The use of fluorescent techniques in combination with flow cytometry for fast counting of *Bifidobacterium longum* ATCC BAA-2753 in BIFICO capsule

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Abstract BIFICO is a main microecological drug that the main ingredient is *Bifidobacterium longum* ATCC BAA-2753. It's necessary to detect the number of *Bifidobacterium longum* ATCC BAA-2753 for quality control during the production of BIFICO. Plate count assay (PCA) is the most commonly used method for counting microbial cells. However, not all microorganisms can be counted by PCA. (e.g. uncultured microorganisms under specific growth conditions). In this study, a method of fluorescent techniques in combination with flow cytometry (FCM) was established for the counting of *Bifidobacterium longum* BAA-2753. Using SYTO9 and PI stain, flow cytometric analysis could easily differentiate live bacteria and dead bacteria. The number of live bacteria determined by FCM was about fourfold higher than that counted by PCA. Therefore, the FCM method could be a practical tool for the quality control of *Bifidobacterium longum* BAA-2753 in the industrial production of BIFICO.

Keywords Fluorescent techniques · Flow cytometry · Fast counting · *Bifidobacterium longum*

Introduction

Probiotics are widely distributed in healthy people. Nearly a century of study has shown that the types and quantities of probiotics colonized in the human body are closely related to the health status of the host and may even affect human longevity (Zhao et al., 2017). The WHO/FAO defines probiotics as beneficial living microorganisms for the host, and their benefit to human health is obtained by improving the digestive tract system, immune system and cardiovascular system (Williams, 2010). Probiotics mainly includes lactic acid bacteria (e.g., *Lactobacillus* and *Bifidobacterium*). *Bifidobacteria* represents one of the most important probiotic groups colonized in the human gastrointestinal tract (Bukharin et al., 2012). Studies have shown that *Bifidobacteria* plays an important role in maintaining the balance of intestinal flora and is widely used in clinical therapy, where it can effectively decrease the occurrence of infections and ameliorate *Helicobacter pylori* (Huang et al., 2016; Liu et al., 2013; Yu et al., 2015). Recently studies not only focus on its effect to intestinal tract, but also to metabolic related disease (e.g., obesity and diabetes) (Shin et al., 2017) and enhanced efficacy of antibody (e.g., PD-L1 and CTLA-4) (Sivan et al., 2015; Snyder et al., 2015). Thus, *Bifidobacteria* is particularly important to human health.

BIFICO is a main microecological drug used widely in clinic to treat diarrhea in China, which comprises three species of bacteria, namely *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*, with total live bacteria no less than 1.0×10^7 CFU. In its production, it's required to detect the viable cell number so as to determine whether this batch meets the quality control standard. Plate count assay (PCA) is usually used to evaluate cell viability. However, it only indicates how many of

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the cells can replicate under the specific conditions provided for their growth (Joux and Lebaron, 2000). Due to their sensitivity to oxygen, the anaerobic bacteria are easy to die during PCA operation, resulted in incorrect result. In addition to the above-mentioned limitations, forming countable colonies often requires several days of incubation for Anaerobic bacteria (Kell et al., 1998), which adds to the costs of production and reduces the production efficiency. Thus, it is urgent to develop a method to count live bacteria more accurately and efficiently during microecological drug production.

Fluorescent techniques in combination with flow cytometry (FCM) have been extensively used for assessment of the viability of microorganisms from different source samples (Breeuwer and Abee, 2000). Since FCM is a powerful tool for analyzing a cell population at the single-cell level, it can be used both to identify and enumerate bacterial populations of fermented samples and to characterize functional properties of each individual cell (Shapiro, 2000; Suller and Lloyd, 1999). FCM with fluorescence activated cell sorting allows the isolation of cells with selected characteristics at the single cell level, which has been proven very useful in investigating metabolic activity and membrane integrity. FCM has been used to distinguish live and dead cells and estimate cell vitality and spore germination (Hornbaek et al., 2002).

The aim of this study was to establish a FCM-based simple method for the rapid count of live *Bifidobacterium longum* ATCC BAA-2753 in BIFICO capsules. First, the FCM analysis combined with SYTO9 and PI double-staining to distinguish live cells and dead cells was developed and the accuracy was evaluated by linear regression. Then, the accuracy of the FCM method was further compared with the traditional PCA method.

Materials and methods

Probiotic strains and culture condition

The single probiotic strain *Bifidobacterium longum* ATCC BAA-2753 used in this study was obtained from the bacteria library of SPH Sine Pharmaceutical Laboratories Co., Ltd. (Shanghai, China). *Bifidobacterium longum* ATCC BAA-2753 was grown in the MRS broth medium (Merck Serono Co., Ltd., Germany) or MRS agar medium (Oxid Ltd., UK) at 37 °C under anaerobic conditions.

Preparation of live and dead bacterial cells

0.1 g freeze-dried powder (*Bifidobacterium longum* ATCC BAA-2753) was added in 100 mL MRS broth medium, and then cultivated at 37 °C for 10–24 h under the 37 °C

condition of nitrogen and carbon dioxide (4:1, v/v), which was used as the pure suspension of live cells. The pure suspension of dead cells was obtained via killing live cells by heat treatment (100 °C, 15 s). Thus, the accordingly treated cells were further used as live and dead cells for the staining tests. Mixtures of different proportions of live/dead cells as listed in Table 1 were prepared prior to staining.

Viable cell numbers were determined by spotting 100 µL of a 1:10 dilution series on MRS agar.

Double fluorescent staining

The nucleic acid dyes SYTO9 (Life Technologies, Carlsbad, CA, USA) and propidium iodide (referred to hereafter as PI, Life Technologies, Carlsbad, CA, USA) were used in this study. 1 mL of the samples taken from all experiments were immediately diluted (at least 1:100,000, v/v so that there were $\sim 10^4$ cells/mL) with MRS broth medium, respectively. 1 µL of 3.34 mM SYTO9 and 1 µL of 20 mM PI were added per tube before incubating in dark conditions for 10 min at room temperature. This staining protocol was strictly applied so as to achieve comparable data.

Flow cytometric analysis

FCM measurements were performed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser (488 nm). The flow rate was 35 µL/min in all experiments. SYTO9 fluorescence was measured at FL-1 533/30 nm while PI fluorescence was monitored using FL-2 or FL-3 > 670 nm optical detectors. Forward and sideways-scattered light was also collected. All data were recorded and processed with the BD Accuri C6 Flow software, and electronic gating was used to separate positive signals from instrument and medium background. A combination of instrument and gating parameters was used to discriminate cell populations and kept identical for all samples in order to achieve comparable data.

Plate count assay

The ISO (2010) method was used to numerate the *Bifidobacterium longum* ATCC BAA-2753. 1 mL of each sample taken from all experiments was immediately diluted with MRS broth medium, respectively. Inoculations of each appropriate decimal dilution were plated into the MRS agar medium, and then incubated at 37 °C for 36 h under anaerobic conditions of nitrogen and carbon dioxide (4:1, v/v).

Table 1 Preparing mixed suspensions of live and dead cells

Ratio of live:dead cells	Live-cell suspension (mL)	Dead-cell suspension (mL)
0:100	0	1.00
25:75	0.25	0.75
50:50	0.50	0.50
75:25	0.75	0.25
100:0	1.00	0

Fig. 1 Distinguishing dead cells and live cells of *Bifidobacterium longum* by double staining. Dead cells measured by FL-2 (A), Dead cells measured by FL-3 (B), Live cells measured by FL-1 and FL-2 (C), Live cells measured by FL-1 and FL-3 (D)

Results and discussion

Determination of fluorescent detection channel

It is well known that propidium iodide (PI) intercalates to DNA with no sequence preference with one dye molecule per four to five base pairs. When PI binds to DNA, its fluorescence is enhanced 20- to 30-fold. PI is usually used for identifying dead cells in a population and as counter-stain in multicolor fluorescent techniques because it is supposed to penetrate only cells with disrupted membranes and is generally excluded from viable cells (Stocks, 2004). In contrary, the green-fluorescent nucleic acid stain SYTO9

can enter into both live and dead bacterial cells. Its fluorescent signal is also strongly enhanced when bound to nucleic acid and shows low intrinsic fluorescence signal when unbound. The combination of SYTO9 and PI can be a very useful tool to detect the live and dead cells in regard to membrane integrity and has been proven to be a reliable staining technique (Stiefel et al., 2015). In flow cytometric analysis, SYTO9 is generally measured with FL-1 for detecting and counting live cells while PI is usually measured with FL-2 or FL-3 for detection and counting of dead cells, but when the cells are double stained with SYTO9, it is highly recommended to detect PI fluorescence by FL-3 to avoid cross-signal of SYTO9. Since MRS broth medium

Fig. 2 Different ratios of live and dead cell mixtures were analyzed by flow cytometer. Gate P1, dead cells and GateP2, live cells. (A) All dead cells; (B) 25% live cells and 75% dead cells; (C) 50% live cells and 50% dead cells; (D) 75% live cells and 25% dead cells; (E) all dead cells

Fig. 3 Best-fit linear regression analysis generated using double stain. Live (dashed line $R^2 = 0.9979$) and dead (solid line $R^2 = 0.9982$) *Bifidobacterium longum* were mix at various live: dead ratios (Table 1)

is a diluent that includes many impurities that can be stained by SYTO9 or PI, it also needs to choose proper detection channels in order to distinguish cells from these interfering substances. Dead or live *Bifidobacterium*

Table 2 Analysis of *Bifidobacterium longum* using flow cytometry (FCM) and plate count assay (PCA)

Batch no.	Culture time (h)	Total living cells ($\times 10^8$ CFU/mL)	
		PCA	FCM
20170426	24	8.8 ± 0.4	36 ± 1.4
20170503	20	6.0 ± 0.3	29 ± 0.9
20170509	14	4.4 ± 0.1	14 ± 0.1
20170510	10	2.0 ± 0.0	9.7 ± 0.1
20170503	18	5.1 ± 0.3	19 ± 0.5

longum ATCC BAA-2753 cells were double-stained with SYTO9 and PI, and then the fluorescence events were detected at FL-1/FL-2 and FL-1/FL-3 channels, respectively.

As seen in Fig. 1, the signals of the dead cells and the live cells were both better separated from each other as well as from that of the background when measured by FL-1/FL-3 (Fig. 1 B, D) than FL-1/FL-2 (Fig. 1A, C). Thus, FL-1/FL-3 was used in subsequent FCM analysis. Besides, SYTO9 and PI stained samples were kept on ice in the dark

for more than 30 min in a previous study (Amor et al., 2002) while the procedure in this study only took 10 min. The good detection result (shown in Fig. 1) demonstrated the shortened staining time was appropriate. So, it can be assumed that staining for no less than 10 min has little influence on the outcomes, which will accordingly save a lot of analysis time.

Analyzing live and dead cells by FCM

Dead and live cells could be well defined by SYTO9 and PI double stain using flow cytometric analysis. Dead cells are different from living cells in the count regions. The stock solutions of live and dead cell mixtures listed in Table 1 were stained with a combination of SYTO9 and PI for flow cytometric analyzing. As shown in Fig. 2, live cells and dead cells are clearly distributed in different locations and there is an obviously corresponding change in the number of live cells and dead cells as the ratios change. A bacteria/mL-versus-percent live cells graph was accordingly generated and then best-fit linear regression analysis was done to evaluate the accuracy of this method. Figure 3 shows the R^2 of linear regression for live cells measurement is 0.9979 while that for dead cells is 0.9982, indicating SYTO9 and PI double staining combined with FCM is a good and reliable method for fast counting of *Bifidobacterium longum*. Although most cells in probiotic products are live cells (> 99% of the population), strict control on the production of *bifidobacteria* still requires the detection of dead cells in each batch. Thus, this would be a promising method to be applied in the quality control of probiotic production, for example, the production of BIFICO.

Analysis of *Bifidobacterium longum* ATCC BAA-2753 using the FCM method and PCA

To further verify the accuracy of the FCM method developed in this study, this FCM method was compared with PCA traditionally used for counting *Bifidobacterium longum*. Five batches of fermentation broth that contained different number of living *Bifidobacterium longum* cells were analyzed by these two methods, respectively. Table 2 lists the results, namely the number of living bacteria counted by FCM and PCA. In general, the number of living bacteria counted by PCA was ~ 75% lower than that counted by FCM. This big variation could be attributed to several factors. In principle, FCM allows one to determine viability in a retrospective manner, whereas PCA cannot determine “viable but non-culturable” cells from microbial populations, leading to higher bacteria number counted by FCM. Second, a few *Bifidobacterium longum* cells might die during the sampling and PCA operation. Third, PCA cannot distinguish the colonies formed by single cells or

clumps and does take into account the inherent selectivity of bacteria under specific growth conditions. Hence, compared with PCA for determining the number of *Bifidobacterium longum* cells, our FCM method could be more reliable for the production of probiotic capsules.

In addition, PCA often requires several days of incubation to form the countable colonies (Laflamme et al., 2005) while more accurate result of cell count could be directly obtained in 30 min by using this FCM method, which makes it possess overwhelming advantages particularly when there are a large number of samples that need to be analyzed. The advantages of the FCM method is lower labor work, shorter detection time, and higher accuracy. Therefore, this simple FCM method can be potentially applied for monitoring quality control during the production of *Bifidobacterium longum* on the industrial scale.

In this study, a method of counting *Bifidobacterium longum* BAA-2753 in BIFICO's production, namely the SYTO9 and PI double staining technique combined with flow cytometry, was well established. The detection result demonstrated that this FCM method allowed the live cells and dead cells of samples to be well discriminated and counted in shorter time but with higher accuracy. It offers an alternative for the quantification of viable *Bifidobacterium longum* BAA-2753 in industrial production of BIFICO. Therefore, with the development of automated FCM and easily obtained fluorescent dyes, this method developed here will provide a promising opportunity for microbiology analysis in microecological medicine industry.

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