PHENOTYPIC IDENTIFICATION AND WHOLE CELL PROTEIN ANALYSIS BY SDS-PAGE OF DOMINANTS LACTIC ACID BACTERIA ISOLATED FROM ALGERIAN RAW MILK.

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ABSTRACT

A Total of 62 strains of lactic acid bacteria were isolated from raw goat, camel) and Roquefort cheese. Phenotypic identification of 21 representative strains revealed the presence of the following species: Leuconostoc mesenteroides subsp. dextranicum, Weissella paramesenteroides, Lactococcus lattis biovar. diacetylactis, Pediococcus acidilactici, Enterococcus faecium, Lactobacillus rhamnosus, Lactobacillus paracasei subsp. paracasei and Lactobacillus plantarum. 1n order to validate the previous results, whole tell protein patterns were obtained using Sodium Dodecyl Sulphate Gel Electrophoresis (SDS-PAGE) of these lactic acid bacteria strains, were analysed by calculating the coefficients of similarity (x100) for each two strains which were 80.7% and 78% for next couple of strains (6, 13) and (43, L$_4$) that were identified phenotypically as Weissella paramesenteroides and Leuconostoc mesenteroides subsp. dextranicum , respectively. The coefficients of similarity (x100) between one strain of Leuconostoc mesenteroides and one strain of Weissella paramesenteroides were 36%, 48%, 44.4%, 48.6%, 44.4% and 48.6%, lie- the Ibllowing couples (L4, 13), (L4, 6), (43.13), (43,6), (27,13) and (27, 6), respectively, in agreement with distant phylogenetic relationship between Leuconostoc mesenteroides and Weissella paramesenteroides. The SDS-PAGE method allowed clareing some ambiguous points in phenotypic identification. It had corroborate, complete and correct phenotypic identification, although, further study is required to accurate it.

Keywords: Lactic acid bacteria, identification, phenotypic method, SDS-PAGE, raw milk, *Leuconostoc*.

RESUME

Soixante-deux souches de bactéries lactiques ont été isolées d'échantillons de laits crus (vache, chèvre, chamelle) et de fromage de Roquefort. L'identification phénotypiques de 21 souches représentatives a révélé la présence des espèces suivantes: Leuconostoc mesenteroides subsp. dextranicum, Weissella paramesenteroides, Lactococcus lattis biovar. diacetylactis. Pediococcus acidilactici, Enterococcus fitecium, Lactobacillus rhamnosus, Lactobacillus paracasei subsp. paracasei et Lactobacillus plantarum. L'étude du profil des protéines cellulaire totale de ces souches a été réalisé par électrophorèse SDS-PAGE et les résultats analysés par le calcul des coefficients de similarité (x100) pour chaque deux souches. Des coefficients de 80.7% et 78% pour les couples de souches (6, 13) et (43, L$_4$) lesquels ont été identifiés phénolypiquement comme étant Weissella paramesenteroides et Leuconostoc mesenteroides subsp. dextranicum respectivement. Les coefficients de similarité entre une souches de Leuconostoc mesenteroides et une souche de Weissella paramesenteroides ont donné les valeurs suivantes 36%, 48%, 44.4%, 48.6%, 44.4% et 48.6% pour les couples (L$_4$, 13), (L$_4$, 6), (43, 13), (43,6), (27, 13) et (27, 6) respectivement, ce qui est en accord avec le fait que Leuconostoc mesenteroides et Weissella paramesenteroides soit phylogénétiquetnent éloignés.

La méthode SDS-PAGE a permis de clarifier certains points ambigus dans l'identification phénotypique. Elle a corroborée, complétée et corrigée le résultat de l'identification phénotypique, quoiqu'une étude plus approfondie est requise.

Mots clés Bactéries lactiques, identification, méthode phénotypique. SDS-PAGE, Lait cru, *Leuconostoc*.

I. INTRODUCTION

During the last decade studies concerning development of new dairy products have focused on selection of new strains isolated from wild niches that are able to increase biodiversity and restore the unique characteristics of traditional dairy products (De Palencia *et al.,* 2006).

Lactic acid bacte7ia (LAB) are Gram positive and usually catalase negative, grow under anaerobic conJitions but they are aerotolerant, non-spore forming and have a fermentative sugar metabolism with lactic acid as a major final product. They have a great economic importance in dairy and other fermented food industries (Guessas et Kihal. 2004, Badis *et al.,* 2005; Guessas *et al.,* 2005; Thapa *et al.,* 2006).

Lactic acid bacteria consist of a wide range of genera including a considerable number of species. Traditionally, they were divided into four genera, *Streptococcus, Lactobacillus, Leuconostoc* and *Pediococcus.* However, a considerable change in the taxonomy of lactic acid bacteria was observed during the last years (Stiles *et al.,* 1997).

Currently, the group of LAB in foods exist in the following genera *Carnobacterium, Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Oenococcus, Lactobacillus, Pediococcus, Tetragenococcus, Vagococcus, Weissella, Aerococcus, A lloiococcus ,Globicatella* and *Dolosigranulum* (Axelson,1998).

Phenotypic identification of LAB in dairy products are based mainly on morphological, physiological and biochemical characteristics, which are useful and indispensable tools that have been commonly used (Badis *et al.,* 2004a; Guessas *et al.,* 2005).

Although, phenotypic tests provide some evidence of metabolic capabilities, there are some problems, such as a lack of reproducibility and a lack of discriminatory power. Designation of certain neotype strains based only on phenotypic characteristics gave confused results which were resolved only by using molecular technique (De Angelis *et al.,* 2001). Recently, genetic techniques such as the mol % G+C contents of the DNA, DNA: DNA hybridization studies and structures and sequence of rRNA have been developed in order to get more consistent and accurate identification of LAB (Ogier *et al.,* 2004; Catzeddu *et al.,* 2006; Bensalah *et al.,* 2006). Ogier *et al.,* (2004)observed a spatial distribution of bacteria species in cheese, based on metabolite availability and competition between bacteria. The bacteria of the cheese surface generally correspond to high G+C content genomes, whereas bacteria present in the core generally have low G+C content genomes.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell protein is widely used for identification, since it offers the advantage to have a good level of taxonomie resolution at species and subspecies. Molecular methods have been used successfully to identify lactic acid bacteria isolated from different fermented foods (Samelis *et al.,* 1995; Sanchez *et al..* 2003; Sacilik *et al.,* 2000). The interest in LAB occurring in milk is primarily due to the biotechnological potential of new species.

The use of starters improved the technological quality of dairy products, but at the same time limited their biodiversity as well as the organoleptic variation of the end products. Therefore, an increasing demand exists for new strains that show desirable effects on the product characteristics (Zamfir *et al.,* 2006)

The objective of this study is to identify the LAB isolated from Algerian raw milk using phenotypic methods and whole cell proteins fingerprinting.

II-MATERIAL AND METHODS

II-1- Sampling.

Raw milk samples (covv, goat and camel) were collected from Oran, Mascara and Tindouf regions between June 2001 and January 2002. The Roquefort French cheese was also used to isolate LAB.

II-2- Isolation of strains and culture conditions.

In order to select the *Leuconostoc* species, the strains have been isolated on MRS agar at pH 6.5, supplemented with 25µg/m1 of vancomycin (Mathot et al., 1994) (MRSv) and were incubated at 28°C for 48h. Other LAB have been isolated on MRS agar without the presence of vancomycin at pH 6.5 and were incubated at 28°C and 45°C for 24h —72h. One reference strain of *Leuconostoc mesenteroides* subsp. *dextranicum* (L4) obtained from Biotechnology-Microbiology Laboratory of ENSBANA, Dijon (France), has been used in this study. Morphologically, different colonies were selected and purified in the same previous conditions. A total of 62 isolates which were Gram positive and catalase negative, isolated from raw milks and Roquefort French cheese, have been retained.

Stock cultures were routinely maintained in MRS agar slants at 4°C, and sub-cultured every *15* days. For long-term storage, frozen samples (-20°C) were prepared in sterile skim milk with 30% (v/v) glycerol. Enumeration of colonies of LAB in raw coagulate milk samples was performed.

Il-3- Phenotypic identification.

On the basis of morphological criteria, 21 dominant strains were chosen for this study. They were tested for: The morphological aspect, the proteolytic activity on PCA at 2% of skim milk, the production of CO-, from glucose, the growth at different temperatures 10, 15, 37 and 45°C, the thermotolerance at 60°C for 30min, the growth at 6.5% NaC1 and at pH 9.6, the hydrolysis of arginine, the dextran production which was detected at 10 % sucrose agar medium and the hydrolysis of aesculin was tested with 0.5% (w/v) aesculin. The citrate utilization, in the presence of glucose, was performed on the KMK medium (Kempler and Mac Kay, 1980).

The production of acetoin from glucose was determinate using Voges-Proskauer test on Clak and Lubs broth. All isolates were tested for the fermentation of the following carbohydrates: L(+) arabinose, D(-) ribose, D(+) xylose, dulcitol, glucose, galactose, fructose, D-mannose, D-mannitol, sorbitol, lactose, maltose, sucrose, D(+) cellobiose, D(+) raffinose and L(+) rhamnose (Badis *et al.,* 2004a; Badis *et al.,* 2004b, Guessas and Kihal, 2004).

The identification of LAB isolates was referred to the following authors: Garvie (1986b), Collins *et al.(1993)* for genus *Leuconostoc* and *Weissella.* Garvie (1986a) for *Pediococcus.* Farrow and Collins (1984), Schleifer and Kilpper-Bàlz (1984) and Schleifer *et* al. (1985), for *Streptococcus, Lactococcus* and *Enterococcus,* Garvie, (1986a) and Samelis *et al.,* (1995).) for *Lactobacillus.*

II-4- Electrophoresis SDS-PAGE of whole cell protein extracts

Only 14 representative species have been chosen for Gloser phenotypic profiles.

II-4-1-Preparation of whole cell protein extracts

Cultures were grown in MRS broth, for 18h-48h, and were streaked on buffered MRS agar (phosphate buffer 0.1M, pH 6.8) then were incubated for 18h to 48h. Cells were collected and resuspended in distilled water, then, harvested by centrifugation at 10000 rpm for 10min. The pellet was washed twice with distilled water and 50mg from the pellet was recuperated for each sample. Cells were sonicated at 30W for 30 sec and then incubated on ice for 20 sec. This performance was repeated twice. Subsequently, samples were resuspended in 0.9ml phosphate buffer 0.1M (pH 6.8), containing 5% 13-mercaptoethanol plus 5% sucrose, then a 0.1m1 SDS (20%) was added to the suspension and the mixture was heated at 100°C for 2min. After cooling the mixture on ice and centrifuging at (10000 rpm for 10 min), the supernatant was recuperated and preserved at —20°C (Kersters, 1980).

Protein concentration in the supernatant was measured by Bradford assay, using bovin serum albumin as standard. A standard protein solution was prepared using bivine serum albumin (68000 Da), casein (24000 Da) and 3-lactoglobulin (18000 Da) (Sanchez *et al.,* 2002).

1I-4-2-SDS-PAGE Electrophoresis:

Cell-free extracts were subjected to SDS-PAGE electrophoresis on vertical slabs gel. The polyacrylamide gels were consisted of resolving gel 12% and stacking gel 5%. The electrode buffer was 0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) SDS (pH 8.3). A volume of sample containing 50 jtg of proteins was layered on top of gel and 10-200 of loading sample buffer [0.01% (w/v) Bromophenol blue, 2% (w/v) SDS in buffer phosphate 1M (pH 6.8)] was added to each layer as a visible market·. Electrophoresis was performed at current of 50 mA and 120V. Proteins were fixed by immersing the gels in an aqueous of 10% (v/v) trichloracetic solution for lh and stained overnight in coamassie blue stain [0.25% (w/v) coomassie blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid]. The excess stain was washed out by distaining the gel with a solution of 25% (v/v) methanol and 10% (v/v) acetic acid (Kersters, 1980).

II-4-3-Analysis of protein patterns:

Molecular weight of proteins in electrophoregrams and coefficients of similarity (Jaccard coefficient) were calculated.

Sab Ns / Ns+ Nd

Sab: coefficients of similarity between two strains (a) and (b). Ns: Number of proteins having similar molecular weight between two strains (a) and (b) in electrophoregram. **Nd:** Number of proteins having different molecular weight between two strains (a) and (b) in electrophoregram.

III-RESULTS AM) DISCUSSION

Results of the enumeration of lactic acid bacteria on MRS and MRSv have shown that the proportion of mesophilic lactic acid bacteria resisting to vancomycin are respectively: 7.3%, 0.265% and 1.7% from raw milk of Boat, cow and camel. Mathot *et al.* (1994) had mentioned the resistance of *Leuconostoc* strains and the sensitivity of *Lactococcus* strains towards vancomycin at this concentration. However resistance to this antibiotic is among the general features **of** *Leuconostoc,* although it has been shown that *Pediococcus* and *Lactobucfflus* are also vancomycin resistant. The 21 isolates were identified as belonging to the genus *Leuconostoc-Weissela* (10), *Lactobacillus* (8), *Lactococcus (1), Pediococcus* **(1)** and *Enterococcus (1).*

111-1-Phenotypic identification of Lactic acid bacteria:

Firstly, we have limited our research to the group of LAB that can be found in milk and dairy products. Strains numbering 17, 2, 13, 6, 43 and 27 isolated on MRSv and strains 19, 39 on. MRS without vancomycin have the coccoid rod cells and occurred in pairs, in short chains or little mass. They were heterofermentative mesophilic, they did not hydrolyse arginine and they were considered as *Leuconostoc* (Table 1). Production of dextran from sucrose is an important character (Garvie, 1986b) to differentiate *Leuconostoc* species. Only two strains L4 *(Leuconostoc mesenteroides* subsp. *dextranicum)* and 43 had shown this property. A weak dextran production was detected with the 43 strain. Devoyod and Poullain (1988) had also isolated the species of *Leuconostoc mesenteroides subsp. mesenteroides* from dairy products which had also shown a weak dextran production. The *Leuconostoc* dextran (+) can be differentiated by the ability to ferment L-arabinose (Carr *et al.,* 2002 and Singh *et al.,* 2006). This reaction was negative with the strains L4, 43, which were identified as *Leuconostoc mesenteroides* subsp. *dextranicum* (Table 1). On the basis of sugar fermentation patterns, we could differentiate them into 43 ($xylose^-$, $mannitol^-$, $raffinose^+$) and L4 (xyl^+, $mannit^+$, raff).

The *Leuconostoc cremoris* has a limited sugar fermentation profile and does not grow at 37°C. This is not the case of strains 17, 2, 13, 6, 27, 19 and 39 which doesn't produce dextran, and doesn't ferment fructose, sucrose, maltose and mannose (Table 1). It is difficult to distinguish between *Leuconostoc lactis* and *Leuconostoc paramesenteroides* by their sugar fermentation patterns (Carr *et al.,* 2002 and Singh *et al.,* 2006). However, our leuconostocs were assigned to *Leuconostoc* *paramesenteroides* (currentiy named *Weissella paramesenteroides* by Collins *et al.,* 1993). Furthermore, they can be divided into four groups: Groupl: 27 (arabinose$^+$, xylose", Cellobiose$^-$, raffinose$^-$, mannitol$^+$).Group 2: 6, 1, 2, 17, 39 (ara$^-$, xyl$^+$, Celb$^+$, rafr mannif). Group 3 : 13 (ara$^-$, xyl$^-$, Celb$^-$, rafr, mannif). Group 4 : 19 (ara$^-$, xyl$^-$, Celb$^+$, rafr, mannif). Since the Ribose fermentation is a distinguishable character between *Leuconostoc lactis* and *Weissella paramesenteroides* (Carr *et al.,* 2002) , all the strains, men tioned previously, are characterised by ribose fermentation and they belong to *Weissella paramesenteroides.* Moreover, hence the strains 27, 13, 19 and all strains from group 2 are a variable sugar (Cellobiose, xylose, mannitol, arabinose, and raffinose) (Table 1) fermentation, therefore, they cannot belong to the species of *Leuconostoc pseudomesenteroides.*

It should be noted that one *Weissella paramesenteroides* number 27, was citrate defective variant. The instability of plasmid encoded citrate metabolism is known in the case of *Leuconostoc* sp. (Kihal *et al.,* 1996). Strains 20, 21 and 38 were homofermentative cocci.

On the basis of the growth at both 10°C and 45°C, in 6.5% NaC1, pH 9.6, surviving at 60°C for 30min and the hydrolysation of arginine, the strain 20 was identified as *Enterococcus* (Table 1). Notably, the ability of *Enterococcus filecium* to ferment arabinose led to distinct it from *En. faecalis* and *En. durans* (Schleifer and Kilpper Balz, 1984, Carr *et al.,* 2002).

Except, the growth at 10°C and arginine hydrolysation, the above other tests were negative for strain 21, therefore, it is assigned to the genus *Lactococcus. It* is arginine(+) and citrate(+) and VP(+). The latter properties had suggested the identification of this strain as *Le. lactis* subsp. *lactis* biovar. *diacetylactis.* Strain 38 had spherical cells, formed tetrads and was assigned to genus *Pediococcus.* Strain 38 was heat resisting, able to grow at 45°C but did not grow at temperature of 10°C, 6.5% of NaCl and pH 9.6. Contrary to *Streptococcus thermophilus,* it was arginine positive, fermented arabinose, cellobiose and did not fermented sucrose, consequently, the previous definition was confirmed, unlike to *Pc. damnosus,* which is able to grow at pH 7.0, at 37°C and at 45°C. On the other hand, *Pc. acidilactici* and *Pc. pentosaceus* are closely related species that may not be clearly differentiated by phenotypic characteristics, but they are differentiated by ADN-AND homology. On the basis of growth at 50°C, the strain 38 was identified as *Pc. acidilactici* (Stiles *et al.,* 1997; Carr *et al.,* 2002; Singh *et al.,* 2006). The latter was the only *Pediococcus* (isolated from raw mille and dairy products) that tolerate this temperature. All strains 23, 24, 52, 58, 61, 25, 50 and 32 (Table 2) were characterised as mesophilic homofermentative lactobacilli belong to the group (Samelis *et al.,* 1995; Singh *et al.,* 2006). These strains do not hydrolyse arginine.

The rod-shaped cells from strain 25 had a tendency to a curve. Strains 52 and 61 had shown thickest rod cells. Colonies from the strains 58, 61, 52 and 25 were easily distinguished from all the other strains. Strains 58, 61 and 52 were larger, more convex and cheesy white, however, 25 were smaller colonies, lesser convex and transparency white.

The strains 23, 24, 52, 58, 61, 50 and 32 (Table 2) were lactose positive, ribose positive, sucrose positive, and sorbitol positive, that is why it cannot belong to *Lactobacillus casei.*

The fact that ail those strains were sorbitol positive and able to grow at 15°C, their identification was restricted to *Lb. paracasei* subsp. *paracasei, Lb. plantarum, Lb. rhamnosus.* Only *Lb. pentosus* doesn't grow at 15°C. Moreover, fermenting arabinose by strains 52, 58 and 61, it's an other argument in favour. These strains were grown at 15°C and, except 58, did not grow at 45°C. They did not ferment rhamnose, therefore, were assigned to *Lb. plantaruni* or *Lb. paracasei* subsp. *paracasei* and strain 58 to *Lb. paracasei* subsp. *paracasei.*

Strains 23, 24, 50 and 32 were arabinose negative and rhamnose positive exept strain 32 which was rhamnose negative. Also, the ability of their growth on 15°C and 45°C was observed with ail the strains except with strain 32 which did not show a grow on 45°C. Thus, 23, 24, 50 were assigned to *Lb. rhamnosus* and 32 (resistant to 25µg/m1 to vancomycin) to *Lb. plantarum* or *Lb. paracasei* subsp. *paracasei.* Furthermore, the fermentation of raffinose can differentiate strains of *Lb. rhamnosus* into groups; (1) containing strain 50 was raffinose positive and (2) containing strains 23, 24 were raffinose negative.

The following characteristics sorbitol negative and cell-shaped indicated that strain 25 (resistant to 25µg/ml to vancomycin) do not belong to any of the above species of lactobacilli. Sugar fermentation pattern of this strain from it did net perfectly match coincided with the other lactobacilli in group II. Therefore, it was remain to be unidentified.

III-2- Identification by whole cell protein profiles.

Our phenotypic identification is a little ambiguous. In order to resolve this problem and improve it, protein fingerprinting by SDS-PAGE method as complementary analysis to phenotypic characterization has

Iii -2-1-*Leuconostoc* and *Weissella paramesenteroides*

The results of SDS-PAGE confirmed the assignment of strain 43 (isolated from goat milk, xyl^-, $mannit^-$, raff) to *Ln. mesenteroides* subsp. *dextranicum* by coefficient of similarity of 78% (Table 3) with strain L4 (ENSBANA-Dijon, xyl^+, $mannit^t$,raff). This result clarifies the identity of the *Leuconostoc mesenteroides* subsp. *dextranicum* strain 43. The slight dissimilarities between strains 43 and L4 may be are due to the diffèrent engin of the compared strains, as **it** was indicated by Samelis *et al. (1995);* Pérez *et al.* (2000).

The two strains 6 (isolated from camel milk, ara^-, xyl^+, $Celb^+$, raft mannit) and 13 (isolated from cow milk, ara^-, xyl^-, $Celb^-$, rafr, $mannit^-$) have shown an 80.7% of similarity. That corroborated the assignment of them to the same identity, phenotypically defined as *Weissella paramesenteroides.*

been used. The analysis of cell protein extracts by SDS-PAGE of 14 representative strains was studied. It was undertaken by visual comparison of the electrophoretic patterns (Fig. 1, Fig. 2). That was done by the coefficients of similarity (x 100) calculated by using the molecular weight of patterns in electrophoregrams. The coefficients of similarity (x100) were obtained for each two strains of the studied LA B (Table 3).

Sanchez *et* u/.(2003) have observed that the SDS-PAGE technique generated complex and stable patterns that were easy te interpret and compare with the reference strains of lactic acid bacteria. After numerical analysis of the resulting electrophoretic protein patterns the clusters were discriminated with some exceptions and correlated well with different groups phenotypically found on Almagro Spain cheese.

The results obtained by SDS-PAGE of whole-cell proteins discriminates as much as the DNA-DNA hybridization (Ferraz *et al.,* 2000) and present a very gond correlation between the results of numerical analysis of protein patterns and 16 rRNA based on oligonucleotide probe hybridization has been reported (Pot *et al., 1993;* Sacilik *et al.,* 2000; Booysen *et al.,* 2002).

Protein fingerprinting of strains 43 and 27 (isolated from the same goat milk sample) were very similar with a coefficient of 100%. Nevertheless, they were phenotypically identified as *Ln. mesenteroides* subsp. *dextranicum* for the first strain and *Weissella paramesenteroides* for the second. The same phenomenon has been found by Perez *et al.* (2000). The difficulty of the electrophoresis of cell-free extracts is to distinguish between the *Leuconostoc* subspecies which has been reported by Tsakalidou *et al.* (1994) and Villani *et al.* (1997), and it is probably because they are very close phylogenetic subspecies which produce very similar protein patterns (Pérez *et al.,* 2000; Moschetti *et al.,* 2000). These data and the sugar fermentation pattern indicated that it is quite possible to identify strain27 as *Ln. mesenteroides* subsp. *mesenteroides*, which bas lest dextran production and citrate utilisation. Several

Tableau 1 : Physiological and biochemical properties of coccoid and lactic acid bacteria.

Stratus	1	39							3	27	I	L,	20
Tests													
Fermentative metabolism	ht	ht	lit	lit	ht	lit	hm	hm	ht	lit	ht	ht	h m
Growth in NaCI 6,5%													
Growth at 45°C	- -												
Growth at 37°C											–	-	+
Growth at 10°C			-	-	-		+	-			-	-	+
	±	±	+	+	+	+	+ ++	+ +	+				
Growth at 50°C													
Thermotolerance	+	++		+	+	+	-	+	+	+	+	+	+
Growth in pH 9,6	Nt	Nt	Nt	Nt	Nt	Nt	+	Nt	Nt	Nt	Nt	Nt	Nt
Hydrolysis of arginine				–	-		+	- -			-	+	
Citrate (KMK)								+ -			-	++	-
VP											-	+	
Dextran	+	+		++			+	++			+	+	+
Hydrolysis of aesculin			-	-			+	+	+		-	+	
Proteolytic activity			-	-	-		Nt	Nt	+		-	+	Nt
Sugar fermentation :			-	-		+	-	-			+		+
Fructose													
Dulcitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+		+	+	+
Glucose													
D(+) xylose	+	+	+	+	+	+	+	+	+	+	+	+	+
L(+) arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+			+	±	-				++	
Lactose							+	+		+	-	-	+
L(+) rhamnose	+	+	+	+	+	+		+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	++±+++					
Sucrose													
D mannose	±	±	+	±			++	+	+		±	+	+
D mannitol	+	+	+	+	±	+	_	+	+	+	+	+	+
D(-) ribose	±		±	+	±	+	+	+	+	+	+	+	+
D(+) raffinose			-	_				+		±	-	+	±
Sorbitol	+	+	Ɨ	±	+	+	+	+	+	+	+	+	+
	+	+	+	+ +		+		±	+		+	-	_

Symbols : +; positive reaction at 24h or 48h , -; negative reaction, ±; weak and / or delayed reaction (the time of reaction exceed 48h), ht; heterofermentative metabolism, hm; homofenmentative metabolism, Nt; not tested, KMK; Kempler and Mac Kay media contained 3g/l of yeast extract. VP; Voges Proskauer test (positive result detected after incubation 2-3 days, except, strain 21 at 6h)

Tableau 2: Physiological and biochemical properties of lactobacilli

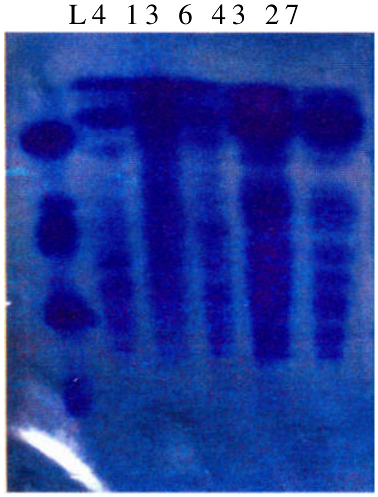
Tests	:trains							
	23	24	52	58	61	25	50	32
Fermentative metabolism	hm	H ni	hm	h ni	hm	hm	h ni	hm
Growth at 45°C	+	+	-	+	-		+	–
Growth at 15 °C	+	+	+	+	+	+	+	+
Arginine hydrolysis							-	-
Citrate (KMK)			+	+	+			-
Vp	+		+	+	+			
Aesculin hydrolysis			+	+	+	-		
Proteolytic activity	+	+	+	+	+	+	+	+
Sugar fermentation :								
Fructose	+	+	+	+	+	+	+	+
Dulcitol		-				-		-
Galactose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
D(+) xylose	-	-				-		-
L(+) arabinose	-	-	+	+	+	+	-	.
Maltose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	i	+
L(+) rharrmose	+	+					±	-
Cellobiose	+	+	+	+	+		+	+
Sucrose	±	+	+	+	+	+	+	+
D mannose	+	+	+	+	+	+	+	+
D mannitol	+	+	+	+	+	+	+	+
D(-) ribose	+	+	+	+	+	+	+	+
D(+) raffinose		-	+	+	+		+	-
Sorbitol		+	+	+	+		+	

Symbols : +; positive reaction at 24h or 48h, - ; negative ; reaction, ±; weak and / or delayed reaction (the time of reaction exceed

48h), ht; heterofermentative metabolism, hm; homofermentative metabolism, KMK; Kempler and Mac Kay media contained 3g/l of yeast extract, VP ; Voges Proskauer test (positive rcsult detected after incubation 2-3 days).

Tableau 3: Coefficients of similarity (x 100) between each two strains of LAB.

Strains	L4	13	6	43	27	52	58	32	50	23	25	38	20
13	36												
6	48,6	80,7											
43	78	44,4	48,6										
27	78	44,4	48,6	100									
52	27,1	30,2	37,2	30,6	30,6								
58	26,8	22.5	25	30.9	30,9	55,5							
32	29.5	18,2	27.9	30,4	30.4	77,8	52						
50	35,9	23	27.5	33,3	33,3	36,4	37,8	36,6					
23	27.5	21	19,5	28,6	28,6	31,8	36	31,7	85,2				
25	35,2	9.3	11,7	7,5	7,5	7	8.6	10.2	8,3	8,8			
38	19,1	18,6	22,7	11,3	11,3	31,6	22,7	22,9	14,6	6,5	2,4		
20	25,7	25,8	23,5	20,5	20,5	18,6	16,6	20,5	22,8	17.1	11,11	17,9	
21	21.1	20,8	22	22,2	22,2	29,6	32,6	26,9	21,5	22,4	4.2	35,4	26,2



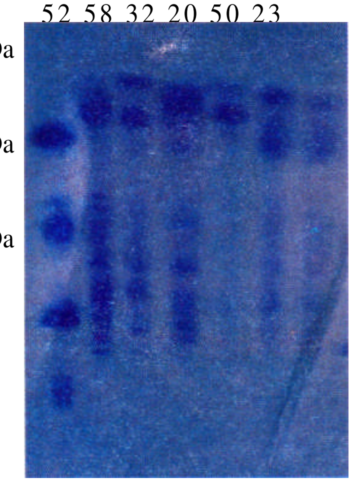


Fig. 1 Whole cell protein profiles of *Leuconostoc* (L4, 43, 27) and *Weissella paramesenteroides* (13, 6).

Fig. 2 Whole cell protein profiles of *Lactobacillus* (52, 58, 32, 50, 23) and *Enterococcus faecium (20).*

authors have mentioned this specificity in *Leuconostoc mesenteroides* (Devoyod and Poullain, 1988; Kihal *et al.,* 1996, Kihal *et al.,* 2006).

The difference from two characters (arabinose, dextran) between strains 43 and 27 could not be demonstrated by the SDS-PAGE protein profile analysis, the same fact has been reported by Sanchez *et al.* (2003).

The strain 27, and the strains 13 and 6, which were previously identified as *Weissella paramesenteroides„* had shown a low coefficient of similarity (Table 3). Hence, the protein profile revealed the atypical character of the strain 27.

The strain couples (L4, 13), (L4, 6), (43,13), (43,6), (27,13) and (27, 6) had given the following coefficients of similarity 36%, 48%, 44.4%, 48.6%, 44.4% and 48.6% respectively. These low levels of similarity are in agreement with distant phylogenetic relationship between the genus of *Leuconostoc* sp. and *Weissella* (Collins *et al.,* 1993).

111-2-2-Lactobacilli:

The two strains 23 and 50 (respectively raff and raft, isolated from the same sample of goat milk) have shown a high level of similarity with 85.2% (Table 3) and were defined as *Lb. rhamnosus.*

The strains 58, isolated from goat milk, and 52, isolated from camel milk, except, the growth at 45°C for the first strain, they presented similar phenotypic profile. Strain 58 was previously identified as *Lb. paracasei* subsp. *paracasei.* The coefficent of similarity 55.5% between the two strains, have shown different identity of theni. Consequently, if strain 58 had previously identified as *Lb. paracasei* subsp. *paracasei,* the strain 52 had assigned as *Lb. plantarum* rather than *Lb. paracasei* subsp. *paracasei.* On the other hand, the coefficient of similarity between strains 52 (isolated from camel milk, ara^{k}, rafr), and 32 (isolated from roquefort cheese, ara^{-}, raff) was 77.8%. In regard to the level of similarity 52% between strains 58 and 32, the latter strain is closely related to 52 rather than 58 and could be named *Lb. plantarum.*

The low coefficients of similarity between strains 50 and 52. 50 and 58, 50 and 32, 23 and 52, 23 and 58, 23 and 32 (Table 3) are in agreement with the precedent propositions.

Therefore, protein electrophoresis SDS-PAGE had allowed the separation of strains possessing very high or similar phenotypical profiles. On the other hand, the possible mistake to the assignment of strain 52 to the genus *Lactobacillus* or *Lactococcus,* which can be caused by one's rod cell shaped (making the morphological determination difficult), had been excluded by the above result. The very low coefficients of similarity between *Lactobacillus* 25 and other *Lactobacillus* (Table 3) confirmed the phenotypic results, so, it is clearly that strain 25 is distant to the group of *Lb. plantarum, Lb. paracasei, Lb. rhamnosus.*

Simova *et al.,* (2006) described the clustering of kefir lactic acid bacteria strains by using amino acid profiles.

The SDS-PAGE technique generated complex and stable patterns that were easy to interpret and compare the profiles of the strains used in this study. After numerical analysis of the resulting electrophoretic protein pattern, the clusters were discriminated and correlated well with the different groups phenotypically found (Sanchez *et al.,* 2003).

III-2-3 Hom-ofermentative eoccoid:

The very low coefficient of similarity 17.9% (Table 3) between strains 38 and 20 indicated that strain 38 can not be identified as *Enterococcus faecium.*

The microscopie observation of strain 52 had shown the thickest rod-shaped cells, which is easy to be confused with cocci. If this strain was assigned to the genus *Lactococcus,* therefore, it could be identified phenotypically as *Lactococcus lactis* biovar. *diacetvlactis.* However, the low coefficient of similarity 29.6% between strains 52 and 21 showed that the previous proposition was not correct. The reason of this problem is that lactobacilli may produce very short or ellipsoid cells, under certain growth conditions. Pérez *et al.* (2000) had indicated that the major problem encountered in the classical identification of LAB was the assignment to the genera *Lactococcus* or *Lactobacillus* which was based on the microscopie observation of the strains.

All strains occurring coccoid rod cells isolated from MRS supplemented with 25µg/ml of vancomycin were identified as *Leuconostoc* or *Weissella paramesenteroides*. *Weissella paramesenteroides* was dominante heterofermentative cocci in raw milk samples. De Vuyst et Vancanneyt (2006) shows that the isolation of novel taxa mainly depends on the cultivation approach used selective incubation media and conditions.

The identification ot' isolated strains needs a polyphasic approach, including a combination of phenotypic and genotypic methods. The results of ecological study obtained by Ampe *et al.,* (1999) demonstrate that the combination of fingerprinting and RNA quantification should allow workers to precise and rapid characterize the identification of lactic acid bacteria in fermented food.

The biochemical and physiological tests are unsatisfactory for the identification of isolated LAB. The use of SDS-PAGE method had allowed the clarification of some arnbiguous points in phenotypic identification, for example, it allowed the correction of the misidentification which is due to the presence of atypical characters, separation between strains which have doser phenotypic profiles, resolve the problem **of** microscopie determination of cell shape. Consequently, our results show that, protein fingerprinting analysis corroborate, complete and confin-n the phenotypic identification

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