Food Microbiology 28 (2011) 76–83

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

FTIR-based polyphasic identification of lactic acid bacteria isolated from traditional Greek Graviera cheese

John Samelis [a,*], Anne Bleicher [b,1], Céline Delbès-Paus [c], Athanasia Kakouri [a], Klaus Neuhaus [b], Marie-Christine Montel [c]

[a] National Agricultural Research Foundation, Dairy Research Institute, Katsikas, 45221 Ioannina, Greece
[b] Technische Universität München, ZIEL, Abt. Microbiologie, Weihenstephaner Berg 3, 85356 Freising, Germany
[c] Unité de Recherches Fromagères, URF-545 INRA, 20 Côte de Reyne, F-15000 Aurillac, France

ARTICLE INFO

Article history:
Received 3 May 2010
Received in revised form
16 August 2010
Accepted 17 August 2010
Available online 21 August 2010

Keywords:
Graviera cheese
Lactic acid bacteria
Polyphasic taxonomy
FTIR
RFLP
SSCP

ABSTRACT

This study used a combination of phenotypic, physical (Fourier Transformed Infra-Red [FTIR] spectroscopy) and molecular (RFLP and SSCP analysis of 16S rRNA genes) methods to identify the lactic acid bacteria (LAB) flora present in traditional Greek Graviera cheese after five weeks of ripening. A total of 300 isolates collected from high dilution plates of TSAYE (incubated at 30 °C), M-17 (22 °C) and M-17 (42 °C) agar media were clustered by FTIR and then representative strains of each cluster were cross-identified blindly by all methods. Based on their FTIR spectra, 282 isolates were LAB grouped in 28 clusters. The LAB species identified and their prevalence in the cheese samples were: *Lactobacillus casei/paracasei* (68.8%), *Lactobacillus plantarum* (19.5%), *Streptococcus thermophilus* (8.9%), *Enterococcus faecium* (2.1%), and *Lactococcus lactis* (0.7%). Also, *Staphylococcus equorum* (11 isolates), *Corynebacterium* sp. (5 isolates) and *Brevibacterium* sp. (1 isolate) were recovered from TSAYE. Comparative identification results showed that phenotypic and molecular methods were in mutual agreement as regards the LAB species identified. The present polyphasic identification approach based on rapid FTIR screening of 10-fold more isolates than a previous classical identification approach allowed or improved detection of few sub-dominant species; however the predominant LAB species in the cheese samples were the same with both approaches.

1. Introduction

Polyphasic taxonomy has been recognized as a consensus approach to bacterial systematics, and is particularly useful for identification and classification of lactic acid bacteria (LAB) involved in food-associated ecosystems (Vandamme et al., 1996). Various culture-dependent, phenotypic, including chemotaxonomic, and genotypic methods have been applied in different combinations for studying LAB ecology and diversity in raw milk and traditional cheeses made mainly from raw milk (Fitzsimons et al., 1999; Bizzarro et al., 2000; De Angelis et al., 2001; Weinrichter et al., 2001; Bouton et al., 2002; Callon et al., 2004; Georgieva et al., 2008). In recent years, DNA-based, culture-independent methods, such as Single Strand Conformation Polymorphism (SSCP), are used as powerful tools for evaluating microbial (LAB) communities in foods (Giraffa and Neviani, 2001), including raw milk (Callon et al., 2007) and traditional cheeses (Randazzo et al., 2002; Duthoit et al., 2003; Dolci et al., 2008; Martin-Platero et al., 2009).

Despite their existing limitations, culture-independent methods have attracted significant attention because culture-dependent ones, either phenotypic or genotypic, are laborious and time-consuming requiring bacterial isolations, purification and maintenance of purified stock isolates prior to identification. The greater the number of isolates, the more comprehensive and reliable a microbiological ecology study is, and most culture-dependent methods are hampered because of this requirement. Besides, by the classical phenotypic identification methods, isolates belonging to bacterial (LAB) genera or species of high phenotypic heterogeneity may be misidentified or unidentified requiring chemotaxonomic or molecular approaches for confirming and/or resolving their taxonomy (Vandamme et al., 1996; Moschetti et al., 2001). Thereby, any analytical tool that could simultaneously provide rapid and

* Corresponding author. Tel.: +30 2 651 094789; fax: +30 2 651 092523.
E-mail address: jsam@otenet.gr (J. Samelis).
[1] Present address: Leibniz Institute for Natural Product Research and Infection Biology e.V., Hans-Knöll-Institut, Beutenbergstr. 11a, 07745 Jena, Germany.

0740-0020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2010.08.009

robust screening plus identification of large amounts of isolates would be useful; one such tool is Fourier Transform Infra-Red (FTIR) spectroscopy. Since bacterial FTIR spectra give a global picture of whole cellular components (fatty acids, proteins and polysaccharides plus nucleic acids), they have been proposed as a link between phenotypic and genotypic methods (Amiel et al., 2001). The capacity of FTIR spectroscopy for identification and classification of dairy LAB, even for the discrimination of closely related species, such as *Streptococcus thermophilus* from *Streptococcus salivarius*, or *Lactobacillus casei/paracasei* from *Lactobacillus zeae* and *Lactobacillus rhamnosus*, has been proven (Amiel et al., 2000, 2001). The evolution of *Lactococcus* strains during ripening of Brie cheese has also been followed using FTIR (Lefier et al., 2000). Based on FTIR spectra, *Lactobacillus* isolates from homemade cheeses, Swiss cheeses with different *Lactobacillus* adjunct strains added, and pathogenic from non-pathogenic species of *Listeria* and *Staphylococcus* have been discriminated in more recent studies (Lamprell et al., 2006; Savic et al., 2008; Rebuffo-Scheer et al., 2008; Chen et al., 2009).

Graviera is the most popular traditional Greek hard cooked cheese (Litopoulou-Tzanetaki and Tzanetakis, 2007). Several varieties are produced in different regions of Greece from ewes', ewes' mixed with goats', or cows' milk, and three of them produced in the islands of Crete and Naxos and the mountain area of Agrafa have PDO status (Anonymous, 2004). Recently, we evaluated the microbiological quality and safety of Graviera cheeses traditionally manufactured at semi-industrial plant scale from thermized milk with addition of a product-specific commercial starter culture (CSC). All cheeses were stable and hygienically safe in compliance with the current European Union microbiological regulatory criteria (European Commission, 2007). Several challenge studies showed that neither *Listeria monocytogenes* nor enterotoxigenic *Staphylococcus aureus* strains could grow in the cheese core during ripening and/or on the cheese surface post-ripening (Giannou et al., 2009a; Samelis et al., 2009b, 2009c). A more recent study (Samelis et al., 2010) indicated that the microbial flora of Graviera cheeses after five weeks of ripening was dominated by non-starter (NSLAB) mesophilic lactobacilli, *Lb. casei/paracasei* (67.5%) and *Lactobacillus plantarum* (16.3%). Conversely, *S. thermophilus*, *Lactococcus lactis* and *Leuconostoc* sp. included in the CSC were isolated from the ripened cheeses at frequencies as low as 3.8%, 0.6% and 1.9%, respectively. *Enterococcus faecium* (9.4%) and *Enterococcus durans* (0.6%) were isolated among the main LAB flora from two cheese batches; in general, enterococci were present in all batches at 10- to 100-fold lower populations than mesophilic lactobacilli (Samelis et al., 2010). However, the above results on microbial composition of ripened Graviera cheeses were based on the isolation of a limited number of isolates (10 from each agar medium per batch) followed by their identification by phenotypic methods and criteria only.

The present study was therefore undertaken to validate previous phenotypic identification data on the LAB flora composition of traditional Graviera cheese and recover additional species potentially underlying the dominant species identified by Samelis et al. (2010). For this purpose, a polyphasic approach was applied to Graviera cheeses selected on the basis of their low LAB species phenotypic diversity. Compared to our previous classical approach (Samelis et al., 2010), this study used ten-fold more cheese isolates, which were first clustered by FTIR. Next, isolates representing FTIR clusters were identified by restriction fragment length polymorphism (RFLP), SSCP, and biochemical methods. Further, the FTIR spectra of the cheese isolates were compared to a spectrum library to obtain FTIR identification. All methods were applied blindly within the laboratories participating in this study in order to evaluate the relevance and mutual agreement between methods, and the capacity of each method to accurately identify different LAB species present in Graviera cheese.

2. Materials and methods

2.1. Graviera cheeses

Two Graviera cheeses (T and R) derived from one commercial production run (batch A) after five weeks of ripening were selected for the polyphasic identification study among four batches previously analyzed by phenotypic methods (Samelis et al., 2010). Both cheeses were produced in a local semi-industrial plant (Pappas Bros., Filippiada, Epirus) from thermized (63 °C for 30 s) ewes'/goats' (90:10) milk. A product-specific CSC containing *S. thermophilus*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* strains of natural origin (GR02, Mofin Alce Group, Novara, Italy) was added to the cooled milk before rennet addition and curdling, as described by Samelis et al. (2009b, 2010). After standard cheese cooking, molding, pressing, brining and draining operations, Graviera-T (GR-T) cheese was ripened in the plant's ripening room at temperatures of 17–19 °C and relative humidity (RH) of 90–92% with manual monitoring of the air ventilation, whereas Graviera-R (GR-R) cheese was ripened in a controlled pilot ripening room under constantly monitored conditions of temperature (17.5 ± 0.1 °C), RH (96.0 ± 2.3%) and air ventilation (continuous at 1.5 m/s) (Samelis et al., 2010).

2.2. Colony isolation procedure for FTIR analysis

A total of 50 colonies from one high dilution plate of each of TSAYE (incubated at 30 °C), M-17 (incubated at 22 °C) and M-17 (incubated at 42 °C) agar media (Samelis et al., 2010) were isolated for FTIR analysis. All isolation media were purchased from LAB M (Bury, UK). The colonies were collected randomly from the agar surface with the aid of pre-sterilized tooth sticks. No isolations from MRS agar (LAB M) plates incubated at 30 or 45 °C were made for this study because those colonies had grown inside the agar layer after pour plating of the cheese samples, and thus, it was difficult to pick 50 of them from single plates.

Since bacterial FTIR spectra are known being influenced by culture medium and incubation conditions, two different standardized experimental protocols were applied to the cheese isolates to confirm comparability with reference spectra in the FTIR library. Specifically, isolates from TSAYE plates, considered to represent the total mesophilic cheese flora, were streaked on casein soy (CASO) agar (Merck, Darmstadt, Germany) incubated at 30 °C aerobically, whereas all isolates from M-17 agar plates, considered to represent the mesophilic (22 °C) and thermophilic (42 °C) cheese LAB flora, were streaked on APT agar (Merck) incubated at 34 °C anaerobically.

In the above manner, 300 colonies in total (150 from GR-T and 150 from GR-R) were collected. During previous routine isolation procedures 10-fold less colonies (5 colonies from each medium × 3 media × 2 cheeses = 30 colonies in total) were isolated from the duplicate agar plates of the same cheese samples and subjected to biochemical identification (Samelis et al., 2010). Specifically, the number of colonies grown on high (−6) dilution TSAYE, M-17/22 °C and M-17/42 °C agar plates used for the FTIR isolation procedure were 108, 71 and 66, and 174, 85 and 50 for GR-T and GR-R samples, respectively. Thus, by picking 50 colonies from each plate, the FTIR method was actually based on 28.7 and 46.3%, 58.8 and 70.4%, and 75.8 and 100.0% of the microbial (LAB) populations on TSAYE, M-17/22 °C and M-17/42 °C plates, respectively. Conversely, by random picking of five colonies only from each agar plate, the corresponding percentages by the classical method had been as low as 3.4 and 3.7% (TSAYE), 4.4 and 5.0% (M-17/22 °C) and 6.1 and 10.0% (M-17/42 °C) for GR-T and GR-R cheeses, respectively.