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Short communication

Evaluation of genetic polymorphism among *Lactobacillus rhamnosus* non-starter Parmigiano Reggiano cheese strains

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ABSTRACT

Parmigiano Reggiano (PR) is an Italian cooked, long-ripened cheese made with unheated cow's milk and natural whey starter. The microflora is involved in the manufacturing of this cheese, arising from the natural whey starter, the raw milk and the environment. Molecular studies have shown that mesophilic non-starter lactic acid bacteria (NSLAB) are the dominant microflora present during the ripening of PR. In this study, a characterisation of *Lactobacillus rhamnosus* isolated from a single PR manufacturing and ripening process is reported, using a combination of genotypic fingerprinting techniques (RAPD-PCR and REP-PCR). The intraspecies heterogeneity evidenced for 66 strains is correlated to their abilities to adapt to specific environmental and technological conditions. The detection of biotypes that correlate with specific moments in cheese ripening or differential development throughout this process suggests that these strains may have specific roles closely linked to their peculiar technological properties.

1. Introduction

Parmigiano Reggiano (PR) is a hard-textured, cooked and long-ripened cheese whose denomination "Protected Designation of Origin" is linked to a strictly artisanal manufacturing process in specific areas of Northern Italy (http://www.parmigiano-reggiano.it), which involves the use of unheated, partially skimmed raw cow's milk supplemented with a natural whey starter (Mucchetti and Neviani, 2006). Non-starter lactic acid bacteria (NSLAB), autochthonic of raw milk and arising from the environment, as well the starter lactic acid bacteria (SLAB), added with the natural whey starter, are the protagonists of the different biochemical processes during the manufacturing and ripening stages involved in the development of PR cheese (Neviani et al., 2009). Although the role of NSLAB in ripening has not yet been clarified, different authors have suggested the importance in this process mainly in Cheddar cheese (Beresford et al., 1999; Fox et al., 1998; Peterson and Marshall, 1990) and also in PR cheese (Succi et al., 2005).

In recent years, some studies have demonstrated that *Lactobacillus rhamnosus,* with some other NSLAB microflora, was the dominant species present after a lack of essential nutrients, such as sugars, in PR ripening (De Dea Lindner et al., 2008; Gatti et al., 2008; Lazzi et al., 2007). This microflora originates from raw milk and the environment as a result of contamination during the manufacturing procedure, where it is present at low cell densities. Different from SLAB, after cheese brining, *L. rhamnosus* are able to grow and increase in number, while SLAB cells undergo autolysis (Gatti et al., 2008). When this occurs, most of the residual lactose in the cheese has already been utilised by the SLAB; therefore, this species seems to adjust well to the absence of lactose in cheese, confirming an optimal adaptability to unfavourable growth conditions (Succi et al., 2005). Presumably, this adaptability was due to the ability of this strain to use nitrogen fraction (amino acids and peptides) as an alternative energy source. This finding is confirmed by the optimal growth rate of microflora NSLAB, in particular *L. rhamnosus*, in a ripened cheese medium used instead of traditional cultural media (Lazzi et al., 2007; Neviani et al., 2009). This medium, in fact, allowed for the recovery of the NSLAB population in raw milk and fresh curd when they are present in little amounts (Lazzi et al., 2007; Neviani et al., 2009).

Although different studies have highlighted the importance of NSLAB in the ripening process of PR (Coppola et al., 2000; De Dea Lindner et al., 2008; Gala et al., 2008; Gatti et al., 2008; Neviani et al., 2009), the exact role of these bacteria has not been investigated in greater depth. In particular, the biotypes of the dominant species during the maturation stage of cheese processing have never been studied. Indeed, it can only be hypothesised that the technological pressure determines, at different stages, the potential development of the biotype that may have a specific biochemical role leading to certain flavours and sensorial traits of the PR. Molecular typing may be helpful to understand the adaptation of bacterial strains subjected to selective pressure while growing in milk. Therefore, this study aimed

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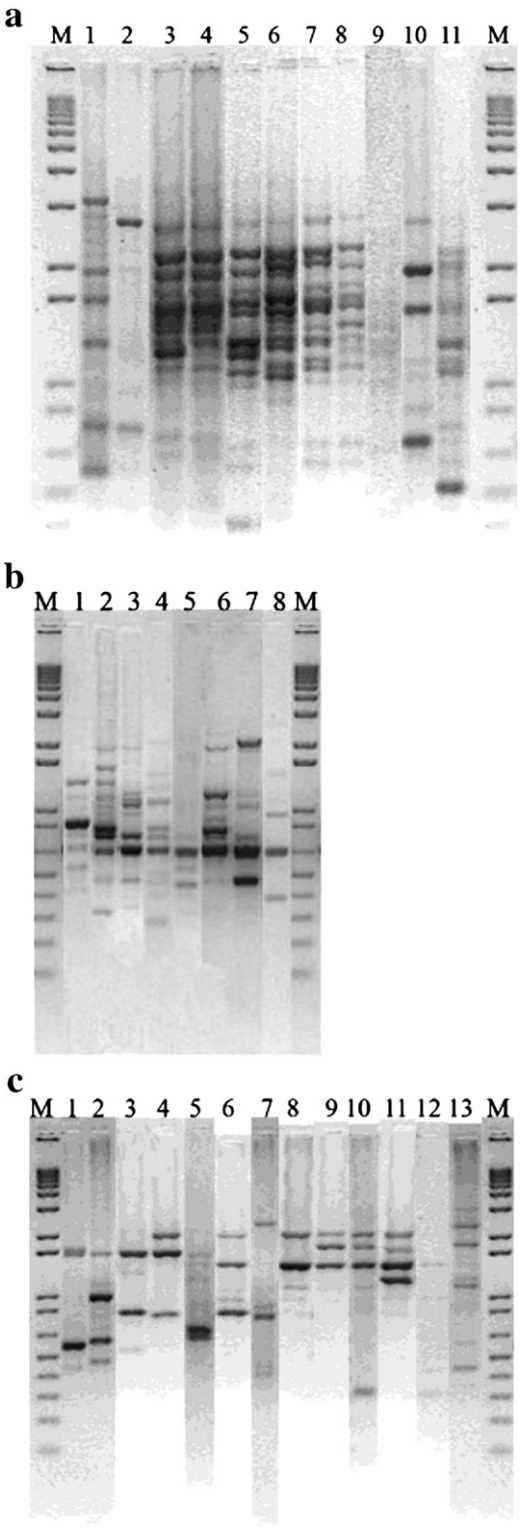

Fig. 1. *L. rhamnosus* REP-PCR patterns obtained with primer GTG_5 (a), and *L. rhamnosus* RAPD-PCR patterns obtained with primers M13 (b) and P1 (c). Each pattern shows the representative fingerprints of the clusters with each primer and was obtained considering 80% similarity level for each single genetic characterisation (data not shown). (a), (b) and (c) M, DNA molecular size standards (1 kb plus DNA ladder; Invitrogen, Carlsbad, CA, USA).

to characterise the diversity of *L. rhamnosus* strains isolated at different steps during the same manufacturing cycle of PR.

Intraspecific genotypic characterisation of *L. rhamnosus* has been already studied by Vancanneyt et al. (2006) using high-resolution DNA fingerprinting techniques (Fluorescent Amplified Fragment Length Polymorphism [FAFLP] and Pulse Field Gel Electrophoresis of macrorestriction fragments). In this work, we determined genotypic relatedness at the intraspecies level for 66 *L. rhamnosus* strains by means of two DNA fingerprinting techniques, i.e., randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and repetitive extragenic palindromic-polymerase chain reaction REP-PCR analysis. The interesting aspect of this study is that the *L. rhamnosus* strains were isolated from samples collected during the same cheese-making process. To our knowledge, no previous studies have investigated the biodiversity of microflora NSLAB present in the ripening process. Therefore, through this study, we aimed to understand if different biotypes are present at different stages of the ripening process.

2. Materials and methods

2.1. Bacterial strains

Sixty-six strains of *L. rhamnosus* isolated from different stages of PR manufacturing and ripening were used in this study. The strains were isolated from twelve samples (curd after 6 and 48 h, cheese at 1, 2, 3, 4, 6, 8, 10, 12, 16, and 20 months of ripening) collected during the same cheese-making period from a cheese factory able to guarantee the production of twin wheels (Gatti et al., 2008). All the strains were isolated, identified and maintained as described in previous work (Neviani et al., 2009).

2.2. Characterisation by RAPD-PCR

RAPD-PCR analysis was performed with the aim of differentiating the strains using the primers M13 and P1 according to Rossetti and Giraffa (2005) and De Angelis et al. (2001), respectively. Primers were used singly in two series of amplification.

Amplification products were separated on a 1, 2% agarose gel in 1X TAE buffer. The repeatability of RAPD-PCR fingerprints was determined by triplicate loading of independent triplicate reaction mixtures prepared with the same strains.

2.3. Strain typing by REP-PCR

The single oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) was applied for REP-PCR. Amplifications were performed as reported by Coudeyras et al. (2008). The repeatability of REP-PCR fingerprints was determined as above for RAPD-PCR.

2.4. Cluster analysis

Conversion, normalisation, and further analysis of the RAPD-PCR and REP-PCR patterns were carried out with Bionumerics software (Version 3.0; Applied Maths, BVBA, Sint-Martens-Latem, Belgium). The two series of RAPD-PCR profiles and the profiles of REP-PCR were combined to obtain a unique dendrogram. Cluster analysis was carried out utilising the unweighted pair group method with arithmetic average (UPGMA).

Fig. 2. UPGMA dendrogram of *L. rhamnosus* strains based on the combined analysis of RAPD-PCR patterns of the two primer P1 and M13 and REP-PCR patterns using the primer GTG_5. The strains and the sources of isolation are indicated on the right side of the dendrogram. The numbers reported in the figure represent the clusters formed at the cut-off point (vertical dotted line).