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.


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 microorganisms are associated with human’s, animal’s, dairy products, fermented beverages and plant material [3-5].

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 pressure 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.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Regions</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil</td>
<td>Remchi</td>
<td>03</td>
</tr>
<tr>
<td></td>
<td>Tlemcen</td>
<td>01</td>
</tr>
<tr>
<td>Sheep’s raw milk</td>
<td>Remchi</td>
<td>02</td>
</tr>
<tr>
<td></td>
<td>El Amria</td>
<td>02</td>
</tr>
<tr>
<td>Goat’s raw milk</td>
<td>Zenata</td>
<td>03</td>
</tr>
<tr>
<td></td>
<td>El Amria</td>
<td>01</td>
</tr>
<tr>
<td>Cow’s raw milk</td>
<td>El Amria</td>
<td>07</td>
</tr>
<tr>
<td></td>
<td>El Amria</td>
<td>01</td>
</tr>
<tr>
<td></td>
<td>Oued Tlelate</td>
<td>02</td>
</tr>
</tbody>
</table>

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 Leesement [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 strains 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 $10^4 - 1.3 \times 10^5$ cfu/ml, $1.2 \times 10^4 - 1.1 \times 10^5$ cfu/ml and $1.8 \times 10^4 - 4 \times 10^4$ 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 acetoin and have ability to utilize citrate and to hydrolyse arginin. 35 strains were identified as Lactococcus lactis subsp. cremorius but with difference in carbohydrate fermentation patterns (Tab. 4).
Lactic acid bacteria of western Algeria. I: characteristics of strains isolated from raw milk and olive oil.

All strains of Lactococcus fermented ribose, starch and trehalose. Three strains of Lactococcus lactis subsp. biivar diacetylactis (LVA8, LVA9, LVA10, LVA24 and LVA27) grew at 6.5%NaCl.

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

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

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<thead>
<tr>
<th>Samples</th>
<th>Regions</th>
<th>Number of strains</th>
<th>Designation of strains</th>
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</thead>
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<td>HOR1, HOR2, .. to HOR17</td>
</tr>
<tr>
<td></td>
<td>Tlemcen</td>
<td>03</td>
<td>HOT1, HOT2 and HOT3</td>
</tr>
<tr>
<td>Sheep’s raw milk</td>
<td>Remchi</td>
<td>07</td>
<td>LBR1, LBR2, .. to LBR7</td>
</tr>
<tr>
<td></td>
<td>El Amria</td>
<td>50</td>
<td>BA1, BA2, .. to BA50</td>
</tr>
<tr>
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<td>Zenata</td>
<td>19</td>
<td>LCH1, LCH2, .. to LCH19</td>
</tr>
<tr>
<td></td>
<td>El Amria</td>
<td>02</td>
<td>CA1 and CA2</td>
</tr>
<tr>
<td>Cow’s raw milk</td>
<td>El Amria</td>
<td>35</td>
<td>LVA1, LVA2, .. to LVA35</td>
</tr>
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<td></td>
<td>El Amria</td>
<td>20</td>
<td>VA1, VA2, .. to VA20</td>
</tr>
<tr>
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<td>Oued Tlelate</td>
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<td>VT1</td>
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Table 3a: Identification of lactococci isolated from raw milk and olive oil.

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<th>LBR</th>
<th>HOR</th>
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<td>10</td>
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<td>spherical to ovoid</td>
<td>ovoid</td>
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<td>+</td>
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<td>(--)**</td>
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</tbody>
</table>

homo: homofermentation.

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.

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to grow 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 10⁶ 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 on 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 thermophilis*.

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

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**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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>BA</th>
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<th>VA</th>
<th>VT</th>
<th>BA</th>
<th>HOT</th>
<th>VA</th>
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<td>spherical to ovoid</td>
<td>pairs or short chains</td>
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<td>Arrangement of cells</td>
<td>chains of pairs</td>
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**Table 4:** Pattern of carbohydrate fermentation by lactococci strains. *Lc.*: Lactococcus
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.

Acknowledgements

This work was supported by a grant of the Ministère de l’Enseignement Supérieur de l’Algérie (project F3101/04/97) and also by a grant of ANDRS (project 02/12/01/99 038).

REFERENCES