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Review

Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: A review

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ABSTRACT

Lactobacillus is among the most important GRAS food lactic acid bacteria, with nearly 140 species at present, mostly of industrial importance. Being part of the natural flora of a range of food products like raw milk, fermented dairy products, fruits, vegetables, meat products they also serve as starters for a number of fermented food products either to enhance the quality or to add health benefits. These groups of economically important species are often alike in phenotypic and physiological characteristics, probably due to their co-evolution in the same ecological niches; hence they are difficult to be differentiated. This demands advanced methods for their proper identification and characterization. With the advancement of molecular biology, a range of DNA-based molecular techniques has replaced the largely cumbersome phenotypic methods. This review summarizes the various molecular techniques available for detection and identification within the genus *Lactobacillus*, with special emphasis on the four groups of closely resembling species: *L. casei* group, *L. acidophilus* group, *L. delbrueckii* subspecies, and *L. plantarum* group. This review also provides insights into current trends for alternative molecular markers other than 16S rRNA to resolve the ambiguity within phylogenetically close species in the genus *Lactobacillus*.

1. Introduction

The genus *Lactobacillus* contains a diverse assemblage of 140 species (Euzéby, 1997) and includes gram-positive, catalase negative, non-motile, non-sporulating, facultative anaerobes, growing under microaerophilic to strictly anaerobic conditions (Klein, Pack, Bonaparte, & Reuter, 1998). Lactobacilli are usually thin slender rods, although they can also attain spiral or coccobacillary forms under certain conditions. They are genetically diverse. With their G + C content ranging from 32% for *L. mali* to 54 mol% for *L. pontis* and *L. fermentum*, they surpass the required threshold limit for a genus, hence giving the impression that *Lactobacillus* is not a well defined genus (Vandamme et al., 1996). Particular interest inflicted in lactobacilli is largely due to (i) the association of these organisms with health promoting properties; (ii) their inclusion in numerous food products from nutritional or quality improvement aspects and (iii) the requirement of legislative and industrial bodies, as well as consumer, with respect to safety, labeling, patenting and strain integrity (Charteris, Kelly, Morelli, & Collins, 1997; Holzapfel et al., 2001; Prassad, Gill, Smart, & Gopal, 1998). The most studied and accepted probiotic strains include *L. acidophilus* LA1, *L. acidophilus* NCFB 1748, *L. rhamnosus* GG, *L. casei* Shirota, *L. gasseri* ADH and *L. reuteri*. Benefits from their consumption, like immune enhancements, reduction in fecal enzyme activity, prevention of intestinal disorders, viral diarrhoea, suggest their use as probiotic agents for the treatment of GI infections and inflammatory bowel disease (Macfarlane & Cummings, 2002; Madsen, 2001). They are also known to produce an important group of natural antibiotics i.e. bacteriocins like Lactacin B, Lactacin F, Brevicin 37, Buchnericin LB, Lacticin A, Helveticin J, Sakacin A, Plantaricin A, Gassericin A (Barefoot & Nettles, 1992; Klaenhammer, 1993; Muriana & Klaenhammer, 1991; Yildirim & Yildirim, 2001) which are being used as natural preservatives for food products.

Owing to their vast range of beneficial properties, 10 draft genome sequences for major *Lactobacillus* species including strains of probiotic potential like *L. acidophilus* NCFB 1748 have been generated by 2006 and at least 11 more sequencing projects are ongoing (Claesson, van Sinderen, & O'Toole, 2007). The resulting information will help to determine the genetic basis for the taxonomy in genus *Lactobacillus*, and more specifically to eliminate inconsistencies in the *Lactobacillus casei* – Pediococcus group.

Although a number of review articles have been published on molecular identification and characterization of lactobacilli (Charteris et al., 1997; Coeuret, Dubernet, Bernardeau, Gueguen, & Vernoux, 2003; Giraffa & Neviani, 2000; Lick, 2003; McCartney, 2002),

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their focus is generally limited to probiotic species, or this issue has been addressed in brief. This present review deals with the discussion of various molecular methods used in the differentiation within the genus *Lactobacillus* and their rate of success especially within the group of closely resembling species.

2. Taxonomy of the genus *Lactobacillus*

The large number of species within this genus and their similar phenotype and physiology along with horizontal transfer of plasmid linked characteristics put the taxonomy of this genus largely in confusion, and many times leads to misidentifications (Dalezios & Siebert, 2001; Hammes & Hertel, 2006; Hammes & Vogel, 1995). In order to make identification and characterization of a range of *Lactobacillus* species easier and comprehensive, various schemes have been proposed, based on distinguishable features/characteristics, such as phenotype, physiological, biochemical characteristics and sequence comparisons of 16S rRNA gene (Collins et al., 1991; Hammes & Vogel, 1995; Kandler & Weiss, 1986; Orla-Jensen, 1919; Stiles & Holzapfel, 1997). Hence, along with these taxonomic classification schemes the taxonomy of the various species within the genus *Lactobacillus* has undergone major changes and various species have been moved in and out of the *Lactobacillus*. Some atypical *Lactobacillus* species like *L. confuses*, *L. halotolerans*, *L. kandleri*, *L. minor*, *L. viridescens*, *L. minutus*, *L. rimae* and *L. uli* have been reclassified and the new genus *Atopobium* has been proposed (Collins, Metaxopoulus, & Wallbanks, 1993; Collins & Wallbanks, 1992). *L. maltaromicus*, *L. carnis* and *L. divergens* have been shifted into the new genus *Carnobacterium* (Collins, Farrow, Phillips, Ferusu, & Jones, 1987). In addition, there are issues of taxonomic dispute and the problem of very minute differences at nucleotide level in the 16S rRNA gene, creating ambiguity among the four prominent *Lactobacillus* groups namely, *L. acidophilus*, *L. casei*, *L. plantarum* and *L. delbrueckii*, well recognized for their use in dairy products as well as neutraceuticals. Before describing the various molecular methods and their potential in general, we will focus on the taxonomic status of these mentioned groups.

The *L. casei* and *L. acidophilus* groups are of special relevance for the pharmaceutical industry due to their important role in promoting human health (Holzapfel, Haberer, Geisen, Bjorkroth, & Schillinger, 2001; Klein et al., 1998; Roy, Ward, Vincent, & Mondou, 2000). A single species *casei* with five subspecies namely *casei*, *alactosus*, *pseudoplantarum*, *tolerans and rhamnosus* was reclassified into three species: (i) *L. casei* including the reference strain of previous *L. casei* ssp. *casei*, (ii) *L. paracasei* with two subspecies *paracasei* and *tolerans* including the former subspecies *alactosus* and *pseudoplantarum* in subspecies *paracasei* and ssp. *tolerans* for the former *L. casei* ssp. *tolerans*, (iii) species *L. rhamnosus* as a replacement of *L. casei* ssp. *rhamnosus* (Collins, Phillips, & Zanoni, 1989).

This classification, however, initiated a new stream of controversial results, largely due to the failure of differentiation between the newly created *L. paracasei* and former *L. casei* strains even by molecular techniques (Chavagnat, Haueter, Jimeno, & Casey, 2002; Vasquez, Ahrne, Pettersson, & Molin, 2001). Various researchers have produced reasonable evidences for the replacement of type strain of *L. casei* ATCC 393 by ATCC 334 and rejection of name *L. paracasei* by using different molecular methods (Chavagnat, Haueter, Jimeno, & Casey, 2002; Chen, Lim, Lee, & Chan, 2000; Dellaglio, Dicks, du Toit, & Torriani, 1991; Dicks, Du Plessis, Dellaglio, & Lauer, 1996; Felis, Dellaglio, Mizzi, & Torriani, 2001; Ryu, Czajka, Sakamoto, & Benno, 2001; Ward & Timmins, 1999). Moreover, a new species *L. zeae* has been proposed for the group of the former *L. rhamnosus* strains with type strain ATCC 15820, and inclusion of ATCC 393 has been suggested in *L. zeae*. Although these proposals have been strongly endorsed by the International Committee on Systematic Bacteriology (Biavati, 2001; Klein, 2001), a final decision has not been made. It is likely that the taxonomy of this group will undergo further changes with more extensive studies providing more evidence in the coming years.

Heterogeneity among *L. acidophilus* strains was first recognized in the 1960s by Lerche and Reuter, who suggested four different biotypes for this species. Subsequently, DNA–DNA hybridization studies (Johnson, Phelps, Cummins, London, & Gasser, 1980; Lauer, Helming, & Kandler, 1980) confirmed this heterogeneity and evidenced the presence of six different species within the group namely, *L. acidophilus*, *L. crispatus*, *L. amylovorous*, *L. gasseri*, *L. gallinarum* and *L. johnsonii* (Cato, Moore, & Johnson, 1983; Fujisawa, Benno, Yaeshima, & Mitsuoka, 1992). However, it is difficult to differentiate unambiguously among some of these species (Holzapfel, Schillinger, Du Toit, & Dicks 1997; Klaenhammer, 1998; Song et al., 1999). There are several reports, regarding the misidentification of a number of strains belonging to this group (Schillinger, 1999; Song et al., 2000; Yeung, Sanders, Kitts, Cano, & Tong, 2002). In fact *L. gasseri* and *L. johnsonii* are difficult to be distinguished from each other sometimes even by molecular techniques (Walter et al., 2000).

The third major group under this category is represented by *L. delbrueckii* species containing three highly resembling subspecies, namely: *L. delbrueckii* ssp. *delbrueckii*, *L. delbrueckii* ssp. *bulgaricus*, *L. delbrueckii* ssp. *lactis*, which are of special relevance in food fermentations. The subspecies *bulgaricus* and *lactis* are common starters used most often in the dairy industry, whereas *L. delbrueckii* ssp. *delbrueckii* is found mainly in vegetable fermentations. These three subspecies are known to share more than 80% of DNA–DNA homology (Weiss, Schillinger, & Kandler, 1983) along with 16S rRNA sequence homology reaching 90.8–99. 3% (Collins et al., 1991; Vandamme et al., 1996).

The fourth group of closely resembling *Lactobacillus* species is the *L. plantarum* group consisting of *L. plantarum*, *L. paraplantarum*, and *L. pentosus* species. They exhibit very high levels of DNA homology, with *L. plantarum* and *L. pentosus* sharing even greater than 99% similarity with only a minute 0.3% difference in their 16S rRNA sequence (Collins et al., 1991; Quere, Deschamps, & Urdaci, 1997). Despite this, Zanoni, Farrow, Phillips, and Collins (1987) demonstrated that they are separate species on the basis of DNA–DNA hybridization studies. Likewise, many attempts to discriminate these species succumbed to failure in the past and only limited success could be achieved (Curk, Peladan, & Hubert, 1994; Van Reenen & Dicks, 1996). However lately, some success has been obtained in discrimination among these species with the use of alternative molecular markers (Berthier & Ehrlich, 1998; Torriani, Felis, & Dellaglio, 2001).

3. Molecular identification methods

The identification of lactobacilli using biochemical methods is notoriously difficult largely due to the need for plenty of cumbersome biochemical tests along with the problems of highly resembling large number of species groups that are prone to transfer of plasmids among them. Hence, they alone are not sufficient for inter- and intra-species differentiation and need to be supplemented with sensitive molecular methods to obtain more reliable identification.

Contrary to the phenotypic methods, molecular identification and characterization tools are far more consistent, rapid, reliable and reproducible and can discriminate even between closely related groups of species, which are otherwise indistinguishable on the basis of phenotype. In fact, many *Lactobacillus* species have been reclassified on the basis of fresh information from advanced molecular techniques and their correct taxonomic status has been determined, such as *L. cellobiosus, L. pastorianus*, *L. arizonensis* have