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Identification of lactic acid bacteria isolated from milk and fermented olive oil in western Algeria

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بكتيرية اللبن من الحليب الطازج وزيت الزيتون في الغرب الجزائري

قمنا بعزل 154 سلالة بكتيرية من عينات مختلفة من زيت الزيتون وحليب البقرة والنعجة والعنزة جمعت من مناطق مختلفة في الغرب الجزائري. تشمل هذه السلالات 50 سلالة من نوع Lactococcus lactis;subsp.lactis، 34 سلالة من نوع Lactococcus lactis subsp.biovar diacetylactis،35 سلالة من نوع Lactococcus lactis subsp.cremoris، 21 سلالة من نوع .Lactobacillus sp، 11 سلالة من نوع Enterococcus faecium و أخيرا 03 سلالات من نوع streptococcus bovis.

الكلمات المفتاحية : بكتيرية البن ـ الحليب ـ زيت الزيتون ـ التشخيص البكتيري

Identification de souches de bactéries lactiques isolées à partir de lait cru et d'huile d'olive dans l'ouest algérien

Un total de 154 souches de *Lactococcus*, *Lactobacillus*, *Enterococcus* ou *Streptococcus* ont été 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 morphologiques et biochimiques 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*. Vingt et une souches ont été identifiées comme étant des *Lactobacillus* sp., 11 souches des *Enterococcus faecium* et 03 souches des *Streptococcus bovis*.

Mots clés: Bactéries lactiques- *Lactococcus*- *Lactobacillus*- Lait- Huile d'olive- Identification - Algérie

Identification of lactic acid bacteria strains isolated from milk and fermented olive oil in western Algeria

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 western Algeria. The lactic acid bacteria strains were phenotypically identified and characterized using morphological 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*. Twenty one strains were identified as *Lactobacillus* sp., 11 strains as *Enterococcus faecium* and 03 strains as *Streptococcus bovis*.

Key words: Lactic acid bacteria - *Lactococcus* - *Lactobacillus* - Milk - Olive oiI - Identification - Algeria

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INTRODUCTION

The lactic acid bacteria represented by lactococci, lactobacilli, leuconostocs, pediococci and streptococci have a great significance for their contribution to food fermentation and preservation (Demazeaud, 1996; Stiles & Holzapfet, 1997).

All these micro-organisms are associated with humans, animals, dairy products, fermented beverages and plant material (Desmazeaud,1992; 1996; Delgado *et al.*, 2001).

Generally, dairy product materials constitute a major source for isolation and screening of lactic acid bacteria (Yoshikazu *et al.*,1994; Van Den Berg *et al.*, 1993).

Raw milk, for example, is the most used product for obtaining useful cultures for food and feed industry. This product is rich in nutrients, has a favourable pH, contains air, has a low salt concentration and a low osmotic pression.

All this makes 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 milk in many countries (Tzanetakis & Litopoulou-Tzanetaki, 1989; Isono *et al*., 1994).

Several vegetable products were used during the last few years for the isolation and the 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 near or below 25°C depending on the nature of the microflora, the fermentation conditions and the type of olive.

Van Den Berg *et al.* (1993) report 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, *Lb. plantarum* was the main representative of the group of lactic acid bacteria (Ruiz Barba *et al*.,1994; Delgado *et al*, 2001).

To our knowledge, the presence of *Lactococcus* species in both fermented olive and olive oil has not been yet reported.

In Algeria, there are a few studies on lactic acid bacteria obtained from cow, goat and sheep (Karam, 1995) and recently from camel's raw milk (Zadi Karam, 1998) but not from olive or olive oil.

Several regions of West Algeria are well known for traditionally fermented olive and olive oil.

The isolation of lactic acid bacteria from fermented olive oil in order to use them as starter cultures for the fermentation of green olive seems to be interesting.

This might preserve the fermented products from the introduction of a new flavour or texture.

To achieve this goal, it will be necessary to characterize the lactic culture and to select the appropriate strains of lactic acid bacteria.

This paper deals with the isolation of new strains of lactic acid bacteria from various raw milks from cow, goat and sheep and from traditional fermented olive oil collected in several regions of West Algeria.

This study has included characterization and identification of isolates based on phenotypic criteria. It constitutes a preliminary study in order to elaborate our a local starter culture collection.

MATERIALS & METHODS

As shown in table 1, different cow's, goat's and sheep's raw milk samples (50 ml each) were collected from four regions of West Algeria (Remchi, El Amria, Zenata and Oued Tlelate).

Traditional fermented olive oil samples (50 ml each) were obtained from small manufactories located in two regions (Remchi and Tlemcen).

After collection, milk and oil samples were transported to the laboratory in thermoflasks containing ice in order to prevent bacterial development during transportation.

1. Counting and isolation of bacteria

One ml of milk or oil was added to a sterile 0.9% NaCl solution to obtain 1:10 dilution and

vigorously mixed. Serial decimal dilutions (10^{-2} - 10^{-4}) were made in 0.9% NaCl solution. 0.1 ml volumes of each dilution were surface plated in triplicate.

Lactobacilli were counted in MRS agar (Unipath, Basingstoke, RG24 OPW, UK) ajusted with acetate (pH 5.4) (de Man *et al.*, 1960).

Plates were incubated under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 24 to 36 hours until growth was evident.

Lactococci were counted in M17 agar (Unipath) (Terzaghi & Sandine, 1975) after incubation for 24 hours at 30°C.

The quantity of bacteria was expressed as cfu/ml. After bacterial count, 8 to 10 colonies belonging to different types were randomly picked from each 30-50 colony count plate of MRS or M17 agar and purified through 3 cycles of single colony cultures.

2. Identification and storage of bacteria

Morphological and cultural properties of the isolated lactic acid bacteria were examined according to the methods and criteria developed by Schleifer *et al.*(1985), Schleifer (1986) and (Devriese *et al.*, 1987).

Cells shape, cells arrangements, catalase activity and Gram-staining were checked for lactic isolates in M17 or MRS broth culture media at 32°C for 18 hours.

Temperature requirement (10°C, 15°C and 45°C) of the isolated cultures as well as, NaCl tolerance (4% and 6,5%) and production of gas from glucose fermentation were studied on M17 broth (Korkeala & Mäkelä, 1989**;** Coppola *et al.*, 2000).

For the identification of isolates, biochemical and physiological tests were performed with the API 20 STREP and API 50 CHS micro-identification systems (API-System, S.A., La Balme Les Grottes, Montalieu-Vercieu, France) at 32°C for 48 hours, under anaerobic conditions. This identification was combined with the table of sugar fermentation in *Bergey's Manual of Systematic Bacteriology* (1986).

Bacterial cultures were stored at 4°C in reconstituted skim milk (10%) or at -20°C in M17 broth supplemented with 20% glycerol.

RESULTS & DISCUSSION

1. Counting, isolation and identification bacteria

In all cow's, goat's and sheep's milk samples the count of viable cells was ranged between 10^4 and 1.3 x 10^5 in M17 agar (lactococci) and 1.2 x 10^4 to 1.1 x 10^5 cfu / ml in MRS agar (lactobacilli).

Within the fermented olive oil samples, the average number of lactococci was estimated to 10^5 cfu/ml.

The Lactobacilli number in MRS broth was much lower and was ranged between 4.8 x 10^2 and 5.6 x 10^2 cfu / ml in the four olive oil samples.

For the characterization of isolates we have attached much importance to a sharp distinction between cocci and rods, Gram staining, catalase activity, acetoïn and arginine dihydrolase reaction.

A total of 154 isolates of lactic acid bacteria were isolated from cows, sheep and goats milk and from olive oil (Table 1).

Table 1. Origin and designation of the 154 isolates of lactic acid bacteria
Isolates were designated by letters and numbers corresponding to the product and region from which they were isolated)

Samples	Regions	No of samples	No of isolates	Designation of isolates
Olive oil	Remchi	03	17	HOR1, HOR2, to HOR17
	Tlemcen	01	03	HOT1, HOT2 and HOT3
Sheep's raw milk	Remchi	02	07	LBR1, LBR2, to LBR7
	El Amria	07	50	BA1, BA2, to BA50
Goat's raw milk	Zenat	03	19	LCH1, LCH2, to LCH19
	El Amria	01	02	CA1 and CA2
Cow's raw milk	El Amria	08	55	LVA1, LVA2, to LVA55
	Oued Tlilate	02	01	VT1

Morphological, physiological and biochemical characteristics of lactococci, enterococci and streptococci are given in full in table 2 (A and B), following the schemes for identifying species developed by Schleifer *et al.* (1985), Schleifer (1986) and Devriese *et al*. (1987).

One hundred ninteen (119) Gram positive, catalase nagative and homofermentative cocci which were capable of growing at 10 and 40°C but not at 45°C or at 6.5% salt were characterized as lactococci.
Of these isolates, 50 strains were identified as *Lactococcus lactis* subsp *lactis*. They did not produce acetoïn and have ability to hydrolyse arginin.

Five of them grew in 6.5% NaCl but tests performed with API 50 CHS confirmed that these isolates have a *Lactococcus lactis* subsp. *lactis* characteristics.

As shown in table 2 A, strains of *Lactococcus lactis* subsp. *lactis* were isolated from cows milk (22 strains designated LVA), goats milk (9 strains, LCH), sheep milk (10 strains, LBR and BA) and from olive oil (10 strains, HOR and one strains designated HOT).

Thirty four isolates were assigned to *Lactococcus lactis* subsp. biovar *diacetylactis* because they produced acetoïn and have ability to hydrolyse arginin.

Table 2A. Identification of lactococci isolated from raw milk and olive oil
All isolates were Gram-positive and catalase negative. Identification of bacteria was performed by the API System and confirmed with API 50 CHS System

Isolates	LVA	LCH	LBR	HOR	LVA	LCH	LBR	HOR	BA	HOT
Number of isolates	11	10	06	07	22	09	01	10	07	01
Cell shape	..... Spherical Chains of pairs				..... Spherical Chains of pairs					
Arrangement of cells										
growth at or in:										
10°C	+	+	+	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	-	-	-	-	-
4%NaCl	+	+	+	+	+	+	+	+	+	+
6.5%NaCl	(-)*	-	-	-	-	-	-	-	-	-
Fermentation type:	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	-	-	-	-	-
β-Galactosidase	+	+	+	+	+	+	+	+	+	+
β-Glucuronidase	-		-	-	-	-	-	-	-	-
Pyrrolidonylarylamidase	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	+	+	+	+	+	+
Acetoin	+	+	+	+	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+		
Arabinose	+	-	+	+	+	+	+	+	+	+
Mannitol	+	-	-	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	+	+
Lactose	+	+	+	+	+	+	+	+	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	-	-
Glycogen	-	-	-	-	-	-	-	-	+	+
Glycerol	-	-	(-)**	(-)**	-	(-)**	-	-	-	-
Identified as:			*Lc. lactis biovar diacetylactis*						*Lc. lactis subsp. lactis*	

Lc: Lactococcus ; homo: homofermemtation ; (*): Five strains of *Lc. lactis* subsp. biovar *diacetylactis* (LVA8, LVA9, LVA10, LVA24 and LVA27) grew at 6.5%NaCl. ; (**): Two strains of *Lc. lactis* subsp. biovar *diacetylactis* (LBR3 and HOR17) and one strain of *Lc. lactis* subsp. *lactis* (LCH10) have ability to utilize glycerol.

Table 2B. Identification of lactococci, streptococci and enterococci isolated from raw milk and olive oil

Isolates	VT	BA	HOT	LVA	CA	BA	LVA	BA	LVA
Number of isolates	01	25	02	05	02	02	01	03	08
Cell shape	Spherical to ovoid Pairs or short chains					Spherical Short chains		Ovoid pairs	
Arrangement of cells									
Growth at or in:									
10°C	+	+	+	+	+	-	-	+	+
15°C	+	+	+	+	+	-	-	+	+
45°C	-	-	-	-	-	+	+	+	+
4%NaCl	+	+	+	+	+	+	+	+	+
6.5%NaCl	-	-	-	-	-	-	-	+	+
Fermentation type:	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho
Hippurate hydrolysis	-	-	-	-	-	+	-	-	-
Esculin hydrolysis	+	+	+	+	+	-	-	+	+
α-Galactosidase	-	-	-	-	-	+	+	-	-
β-Galactosidase	+	+	+	+	+	-	-	+	+
β-Glucuronidase	-	-	-	-	-	-	-	-	-
Pyrrolidonylarylamidase	-	-	-	-	-	-	-	+	+
Arginine hydrolysis	-	-	-	—	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	-	-	+	+
Mannitol	+	+	+	+	+	-	-	+	+
Sorbitol	-	-	-	-	-	-	-	+	+
Acetoin	-	-	-		-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	+
Glycogen	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-			+	+
Identified as:	*Lc lactis subsp. cremoris*		Str. bovis		En.faecium				

Str*:* Streptococcus ; En.*:* Enterococcus

This microorganism was isolated from cows milk (11 strains, LVA), goats milk (10 strains, LCH), sheep milk (06 strains, LBR) and olive oil (7 strains, HOR).

Thirty five isolates were identified as *Lactococcus lactis* subsp. *cremoris.* Of these isolates, 25 strains coded, BA were isolated from sheep milk, 5 strains (LVA) and one strain (VT) from cows milk, 2 strains (CA) from goats milk and 2 strains (HOT) from olive oil. (Table 2B).

All lactococci fermented ribose, starch and trehalose. Three isolates of *Lactococcus lactis* subsp. biovar *diacetylactis* (HOR1, LBR3 and LCH10) showed an exceptional ability to utilize glycerol.

As it is known this property is only occasionally found in certain strains of *Lactobacillus reuterii* which utilize glycerol as hydrogen acceptor (Talarico *et al*., 1990; Desmazeaud, 1996).

Similar results obtained for several strains of *Lactobacillus* have been reported by Claisse & Lonvaud-Funel (2001). Fermentation of glycerol has not been yet described for lactococci.
Eleven Gram-positive, catalase-negative and ovoid cocci grouped in pairs or short chains were isolated from sheep milk (3 isolates designated BA) and cow milk (8 isolates, LVA).

All isolates were considered as enterococci. They did not produce gas from glucose fermentation, grew at 10, 40 and 45°C and grew in a 6.5% salt (Devriese *et al*., 1987).

Tests performed with API 20 STREP confirmed that isolates have a *Enterococcus faecium* critereon. All the isolates hydrolyzed arginin, fermented Arabinose Ribose, Trehalose, Starch and glycerol (Table 2 B).

Three isolates of *Streptococcus* sp. were isolated from sheep milk (2 isolates coded BA) and cow milk (one isolate, LVA). These isolates seemed to belong to the species *Streptococcus bovis* because they grew at 45°C but not in 6.5% NaCl and they did not hydrolyse arginin.

All isolates of *Streptococcus* fermented starch and Raffinose (Table 2B).

Twenty one rods, Gram-positive, catalase negative and homofermentative cells were isolated from sheep milk (13 isolates, BA) and cows milk (8 isolates, LVA).

Isolates were classified as *Lactobacillus* sp.. They grow at 15°C but not at 45°C or at 6.5% NaCl.

All isolates hydrolyzed arginine.These isolates were not identified to species level.

From these findings, we conclude that, the major isolates from all milk and oil samples were identified as *Lc. lactis* subsp. (*lactis*, *cremoris* and biovar *diacetylactis*) which are the species most often occurring in milk (Desmazeaud, 1996).

In our study, we confirmed that *Lactococcus* species are present in olive oil and are capable of growing in this product. In adition, *Lactobacillus* genus was isolated only from cows and sheep milk but not from goat and olive oil.

These results contrast with those of Ruiz Barba *et al.*(1994) who reported the presence of *Lactobacillus* genus in fermented olive.

To our knowledge, the presence of *Lactococcus* species in both fermented olive and olive oil has not been yet reported.

Among enterococci and streptococci respectively, *En. faecium* and *Str. bovis* species were the minor microorganisms isolated from cows and sheep milk.

Respectively, M17 or MRS media, that we used in this study are generally described as selective media on which only typical colonies of lactococci or lactobacilli are selected (Terzaghi & Sandine, 1975; Garcia *et al.*, 1987).

In our study we found that isolates of *Lactococcus* as well as *Lactobacillus* were able to grow either in MRS medium and also that enterococci and streptococci were able to grow in this media.

CONCLUSION

Lactic acid bacteria represented by *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Streptococcus* have been isolated from cows, sheep and goats milk or from olive oil. The identification of isolates on the basis of microscopic analysis and phenotypic characteristics (especially biochemical properties and sugar fermentation abilities) is very useful and remains the most widely recognized approach but, in the future, it would be interesting to conduct a more detailed study on bacterial identification using molecular methods.

Lactococcus species (20 strains) isolated from olive oil might be investigated in order to elaborate an adequate starter culture which would permit the manufacturing an industrial scale of uniform fermented green olives, and preserve the quality characteristics of the artisanal product as much as possible.

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