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Short communication

Comparison of phenotypic (Biolog System) and genotypic (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) methods for typing *Lactobacillus plantarum* isolates from raw vegetables and fruits

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ABSTRACT

The diversity of 72 isolates of *Lactobacillus plantarum*, previously identified from different raw vegetables and fruits, was studied based on phenotypic (Biolog System) and genotypic (randomly amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) approaches. A marked phenotypic and genotypic variability was found. Eight clusters were formed at the similarity level of 92% based on Biolog System analysis. The most numerous clusters grouped isolates apart from the original habitat. Almost all isolates fermented maltose, D,L-lactic acid, N-acetyl-D-mannosamine and dextrin, and other typical carbon sources which are prevalent in raw vegetables and fruits. None of the isolates fermented lactose and free amino acids. At high values of linkage distance, two main clusters were obtained from both UPGMA (unweighted pair group with arithmetic average) dendrograms of RAPD-PCR and AFLP analyses. The two clusters mainly separated isolates from tomatoes and carrots from those isolated from pineapples. At 2.5 linkage distance, a high polymorphism was found and several sub-clusters were formed with both analyses. In particular, AFLP allowed the differentiation of 55 of the 72 isolates of *L. plantarum*. The discriminatory power of each technique used was calculated through the Simpson's index of diversity (*D*). The values of the *D* index were 0.65, 0.92 and 0.99 for Biolog System, RAPD-PCR and AFLP analyses, respectively.

1. Introduction

Lactobacillus is a diverse genus (Stiles and Holzapfel, 1997) including more than 150 species (http://www.dsmz.de/microorganisms). Some of the species are highly specialized and adapted to specific ecological niches such as dairy substrates (Bolotin et al., 2004; van de Guchte et al., 2006), whereas others are typically found in the mammalian gastro-intestinal tract (Russell and Klaenhammer, 2001). *Lactobacillus plantarum* is a highly heterogeneous and versatile species (Bringel et al., 1996; Dellaglio et al., 1975) which is widely used in many food and health applications, including starter cultures for fermentation processes (vegetables, meat and fish, sourdough, and dairy products) (Antara et al., 2004; Di Cagno et al., 2008; Filya et al., 2004; Gardner et al., 2001; Noonpakdee et al., 2004; Kostinek et al., 2005). The broad commercial applications of this species may reflect the remarkable ecological adaptability to different habitats. The genome size (3.3 Mb) of *L. plantarum* strain WCFS1, a human saliva isolate, is the largest genome sequenced to date from lactic acid bacteria (Kleerebezem et al., 2003). The large genome size is probably related to the ability of this bacterium to inhabit diverse environmental niches, allowing the fermentation of a large number of carbohydrates (Bringel et al., 2001). Comparative genome analysis of 20 *L. plantarum* strains isolated from various sources revealed regions with unusual base composition, which indicates evolutionarily recent acquisitions (Molenaar et al., 2005). Although many ecological studies on molecular typing of *L. plantarum* were made (Molenaar et al., 2005; De las Rivas et al., 2006; Siezen et al., 2010), the interest in this topic is still very high. In particular, for understanding the evolutionary adaptation of this species to different ecological niches. Besides, intraspecific differentiation is the important preliminary step to select starter cultures. Indeed, technological, probiotic, sensory and antimicrobial attributes are mainly strain-specific.

Several studies have explored the diversity within *L. plantarum* using different approaches such as PCR-based methods (randomly amplified polymorphic DNA, RAPD-PCR) (Bringel et al., 1996, 2001; Elegado et al., 2004), pulsed-field gel electrophoresis (Sànchez et al., 2004) and multilocus sequence typing (MLST) (De las Rivas et al.,

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2006; Tanganurat et al., 2009). A different discriminatory power was found between these techniques. Although RAPD-PCR was proposed as the rapid genotyping molecular tool to study the biodiversity of a large number of lactic acid bacteria (Di Cagno et al., 2008; Martín-Platero et al., 2009), in some cases it might show low discriminatory power. On the contrary, the Amplified Fragment Length Polymorphism (AFLP) used as genomic fingerprinting was recently considered a useful tool to discriminate either at species or at strain levels (Lazzi et al., 2009). To the best of our knowledge, no studies considered the use of the AFLP to discriminate the genotypic polymorphism of *L. plantarum* strains. Notwithstanding the usefulness of genetic tools, the phenotype characterization is always the ultimate goal to exploit the potential of strains that colonize different environments. The Biolog System was also successfully used to describe the phenotypic features of food related bacteria (De Angelis et al., 2006; Di Cagno et al., 2007; Di Cagno et al., 2009).

This paper describes the phenotypic characterization based on the Biolog System analysis and the genetic diversity of *L. plantarum* isolates from raw vegetables and fruits by comparing RAPD-PCR and AFLP analyses.

2. Materials and methods

2.1. Bacterial isolates

A number of 72 *L. plantarum* isolates belonging to the Culture Collection of the Department of Biologia e Chimica Agro-Forestale ed Ambientale of the University of Bari, Italy, were used in this study (Table 1). All strains were previously identified by partial 16 S rRNA sequencing and using the partial sequencing of the recA gene (Di Cagno et al., 2008, 2009, 2010). All strains were maintained as stock cultures in 15% glycerol (w/v) at −20 °C, and routinely propagated at 30 °C for 24 h in MRS broth (Oxoid, Basingstoke, Hampshire, United Kingdom).

2.2. Phenotype characterization by Biolog System

Three days before the inoculation of Biolog AN plates, containing 95 different carbon sources, (Biolog, Inc., Hayward, CA, USA), the isolates were streaked twice on MRS agar plates and incubated for 24 h at 30 °C. The wells of the Biolog AN plates were inoculated with 150 µl of the bacterial suspensions, adjusted to 65% transmittance as recommended by the manufacturer. Positive reactions were automatically recorded using a microplate reader with a 590-nm wavelength filter.

2.3. DNA extraction

Genomic DNA from the 72 *L. plantarum* isolates was extracted as described by De Los Reyes-Gavilán et al. (1992) from 2 ml samples of overnight cultures grown at 30 °C in MRS (de Man, Rogosa and Sharpe, 1960) medium. The final concentration of lysozyme used for cell lysis was 2 mg/ml (all products were from Sigma-Aldrich, Milan, Italy). The concentration and purity of DNA was assessed by spectrophotometric measurements using NanoDrop® ND-1000 Spectrophotometer (ThermoFisher Scientific Inc., MI., Italy).

2.4. Genotypic characterization by randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis

PCR amplification of each isolate was carried out with PTC-100 Peltier Thermal Cycler (MJ Research Inc. Waltham, Massachusetts). Three primers (Invitrogen), with arbitrarily chosen sequences (M13, 5′-GAGGGTGGCGGTTCT-3′, P7 5′ AGCAGCGTGG 3′ and P4 5′ CCGCAGCGTT 3′), were used singly in three series of amplification (Di Cagno et al., 2008). The molecular weight of the amplified DNA fragments was estimated by comparison with 1 Kb Plus DNA Ladder (Invitrogen). Only reproducible well-marked amplified fragments were scored, with faint bands being ignored. The three series of RAPD-PCR profiles were evaluated and combined to obtain a unique dendrogram.

2.5. Genotypic characterization by amplified fragment length polymorphism (AFLP) analysis

The AFLP analysis was carried out using the AFLP Microbial fingerprinting kit (Applied Biosystem-Pe Corporation, Foster City, Calif.) as described by Vos et al. (1995) and subsequently modified by Lazzi et al. (2009). Aliquots of ca. 200 ng of genomic DNA were digested with EcoRI and MseI (AFLP Microbial fingerprinting kit) and the DNA fragments were bonded to restriction site-specific adaptors provided from the fingerprint kit. The pre-selective PCR was carried out in 20 µl (final volume) mixture. For the selective reaction of PCR, 1.5 µl of the 1:1 dilution of the pre-amplification product was amplified in 10 µl (final volume) mixture. 5′-Carboxy fluorescein (FAM) labelled EcoRI-A and unlabelled MseI-A were used as the selective primers. All amplification were carried out in the Mastercycler Ep Gradient S (Eppendorf, Hamburg, Deutschland) as reported by Lazzi et al. (2009). Samples were loaded and run on the ABI Prism 310 (Applied Biosystem-Pe Corporation Foster City, Calif.), and analyzed using the Genemapper Analysis Software.

2.6. Statistical analysis

The peak matrices corresponding to the Biolog System, RAPD-PCR and AFLP analyses were subjected to clustering. The three series of RAPD-PCR profiles were evaluated and combined to obtain a unique dendogram. Binary 0/1 matrices were created based on the absence or presence of DNA bands or positive reaction. For AFLP, the peak height thresholds were set at 50 bp. Peaks representing AFLP fragments from 50 to 500 bp were considered. Hierarchical cluster analysis was carried out with Statistica 6 software (Statsoft Italia, Padova, Italia). The following setting was used: Euclidean distance and unweighted pair group method with arithmetic mean (UPGMA) for RAPD-PCR and AFLP analyses; an index of similarity by the simple matching coefficient (Sokal and Michener, 1958) for Biolog System analysis.

Table 1
Isolates of *Lactobacillus plantarum* (n = 72) used in this study.

Strains	Source	Number	References
Z1, Z2, Z3, Z4, Z5, Z6, Z7, Z8, Z9, Z10, Z11, Z12, Z15	Marrow	13	Di Cagno et al., 2008
C1, C2, C3, C4, C5, C6, C8, C9, C10, C12, C13	Carrot	11	Di Cagno et al., 2008
POM1, POM8, POM20, POM21, POM24, POM26, POM27, POM28, POM29, POM31, POM32, POM33, POM34, POM35, POM36, POM37, POM38, POM39, POM40, POM41, POM42, POM43	Tomato	22	Di Cagno et al., 2009
1LE1, 1LS5, 1LE20, 1LS16, 1LE8, 1LS19, 1LS20, 2LE18, 2LE19, 2LC3, 2LS15, 1LE12, 1LE16, 1LS18, 1LS17, 2LE17, 2LE20, 2LS4, 2LS13, 2LE6, 2LE14, 2LC11, 2LC21, 3XLC2, 3XLC1, 3XLC4	Pineapple	26	Di Cagno et al., 2010