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Protein fingerprinting as a complementary analysis to classical phenotyping for the identification of lactic acid bacteria from Tenerife cheese

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Abstract — A total of 125 lactic acid bacteria (LAB) from the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* isolated from Tenerife cheese were identified by both classical phenotypic and protein fingerprinting methods. Classical identification revealed the presence of 11 different species and subspecies of LAB. Lactobacilli and leuconostocs identification was easy to achieve with the API 50 CH system. By contrast, the identification of lactococci was difficult due to the heterogeneous and atypical profiles displayed by our strains. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of cell-free extracts (protein fingerprinting) has proved to be an efficient identification method for LAB from Tenerife cheese. It generated complex and stable patterns that are easy to interpret and compare with those of the reference strains. SDS-PAGE analysis could discriminate well between the 8 different species and subspecies of LAB. However, the assignment of LAB strains to one of the *Leuconostoc mesenteroides* subspecies required, in addition to protein fingerprinting, the performance of two biochemical tests. When results of both identification methods were compared, 23% of Tenerife cheese LAB isolates turned out to have been misclassified by the classical technique.

lactic acid bacteria / cheese / identification / protein fingerprinting

Résumé — L'empreinte digitale des protéines comme analyse complémentaire aux méthodes phénotypiques classiques pour l'identification des bactéries lactiques du fromage de Tenerife. Cent-vingt-cinq bactéries lactiques (LAB) des genres *Lactococcus*, *Lactobacillus* et *Leuconostoc*, isolées à partir du fromage de Tenerife, ont été identifiées par les méthodes phénotypiques classiques et par la méthode d'empreinte digitale des protéines. Par identification classique, 11 espèces et sous-espèces différentes des LAB ont été mises en évidence. Les lactobacilles et leuconostocs ont été facilement identifiés à l'aide du système API 50 CH. Par contre, l'identification des lactocoques s'est révélée difficile en raison des profils hétérogènes et atypiques des souches. L'électrophorèse en SDS polyacrylamide (SDS-PAGE) de l'extrait cellulaire (l'empreinte digitale des protéines) s'est

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montrée efficace pour l'identification des LAB du fromage de Tenerife, car elle a conduit à des profils complexes et stables faciles à interpréter et à comparer à ceux des souches de référence. L'analyse par SDS-PAGE a permis de distinguer clairement 8 espèces et sous-espèces différentes de LAB. Cependant, l'identification des sous-espèces de *Leuconostoc mesenteroides* a nécessité la réalisation de 2 tests biochimiques complémentaires. Quand on compare les deux méthodes d'identification, on constate que 23 % des LAB isolées du fromage de Tenerife sont incorrectement identifiées par la technique classique.

bactérie lactique / fromage / identification / empreinte digitale des protéines

1. INTRODUCTION

Lactic acid bacteria (LAB) are of great economic importance for the dairy and other fermented food industries. LAB are Gram-positive, catalase negative, non-spore forming and have a fermentative sugar metabolism with lactic acid as a major end product. The LAB found in cheeses consist primarily of *Lactococcus*, *Leuconostoc*, and homofermentative and heterofermentative *Lactobacillus* species.

Classical phenotypic identification of LAB in dairy products depends mainly on physiological and biochemical criteria. Identification at the species level however, is time consuming and often ambiguous. Moreover, distinguishing between groups of LAB such as leuconostocs and gas forming heterofermentative lactobacilli or between subspecies such as *Lactococcus lactis* ssp. *lactis* and ssp. *cremoris*, is difficult and there have been many misclassifications [11, 23]. Due to this fact, much research has been directed towards the development of new phenotypic methods that improve the identification of these microorganisms [15].

Modern taxonomy methods applied to LAB are based on molecular typing methods and include both phenotypic and genotypic analysis. Among phenotypic methods used, protein fingerprinting by SDS-PAGE of cell free extracts or of cell wall proteins has revealed itself as a useful tool in the identification of species and subspecies of LAB [10, 12, 27, 30]. With regard to genotypic techniques, plasmid profile patterns ascertained by agarose gel electrophoresis [3, 6], ribotyping and randomly amplified polymorphic DNA (RAPD) [5, 9, 13] have been successfully used to resolve the taxonomic status of LAB.

Tenerife cheese is a traditional farmhouse variety produced on the island of Tenerife (Spain) from raw goat's milk with the action of natural microflora, the identification of which is the first step towards the preparation of a starter culture for use in large-scale manufacture. Since the identification of this flora on the basis of classical phenothypic characteristics has proved to be ambiguous [29], we decided to use a molecular typing method to improve its identification.

The aim of this study was to evaluate the efficiency of protein fingerprinting when used as a complementary analysis to classical phenotyping for the identification of 125 LAB (lactococci, lactobacilli and leuconostocs) strains isolated from Tenerife cheese.

2. MATERIALS AND METHODS

2.1. Strains, media and cultivation conditions

One hundred and twenty-five strains of lactic acid bacteria (LAB) from the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* were used in the present study. The strains have been isolated from 4 different

Tenerife cheeses at different maturation times as described previously [29] and assigned to genus by means of the following tests: microscopic appearance in Gram stained preparations, catalase activity, CO_2 production from glucose in MRS broth without citrate using inverted Durham tubes and hydrolysis of arginine determined with the Nessler reagent [14]. Reference strains used for protein fingerprinting (Tab. I) were obtained from the Colección Española de Cultivos Tipo (CECT) and included representative members of the species and subspecies of LAB that were phenotypically identified from Tenerife cheese, as well as other LAB and enterococci usually isolated from Spanish raw goat's milk cheeses [7, 26]. All strains were maintained as frozen stocks at –80 °C in Man-Rogosa-Sharpe medium [4] (MRS, Unipath, Basingstoke, UK) containing 20% (v/v) glycerol. Working cultures were prepared from frozen stock cultures by two consecutive transfers in MRS broth at 30 °C.

Table I. Reference strains used for identification of LAB isolates by protein fingerprinting.

Tableau I. Souches de référence utilisées pour l'identification des LAB isolées par l'empreinte digitale des protéines.

Name	CECT nb.	Type strain	Strain number as received
Enterococcus avium	968	T	ATCC 14025
Enterococcus faecalis	481	T	ATCC 19433
Enterococcus faecium	410	T	ATCC 19434
Enterococcus gallinarum	970	T	ATCC 35038
Lactobacillus acidophilus	903	T	ATCC 4356
Lactobacillus brevis	216		CCRC 14060
Lactobacillus casei	475		ATCC 393
Lactobacillus cellobiosus	562	T	ATCC 11739
Lactobacillus curvatus ssp. *curvatus*	904	T	ATCC 15601
Lactobacillus fermentum	4 007	T	ATCC 14931
Lactobacillus paracasei ssp. *paracasei*	4 022	T	ATCC 25302
Lactobacillus paracasei ssp. *paracasei*	277		ATCC 25598
Lactobacillus plantarum	748	T	ATCC 14917
Lactobacillus plantarum	220		ATCC 8014
Lactobacillus rhamnosus	278	T	ATCC 7469
Lactococcus lactis ssp. *cremoris*	697	T	ATCC 19257
Lactococcus lactis ssp. *lactis*	185	T	ATCC 9936
Lactococcus lactis ssp. *lactis*	4 041		IFPL 361
Lactococcus raffinolactis	988	T	ATCC 43920
Leuconostoc lactis	4 173	T	ATCC 19256
Leuconostoc mesenteroides ssp. *cremoris*	872	T	ATCC 19254
Leuconostoc mesenteroides ssp. *dextranicum*	912	T	ATCC 19255
Leuconostoc mesenteroides ssp. *mesenteroides*	219	T	ATCC 8293
Leuconostoc mesenteroides ssp. *mesenteroides*	4 046		IFPL 704

ATCC, American Type Culture Collection, Rockville, Maryland, USA; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CCRC, Culture Collection and Research Center, Taiwan; IFPL, Instituto del Frío, Ciencia y Tecnología de los Productos Lácteos, Madrid, Spain.

2.2. Identification of strains by classical phenotypic criteria

Identification at the species level of *Lactococcus* was performed according to the criteria of Schleifer and Kilpper-Bälz [21], Schleifer et al. [22] and Schleifer [20]. The API 50CH galleries with API 50 CHL Medium (Bio Merieux, Marcy-l'Étoile, France) were used to identify the leuconostocs and lactobacilli due to the metabolism of 49 carbohydrates. Identification results were obtained with the aid of the APILAB Plus (API, Bio Merieux) identification software.

2.3. Identification of strains by SDS-PAGE of cell-free extracts (protein fingerprinting)

Cells were grown in 5 mL of MRS broth at 30 °C for 18 h. 1×10^{10} cfu were harvested by centrifugation at 13000 *g* for 10 min and washed twice with 1 mL of 50 mmol·L^{-1} Tris-HCl, pH 7.0. 50 mg of glass beads (150-200 μm, Sigma, St.-Louis, MO, USA) were added to the pellet. After mechanical disruption of the cells by vortexing during 1 min, 100 μL of SDS-PAGE sample treatment buffer (150 mmol·L^{-1} Tris-HCl, pH 6.8; 4% SDS; 20% glycerol, 10% β-mercapthoethanol; 0.005% bromophenol blue) was added and the samples were boiled for 10 min. Whole cells and cell fragments were separated by centrifuging twice at 13 000 *g* for 10 min in order to obtain the cell free extracts.

Cell free extracts (20 μL) were loaded into vertical slab gels ($80 \times 70 \times 0.75$ mm) using a Mini Protean II electrophoresis cell (Bio-Rad, Richmond, CA, USA) and analysed by SDS-PAGE (10% acrylamide (w/v) in the resolving gel and 4% acrylamide (w/v) in the stacking gel) by the method of Laemmli [16]. Molecular weight markers purchased from Sigma were: myosin (205 kg·mol^{-1}), β-galactosidase (116 kg·mol^{-1}), phosphorylase b (97.4 kg·mol^{-1}), bovine albumin (66 kg·mol^{-1}), egg albumin (45 kg·mol^{-1}) and carbonic anhydrase (29 kg·mol^{-1}). Gels were run for 1 h at 20 mA and stained for 30 min with 0.1% (w/v) Coomassie blue R-250, 40% (v/v) methanol and 10% (v/v) acetic acid. Gels were destained with 40% (v/v) methanol and 10% (v/v) acetic acid and photographed using a Mitsubishi CCD-400E video camera with P90 video printer and K65HM thermal paper.

3. RESULTS

3.1. Identification of lactic acid bacteria by classical methods

On the basis of classical phenotypic analysis, 22 strains of lactic acid bacteria that were homofermentative, Gram-positive, catalase-negative cocci were considered lactococci (Tab. II). Within the three tests commonly used to differentiate enterococci and lactococci (growth at 45 °C, at pH 9.6 and in the presence of 6.5% NaCl; positive for enterococci and negative for lactococci), most of the lactococci isolates were able to grow in 6.5% NaCl and some of them were even able to grow at 45 °C or at pH 9.6. Strains were considered to be lactococci when up to two of these physiological tests were positive and to the genus *Enterococcus* when they were all positive. The identification to the subspecies level of *Lactococcus lactis* was done according to the inability of *Lc. lactis* ssp. *cremoris* to hydrolyse arginine, in contrast to *Lc. lactis* ssp. *lactis*. Among *Lc. lactis* ssp. *lactis* one strain was assigned to biovar *diacetylactis* because of its ability to produce acetoin from citrate by the Voges-Poskauer reaction. Finally, one strain that produced acid from raffinose and sorbitol and did not hydrolyse arginine was identified as *Lactococcus raffinolactis*.

Gram-positive, catalase-negative, heterofermentative cocci that did not hydrolyse arginine were considered leuconostocs, while Gram-positive, catalase-negative rods were considered lactobacilli. Table III

Table II. Physiological and biochemical characteristics of lactococci isolated from Tenerife cheese.

Tableau II. Caractéristiques physiologiques et biochimiques des lactocoques isolés à partir du fromage de Tenerife.

Number of isolates	13	1	7	1
Growth at				
10 °C	13[a]	1	7	1
45 °C	0	0	1	0
Growth in				
pH 9.6	4	0	0	0
4% NaCl	13	1	7	1
6.5% NaCl	7	0	7	0
0.1% methylene blue	13	1	6	1
0.04% Ktellurite	8	1	0	0
Survives 60 °C·30 min^{-1}	13	1	6	1
Arginine hydrolysis	13	1	0	0
Acetoin from citrate	0	1	5	1
β-haemolysis	0	0	0	0
Acid from				
L-Arabinose	2	0	2	1
Glucose	13	1	7	1
Lactose	13	1	7	1
Maltose	13	1	7	1
Mannitol	5	0	6	1
Melezitose	1	0	6	1
Melibiose	2	0	6	1
Raffinose	1	0	2	1
Ribose	13	1	7	1
Sorbitol	8	1	4	1
Litmus milk test	RAC[b] 8 AC 5	RAC 1	RAC 6	RAC 1
Identification	*Lc. lactis* ssp. *lactis*	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*	*Lc. lactis* ssp. *cremoris*	*Lc. raffinolactis*

[a] Number of positive isolates in each test; [b] R = reduction, A = acidification, C = coagulation.

[a] Nombre d'isolats positifs pour chaque test ; [b] R = réduction, A = acidification, C = coagulation.

summarises the identification of both genera at the species level. Since the API 50 CH system does not distinguish between *Leuconostoc mesenteroides* ssp. *mesenteroides* and *Ln. mesenteroides* ssp. *dextranicum*, the criterion followed to differentiate them is the ability of the first subspecies to produce acid from L-arabinose (one of the carbohydrates included in the API 50 CH gallery) in contrast to the second one [20]. According to this, 71.9% of strains were *Ln. mesenteroides* ssp. *mesenteroides*, 15.6% were *Ln. mesenteroides* ssp. *dextranicum* and one strain was identified as *Ln. lactis.*

Phenotypic characterisation of lactobacilli revealed a predominance of the species *Lactobacillus plantarum* (52.1%) and *Lb. paracasei* ssp. *paracasei* (36.6%) and the presence in smaller numbers of *Lb. curvatus*, *Lb. brevis*, and *Lb. pentosus.*

Table III. Lactobacilli and leuconostocs from Tenerife cheese identified by the API 50 CH system.
Tableau III. Souches de lactobacilles et leuconostocs du fromage de Tenerife, identifiées par le système API 50 CH.

Isolates	Number	%
Lactobacilli	71	100
Lactobacillus plantarum	37	52.1
Lb. paracasei ssp. *paracasei*	26	36.6
Lb. curvatus	2	2.8
Lb. brevis	1	1.4
Lb. pentosus	1	1.4
Unidentified	4	5.7
Leuconostocs	32	100
Leuconostoc mesenteroides ssp. *mesenteroides*	23	71.9
Ln. mesenteroides ssp. *dextranicum*	5	15.6
Ln. lactis	1	3.1
Unidentified	3	9.4

3.2. Identification of lactic acid bacteria by protein fingerprinting

The analysis of cell protein extracts of the 125 strains of Tenerife LAB cheese isolates by SDS-PAGE showed six clearly different profiles. After visual comparison of these electrophoretic patterns with those of the reference strains, the following species and subspecies were identified: *Lactobacillus plantarum*, *Lb. paracasei* ssp. *paracasei*, *Lb. curvatus*, *Lactococcus lactis* ssp. *lactis*, *Lc. lactis* ssp. cremoris and *Leuconostoc mesenteroides*.

Figures 1 and 2 show the protein fingerprinting identification of 14 of our LAB isolates (TF strains). Protein fingerprints of *Lactobacillus plantarum* and *Lb. paracasei* ssp. *paracasei* isolates from Tenerife cheese were identical to that of their respective type strains (Fig. 1). On the other hand, *Lactobacillus curvatus* ssp. *curvatus* isolates were slightly different when compared to the type strain since the latter exhibited two clear bands below 45 $kg \cdot mol^{-1}$ while the former lacked the lowest one (Fig. 1a, lanes 6 and 7).

Protein profiles of our *Lactococcus lactis* ssp. *lactis* strains were almost the same as the one exhibited by the type strain, except that our isolates showed an additional band between 45 and 29 $kg \cdot mol^{-1}$ (Fig. 2a, lanes 2 to 5). In addition, no significant differences were found between the protein profiles of our *Lactococcus lactis* ssp. *cremoris* strains and the type strain, though the clear band between 97.4 and 66 $kg \cdot mol^{-1}$ was more intense in our isolates (Fig. 2a, lanes 6 and 7).

Protein fingerprints of type strains of *Leuconostoc mesenteroides* subspecies were very similar (Fig. 2b, lanes 3, 5 and 7), making the identification of our strains by visual comparison very difficult. In this case, the following biochemical criteria were taken into consideration to finally assign the *Ln. mesenteroides* to one of the three subspecies: *Ln. mesenteroides* ssp. *cremoris*, inability to produce dextran when grown on solid media containing 0.5% sucrose; *Ln. mesenteroides* ssp. *mesenteroides*, production of dextran and of acid from L-arabinose; and *Ln. mesenteroides* ssp. *dextranicum*,

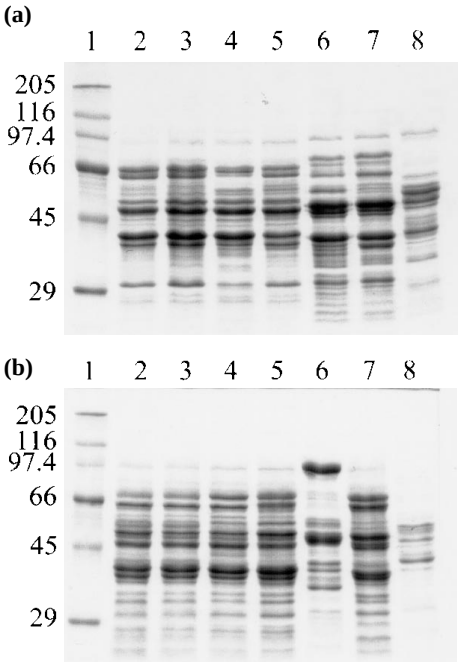

Figure 1. SDS-PAGE of cell-free extracts of lactic acid bacteria. **(a)** Lane 1, molecular weight markers; lane 2, *Lactobacillus plantarum* TF318; lane 3, *Lb. plantarum* TF236; lane 4, *Lb. plantarum* TF845; lane 5, *Lb. plantarum* type strain; lane 6, *Lb. curvatus* ssp. *curvatus* TF176; lane 7, *Lb. curvatus* ssp. *curvatus* type strain; lane 8, *Lb. brevis* type strain. **(b)** Lane 1, molecular weight markers; lane 2, *Lb. paracasei* ssp. *paracasei* TF672; lane 3, *Lb. paracasei* ssp. *paracasei* TF648; lane 4, *Lb. paracasei* ssp. *paracasei* TF271; lane 5, *Lb. paracasei* ssp. *paracasei* type strain; lane 6, *Lb. fermentum* type strain; lane 7, *Lb. casei* type strain; lane 8, *Lb. acidophilus* type strain.

Figure 1. SDS-PAGE de l'extrait cellulaire des bactéries lactiques. **(a)** 1, Marqueurs de masse moléculaire ; 2, *Lactobacillus plantarum* TF318 ; 3, *Lb. plantarum* TF236 ; 4, *Lb. plantarum* TF845 ; 5, *Lb. plantarum* souche type ; 6, *Lb. curvatus* ssp. *curvatus* TF176 ; 7, *Lb. curvatus* ssp. *curvatus* souche type ; 8, *Lb. brevis* souche type. **(b)** 1, Marqueurs de masse moléculaire ; 2, *Lb. paracasei* ssp. *paracasei* TF672 ; 3, *Lb. paracasei* ssp. *paracasei* TF648 ; 4, *Lb. paracasei* ssp. *paracasei* TF271 ; 5, *Lb. paracasei* ssp. *paracasei* souche type ; 6, *Lb. fermentum* souche type ; 7, *Lb. casei* souche type ; 8, *Lb. acidophilus* souche type.

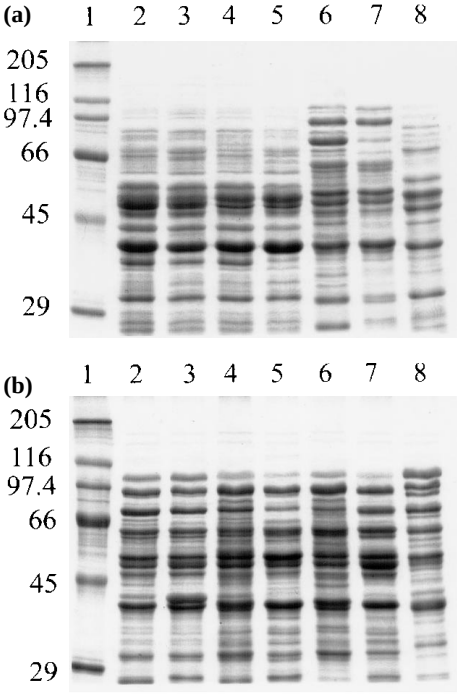

Figure 2. SDS-PAGE of cell-free extracts of lactic acid bacteria. **(a)** Lane 1, molecular weight markers; lane 2, *Lactococcus lactis* ssp. *lactis* TF400; lane 3, *Lc. lactis* ssp. *lactis* TF53; lane 4, *Lc. lactis* ssp. *lactis* TF61; lane 5, *Lc. lactis* ssp. *lactis* type strain; lane 6, *Lc. lactis* ssp. *cremoris* TF165; lane 7, *Lc. lactis* ssp. *cremoris* type strain; lane 8, *Lc. raffinolactis* type strain. **(b)** Lane 1, molecular weight markers; lane 2, *Leuconostoc mesenteroides* ssp. *mesenteroides* TF756; lane 3, *Ln. mesenteroides* ssp. *mesenteroides* type strain; lane 4, *Ln. mesenteroides* ssp. *dextranicum* TF275; lane 5, *Ln. mesenteroides* ssp. *dextranicum* type strain; lane 6, *Ln. mesenteroides* ssp. *cremoris* TF197; lane 7, *Ln. mesenteroides* ssp. *cremoris* type strain; lane 8, *Ln. lactis* type strain.

Figure 2. SDS-PAGE de l'extrait cellulaire des bactéries lactiques. **(a)** 1, Marqueurs de masse moléculaire ; 2, *Lactococcus lactis* ssp. *lactis* TF400 ; 3, *Lc. lactis* ssp. *lactis* TF53 ; 4, *Lc. lactis* ssp. *lactis* TF61 ; 5, *Lc. lactis* ssp. *lactis* souche type ; 6, *Lc. lactis* ssp. *cremoris* TF165 ; 7, *Lc. lactis* ssp. *cremoris* souche type ; 8, *Lc. raffinolactis* souche type. **(b)** 1, Marqueurs de masse moléculaire ; 2, *Leuconostoc mesenteroides* ssp. *mesenteroides* TF756 ; 3, *Ln. mesenteroides* ssp. *mesenteroides* souche type ; 4, *Ln. mesenteroides* ssp. *dextranicum* TF275 ; 5, *Ln. mesenteroides* ssp. *dextranicum* souche type ; 6, *Ln. mesenteroides* ssp. *cremoris* TF197 ; 7, *Ln. mesenteroides* ssp. *cremoris* souche type ; 8, *Ln. lactis* souche type.

Table IV. Comparison analysis of the identification of LAB from Tenerife cheeses by classical and protein fingerprintig methods.
Tableau IV. Analyse comparative de l'identification des LAB du fromage de Tenerife par la méthode classique et l'empreinte digitale des protéines.

Nb. isolates	Name after classical phenotypic analysis	Nb. isolates	Name after SDS-PAGE analysis	% of coincidence
14	*Lactococcus lactis* ssp. *lactis*	13	*Lactococcus lactis* ssp. *lactis*	92.8
		1	*Lactobacillus plantarum*	
7	*Lactococcus lactis* ssp. *cremoris*	1	*Lactococcus lactis* ssp. *cremoris*	14.2
		5	*Lactobacillus plantarum*	
		1	*Lactobacillus paracasei* ssp. *paracasei*	
1	*Lactococcus raffinolactis*	1	*Lactobacillus paracasei* ssp. *paracasei*	0.0
37	*Lactobacillus plantarum*	29	*Lactobacillus plantarum*	78.4
		6	*Lactobacillus paracasei* ssp. *paracasei*	
		1	*Lactobacillus curvatus* ssp. *curvatus*	
		1	*Lactococcus lactis* ssp. *lactis*	
26	*Lactobacillus paracasei* ssp. *paracasei*	25	*Lactobacillus paracasei* ssp. *paracasei*	96.1
		1	*Lactobacillus curvatus* ssp. *curvatus*	
2	*Lactobacillus curvatus*	1	*Lactobacillus curvatus* ssp. *curvatus*	50.0
		1	*Lactobacillus paracasei* ssp. *paracasei*	
1	*Lactobacillus brevis*	1	*Lactobacillus curvatus* ssp. *curvatus*	0.0
1	*Lactobacillus pentosus*	1	*Lactobacillus plantarum*	0.0
4	Unidentified lactobacilli	4	*Lactococcus lactis* ssp. *lactis*	
23	*Leuconostoc mesenteroides* ssp. *mesenteroides*[a]	22	*Leuconostoc mesenteroides* ssp. *mesenteroides*[b]	95.6
		1	*Leuconostoc mesenteroides* ssp. *cremoris*[b]	
5	*Leuconostoc mesenteroides* ssp. *dextranicum*[a]	5	*Leuconostoc mesenteroides* ssp. *dextranicum*[b]	100
1	*Leuconostoc lactis*	1	*Leuconostoc mesenteroides* ssp. *dextranicum*[b]	0.0
3	Unidentified leuconostocs	2	*Leuconostoc mesenteroides* ssp. *dextranicum*[b]	
		1	*Leuconostoc mesenteroides* ssp. *cremoris*[b]	

Tests performed for subspecies identification: production of: [a] acid from L-arabinose; [b] dextran and acid from L-arabinose.

Tests pour l'identification des sous-espèces : production de : [a] acide à partir du L-arabinose ; [b] dextrane et acide à partir du L-arabinose.

production of dextran and inability to produce acid from L-arabinose [20].

Tables IV and V show respectively the comparison analysis of the identifications of LAB from Tenerife cheese ascertained by classical and protein fingerprinting methods and the coincidence between both.

Lactococci were the LAB isolates that gave the worse results in the comparative study since only 63.6% of the identifications obtained by classical methods were confirmed by protein fingerprinting analysis (Tab. V). Among lactococci, only 14.2% of the classical phenotypically identified *Lactococcus lactis* ssp. *cremoris* remained unchanged after SDS-PAGE analysis, while the rest of the strains turned out to be lactobacilli (Tab. IV). In addition, the *Lc. raffinolactis* strain identified by the classical method proved to be *Lb. paracasei* ssp. *paracasei* after protein fingerprinting.

Fifty-five of sixty-one lactobacilli (77.5%) were correctly identified by API 50 CH, as the identifications were confirmed by SDS-PAGE analysis. Apart from some classical misidentifications within the genera, it is remarkable that the four presumptive lactobacilli that gave an unacceptable profile in the API system were finally assigned to *Lactococcus lactis* ssp. *lactis* by protein fingerprinting analysis (Tab. IV).

With regard to leuconostocs, API 50 CH and protein fingerprinting identifications were coincident in 84.4% of isolates. The five phenotypic misclassifications found by classical methods were finally assigned to one of the three subspecies of *Leuconostoc mesenteroides* after SDS-PAGE analysis followed by the determination of the production of dextran and of acid from L-arabinose.

4. DISCUSSION

Classical identification of Tenerife cheese LAB strains revealed the presence of 11 different species and subspecies. The identification of leuconostoc and lactobacilli by the API 50CH system and the APILAB plus program was easy to perform. This traditional standardized identification method is still very useful and routinely used [17, 25] although it is relatively expensive, especially when a great number of isolates are to be identified, and it does not take into account the recent progress in LAB taxonomy [24]. In contrast, the identification of species and subspecies of lactococci by classical physiological and biochemical methods has been difficult because of the heterogeneous and doubtful profiles obtained, which complicates the comparison with the

Table V. Coincidence of the identifications of LAB from Tenerife cheese by classical and protein fingerprinting methods.

Tableau V. Coïncidence entre les identifications des LAB du fromage de Tenerife par la méthode classique et l'empreinte digitale des protéines.

Group after classical phenotypic analysis	Nb. isolates	Coincidence of classical and SDS-PAGE analysis identifications	
		Nb. isolates	%
Lactococci	22	14	63.6
Lactobacilli	71	55	77.5
Leuconostocs	32	27	84.4
Total	125	96	76.8

phenotypic characteristics reported in the literature. Thus, our *Lactococcus lactis* ssp. *cremoris* strains showed a very atypical profile, growth at 6.5% NaCl, in 0.1% methylene blue and production of acetoin from citrate (Tab. II) when compared to the type strain. Furthermore, this lactococci subspecies was present in an unexpectedly high proportion (7 out of 22 lactococci) considering the difficulty of isolating the cremoris phenotype from natural sources [1]. These disagreements, which were initially attributed to the different environmental pressures of our isolates and the type strains as has been previously reported [2, 8], were finally considered to be a consequence of the misclassification of the strains.

Analysis of cell-free protein profiles performed as a complementary identification method to classical phenotyping has been successfully used in this study. Each LAB species gave a complex and stable pattern that could be visually compared, for identification purposes, with those displayed by the reference strains. In strains such as *Lactobacillus plantarum* and *Lb. paracasei* ssp. *paracasei*, the protein pattern generated after SDS-PAGE was identical to that of their respective type strains. The protein fingerprints of the rest of lactobacilli and lactococci did not perfectly match with those of their type strains, though the differences found did not affect the identification by visual comparison. These slight dissimilarities may be due to the different origin of the type strains and our strains, and they have also been noticed by other authors when identifying lactobacilli species isolated from naturally fermented Greek dry salami and cheese [19, 30].

Leuconostoc mesenteroides subspecies identification by SDS-PAGE was very difficult since the type strains displayed very similar protein patterns which complicates the visual comparison with our strains. In this case, two additional biochemical tests have to be performed in order to assign the subspecies: dextran production from sucrose which is negative for *Leuconostoc mesenteroides* ssp. *cremoris* and positive for the other two subspecies, and acid production from L-arabinose that is positive for *Ln. mesenteroides* ssp. *mesenteroides* and negative for subspecies *dextranicum*. The difficulty of the electrophoresis of cell free extracts to distinguish between the *Leuconostoc mesenteroides* subspecies has been previously reported [27, 28], and it is probably because they are very close phylogenetic subspecies [18] that they give very similar protein patterns.

When the comparison was done between the identification of Tenerife cheese LAB by classical phenotypic and protein fingerprinting methods, differences were found among the three genera under study. A good coincidence was found between the two identification methods in leuconostocs isolates (84.4%). The leuconostocs misclassifications obtained by the API system were confirmed to be *Leuconostoc mesenteroides* after SDS-PAGE and finally assigned to one of the subspecies by determining the production of dextran and of acid from L-arabinose. A coincidence of 77.5% was found for lactobacilli isolates. The majority of phenotypically misclassified lactobacilli were confirmed to be different *Lactobacillus* species by protein fingerprinting, while one strain of *Lactobacillus plantarum* as well as the four presumptive lactobacilli that could not be identified by the API system proved to be *Lactococcus lactis* ssp. *lactis* (Tab. IV).

After protein fingerprinting, only 63.6% of classical phenotypically identified lactococci were confirmed. It is remarkable that only one of seven isolates of *Lactococcus lactis* ssp. *cremoris* remained unchanged while the other six turned out to be *Lactobacillus plantarum* or *Lb. paracasei* ssp. *paracasei*.

The fact that the misclassifications of lactococci were finally assigned to different species of the genus lactobacilli and that

five lactobacilli turned out to be lactococci, indicates 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 microscopic observation of the strains (cocci cells were considered *Lactococcus* while rod shaped cells were considered *Lactobacillus*). The reason for this mistake is that, under certain growth conditions, lactococci may form ovoid or even rod shaped cells and that lactobacilli may produce very short rods or ellipsoid cells [14] which makes the morphological determination difficult. When the strains that were phenotypically missclassified at the genus level were subject to new phenotypic identification, the results obtained were in agreement with those obtained after SDS-PAGE analysis (not shown).

The results of this work indicate that classical biochemical and physiological tests are unsatisfactory for the identification of LAB from Tenerife cheese, since 23.2% of isolates were incorrectly identified following these methods. By contrast, the analysis of cell-free extracts provides an effective and reliable molecular-based typing method that generates complex and stable patterns that are easy to interpret and compare. When protein fingerprinting was performed as a complementary analysis to classic phenotyping the totality of the strains could be correctly identified.

In conclusion, our results show that protein fingerprinting analysis allows the reliable differentiation of Tenerife cheese LAB and can be applied to complement preliminary identifications by classical phenotypic tests.

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