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A selective medium for isolation and accurate enumeration of *Lactobacillus casei*-group members in probiotic milks and dairy products

Maria Di Lena [a], Grazia M. Quero [a], Elisa Santovito [a], Joanna Verran [b], Maria De Angelis [c], Vincenzina Fusco [a, *]

[a] National Research Council, Institute of Sciences of Food Production (CNR-ISPA), Bari, Italy
[b] School of Health Care Science, Manchester Metropolitan University, Chester Street, Manchester, M1 5GD, United Kingdom
[c] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy

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ABSTRACT

The correct identification and quantitative detection of *Lactobacillus casei*-group members continues to be a key issue due to the extensive use of members of this group as (probiotic) adjuncts, corrective and/or reinforcement cultures. Numerous selective/differential media use selective agents to which injured or stressed cells are sensitive, resulting in an underestimation of the actual number of target bacteria. We developed a selective medium and applied it to detect members of the *Lb. casei*-group reliably, regardless of their physiological condition, quantitatively detect and isolate strains of this group from probiotic milk and cheeses, and monitor the probiotic *Lb. paracasei* CRL 431 strain in Caciotta cheeses made either from goats' or cows' milk inoculated with this probiotic strain and a multiple strain culture. This approach could be useful for control bodies, agro-food and pharmaceutical industries to isolate *Lb. casei*-group strains, develop probiotic food/supplements and demonstrate their compliance with the species labelling.

1. Introduction

Microorganisms belonging to *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* species are a taxonomic group of closely related facultative heterofermentative rod-shaped lactic acid bacteria (LAB) known as the *Lactobacillus casei*-group. Several strains of these species are members of the normal intestinal microbiota and may exert probiotic properties thus providing benefits to human and animal health (Buriti & Saad, 2007). In milk and its derivatives, *Lb. casei*-group lactobacilli may naturally occur as part of the non-starter LAB or might be intentionally added for technological or probiotic purposes (Bergamini, Hynes, Palma, Sabbag, & Zalazar, 2009; Buriti & Saad, 2007).

Fermented milks and yoghurt have been the food matrices mainly used as probiotic carriers, but recently cheese has been indicated as a reliable alternative to deliver probiotics due to the limited acidity, the buffering capacity, the low level of oxygen as well as the embedding of probiotic bacteria in the fat-protein matrix of cheese that may support the survival of probiotics during cheese production and storage as well as during the gastrointestinal passage more effectively than a fluid environment (Heller, Bockelmann, Schrezenmeir, & deVrese, 2008).

In food matrices, as well as in the gastrointestinal tract, probiotics are exposed to abiotic and biotic stresses that may sublethally damage them, resulting in a population of live, dead and stressed cells. In this way, the survival rate and functional performance or effectiveness of probiotics may be affected (Davis, 2014). Indeed, it is recommended that, up to the end of its shelf-life, the probiotic food/supplement contains an amount of viable cells of the relevant probiotic(s) at least equal to that used in efficacy trials (EFSA, 2010; FAO/WHO, 2002; Italian Ministry of Health, 2005; Lourens-Hattingh & Viljoen, 2001). This prompts the need for methods to quantitatively detect viable, stressed, injured or viable but non-culturable (VBNC) probiotic cells.

From this perspective, solid- and liquid-based enumeration methods allowing the revivification and cultivation of the greatest number of VBNC injured bacteria could be the most adequate (Fusco & Quero, 2012, 2014; Fusco, Riccardi, & Quero, 2012; Quero,

* Corresponding author. Tel.: +39 0805929322.
E-mail address: vincenzina.fusco@ispa.cnr.it (V. Fusco).

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Santovito, Visconti, & Fusco, 2014a). Nonetheless, the species and strain specific DNA-based identification is crucial to ascertain that the strain used belongs to species generally recognised as safe (GRAS; USFDA, 1999) or as quality presumption of safety (QPS; EFSA, 2004). Moreover, since different strains may exert different probiotic activities, the identification at strain level is of utmost importance even if the probiotic is not yet certified (Aureli et al., 2011). For this purpose, it is recommended the use of genetic-besides phenotypic-based methods (Aureli et al., 2011), which have been recently specified (FAO/WHO, 2002; Italian Ministry of Health, 2005). Less clear and less defined is the situation relevant to the enumeration methods for probiotics: apart from those that enumerate bifidobacteria (IDF/ISO, 2010) and *Lactobacillus acidophilus* (IDF/ISO, 2006) in fermented milks, no standard method is to date available to fulfil this purpose.

To the best of our knowledge, few studies have focused on the development of an alternative medium to selectively enumerate *Lb. casei*-group members in the presence of out-numbering competing bacteria (Champagne, Roy, & Lafond, 1997; Colombo, de Oliveira, de Carvalho, & Nero, 2014; Hartemink, Domenech, & Rombouts, 1997; Lima et al., 2009; Ravula & Saha 1998; Sakai et al., 2010; Sutula, Coulthwaite, Thomas, & Verran, 2012b, 2013; Vinderola & Reinheimer, 2000). However, since they used selective agents to which injured or stressed cells resulted sensitive, they failed to estimate the actual loads of viable and VBNC target bacteria, especially in complex matrices with a numerous and heterogeneous microbiota such as milk and dairy products (Fusco & Quero, 2012, 2014; Fusco et al., 2012; Quero et al., 2014a, b).

Sutula, Coulthwaite, and Verran (2012a) recently developed a differential medium containing MRS added with L-cysteine, vancomycin, and bromocresol purple, which provided optimum conditions to isolate and count the probiotic *Lb. casei* Shirota. Herein, we explored the possibility to improve the 'LcS select' medium of Sutula et al. (2012a) using all components of MRS except glucose and meat extract and amending it with maltose as sole carbohydrate source, L-cysteine, vancomycin, Tween 80 and bromocresol purple. Thereafter, we coupled it with a well-established and widely used PCR-based approach to quantitatively detect and isolate lactobacilli of the *Lb. casei*-group in milk and dairy probiotic products as well as to monitor healthy and sub-lethally damaged cells of a probiotic *Lb. paracasei* strain during the ripening of Caciotta cheeses made either from cows' or goats' milk.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Working cultures were obtained as described by Fusco et al. (2011b). The probiotic *Lb. paracasei* CRL 431 (Christian Hansen, Hørsholm, Denmark) strain (Castillo, de Moreno de LeBlanc, Galdeano, & Perdigón, 2013) was used as test microorganism.

2.2. Preparation of MMV agar medium for the isolation and enumeration of Lactobacillus casei-group members

We used as basal medium a mix containing all the components of MRS (purchased from Sigma–Aldrich, Milan, Italy) except glucose and meat extract (as suggested by the Bergey's Manual of Systematic Bacteriology (Ludwig, Schleifer, & Whitman, 2009) for screening the carbohydrate fermentation in lactic acid bacteria) and supplemented it with (final concentrations; Table 2): maltose (1%, w/v; filter sterilised solution added after autoclaving the agar medium), vancomycin (10 g L^{-1}), Tween 80 (1 mL L^{-1}), L-cysteine (0.05%, w/v) and bromocresol purple (3 mL L^{-1}) all purchased from Sigma–Aldrich. Maltose-MRS-Vancomycin (MMV) and MRS (reference medium) plates were spread-inoculated (in duplicate) with 100 μL of ten-fold dilution/suspensions in maximum recovery diluent (MRD, (Oxoid, Milan, Italy) of a *Lb. paracasei* CRL 431 broth culture in stationary phase. Inoculated plates were anaerobically (AnaeroGene kit, Oxoid) incubated at 37 °C for 48 and 72 h, respectively.

2.3. MMV agar medium analytical specificity

The selectivity, i.e., the capability of the MMV agar medium to discriminate between non-target (exclusivity) and target bacteria (inclusivity) (Fusco, Quero, Morea, Blaiotta, & Visconti, 2011a) was evaluated against 25 strains (Table 1). These strains were already identified by 16S rRNA gene sequencing and characterised in previous studies (Fusco et al., 2011a; Quero, 2006; Sisto, De Bellis, Visconti, Morelli, & Lavermicocca, 2009). Overnight broth cultures of each strain were inoculated on MMV plates and incubated at 37 °C for 72 h in anaerobiosis. The colonies were photographed using a digital camera and their morphological features were recorded.

2.4. MMV agar diagnostic specificity

MMV was also used to isolate *Lb. casei*-group members from locally purchased probiotic cheeses (n = 2) and probiotic milk-based beverages (n = 6). For this purpose, samples, in triplicate, were ten-fold diluted in MRD (Oxoid), following the IDF/ISO:122/8261 method (IDF/ISO, 2001), and plate counted on MMV and MRS, as described above. One hundred twenty-eight colonies (64 per medium) were randomly isolated from MMV and MRS agar plates (highest sample dilutions) and purified by repeated streaking on the relevant media. Colony morphological features were registered as reported above. For a preliminary characterisation, Gram staining and catalase activities of all isolates were carried out. Isolates were then subjected to DNA extraction and PCR-based identification and typing, as described below.

2.5. Evaluation of survival and viability of Lb. paracasei CRL 431 during the production and ripening of Caciotta cheeses by culture-dependent PCR

2.5.1. Cheese manufacture

Experimental production of cows' and goats' Caciotta cheeses was performed in a local dairy farm, starting from either cows' milk or goats' milk (15 L for each batch). In both cases, batches of 15 L of cows' and goats' milk, respectively, without the probiotic strain adjunct, were also processed (controls). Each manufacture was performed twice (n = 2). Raw milk was weighted, filtered, heated to 72 °C for 15 s and subsequently cooled to approximately 38 °C. Afterwards, the probiotic strain *Lb. paracasei* CRL 431 was added (as a pellet obtained from a broth culture in MRS broth previously washed and centrifuged) at a final concentration of approximately 10^8 cfu g^{-1} in milk. Liquid rennet (Clerici s.p.a., Cadorago, Italy) (concentration 1:15000; 20 g per 100 kg of milk) and the commercial (Cadorago, Como, Italy) microbial starter comprising a multiple strain culture of *Lactobacillus helveticus*, *Lactococcus lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* (ratio 1:1:1; final concentration per each strain of 10^7 cfu mL^{-1} of milk) were also added. Curdling was achieved in approximately 30 min. Curds were manually cut using a metallic stick till a dimension of rice grains was reached. Thereafter, the curds were drained and transferred into circular plastic moulds and manually pressed also to achieve the desired shape. After approximately 1 h at room temperature,