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Novel method based on chromogenic media for discrimination and selective enumeration of lactic acid bacteria in fermented milk products

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ABSTRACT

Microbial analyses of fermented milk products require selective methods to discriminate between close species simultaneously present in high amounts. A culture-based method combining novel chromogenic agar media and appropriate incubation conditions was developed to enumerate lactic acid bacteria (LAB) strains in fermented milk. M1 agar, containing two chromogenic substrates, allowed selective enumeration of *Lactobacillus rhamnosus*, two strains of *Lactobacillus paracasei* subsp. *paracasei* and *Streptococcus salivarius* subsp. *thermophilus* based on differential β-galactosidase and β-glucosidase activities. Depending on the presence of some or all of the above strains, M1 agar was supplemented with L-rhamnose or vancomycin and incubations were carried out at 37 °C or 44 °C to increase selectivity. A second agar medium, M2, containing one chromogenic substrates was used to selectively enumerate β-galactosidase producing *Lactobacillus delbrueckii* subsp. *bulgaricus* at 47 °C. By contrast with the usual culture media, the chromogenic method allowed unambiguous enumeration of each species, including discrimination between the two *L. paracasei*, up to 10^9 CFU/g of fermented milk. In addition, the relevance of the method was approved by enumerating reference ATCC strains in pure cultures and fermented milk product. The method could also be used for enumerations on non-Danone commercial fermented milk products containing strains different from those used in this study, showing versatility of the method. To our knowledge, this is the first description of a chromogenic culture method applied to selective enumeration of LAB.

1. Introduction

Yoghurt has a long history of consumption as dietary product. It presents interesting nutritional and organoleptic properties (Astrup, 2014; El-Abbadi et al., 2014; Rizzoli, 2014) conferred by milk fermentation by starter bacteria, the most commonly employed being *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. During the past three decades, yoghurt has also received considerable attention as vehicle for probiotic bacteria and more and more probiotic yoghurts are now available on the market (Boyer and Geng, 2014). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO, 2006). Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly employed as probiotics, and among lactic acid bacteria (LAB) *Lactobacillus rhamnosus* and *Lactobacillus casei/paracasei* are frequently used and therefore, well studied (Bron et al., 2012). The suggested minimal dose is generally agreed to be around 10^6–10^7 colony forming units CFU/g (Ashraf and Shah, 2011; Karimi et al., 2012; Vasiljevic and Shah, 2008; Vinderola and Reinheimer, 2000). In order to ensure microbiological and nutritional quality and product compliance, manufacturers have to be able to discriminate and selectively quantify each strain. This is

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generally achieved using culture-based and/or molecular methods. Traditional culture-based methods are routinely used in analytical microbiology laboratories to identify and quantify viable microbes in dairy products (Sohier et al., 2014). Many agar plate media and protocols have been proposed to selectively enumerate LAB and bifidobacteria in yoghurt (Ashraf and Shah, 2011; Saccaro et al., 2011; Talwalkar and Kailasapathy, 2004; Tharmaraj and Shah, 2003; Van de Casteele et al., 2006; Vinderola and Reinheimer, 2000). Most of the methods were based on the use of standard non selective LAB culture media, with variations in NaCl concentration, carbon sources, pH, inclusion of antibiotics and different incubation temperatures in order to improve selectivity. However, discriminating power generally remained limited to the genus, and at best to the species (Saccaro et al., 2011; Tharmaraj and Shah, 2003; Van de Casteele et al., 2006). Moreover, in mixed matrices such as yoghurt, it is often difficult to selectively enumerate each individual strain in the presence of the others (Ashraf and Shah, 2011). Despite the profusion of culture-based methods described in the literature, many have been evaluated on isolated cultures only, while evaluation on mixed fermented products is necessary to assess their real performances. Currently, a few internationally validated methods are available for enumeration of bifidobacteria (ISO, 2010), *Lactobacillus acidophilus* (ISO, 2006a), citrate-fermenting bacteria (ISO, 2006a,b) and the characteristic yoghurt fermenters *Lactobacillus bulgaricus and Streptococcus thermophilus* (ISO, 2003) in milk or yogurt products, but their discriminating power towards other LABs present in mixtures is sometimes low (Boyer and Combrisson, 2013). Today, there is a clear need for specific and reliable selective methods, and when the available culture methods are employed, it is generally recommended to use additional molecular methods (mostly PCR-based methods) to confirm identification of the analyzed strains. A recent review has summarized the existing methods as well as those with interesting potentialities for dairy analytical purposes (Sohier et al., 2014). Among the molecular methods, PCR-based methods, flow cytometry and other fluorescent labeling-based methods are the most promising. However, as a drawback of their high sensitivity and specificity, these methods are very sensitive to small experimental variations that may lead to inaccurate results. They require a strong standardization and performances evaluation, and few of them like Flow cytometry method, have been validated yet for quality control use in analytical facilities. In addition, molecular methods are more expensive and often necessitate a more qualified staff than culture-based methods. Thus, culture methods remain very valuable as they are simpler to use, cheaper and provide visual results.

The reference medium for enumeration of *L. paracasei* and *L. rhamnosus* from milk products is MRS (de Man, Rogosa and Sharpe) containing 1 μg/ml vancomycin (MRS-V) (Ashraf and Shah, 2011; Colombo et al., 2014; Tharmaraj and Shah, 2003). The fermenting strains *L. bulgaricus* and *S. thermophilus* can be enumerated on acidified MRS (MRSa) at pH 5.4 ± 0.1 and M17, respectively, according to ISO 7889:2003 (ISO, 2003b). However, when these species are all present in milk products a combination of the above media and variations of incubation conditions have to be employed to deduce the CFU numbers for each species. Even then, individual strains of the same species/subspecies cannot be distinguished and enumerated from mixed samples.

One strategy to improve selectivity of the existing culture media and methods is the development of chromogenic media (Manafi, 2000; Druggan and Iversen, 2014). They are based on the cleavage of a colorless substrate (chromogen) by target bacteria's enzymatic activity, which releases a chromophore resulting, usually after oxidation, in colored colonies on agar plates. While chromogenic media have been regularly developed, validated and commercialized for analyses of pathogenic and spoilage bacteria starting from the 80s, they have been totally neglected for analyses of LABs and probiotics (Sohier et al., 2014). The development of chromogenic media relies on the knowledge of bacterial metabolism, and thus, is particularly appropriate to the case of LABs and probiotics that are generally well characterized. Moreover, chromogenic media represent an innovative alternative to selective media using antibiotics, as LABs are generally selected to be the least possible antibiotic-resistant due to food safety considerations (EFSA, 2012). These media are also well adapted to selective enumerations from mixed samples, as demonstrated for instance in clinical and environmental microbiology (Akter et al., 2014; Noble and Weisberg, 2005; Perry and Freydiere, 2007).

This study describes a method based on new chromogenic media to selectively enumerate LABs in fermented milk products. The objective was to discriminate between *L. rhamnosus*, two close strains of *L. paracasei*, *L. bulgaricus* and *S. thermophilus*. The media and culture conditions were evaluated on pure cultures but also on mixtures from fermented milk products. The results were compared to those obtained with reference media, when applicable, in order to establish the new medias' performances.

2. Materials and methods

2.1. Bacterial strains and cultures

L. rhamnosus CNCM (Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, France) I-3690, *L. rhamnosus* ATCC (American Type Culture Collection) 53103, *L. paracasei* subsp. *paracasei* CNCM I-1518 (hereafter named *L. paracasei* 1, for simplification), *L. paracasei* subsp. *paracasei* CNCM I-3689 (hereafter named *L. paracasei* 2), *L. casei* subsp. *casei* ATCC 393, *L. delbrueckii* subsp. *bulgaricus* CNCM I-2787, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *S. salivarius* subsp. *thermophilus* CNCM I-2773, CNCM I-2835, CNCM I-2778 and *S. salivarius* subsp. *thermophilus* ATCC 19258 were used in this study. All strains were stored at −80 °C in MRS broth (BD Difco, New Jersey, USA) for *Lactobacillus* strains or Elliker (BD Difco) for *S. thermophilus*, supplemented with 5.3% DMSO (v/v). *Lactobacillus* pure cultures were grown from frozen stocks inoculated in MRS broth and *S. thermophilus* was grown on M17 + 0.5% lactose.

2.2. Characterization of bacterial enzymatic activities

Phenotypical comparison of the strains was carried out at the Laboratory Veterinary Agency (London, UK) using Phenotype Microarrays (Biolog, Hayward CA, USA), according to the manufacturer's specifications. Based on the use of different carbon sources, nitrogen, phosphate, sulfur, on the inclusion of nutritional additives or stress-inducing agents (salts, pH, antimicrobial agents), about 2000 phenotypes were compared. This resulted in strain-specific phenotypical profiles for each targeted strain. Differential carbohydrate fermentation patterns were observed and selected for preliminary testing of the corresponding enzymatic activities. USB chromogenic agar (bioMérieux, Marcy l'Etoile, France) was used as non-selective medium to distinguish the strains based on their β-galactosidase and/or β-glucosidase activities. Pure cultures from MRS or M17 broth were plated on USB non selective agar medium and incubated aerobically, microaerobically (using GasPak EZ Container Systems, Becton Dickinson) or anaerobically (using Oxoid™ Anaerogen™, Thermo Scientific) at 37 °C. Colony color was observed after 48 h incubation.