Polyphasic Approaches to *Lactobacilli* Identification: Comparison of Phenotypic, Genotypic and Spectroscopic Methods

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ABSTRACT

Lactobacillus forms a large and diverse group of lactic acid bacteria (LAB). In raw milk and dairy products such as cheeses, yoghurts and fermented milks, *lactobacilli* are either naturally present or added intentionally for technological reasons or to generate a health benefit for the consumer. The *Lactobacillus* taxonomy has continued to evolve over the last twenty years, and currently consists of over 170 species.

We employed API 50CH (Apparatus and Procedure of Identification) and SDS-PAGE (sodium dodecyl sulfate-–-polyacrylamide gel electrophoresis) as phenotypic techniques to establish the identity of 52 wild *lactobacilli* isolated from artisanal dairy products. The identifications were confirmed using repetitive genomic element-PCR (Rep-PCR) fingerprinting and spectral methods including fluorescence spectroscopy. The SDS-PAGE results confirmed about 90% of API identification results. PCR using Boxair primers discriminated tested strains into *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus fermentum*, *Lactobacillus paracasei* subsp. *paracasei,* and *Lactobacillus plantarum*. This genetic method confirmed all the SDS-PAGE phenotyping results. Synchronous fluorescence spectra (SyFS) were recorded from 31 non-transparent bacterial colonies using a FluoroMax-2 spectrofluorometer linked to an optic fiber, and the results confirmed all the genotypic results, excluding certain strains: *Lb. plantarum* (217N and 1025RM) and *Lb. paracasei* subsp. *paracasei* 286KC as identified by the fluorescence method were genetically reclassified as *Lb. delbrueckii* subsp. *lactis* and *Lb. plantarum*, respectively.

Our study therefore highlights that spectral data provide real phenotypic fingerprints of the bacteria and can thus be used for taxonomic purposes. This study found that BOXPCR is a good tool for confirming most of the phenotypic identifications, making it possible to taxonomically characterize and differentiate wild-type lactic acid bacteria isolated from traditional dairy products.

Key words: *Lactobacillus* sp, artisanal dairy products, API 50CH, SDS-PAGE, Rep-PCR, intrinsic fluorescence spectroscopy.

INTRODUCTION

Among lactic acid bacteria, the genus *Lactobacillus*, whose classification is constantly being reshuffled, today encompasses more than 171 species and 27 subspecies (www.bacterio.cict.fr). It forms a diverse and heterogenic group of strictly-fermentative Gram-positive bacteria which exhibit remarkable adaptability to diverse habitats including vegetation, mucosal membranes, and the gastrointestinal tracts of terrestrial and marine animals. Domestication of *lactobacilli* for milk preservation originates in the Ptolemaic period of ancient Egypt (Allen, 1936). Today, various species are used worldwide as main or supplementary ingredients in commercial preparations for manufacturing fermented products. Indeed, lactobacilli have well-documented probiotic effects in humans and animals and their use has been rapidly extended to the animal feed and self-care health industries. The European Food Safety Authority (EFSA) proposed the qualified presumption of safety (QPS) concept to provide a generic risk assessment approach for biological agents authorized for use in the food and feed chain, and 35 *lactobacillus* species currently appear on the approval positive list (EFSA, 2010). The introduction of new isolates and basic studies both hinge on reliable taxonomic positioning of the isolates among other safety criteria. For taxonomic

purposes, polyphasic approaches are recommended (Vandamme, 1996), combining phenotypic and genotypic data collection (Bernardeau *et al*., 2008).

Phenotypic identification of *Lactobacillus* at species level mainly uses microscopic descriptors, biochemical trait descriptions. Other biomarkers can complete the analysis (Montel *et al.*, 1991), although these methods have their limitations due to relatively poor reproducibility, and ambiguous identification of closely-related species (Wijtzes *et al.*, 1997), they can now be supplemented with a large range of genotyping analyses (Tailliez *et al.,* 2002, Coeuret *et al.,* 2003, Naser *et al.***,** 2007). Among them, species-specific PCR is able to discriminate closely-related species (i.e. *Lb. plantarum* and *Lb. para plantarum*) that 16S and 23SRNA sequencing fail to discriminate **(**Berthier and Ehrlich, 1998, Delfederico *et al.,* 2006). However, these methods are time-consuming and expensive to implement.

Alternative tools based on vibrational spectroscopy are in development, and are gaining increasing interest. Among them, Raman and Fourier transform (FT)-infrared spectroscopy (Amiel *et al.,* 2000, Mariey *et al.,* 2001), have been effectively applied for lactic acid bacteria discrimination at species level (Bosch *et al.,* 2006, Gaus *et al.,* 2006, Dziuba *et al.,* 2007, Savi *et al.,* 2008). Other techniques such as mass spectrometry and related methodologies including pyrolysis-mass spectrometry, gas chromatography/mass spectrometry, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) have also been successfully used (Sauer & Kliem, 2010) but are relatively laborious, involving sample preparation steps and reagent inputs (Bosch *et al.,* 2006, Postnikova *et al.,* 2008). Nevertheless, in the spectroscopy field, fluorescence spectroscopy does offer inherent advantages compared to FT-infrared, such as no signal noise from water, high sensitivity, and short collection-time requirements (Estes *et al.,* 2003). In taxonomic applications, this method relies on the fact that bacteria retain several intrinsic fluorophores which emit photons according to excitation in the UV region. Tryptophan, phenylalanine and tyrosine are some of the most commonly-used fluorescent molecules. The nucleotides could also fluoresce, but their quantum yields are about 100 times lower than with tryptophan (Cantor & Schimmel, 1980, Lakowicz, 1999). Since the middle of the last decade, several authors have demonstrated that fluorescence spectra collected from bacterial cells could be considered fingerprints of the bacteria, thus discriminating microorganisms belonging to different taxonomical families (Leblanc & Dufour, 2002, Leriche, 2004, Bhatta *et al.,* 2006) or bacteria at family, genus, species, and subspecies level (Ammor, 2007). This paper describes the use of phenotyping, molecular methods and fluorescence spectroscopy to discriminate and identify *Lactobacillus* strains isolated from traditional Egyptian dairy products at genus, species and subspecies levels.

MATERIALS AND METHODS

Bacterial strains and cultivation

Fifty-two cultures were isolated from 20 samples of raw milk, Zabady (Egyptian fermented milk), Kareish cheese and French cheeses. All isolates were identified to genus level according to Sharpe (1979) using morphological, phenotypic and biochemical methods. Cultures of these isolates were stored at -80°C in reconstituted skimmed milk supplemented with 15% (v/v) of glycerol for storage. Working cultures were prepared from frozen stocks through two transfers in MRS broth.

Phenotypic methods

In addition to preliminary tests, carbohydrate fermentation profiles were evaluated using the API 50CH system (bioMérieux, Marcy-l'Étoile, France) following the manufacturer's instructions. Fermentation profiles were interpreted using the APILAB Plus V.3.2.2 computer-aided database.

For SDS-PAGE analysis of the whole-cell proteins, preparation of cell extracts and polyacrylamide gel electrophoresis were done according to Pot *et al.* (1994). Gel-Compar software (Applied Maths, Sint-Martens-Latem, Belgium, V. 4.0) was utilized to compare the protein patterns of the isolates against the intern reference database built from reference strains from the Laboratory of Microbial Biochemistry (LMB, ROM and ES) at the Faculty of Agriculture, Alexandria University, Egypt and the Centre National de Recherche Zootechnique Jouy-en-Josas, France (CNRZ). These reference strains were *Lb. del. bulgaricus* ES0036, *Lb. del. lactis* CNRZ245, *Lb. fermentum* 9LMB, *Lb. paracasei paracasei* 2ROM, and *Lb. plantarum* CNRZ739.

Hierarchical agglomerative clustering of isolates was performed using the Pearson's product moment correlation coefficient (r) and the un-

weighted pair group method using average linkage (UPGMA), as described by Pot *et al.* (1994).

Genotypic identification method

DNA Extraction

Total DNA was extracted from 1.6 ml of fresh cultures in the exponential phase using the Wizard DNA purification Kit following the manufacturer's instructions (Promega, Madison, WI., USA).The DNA concentration was assessed by determining O.D at 260 and 280 nm using an Ultrospectro 3000 system (Amersham Biosciences, Sweden).

Rep-PCR genomic fingerprinting

The DNA concentration of each sample was adjusted to 25 ng/µl in a 25 µl PCR mixture. Amplification was performed in a 25 µl reaction volume typically containing 20 ng genomic DNA, 0.3 µM Boxair primers (5'-CXTAXCGGCAAGGCGACGCTGACG-3') and puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Sweden).

The PCR program was 35 thermal denaturation cycles for 30 sec at 92°C, annealing for 1 min at 40°C and extension for 2 min at 72°C. The cycles were preceded by denaturation at 92°C for 2 min and followed by extension at 72°C for 5 min The PCR products were separated by electrophoresis at 50 V on a 2% (w/v) agarose (Amersham Biosciences. Sweden), and DNA was detected by UV transillumination after staining with ethidium bromide (l0 mg/ml). The molecular sizes of the amplified DNA fragments were estimated by comparison with a 100 bp DNA ladder (Promega, Madison, Wiscon. USA). Conversion, normalization and further analysis of Rep-PCR patterns were carried out using Gel Compar V.4.0 software (Applied Maths, Kortrijk, Belgium). Similarity coefficients for pairs of tracks were calculated using Pearson's product moment correlation coefficient (r). Strains were grouped by UPGMA. Reference strains were obtained from the different LAB culture collections in the reference databases cited earlier.

Optic-fiber based synchronous fluorescence spectroscopy

Measurements

Synchronous fluorescence spectra (SyFS) were recorded from the bacterial colonies using a FluoroMax-2 spectrofluorometer (Spex-Jobin Yvon, Longjumeau, France) linked to an optic fiber. The optic fiber was set at 9 mm above the colonies to keep the light beam diameter at less than 2 mm. SyFS collected in the excitation wavelength range was 250-500 nm (resolution: 1 nm, slits 18 nm), excitation wavelength λ_{ex}, and emission wavelength λ_{em} were scanned synchronously with a fixed offset $\Delta\lambda$ of = 30 nm. For each strain, three SyFS spectra were acquired for three independent colonies resulting from culturing on MRS agar plates at 30 or 37ºC for 48 h depending on the growth conditions of each microorganism. As fluorescence spectroscopy is not suitable for characterizing transparent colonies, the opaque colonies of *Lactobacillus* cultures were only tested.

Thirty one wild strains of *lactobacillus* were studied (5 *Lb. delbrueckii* subsp. *lactis*, 4 *Lb. delbrueckii* subsp. *bulgaricus*, 16 *Lb. plantarum*, 4 *Lb. paracasei* subsp. *paracasei* and 2 *Lb. fermentum.*). The reference strains were *Lb. delbrueckii* subsp. *lactis* CIP101028T (Collection de l'Institut Pasteur Paris), *Lb. delbrueckii* subsp. *bulgaricus* CIP101027T (Collection de l'Institut Pasteur Paris), *Lb. plantarum* ATCC14917 ATCC (American Type Culture Collection) and *Lb. paracasei* subsp. *paracasei* ATCC11978 (American Type Culture Collection).

Spectral data analysis

The data were analyzed using XLStat-pro software (Addinsoft, Paris, France) and Statistica software build 6.1 (Statsoft, Maisons-Alfort, France). In order to minimize scattering effects, all fluorescence spectra were normalized by reducing the area under each spectrum to a value of 1 according to Bertrand & Scotter (1992).

Factorial discriminant analysis (FDA) was chosen as the most appropriate multivariate analysis tool for emphasizing differences between experimental groups (Wittrup, 2000). Before running the FDA analysis, information contained in the spectra was first compressed using principal component analysis (PCA) as described by Jolliffe (1986). The PCA allows the variables (wavelengths) to be transformed into new orthogonal variables called principal components (Bertrand *et al.,* 2006), while conserving an overview of all the information in the dataset. The PCA was computed from the variance/covariance matrices. The first 10 PCs contained more than 99.9% of the information in the spectral datasets, and were used to perform FDA to discriminate the *lactobacilli* strains at different levels. For each analysis, the data were introduced into predefined groups or classes (one class was one taxo-

nomic cluster, species, or subspecies) according to the taxonomic level chosen for discrimination level between strains. For example, for discrimination of reference strains at species level, three groups were created (*Lb. delbruecki*, *Lb plantarum*, *Lb paracasei*), while for discrimination of all tested strains, five groups were created *(Lb. delbrueckii* subsp. *lactis*, *Lb*. *delbrueckii* subsp. *bulgaricus*, *Lb. plantarum*, *Lb. paracasei* subsp. *paracasei* and *Lb. fermentum*). The validation step used leave-one-out cross-validation (LOOCV), where a single observation from the original sample is used as the validation data and the remaining observations as training data. Repetitions ensure that each observation in the sample is used once as validation data (Kohavi, 1995).

RESULTS AND DISCUSSION

Phenotypic methods

Looking at galerie API LAB results, 41 of the 52 strains analyzed were satisfactorily identified. Doubts remains over the identification of 11 strains, as shown in Table (1).

These results further support numerous authors who have questioned the reliability of the commercial tests for the identification of lactobacilli at the species and subspecies levels (Sanchez *et al.*, 2004, Boyd *et al.,* 2005).

The results from numerical analysis of SDS-PAGE whole-cell protein patterns were coherent with the API results for about 90% of the isolates. The few discrepancies concerned isolates classified as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacilus. delbrueckii* subsp. *Lactis* using SDS-PAGE but as *Lactobacilus salivarius* (497N) and *Lactobacilus acidophilus* (137FR), respectively, using the API system. Similar results have been observed by El Soda *et al.* (2003) and Mohammed *et al.* (2009).

Strain typing using Rep-PCR

Rep-PCR fingerprinting patterns generated from Boxair primer amplification were able to differentiate the 52 isolates initially assigned to the *Lactobacillus* genus on the basis of phenotypic methods. Nine strains were identified as *Lb. delbrueckii* subsp. *bulgaricus,* 18 strains were *Lb. delbrueckii* subsp. *lactis,* 3 strains were *Lb. fermentum,* 4 strains were *Lb. paracasei* subsp. *paracasei,* and 18 strains were *Lb. plantarum.* The amplification profiles indicated that *Lb. delbrueckii* subsp. *lactis* shared two common bands around 0.3 and 1 Kb, *Lb. delbrueckii* subsp. *bulgaricus* had three common bands around 0.3, 1 and 2 Kb, *Lb. paracasei* subsp. *paracasei* gave bands in the range of 0.3-0.8Kb, *Lb. fermentum* gave three intense bands around 0.7, 0.4 and 0.3 Kb, while *Lb. plantarum* exhibited four intense bands around 0.3, 0.6, 0.8 and 1 Kb and faint bands around 0.9 and 1.5 Kb. These results confirm the observations of Mohammed *et al.* (2009).

According to UPGMA analysis, the strains were grouped into 5 clusters at a similarity level of about 20%, as shown in Figure (1). Cluster A grouped the type strains of *Lb. delbrueckii* subsp. *bulgaricus* with 80% similarity, Cluster B grouped the type strains of *Lb. delbrueckii subsp. lactis* with 85% similarity, Cluster C grouped the type strains of *Lb. fermentum* with 65% similarity, Cluster D grouped the type strains of *Lb. paracasei* subsp. *paracasei* with 70% similarity, and *Lb. plantarum* strains were grouped into two main clusters (Clusters I and II respectively) with 65% similarity.

Analysis of the DNA fingerprints generated by the BOX primer allowed a reliable grouping of the wild-type *Lactobacillus* isolates at species or subspecies level. Characteristic amplification patterns were obtained from strains of *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. fermentum*, *Lb. paracasei* subsp. *paracasei* and *Lb. plantarum*. For all 52 isolates, taxonomic positioning was consistent with SDS-PAGE results (Table 1).

Intrinsic fluorescence spectra of tested strains

As described previously (Ammor 2007, Tourkya *et al.,* 2009), spectra shapes varied substantially through LAB and could be considered as real fingerprints of each bacteria, allowing an in-depth comparison of strain profiles

A first step led to verify the discrimination of the reference strains. Spectral data collected from *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis, Lb. plantarum* and *Lb. paracasei* subsp. *paracasei* were pooled in one matrix and analyzed by PCA. Before applying FDA, groups were created as described in the materials and methods section. On the factorial map (not shown) corresponding to FDA performed on synchronous spectra, the first two discriminant factors (F1 and

Table 1: Identification of Lactobacillus isolates using phenotypic, genotypic and spectroscopic methods.

Strain	Strains identified by API	Strains identified by SDS-PAGE	Strains identified by rep-PCR	Strains identified by spectroscopic method
610N	*Lb. del. bulgaricus* *Lb. helveticus*	*Lb. del. bulgaricus*	*Lb. del. bulgaricus*	N.T.
572N	*Lb.del. lactis* *Lb. del. bulgaricus* *Lb.helveticus*	*Lactobacillus del. bulgaricus*	*Lb. del. bulgaricus*	*Lb. delbrueckii*
702N	*Lb. del. bulgaricus* *Lb.helveticus*	*Lb. del. bulgaricus*	*Lb. del. bulgaricus*	N.T.
722N	*Lb. del. bulgaricus* *Lb. helveticus*	*Lb. del. bulgaricus*	*Lb. del. bulgaricus*	N.T.
726N	*Lb. del. bulgaricus* *Lb.helveticus*	*Lb. del. bulgaricus*	*Lb. del. bulgaricus*	N.T.
725N	*Lb. del. bulgaricus* *Lb.helveticus*	*Lb. del. bulgaricus*	*Lb. del. bulgaricus*	*Lb.delbrueckii*
497N	*Lb.salivarius*	*Lb. del. bulgaricus*	*Lb.del. bulgaricus*	N.T.
635N	*Lb.del. bulgaricus* *Lb.helveticus* *Lb. del. delbrueckii* *Lb. del. lactis*	*Lb.del. bulgaricus*	*Lb.del. bulgaricus*	*Lb.delbrueckii*
722RM	*Lb.del. lactis* *Lb.helveticus* *Lb.del. bulgaricus*	*Lb.del. bulgaricus*	*Lb.del. bulgaricus*	*Lb.delbrueckii*
157FR	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
153FR	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
178N	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.delbrueckii*
697RM	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
712N	*Lb.helveticus* *Lb.del. bulgaricus* *Lb.del. lactis*	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.delbrueckii*
164FR	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
162FR	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
217N	*Lb.helveticus* *Lb.del. bulgaricus* *Lb.del. lactis*	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.plantarum*
156FR	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
223FR	*Lb.del. delbrueckii* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
137FR	*Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
185FR	*Lb.del. delbrueckii* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
270N	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
416N	*Lb.del. bulgaricus* *Lb.del. lactis*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.

Strain	Strains identified by API	Strains identified by SDS-PAGE	Strains identified by rep-PCR	Strains identified by spectroscopic method
1025RM	*Lb.del. lactis* *Lb.del. bulgaricus* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.plantarum*
701RM	*Lb.del. lactis* *Lb.acidophilus*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
511KC	*Lb.del. delbrueckii*	*Lb.del. lactis*	*Lb.del. lactis*	N.T.
335N	*Lb.acidophilus* *Lb. del. lactis*	*Lb.del. lactis*	*Lb.del. lactis*	*Lb.delbrueckii*
260KC	*Lb.fermentum*	*Lb.fermentum*	*Lb.fermentum*	N.T.
247KC	*Lb.fermentum*	*Lb.fermentum*	*Lb.fermentum*	*Lb.fermentum* *Lb.plantarum* *Lb.delbrueckii*
334N	*Lb.fermentum*	*Lb.fermentum*	*Lb.fermentum*	*Lb.fermentum*
67FR	*Lb.paracasei*	*Lb.paracasei*	*Lb.paracasei*	*Lb.paracasei*
158FR	*Lb.paracasei*	*Lb.paracasei*	*Lb.paracasei*	*Lb.paracasei*
190FR	*Lb.paracasei* *Lb.rhamnosus*	*Lb.paracasei*	*Lb.paracasei*	*Lb.paracasei*
393KC	*Lb.paracasei* *Lb.plantarum*	*Lb.paracasei*	*Lb.paracasei*	*Lb.paracasei* *Lb.plantarum*
583N	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
420KC	*Lb.plantarum* *Lb.pentosus*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
404KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
446KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
284KC	*Lb.plantarum* *Lb.pentosus*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
481KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
475KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
586N	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	N.T.
493KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
432KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	N.T.
257KC	*Lb.pentosus* *Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
286KC	*Lb.pentosus* *Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.paracasei*
340KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
662N	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
326KC	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
277KC	*Lb.plantarum* *Lb.paracasei*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
542RM	*Lb.pentosus* *Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*
832RM	*Lb.pentosus* *Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*

N.T: not tested

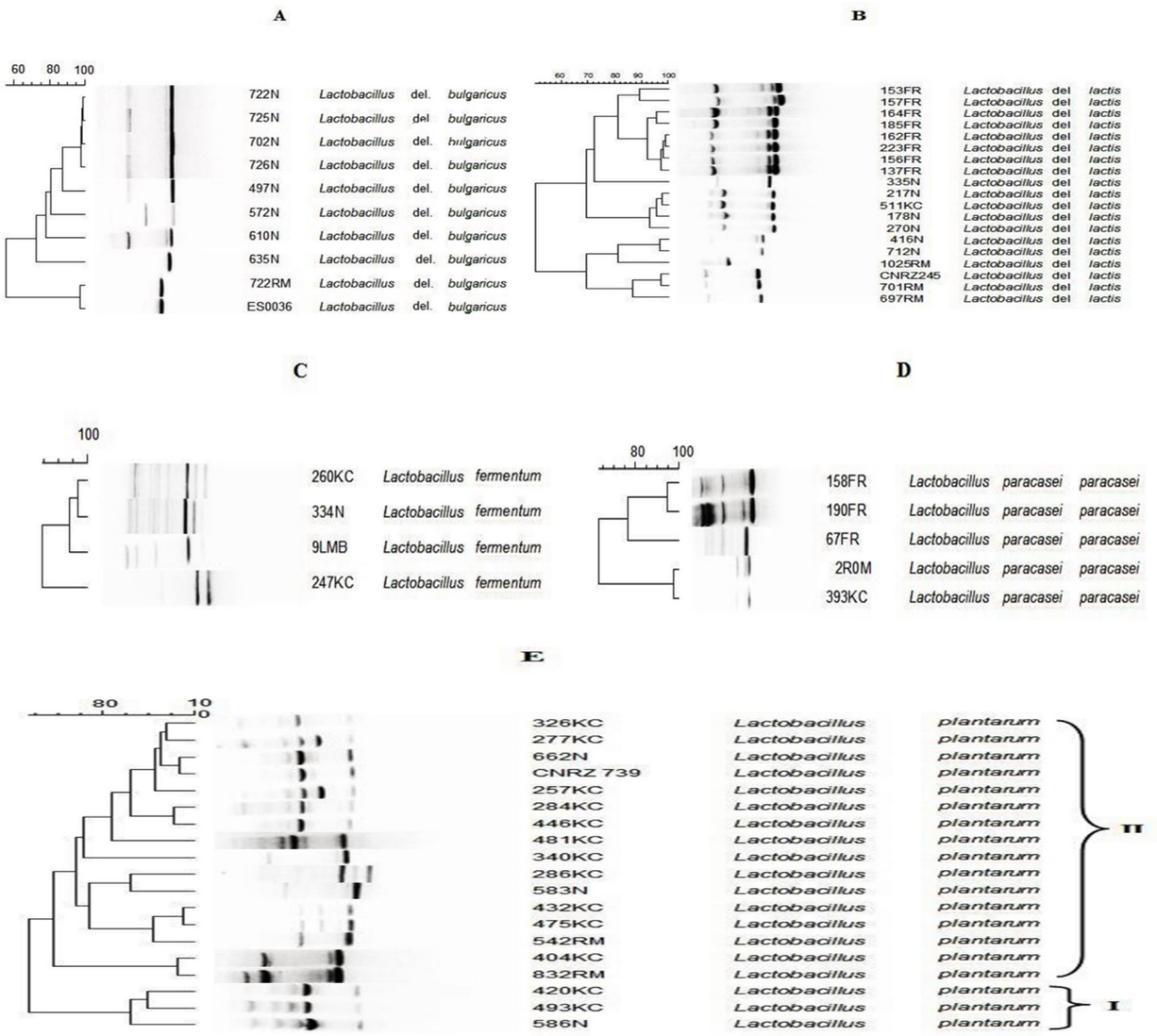

Fig. 1: Dendrogram generated from BOX-PCR fingerprints of lactobacilli strains: (A) *Lactobacillus delbrueckii* subsp. *bulgaricus*, (B) *Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus fermentum* (C), *Lactobacillus paracasei* subsp. *paracasei* (D) and (E) *Lactobacillus plantarum*. The dendogram was constructed using the unweighted pair group method using arithmetic mean, with correlation levels expressed as percentages

***Lb. del. bulgaricus* ES0036, *Lb. del. lactis* CNRZ245, *Lb. fermentum* 9LMB, *Lb. paracasei paracasei* 2ROM and *Lb. plantarum* CNRZ739 are reference strains.**

F2) explained 99.91% of total variance. The four groups were perfectly separated, and the analyses revealed the close relatedness of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*. Cross-validation results from the FDA confusion matrix showed 100% correct classification.

In a second step, a similar analysis was performed on the spectral data collected from all isolates and reference strains included in this part of the study.

To perform FDA analysis, the groups were created on the basis of phenotypic and molecular based-results. The seven groups were *Lb. delbrueckii* subsp. *bulgaricus and Lb. delbrueckii* subsp. *lactis (*isolates and reference strains*)*, *Lb. fermentum*, *Lb. paracasei* subsp *paracasei* (isolates and reference strains) and *Lb. plantarum* (isolates and reference strains). On the factorial map (Fig. 2) corresponding to this FDA, the first two discriminant factors (F1 and F2) explained 65.69% of total variance and allowed the differentiation of seven distinct groups, despite the dispersion of a few spectra. *Lb. fermentum* strains were well separated from other strains according to factor F2, which accounted for 29.87% of total variance. On this axis, *Lb paracasei* were also well separated from *Lb. delbrueckii subsp. lactis* and *bulgaricus.* Note that the method failed to discriminate these latter two subspecies. According to factor F1 which accounted for 35,82% of total variance, *Lb. paracasei* subsp. *paracasei* was clearly distinct from *Lb. plantarum* but close to *Lb. fermentum*.

The cross-validation analysis was managed by poling *Lb. delbrueckii* subsp*. lactis and Lb. delbrueckii* subsp*. bulgaricuss* into a species group. Percent correct classification varied from 66.67% for *Lb. plantarum* to 100% for *Lb. delbrueckii, Lb. paracasei* subsp. *paracasei* and *Lb. plantarum* reference strains*.* The model attributed one *Lb. plantarum*286KC strain to *Lb paracasei*, while two strains of *Lb. delbrueckii* subsp. *lactis* (1025RM and 217N) were reclassified as *Lb. plantarum* (Tables 1 and 2).

Synchronous fluorescence spectroscopy is a fast, simple and low-cost reagent less tool for studying strain relatedness, and can be considered a reliable way to study the bacterial taxonomy of complex clades such as *Lactobacilli*.

CONCLUSION

From this investigation, phenotypic, genomic and spectroscopic methods have been combined and compared to identify wild-type lactic acid bacteria isolated from traditional dairy products and preliminarily identified as *Lactobacillus*. The 52 isolates studied belonged to the species *delbrueckii*, (subsp. *lactis, bulgaricus)*, *plantarum*, *paracasei* and *fermentum*.

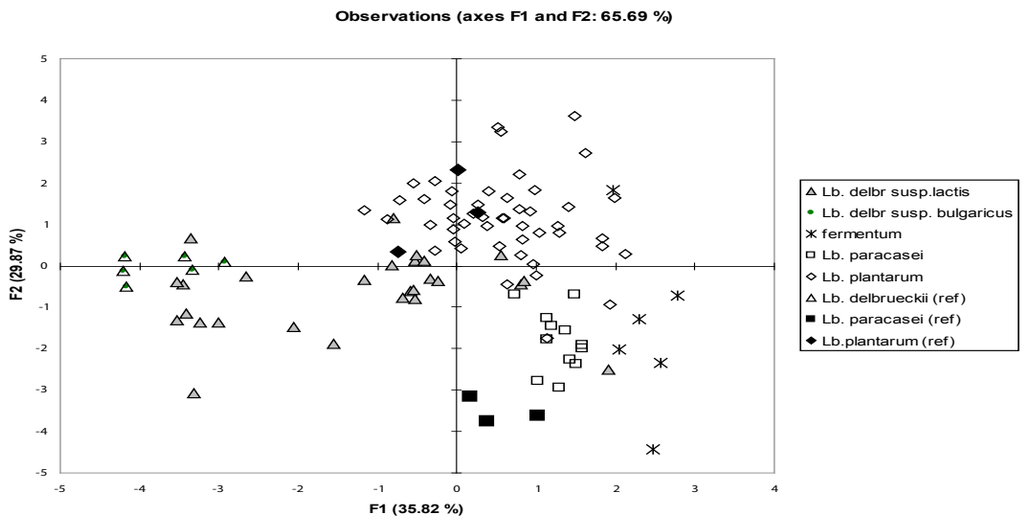

Fig. 2: Discriminant analysis similarity map determined by discriminant factors F1 and F2 for the synchronous fluorescence (Δλ = 30 nm) spectral data of *lactobacilli*
(ref: reference strains)

Table 2: Percentage of correct classification according to the confusion matrix resulting from FDA analysis performed on the synchronous spectra collected from *lactobacilli* isolates and reference strains

from \ to	delb	delb	fermentum	paracasei	paracasei	plantarum	plantarum	Total	% correct
Lb. delbrueckii	19	3	1	0	0	4	0	27	70.37
Lb.delbrueckii^{R}	0	6	0	0	0	0	0	6	100.00
Lb.fermentum	1	0	4	0	0	1	0	6	66.67
Lb.paracasei	0	0	0	11	0	1	0	12	91.67
Lb.paracasei^{R}	0	0	0	0	3	0	0	3	100.00
Lb.plantarum	0	0	0	2	0	45	1	48	93.75
Lb.plantarum^{R}	0	0	0	0	0	0	3	3	100.00
Total	20	9	5	13	3	51	4	105	86.67

R: reference strains

Discrimination of the isolates at species level was excellent using SDS-PAGE rep-PCR and spectroscopic analysis, which revealed a close relatedness of *Lb. paracasei* subsp. *paracasei* and *Lb. fermentum* isolates that had not been described for the reference strains, suggesting an ecological adaptation revealed by whole-cell component analysis.

Rep-PCR based on Boxair primers and fluorescence-based spectroscopy was able to differentiate species and subspecies, even for the two *Lactobacillus delbrueckii* subspecies which form a fairly heterogenic taxon from a metabolic point of view but are barely distinguishable by genomic studies such as 16S rDNA sequence comparison (Canchaya *et al.,* 2006). It was able to identify isolates that the API system had failed to assign to a defined taxonomic position (Table 1).

Working with colonies on agar plates, rep-PCR analysis is fairly straightforward but still requires several steps, toxic reagents, and primers. In contrast, optical fiber-based synchronous fluorescence spectroscopy of colonies on agar plates requires no manual preparation of the cells and no reagents, and spectra are obtained within just a few minutes. Suitable chemometric treatments make it easy to study strain relatedness. This study yields very encouraging results for *Lactobacillus* isolates, although further investigations are needed since there was no reliable way to analyze transparent colonies. For rapid isolate identification, the investigation hinges on access to robust databases that include as many reference and wild-type strains as possible. This investigation is work in progress. Nevertheless, this tool has already proven its reliability for other taxons (Tourkya *et al.,* 2011, in press) and should be rapidly integrated into the panel of techniques for a polyphasic approach to lactic acid bacteria taxonomy.

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طرق التعرف المتعددة لسلالات من اللاكتوبسيلس : مقارنة مابين الطرق المظهرية والوراثية والسبكتروسكوبية

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تشكل البكتريات العصوية مجموعة كبيرة ومتنوعة من بكتيريا حمض اللاكتيك. في اللبن الخام ومنتجات الألبان مثل الجبن و الالبان المتخمرة. واللاكتوبسيلس تكون موجودة اما طبيعيا أو مضافة عمدا لأسباب تكنولوجية أو لاكساب المنتج قيمة صحية للمستهلك. وفي خلال السنوات العشرين الماضية تطور تصنيف هذه العصويات الي ان وصلت حاليا الي أكثر من ١٧٠ نوعا.

تم استخدام كل من الـ 50APICH (جهاز وإجراءات التعرف علي البكتريا)، طريقة الهجرة الكهربية SDS-PAGE كوسيلة للتعرف على ٥٢ من اللاكتوبسيلس المعزولة من منتجات الألبان التقليدية. وتم تأكيد التعرف باستخدام أخذ البصمات للعنصر الجيني المتكرر (Rep-PCR) الي جانب طرق التحليل الطيفية متمثلة في السبكتروسكوبي الفلورسنسي.

اكدت نتائج الـ SDS-PAGE حوالي ٩٠ ٪ من نتائج التعرف بواسطة الـ API. تم تمييز السلالات المختبرة بواسطة PCR- Boxair إلى sub- *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* sp. *lactis, Lactobacillus fermentum, Lactobacillus paracasei subsp. paracasei, Lactobacillus plantarum*.

وتم تأكيد كل نتائج SDS-PAGE المظهرية بواسطة الطريقة الوراثية. ولقد تم تسجيل متزامن للأطياف الفلورنسيية SyFS لـ ٣١ مستعمرة بكتيرية غير شفافة باستخدام FluoroMax-2 مقياس التألق الطيفي المرتبطة بالألياف البصرية، وتم تأكيد كل نتائج التعرف بالطرق الوراثية، باستثناء بعض السلالات: (217N-1025RM) *Lb. plantarum* والـ *Lb. paracasei* subsp. *paracasei* 286KC المتعرف عليهم بواسطة الطريقة الفلورنسية التي تم اعادة تقسيمهم وراثيا الي *Lb. plantarum* و *Lb. delbrueckii* subsp. *lactis* علي التوالي.

تسلط هذه الدراسة الضوء علي توفير البيانات الطيفية كبصمات مظهرية حقيقية للبكتيريا وبالتالي يمكن استخدامها لأغراض التصنيف. وتبين من هذه الدراسة أن BOXPCR هو أداة جيدة لتأكيد معظم التعرف المظهري ، مما يجعل من الممكن تصنيف وتفرقة الانواع البرية من نوع بكتريا حمض اللاكتيك والمعزولة من منتجات الألبان التقليدية.