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Short Communication

Phenotypic and genotypic characterization of lactic acid bacteria isolated from cow, ewe and goat dairy artisanal farmhouses.

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Abstract

Lactic acid bacteria collected from artisanal farmhouses were characterized using a polyphasic approach. Phenotypic methods including biochemical assays, ribosomal DNA restriction analysis and 16S rDNA sequence analysis were performed. This approach provides accuracy for identification, and helps to avoid the loss of natural biodiversity including potentially valuable strains.

Key words: Native lactic acid bacteria, polyphasic identification, artisanal dairy products.

Lactic acid bacteria (LAB) are microorganisms of great economic importance for artisanal and industrial dairy fermentation processes. There is an ongoing need for improving useful properties of starter cultures, and natural microbial populations present in artisanal dairy environments provide a resource of genetic diversity. This is particularly evident in environments where cheeses are produced without pasteurization or commercial starters (Coppola *et al.*, 2001). LAB isolated from natural habitats may possess unusual characteristics including phenotypic differences and intraspecies variability compared with standard type strains (Fortina *et al.*, 1998). Identification of suitable strains as starter cultures for production of fermented dairy-based foods requires identification and consideration of some important technological properties, including acidification rate, exopolysaccharide production, aminopeptidase activity, antibacterial activity against pathogens and antibiotics resistance (Marroki *et al.*, 2011). The occurrence of antibiotic resistance should be taken into account as potentially adverse property that would prohibit their application in food and feed.

Conventional phenotypic identification of LAB depends mainly on morphological, physiological and biochemical criteria. A broad range of DNA-based methods are available for identification and typing of LAB isolates (Temmerman *et al.*, 2004), and current taxonomic polyphasic methods include both phenotypic and genotypic analysis. 16S rRNA gene sequencing is one of the most commonly used techniques for identification of LAB species, but the method often fails to discriminate between phylogenetically closely related LAB species or between subspecies (Temmerman *et al.*, 2004). Although, phenotypic tests provide evidence for different metabolic capabilities among isolates, the complex and sometimes graded differences make distinctions difficult. Designation of certain new type strains based solely on phenotypic characteristics has resulted in ambiguities which have ultimately been resolved using molecular techniques (Van Hoorde *et al.*, 2008). The aim of the present study is to use a polyphasic approach to identify selected strains of LAB isolated from raw milk, cheese and cheese whey starter by characterization of phenotype, amplified ribosomal DNA restriction analysis (ARDRA) and 16S rDNA sequence analysis.

Twelve cocci LAB isolates from cow (8), ewe (1) and goat (1) raw milk, artisanal cheese (1) and cheese whey starter (1) from our laboratory culture collection and three type strains of the American Type Culture Collection (ATCC) were analyzed in this study. The strains to be identified were isolated within the last ten years from samples collected at different farmhouses which process cow, goat or ewe raw milk to cheese without adding commercial starters. The main technological properties of the isolates selected were fast acidification rate, aroma compounds production or bio-protective activity. Working cultures were prepared from isolate and type strain frozen stocks after growth and transfer twice in fresh MRS broth (Oxoid, UK)

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at 32 °C or 45 °C. Selected strains were assigned to the genus level based on phenotype including cell morphology, Gram staining, catalase reaction, CO_2 production from glucose in modified MRS broth (excluding ammonium citrate) containing inverted Durham tubes, arginine hydrolysis, growth at different temperatures (10 °C and 45 °C), medium pH (4.4 and 9.6), and salt content (6.5% NaCl), according to Sharpe (1979). The Voges-Proskauer test was used for further discrimination within the *Lactococcus* genus.

Acid production after fermentative growth of isolates/type strains on selected carbohydrates (ribose, esculin, melezitose, salicin, maltose, lactose, fructose, galactose, arbutin, raffinose, glucose, sucrose, arabinose, xylose, mannose, melibiose, rhamnose, mannitol, sorbitol and trehalose) was evaluated using a miniaturized microplate assay. Each 96-well microplate was prepared by addition to individual wells 10 µL of each filter sterilized sugar solution (20%, w/v) (Marroki *et al.*, 2011), 100 µL LAPTg media (peptone 15 g/L, tryptone 10 g/L, glucose 10 g/L, yeast extract 10 g/L , Tween 80 1 mL/L, and phenol red 2 mg/L) without glucose (Petrov *et al.*, 2008), and inoculated with a type strain or isolate to be identified. Microaerophilic conditions were generated by sealing the well with 50 µL sterile vaseline. Isolates analyzed by biochemical assays were grown overnight, harvested and dispensed to microplates as in Gussils (2001). Microplates were incubated at 34 °C for 48 h, and sugar fermentation was considered as positive by a color change to yellow in medium of the corresponding well. A weak fermentation was associated with a color change to orange.

LAB isolates and type strains were grown in MRS broth at 34 °C and 42 °C to an $OD_{600\ nm}$ of 1.0. Cultures were harvested, washed twice, and suspended in 200 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was purified using a Genomic DNA purification kit (Fermentas International Inc., USA) following manufacturers instructions. DNA was used as template in amplification reactions and concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Wilmington, MA). 16S rRNA gene amplification was performed as described by Weisburg *et al.* (1991). PCR was carried out in a Corbett CG1-96 thermal cycler (Corbett Research Ltd., Cambridge, UK).

A 20 µL sample of purified PCR amplicon was digested with individual restriction enzymes in 40 µL at 37 °C, using 2U HhaI*,* 1U Hinf I (New England Biolabs, USA), or 10U AluI (Fermentas, USA) following conditions recommended by the manufacturer. Digestions using MspI (BIORON, GmbH, Ludwigshafen, Germany) were done at 55 °C. Restriction patterns were analysed on 2% agarose gels using 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0) as running buffer at 10 V/cm for 2 h, stained with 0.5 µg/mL ethidium bromide and visualized and photographed on a UV transilluminator. The amplified DNA fragment of the16S rRNA gene of each isolate was purified and then sequenced by Macrogen Sequencing Service, Korea. DNA sequences were compared by Blast analysis using non-redundant databases at the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine which type strains had highest similarity.

Isolates under study were initially classified based on phenotypic characteristics and acid production from carbohydrates. All LAB isolates were homofermentative, Gram positive and catalase-negative cocci. Growth at 45 °C, pH 9.6 or with 6.5% NaCl was tested for all isolates and type strains. Five isolates (F-11, F-22, F-130, F-131, and F-B2) were not able to grow under these conditions but were able to growth at 10 °C as described by Axelsson (2004) for *Lactococcus* sp. Other studies, however, found variation in salt resistance among wild *L. lactis* spp *lactis* isolates in that a high proportion could grow with 6.5% NaCl (Aquilanti *et al.*, 2007, Nomura *et al.*, 2006). Four isolates (F-53, F-57, F-B1, and F-B3) were classified as *Streptococcus* sp. in accordance with the criteria established by Devriese and Pot (1995). These isolates grew at 45 °C but were unable to grow at 10 °C, extreme pH (4.4 or 9.6) or with 6.5% NaCl. Three other isolates (F-45, F-60, and F-63) having a characteristic cocci-tetrad cell arrangement were identified as *Pediococcus* sp. These were able to grow at 45 °C, in 6.5% NaCl and at pH 4.4, but did not grow at pH 9.6 or 10 °C.

Carbohydrate fermentation using the microplate assay confirmed differences among isolates at the species level (Axelsson, 2004). All isolates and type strains were able to ferment glucose, fructose, mannose and ribose, but unable to ferment melezitose, raffinose, melibiose and sorbitol. The carbohydrate utilization of wild isolates matched those of type strains, except for strains F-63 and F-60 whose carbohydrate fermentation patterns differed from the corresponding type strain, *P. pentosaceus* (ATCC 25745). Isolates F-63 and F-60 did not ferment salicin, maltose, arbutin, rhamnose, and threalose, while F-45 and the *P. pentosaceus* type strain produced acid from these carbohydrates. Bhunia and Johnson (1992) found that isolates unable to ferment rhamnose, arbutin and maltose could be identified as *P. acidilactici*. These are key sugars used for phenotypic identification of these species, and were consistent with 16S rDNA sequencing. Phenotypic and biochemical characterization of the isolates enabled grouping them into four species. Four isolates (F-53, F-57, F-B1, and F-B3) and *S. salivarus subsp. thermophilus* (ATCC 19258) were able to ferment lactose, sucrose and glucose, but unable to ferment galactose. However, Giraffa *et al.* (2001) found that acid production from these sugars was inconsistent within wild isolates of *S. thermophilus*. Five isolates (F-11, F-22, F-130, F-131, and F-B2) and *L. lactis* subsp *lactis* biovar. *diacetylactis* (ATCC 13675) were able to ferment all sugars tested except melezitose, raffinose, xilose, melibiose, rhamnose and sorbitol. All iso-

lates of this group were assigned to biovar *diacetylactis* because of the ability to produce acetoin in the Voges-Poskauer reaction.

Digestion of 16S rDNA amplicons with restriction enzymes HinfI, AluI or MspI resulted in clear banding patterns but these could not be used to discriminate isolates at the species level. Aquilanti *et al.* (2007) working with AluI double digestions (using either HaeIII or FokI as the second enzyme), however, could discriminate subgroups among 112 isolates from Pecorino cheese. In our study, use of HhaI allowed for generation of five banding patterns found within four species (Figure 1). Isolates were classified in the following groups: Group 1, *L. lactis* subsp. *lactis* corresponding to isolates F-22, F-11, F-130 and F-131; Groups 2 and 3, *S. salivarius* subsp. *thermophilus* corresponding to isolates F-53 and F-57, and F-B1 and F-B3, respectively, Group 4, *P. pentosaceus* corresponding to isolate F-45 and Group 5, *P. acidilactici* corresponding to isolates F-60 and F-63. The different profiles found for *S. salivarius* subsp *thermophilus* could be associated with intra-subspecies variation as previously reported by Schlegel *et al.* (2003), however other differences between these isolates were not found in this study. Our results are in accordance with Petrov *et al.* (2008) who found that HhaI alone could be used to discriminate *L. lactis* subsp. *lactis*, *L. lactis* subsp *cremoris*, *E. faecium*, *E. faecalis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *L. lactis* and *S. thermophilus*. However, Yousif *et al.* (2010) required HhaI and HinfI double digestions to discriminate *P. acidilactici*, *P. pentosaceus* and other *Pediococcus* sp.

Species designations of LAB isolated from milk and dairy products by phenotypic and genotypic characterization was confirmed by 16S rRNA gene sequencing (Table 1). All the isolates, except F-131, identified by partial 16S rRNA gene sequence analysis had an alignment identity of 99-100%. Since, isolate F-131 had an alignment identity of 96%, which is below species demarcating criteria, additional criteria for identification were established by phenotypic and ARDRA analysis.

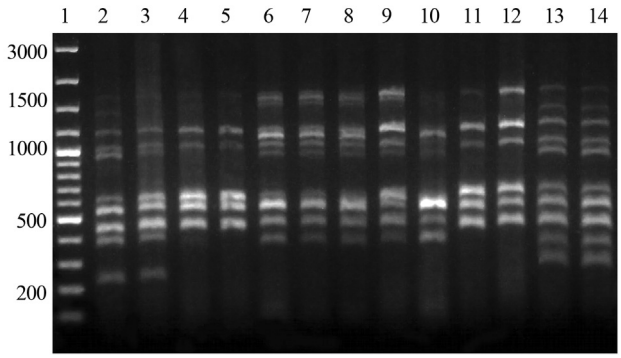

Figure 1 - ARDRA profiles of indigenous isolates and type strains using HhaI as restriction enzyme. Lane 1, molecular weight marker 100 bp to 3000 bp (Fermentas, USA), Lane 2. *S. salivarius* subsp. *thermophilus* ATCC 19258, Lane 3 – 5. F- 57, F-B1, F-B3; Lane 6. *L. lactis subsp. lactis biovar. diacetylactis* ATCC 13675; Lane 7 – 10, F-22, F-11, F-130, F-131; Lane 11. *P. pentosaceus* ATCC 25745*;* Lane 12 - 14 strain F-45, F-60, F-63.

Wild strain selection and accurate identification of LAB is of great artisanal and industrial interest and promises to provide resources for development of innovative processes and products. New strain availability is needed for development of customer-designed starters with the objective of providing "uniqueness" to value added products. Additionally, wild strains provide phenotypic and genetic variation resources for improvement of current commercial starter strains. The polyphasic approach for strain identification provides accuracy that will greatly facilitate developing and improving new starter systems. LAB identification using a polyphasic strategy clearly helps to overcome problems with inherent variation in wild isolates from dairy

Table 1 - Identification of isolates from different dairy sources by partial 16S rRNA gene sequence analysis.

Strain	Isolate number	Length (bp)	Alignment identity (%)	GenBank Accession number
S. thermophilus	F- 53*	903	99	JF749186
	F-57	866	99	JF749187
	F-B1	896	99	JF749188
	F-B3	906	99	JF749189
L. lactis spp. *lactis*	F-11	908	100	JF749190
	F-22	873	100	JF749191
	F-130	875	100	JF749192
	F-131	649	96	JF749193
	F-B2	899	99	JF749194
P. pentosaceus	F-45	871	100	JF749195
P. acidilactici	F-60	914	99	JF749196
	F-63	875	99	JF749197

*F abbreviate the term "FAGRO" of all the isolates.

sources using either phenotypic tests including biochemical analyses (Moschetti *et al.*, 1998) or genetic heterogeneity (Giraffa *et al.*, 2001).

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References

Aquilanti L, Silvestri G, Zannini A, Osimani A, Santarelli S, Clementi F (2007) Phenotypic, genotypic and technological characterization of predominant lactic acid bacteria in Pecorino cheese from central Italy. J Appl Microbiol 103:948-960.

Axelsson L (2004) Lactic Acid Bacteria: Classification and Physiology. *In:* Salminen, S., von Wright, A., Ouwehand, A. C. (eds). Lactic Acid Bacteria. Microbiology and Functional Aspects, 3rd Edition. Marcel Dekker Inc. New York, p.1-66.

Bhunia AK, Johnson MG (1992) A modified method to directly detect in SDS-PAGE the bacteriocin of *Pediococcus acidilactici*. Lett Appl Microbiol 15:5-7.

Coppola S, Blaiotta G, Ercolini D, Moschetti G (2001) Molecular evaluation of microbial diversity occurring in different types of mozzarella cheese. J Appl Microbiol 90:414-420.

Devriese LA, Pot B (1995). The genus *Enterococcus*. *In:* Wood, B.J.B., Holzapfel, W. H. (eds). The Genera of Lactic Acid Bacteria. Blackie Academic & Professional, London, UK. p.327-367.

Fortina MG, Nicastro G, Carminati D, Neviani E, Manachini PL (1998) *Lactobacillus helveticus* heterogeneity in natural cheese starters: The diversity in phenotypic characteristics. J Appl Microbiol 84:72-80.

Giraffa G, Paris A, Valcavi L, Gatti M, Neviani E (2001) Genotypic and phenotypic heterogeneity of *Streptococcus thermophilus* strains isolated from dairy products. J Appl Microbiol 91:937-943.

Gusils C, Figueroa R, Pérez Chaia A, González S, Oliver G (2001) Identification of lactic acid bacteria and enterobacteria. A technique in microplates. INCI 26:563-566.

Marroki A, Zuñiga M, Kihal M, Pérez-Matínez G (2011) Characterization of *Lactobacillus* from Algerian goats milk based on phenotypic, 16S rDNA sequencing and their technological properties. Braz J Microbiol 42:158-171.

Moschetti G, Blaiotta G, Aponte M, Catzeddu P, Villani F, Deiana P, Coppola S (1998) Random amplified polymorphic DNA and amplified ribosomal DNA spacer polymorphism: powerful methods to differentiate *Streptococcus thermophilus* strains. J Appl Microbiol 85:25-36.

Nomura M, Kobayashi M, Narita T, Kimoto-Nira H, Okamoto T (2006) Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. J Appl Microbiol 101:396-405.

Petrov K, Urshev Z, Petrova P (2008) L(+)-Lactic acid production from starch by a novel amylolitic *Lactococcus lactis* subsp. *lactis* B84. Food Microbiol 25:550-557.

Schlegel L, Grimont F, Grimont PAD, Bouvet A (2003) Identification of major streptococcal species by *rrn*-amplified ribosomal DNA restriction analysis. J Clin Microbiol 41:657-666.

Sharpe ME (1979) Identificación of the lactic acid bacteria. Identification Methods for Microbiologists. Academic Press, London, UK.

Temmerman R, Huys G, Swings J (2004) Identification of lactic acid bacteria: culture dependent and culture-independent methods. Trends Food Sci Technol 15:348–349

Van Hoorde K, Vandamme P, Huys G (2008) Molecular identification and typing of lactic acid bacteria associated with the production of two artisanal raw milk cheeses. Dairy Sci Technol 88:445-455.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697-703.

Yousif NMK, Huch M, Schuster T, Cho G-S, Dirar HA, Holzapfel WH, Franz MAP (2010) Diversity of lactic acid bacteria from *Hussuwa*, a traditional African fermented sorghum food. Food Microbiol 27:757-768.