of *Lactococcus lactis* subsp. biovar *diacetylactis* (HOR1, LBR3 and LCH10) showed exceptional ability to utilize glycerol. As we know this property is only occasionally found in some strains of *Lactobacillus reuterii* which utilize glycerol as hydrogen acceptor [2-20]. Fermentation of glycerol has not been yet described for *Lactococcus*.

Characteristics of the lactobacilli, streptococci and enterococci are shown in table 5.

21 strains of *Lactobacillus* sp., 03 strains of *Streptococcus thermophilus* and 11 strains of *Enterococcus* sp. were isolated only from two samples of cow's and sheep's raw milk obtained from El Amria. Tests performed with API 20 STREP confirm that strains of *Enterococcus* sp. have a *Enterococcus faecium* criteria, they have ability

Strains	BA	HOT	VA	VT	BA	HOT	VA	CA
Number of strains	07	01	02	01	25	02	05	02
Cell shape	spherical to ovoid			spherical to ovoid				
Arrangement of cells	chains of pairs			pairs or short chains				
Growth at or in:								
10°C	+	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	-	-	-
4%NaCl	+	+	+	+	+	+	+	+
6,5%NaCl	-	-	-	-	-	-	-	-
Fermentation type:	homo	homo	homo	homo	homo	homo	homo	homo
Esculin hydrolysis	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	-	-	-
β-Galactosidase	+	+	+	+	+	+	+	+
β-Glucuronidase	-	-	-	-	-	-	-	-
Arginin hydrolysis	+	+	+	-	-	-	-	-
Acetoin	-	-	-	-	-	-	-	-
Fermentation of:								
Ribose	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+
Mannitol	+	+	-	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+
Glycogen	-	-	-	+	+	+	+	+
Glycerol	-	-	-	-	-	-	-	-
Identified as:	*Lc. lactis* subsp. *lactis*			*Lc. lactis* subsp. *cremoris*				

Table 3b: Identification of lactococci isolated from raw milk and olive oil.
All strains examined were Gram-positive, and catalase negative.
Lc.: Lactococcus
homo: homofermentation.

to grew in 6,5% NaCl and at 45°C. All the strains hydrolyse arginin and utilize glycerol.

Three isolates of *Streptococcus* sp. seemed to belong to the species *Streptococcus thermophilus* because they grew at 45°C but not in 6,5% NaCl and they did not hydrolyse arginin.

From this findings, we concluded that *Lactococcus lactis* subsp *lactis Lactococcus lactis* subsp. biovar *diacetylactis, Lactococcus lactis* subsp. *cremoris* and *Lactobacillus* strains were the mainly species of lactic acid bacteria isolated in our study. Hence, *Streptococcus* and *Enterococcus* may not be essential micro-organisms in both cow's, goat's and sheep's raw milk samples or olive oil samples.

M17 or MRS medium are generally described as elective media on which only typical colonies of *Lactococcus or Lactobacillus* strains are respectively selected. In our study we found that *Lactococcus* as well as *Lactobacillus* strains were able to grow either in M17 or MRS and also that *Streptococcus* and *Enterococcus* strains were able to grow in these media.

One of first questions concerned the energy source utilized by the lactococci strains for their growth in fermented olive oil. As three isolates are able to grow in oil and are able to reach counts in the range of 10^6 cfu/ml, they must obviously utilize an available energy source other than ribose or other sugar. We observed strains that utilize glycerol. Glycerol is possibly the sole energy source still available after fermentation of the others sugars by oil microflora. Hence to any of these approaches, more information is needed about fermentation of glycerol by *Lactococcus* strains.

CONCLUSION

Based an the conventional culturing and biochemical characterization in this study, it was found that all the strains isolated from cow's, goat's and sheep's milk as well as from olive oil could be assigned to *Lactococcus lactis*, *Lactobacillus* and *Enterococcus* genus. The dominant strains were *Lc. lactis* subsp. *lactis*. *Streptococcus thermophilus* strains were isolated as minor strains from sheep's and cow's samples obtained from El Amria. Among 119 isolates of *Lactococcus*, 50 strains were identified as *Lc. lactis* subsp. *lactis*, 34 strains were identified as *Lc. lactis* subsp. biovar. *diacetylactis* and 35 strains were identified as *Lc. lactis* subsp. *cremoris,* 21 strains were identified as *Lactobacillus* sp., 11 strains were identified as *Enterococcus faecium* and 03 strains were identified as *Streptococcus thermophilus.*

Both in milk as well in olive oil, selected bacteria grew

Strains / Carbohydrates	LVA8	LBR3	HOR17	BA40	VA14	LCH	VT1	BA49	CA1
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	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	+	-
2-Ceto-gluconate	-	-	-	-	-	-	-	-	-
5-Ceto-gluconate	-	-	-	-	-	-	-	-	-
Identifed as: *Lc.lactis*	subsp. biovar *diacetylactis*			subsp. *lactis*			subsp. *cremoris*		

Table 4: Pattern of carbohydrate fermentation by lactococci strains. *Lc* : *Lactococcus*

Strains	BA	VA	BA	VA	BA	VA	LVA
Number of strains	13	08	02	01	03	04	04
Cell shape	Rods		Spherical		ovoid		
Arrangement of cells	short chains		chains		pairs		
Growth at or in:							
15°C	+	+	-	-	+	+	+
45°C	-	-	+	+	+	+	+
6.5%NaCl	-	-	-	-	+	+	+
Fermentation type:	homo	homo	homo	homo	homo	homo	homo
Arginine hydrolysis	-	-	-	-	+	+	+
Acetoïn	-	-	-	-	-	-	-
Identified as:	*Lactobacillus* sp.		*Streptococcus* sp.		*Enterococcus* sp		

Table 5: Physiological and biochemical characteristics of lactobacilli, streptococci and enterococci.

to high levels, and show a variations in their carbohydrate fermentation profile.

In this study, it is also found that 3 strains of lactococci can utilize glycerol as sole source of carbon, while 5 isolates were able to grow in media supplemented with 6.5% of NaCl.

We attempt now to explain the unexpected abilities of the lactococci to ferment glycerol or to express salt tolerance.

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