International Dairy Journal 37 (2014) 1–4

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Short communication

Rapid and specific enumeration of viable Bifidobacteria in dairy products based on flow cytometry technology: A proof of concept study

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ARTICLE INFO

Article history:
Received 12 May 2013
Received in revised form
29 January 2014
Accepted 3 February 2014

ABSTRACT

A rapid and specific analytical method to enumerate viable Bifidobacteria in dairy products containing a mix of lactic acid bacteria was developed to proof of concept. A polyclonal antibody against *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) was used for specific detection by flow cytometry (FCM). The antibody, which targeted the cell wall of *B. lactis*, had a high labelling efficiency of 95.5 ± 1.9% of the population. A viability probe, 5,6-carboxyfluorescein diacetate, was combined with the antibody for real-time quantification of viable *B. lactis*. Enumeration results from FCM on four different commercial dairy products that contain *B. lactis* showed a good correlation with the standard culture-based method. Moreover, it provided a species-specific method to enumerate viable *B. lactis* within 2 h; the standard method counts the total cultivable Bifidobacteria genus on plates after 72 h. This study demonstrated that FCM could be a rapid solution for probiotic analysis in dairy products.

1. Introduction

Bifidobacteria have been widely used as probiotics in dairy products in combination with other lactic acid bacteria (LAB). Many studies have revealed that Bifidobacteria may contribute significant beneficial effects on human and animal health (Imaoka & Umesaki, 2009; Veiga et al., 2010; Ventura et al., 2007; Vyas & Ranganathan, 2012). According to the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO, 2002), the current definition of probiotics is: "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host". Therefore, quantitative measurement of viable Bifidobacteria in a dairy product is particularly important.

Currently, a classical plate count approach is recommended and is employed in the dairy industry as the standard method for the quantification of Bifidobacteria (ISO, 2010). This culture-based method is labour-intensive and time consuming. It requires a long incubation time (72 h) before results are available; this delays decision making and may become a bottleneck in production optimisation and quality control. In addition, due to the non-specificity of the method, most of the *Bifidobacterium* will grow on the plates, thus it is not possible to distinguish between different species of *Bifidobacterium*. Moreover, instead of measuring Bifidobacteria viability, such method only indicates how many of the bacteria can replicate under the conditions provided for growth (Davey, 2011). It is thus desirable to develop a rapid and specific method to identify and enumerate viable Bifidobacteria in dairy products containing mixed LAB.

In recent years, culture-independent methods for microbiological analysis of food products have been extensively developed (Boyer & Combrisson, 2013; Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010). Among these alternative methods, flow cytometry (FCM) in combination with fluorescent techniques offers a powerful tool for the multiparameter analysis of cell populations in dairy products. FCM has been applied for the evaluation of somatic cell load in milk (Gunasekera, Veal, & Attfield, 2003), identification of potential pathogens in milk (Holm, Mathiasen, & Jespersen, 2004), and enumeration of total viable bacteria in milk powder (Flint et al., 2006). In addition, FCM has been successfully introduced for characterising probiotics (Bunthof & Abee, 2002), monitoring LAB cell damage during processing and storage (Rault, Beal, Ghorbal, Ogier, & Bouix, 2007; Volkert, Ananta, Luscher, & Knorr, 2008) and assessment of probiotic viability after stress treatments (Amor et al., 2002; Ananta, Heinz, & Knorr, 2004). In spite of real-time analysis and quantification of microbial population, none of these studies demonstrated species-specific detection in mixed preparations.

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http://dx.doi.org/10.1016/j.idairyj.2014.02.002
0958-6946/

In the present study, a polyclonal antibody against *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) was used for specific detection by FCM. A viability probe, 5,6-carboxyfluorescein diacetate (5,6-cFDA), indicating esterase activity was used with the antibody for a rapid and specific enumeration of viable *B. lactis* in commercial fermented dairy products. To our knowledge, this is the first immunology application of FCM for the study of probiotics in dairy products.

2. Materials and methods

2.1. Bacterial culture and dairy products

B. lactis CNCM I-2494, *Lactobacillus delbrueckii* spp. *bulgaricus* CNCM I-1519, *Streptococcus thermophilus* CNCM I-1630 and *Lactococcus lactis* CNCM I-1631 from an internal strain collection were used in the study. After inoculation from the freezer stocks, cultures were cultivated overnight (16–20 h). *B. lactis* was grown at 37 °C in MRS broth (Oxoid, Hampshire, England) supplemented with 0.03% L-cysteine hydrochloride monohydrate (Sigma Aldrich, St. Louis, MO, USA). *Lb. bulgaricus* was grown at 37 °C in MRS broth. *S. thermophilus* and *Lc. lactis* were cultivated at 37 °C in M17 broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.5% lactose (Oxoid).

Four commercial fermented milk products containing *B. lactis* and two fermented milk products without *B. lactis* from the French market were also analysed.

2.2. Antibodies

Primary anti-Bifidobacteria polyclonal antibodies and secondary anti-rabbit IgG fragment Alexa Fluor® 647 F(ab′)2 (Invitrogen, Carlsbad, CA, USA) were used in the test. Production procedure and specificities of the primary antibodies have been described elsewhere (Duez et al., 2000). Briefly, this polyclonal antibody was found to be specific for *B. lactis* and *B. animalis* species with no cross-reactivity with 27 other species of *Bifidobacterium* (89 strains were tested in all). Purification of the primary antibody was performed according to the instruction of NAb™ protein A Spin Kits (Thermo scientific, Rockford, IL, USA), and concentration of the purified antibodies was measured by absorbance at 280 nm in spectrophotometer (Nanodrop, Wilmington, DE, USA).

2.3. Double fluorescent staining

A double fluorescent staining protocol was used in this study. First, for the immunolabelling of the Bifidobacteria, a primary antibody–secondary F(ab′)2 fragment complex was formed: 5 μL purified antibodies (0.23 g L^{-1}) and 0.6 μL F(ab′)2 fragments (2 g L^{-1}) were incubated in dark at 25 °C for 1 h under agitation. The bacteria culture or dairy products were diluted to 10^6 cells mL^{-1} using phosphate buffered saline (PBS; Invitrogen,), followed by a 1/10 dilution in PBS-Tween solution (0.2% Tween 20). Then 125 μL of the sample dilution in PBS-Tween was mixed with this red fluorescent antibody–Fab fragment complex at 25 °C in the dark for 30 min under agitation.

Second, a green fluorescent DNA probe Syto®24 (Invitrogen) was used for the quantification of total bacteria in the bacteria culture: 100 μL of immunolabelled sample was stained with 1 μL of 1 mM Syto®24 in 1 mL PBS buffer in the dark at 37 °C for 15 min prior to analysis. Meanwhile, for the tests on fermented dairy products, viability probe 5,6-cFDA (Invitrogen) replaced Syto®24 for the detection of the viable population in the products: 100 μL antibody labelled samples were incubated with 10 μL 5,6-cFDA (1 mM) in 1 mL PBS buffer in the dark at 37 °C for 15 min prior to analysis.

2.4. Flow cytometry

Fluorescent labelled samples were analysed using a Cyflow space® flow cytometer (Partec, Münster, Germany) equipped with two lasers (488 nm and 635 nm). Four parameters were recorded: forward scatter (FSC), side scatter (SSC), green fluorescence (FL1) and red fluorescence (FL4). Different populations of the bacteria were identified and gated according to the fluorescent intensity. Data acquisition was performed and analysed using FlowMax software (Partec). For each sample analysed in the flow cytometer, at least 10,000 events were acquired.

2.5. Plate count

The ISO (2010) method was used to numerate Bifidobacteria. In short, inoculations of appropriate decimal dilutions of the homogenised sample were plated into selective agar medium consists of transgalactosylated oligosaccharide (TOS; Yakult Pharmaceutical Industry Co., Ltd, Tokyo, Japan) and Lithium mupirocin (Sigma Aldrich). Plates were incubated at 37 ± 1 °C under anaerobic conditions for 72 ± 3 h.

3. Results and discussion

3.1. Method development on bacterial culture

A double fluorescent staining protocol was used to evaluate the binding efficiency of the antibody. All the bacteria in the sample were labelled with the green fluorescent probe Syto®24 (excitation 515 nm), while *B. lactis* were also labelled with the red fluorescent antibody complex (excitation 647 nm) at the same time. Therefore, the population appearing in the parameters of FSC and FL1 was the total population of the bacteria in the samples (Fig. 1a). The bacteria identified in the parameters of FL1 and FL4 were the population of *B. lactis* (Fig. 1a′). Result showed that 95.5 ± 1.9% of the *B. lactis* was labelled with antibody complex in pure culture, indicating a high binding efficiency of the antibody. Under confocal fluorescent microscopy, the antibody seemed to target on the entire cell surface of the Bifidobacteria (supplementary data Fig. S1).

To verify the specificity of the antibody, the same protocol was applied to the LAB cultures (*Lb. bulgaricus*, *S. thermophilus* and *Lc. lactis*) that was present in the fermented milk products. FCM results indicated that there was no cross reaction between this antibody complex and any pure culture of these LAB (data not shown), which was in accordance with the microscopy observation (supplementary data Fig. S1). The non-specific binding of this antibody complex with the mixed LAB culture is less than 0.1% (Fig. 1b,b′).

In the traditional two-step immunolabelling protocol, cells were first incubated with primary antibody, then with a compatible secondary antibody, which required duplicate washing and centrifugations. We observed a considerable quantity of bacterial loss during centrifugation and bacterial aggregation between the two antibodies during incubation. In this study, a one-step immunolabelling protocol was introduced by use of pre-formed primary antibody–secondary Fab fragment complex to reduce cell loss and aggregation during antibody labelling. This alternative protocol enabled more precise quantification of the bacteria with less non-specific interference from the secondary antibody.

3.2. Analysis of fermented dairy products

For the quantification of viable Bifidobacteria in the dairy products, probe 5,6-cFDA based on intracellular esterase activity replaced Syto®24 in the double fluorescent staining protocol.