The use of a simple flow cytometry method for rapid detection of spores in probiotic *Bacillus licheniformis*-containing tablets

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Received June 30, 2016
Revised October 25, 2016
Accepted November 19, 2016
Published online February 28, 2017

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pISSN 1226-7708
eISSN 2092-6456

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Abstract Detection of the number of vegetative cells and endospores is necessary for quality control during the production of orally administered probiotic *Bacillus licheniformis*-containing tablets (BCT). However, there is no standard method for the rapid detection of vegetative cells and endospores in China. In this study, a simple flow cytometry (FCM) method was used to monitor the population dynamics of BCT. Using a specific fluorescent stain, SYBR green I, flow cytometric analysis could easily differentiate two morphological states of *B. licheniformis*. Compared with plate count assay (PCA) for determining the number of vegetative cells and endospores, the percentage of endospores determined by FCM was ~10% higher than that by PCA. Advantages of the FCM method over conventional methods include lower labor work, shorter detection time, and higher accuracy. Therefore, this simple FCM method could be a practical tool for monitoring quality control during the production of probiotic BCT.

Keywords: *Bacillus licheniformis*-containing tablets, probiotics, flow cytometry, rapid detection of spores

Introduction

Probiotics are microbial dietary supplements with beneficial effects to the host, e.g., humans and animals. The beneficial effects include enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activities, growth-promoting factors, increased immune response, and improved feed value (1). Probiotics mostly include lactic acid bacteria (e.g., *Lactobacillus* and *Bifidobacterium*), the genus *Bacillus*, photosynthetic bacteria, and yeasts. Among the *Bacillus* species, *B. licheniformis*, a gram-positive, spore-forming soil bacterium closely related to *Bacillus subtilis*, has been used as a probiotic for alleviating enteritis and diarrhea and widely applied in various aspects of biotechnology, e.g., production of nanoparticles, enzymes, antibiotics, and biochemicals (2,3). Similar to *B. subtilis*, *B. licheniformis* undergoes two interesting morphological states: vegetative cells and endospores. Endospores is the preferred biomass form for probiotics, which can be resistant to extreme environments and stored at room temperature for many years (4).

During the production of probiotic *B. licheniformis*-containing tablets (BCT), it is necessary to detect the viable cell number and spore count for quality control. Plate count assay (PCA) is the most

commonly used method for evaluating cell viability (5). However, PCA does not take into account (a) colony formed by single cells or clumps, (b) “viable but non-culturable” (VBNC) cells of the microorganism populations, and (c) inherent selectivity under specific growth conditions (6,7). In addition to these limitations, forming countable colonies often requires several days of incubation for bacteria (6). Recently, new viability assays have been developed for bacteria on the basis of respiratory activity, external membrane integrity, intracellular pH, and membrane potential, some of which have been validated directly on *Bacillus* spores (6).

Among the assays directly on spores, flow cytometry (FCM) is a rapid method to acquire multiparametric data from thousands of individual cells within a sample (5). FCM with fluorescence activated cell sorting allows for the isolation of cells with selected characteristics and enables rapid vitality assessments with high sensitivity at the single cell level, which have proven very useful in single cell analysis to study metabolic activity and membrane integrity (7). FCM has been used to distinguish live and dead cells and estimate cell vitality and spore germination (5–7).

Although the market of orally administered probiotic of *B. licheniformis* is ~3 billion renminbi in China, there is no standard method for counting the spore number of probiotic BCT in the

Chinese Pharmacopoeia. Hence, it is very useful to develop a simple assay for the rapid detection of spores in probiotic tablets. In this study, we developed a simple FCM method for rapidly distinguishing live and dead cells and counting the number of endospores in the commercial batches of probiotic BCT from Jingxin Pharmaceutical Co. Ltd. and compared the accuracy with the traditional PCA method.

Materials and Methods

Probiotic tablets use and culture condition Commercial batches of orally administered probiotic BCT (Batch No. 20140505, 20141003, 20141003-1, 20141003-3, and 20141003-4) from Jingxin Pharmaceutical Co. Ltd. (Zhejiang, China) were used in this study. *B. licheniformis* from commercial batches was grown in the nutrient broth medium (Batch no. 3102499; Guangdong Huankai Microbial Sci. & Tech. Co. Ltd., Guangzhou, China) or Nutrient agar medium (Batch no. 20130922; Qingdao Hope Biol-Technology Co. Ltd., Shandong, China) at 37°C.

Preparation of pure suspensions of vegetative cells Three grams of the samples from commercial batches were added in 27 mL of sterile 0.9% NaCl and mixed with agitation (200 rpm) at 37°C for 30 min as the stock solutions. One milliliter of the stock solution was added in 100 mL of nutrient broth medium and incubated in a rotating shaker at 250 rpm and 37°C. Direct microscopic count (DMC) observation of the samples at different culture times (0–31 h of cultivation) was used to determine the culture time for pure suspensions of vegetative cells without spores. Endospore-free suspensions of vegetative cells were prepared and harvested prior to the appearance of endospores.

Preparation of pure suspensions of endospores by heat treatment at 100°C Stock solution contains vegetative cells and endospores. To prepare a pure suspension of endospores, vegetative cells in the stock solution were killed by heat treatment. To determine the suitable treatment time, pure suspensions of vegetative cells and stock solutions were heated at 100°C. To count the surviving vegetative cells or endospores, a 100 µL aliquot of each treated suspension was serially diluted with sterile 0.9% NaCl and plated in triplicate on nutrient agar. When the plates were incubated for 18 h at 37°C, the colony count was determined and expressed as the average number of surviving vegetative cells or endospores. The number of colony forming units (CFU) per gram or milliliter of samples from commercial batches was determined, and the percentage of (surviving) vegetative cells or endospores was calculated using the average number of (surviving) vegetative cells or endospores divided by the total number of cells containing vegetative cells and endospores without heat treatment. All experiments were performed in triplicate.

Direct microscopic count of vegetative cells and endospores of *B. licheniformis* Vegetative cells or endospores from samples collected from all experiments were observed by crystalviolet staining and measured in a counting chamber under an optical microscope (ECLIPSE 50i; Nikon, Tokyo, Japan).

Flow cytometric analysis of physiological status The nucleic acid dyes SYBR green I (referred to hereafter as SYBR-I, purchased from Life Technologies, Carlsbad, CA, USA) and propidium iodide (referred to hereafter as PI, purchased from Sangon Biotech. Co. Ltd., Shanghai, China) were used in this study. The SYBR-I and PI commercial stock solutions corresponding to 10,000-fold and 100-fold, respectively, were diluted to the recommended concentration for staining. The 100–200 µL aliquots of samples taken from all experiments were immediately diluted (at least 1:1000, v/v so that there were ~10⁶ cells/mL) with sterile 0.9% NaCl and stained with the fluorescent dyes in dark conditions for 20 min at room temperature. FCM measurements were obtained using an Accuri C6 cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser (488 nm). The flow rate was 20 µL/min in all experiments. Vegetative cells and endospores (~30,000) were gated by forward-scatter and side-scatter parameters. SYBR-I fluorescence or PI fluorescence was measured at 530 and 675 nm. All flow cytometric parameters were set up by the systems software BD Accuri™ C6 Software (BD Biosciences). Data from the flow cytometer were recorded, processed, and drawn as graphs with the software Flow Jo 7.6.1 that discriminates cell populations using a combination of all the recorded parameters.

Statistical analysis Data were presented as mean±SD of triplicate experiments. All variances were checked for normality and homogeneity. All statistical analyses were performed using the software program SPSS version 20 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Preparation of pure suspensions of vegetative cells and endospores as the controls The *B. licheniformis* samples taken from different culture times (0–31 h of cultivation) were observed and counted using the DMC method. The percentage of endospores was decreased from 50 to 0.2% during 0–8 h, increased from 0.2 to 40% during 8–27 h, and then decreased again from 27 to 31 h (Fig. 1). The result suggested a spore germination phase during 0–7 and 27–31 h and spore formation phase during 8–27 h under our culture condition. Based on the result of spore percentage (Fig. 1), 7 h was the optimal culture time for preparing the pure suspensions of vegetative cells without spores because endospore-free suspensions of vegetative cells should be harvested prior to the appearance of endospores.

Fig. 1. The percentage of endospores in *B. licheniformis* samples taken from different cultivation times.

Probiotic BCT contain vegetative cells and endospores. To prepare a pure suspension of endospores, the heat treatment method (8) was used to kill vegetative cells in the stock solution. Under the suitable heat treatment, vegetative cells could be killed and endospores were still viable, so endospore suspensions of greater than 99% purity were yielded when examined using dilution plating techniques. To determine the suitable treatment time, pure suspensions of vegetative cells and stock solutions were heated at 100°C. On heat treatment at 100°C for more than 15 s, most vegetative cells (greater than 99% cells in a population) were killed in pure suspensions of vegetative cells (Fig. 2A). However, surviving cells in stock solutions were maintained constant by heat treatment from 15 to 30 s (Fig. 2B), suggesting that the live cells in the samples are endospores. Thus, endospore suspensions of greater than 99% purity could be yielded by 15 s of heat treatment at 100°C.

Distinguishing vegetative cells and endospores by FCM To investigate the changes in the physiological state of *B. licheniformis* populations throughout batch production of probiotic tablets, single staining with SYBR-I or double staining with SYBR-I and PI were used for flow cytometric analysis. SYBR-I has a strong binding affinity for double-stranded DNA, but it also binds with single-stranded DNA and RNA with lower affinities (9). SYBR-I staining was used to distinguish between vegetative cells and endospores. Since nucleic acids of endospores are not accessible to the nucleic acid fluorescence dye, whereas those of vegetative cells are efficiently stained (10), vegetative cells indicated high fluorescence and endospores showed very low fluorescence. Sample of the pure suspension of vegetative cells was used as the control. Based on flow cytometric analysis, the pure suspension of vegetative cells was also greater than 99% purity (Fig. 3A). FCM result showed that 59.2% of cells in the stock solution were endospores and 36.8% of cells were vegetative cells (Fig. 3B). Sample at exponential phase (4 h of cultivation) contains 89.0% of vegetative cells and 7.9% of endospores. All data determined by FCM were similar with the above data obtained using the DMC method dilution plating methods, indicating that SYBR-I staining could distinguish between vegetative cells and endospores in probiotic BCT.

Fig. 2. Percentage of surviving cells by heat treatment at 100°C. The pure suspensions of vegetative cells (A) and the stock solutions (B).

Distinguishing live and dead cells by FCM Both SYBR-I and PI bind to DNA that are able to distinguish between live and dead cells (11), but PI cannot cross an intact cytoplasmic membrane (12). PI only stains the cells with broken cytoplasmic membranes. Regions corresponding to dead cells could be defined by PI staining using flow cytometric analysis. Stock solutions were stained with a mixture of SYBR-I and PI (Fig. 4A) or SYBR-I alone (Fig. 4B). The percentage and CFU of vegetative cells and endospores were almost same using SYBR-I and PI or SYBR-I alone, and the percentage of dead cells was very low (<1%). Steady-state cells of *B. licheniformis* containing live and dead cells were also stained with a mixture of SYBR-I and PI (Fig. 4C) or SYBR-I alone (Fig. 4D). Compared with SYBR-I staining, a region of dead cells from a steady-state sample of *B. licheniformis* was found using double staining of SYBR-I and PI (Fig. 4C and 4D). Although PI staining could detect dead cells, our simplified FCM method used SYBR-I staining alone and not double staining of SYBR-I and PI because most cells in probiotic BCT are live cells (>99% of the population).

Analysis of probiotic tablets using the FCM method and PCA To validate the accuracy of our developed FCM method, we compared this FCM method with traditional PCA. Samples of five commercial batches of orally administrated probiotic BCT were analyzed in terms of the number of total viable cells, vegetative cells, and endospores by these two methods. Using PCA for counting the number of endospores, the samples were heated at 100°C to kill vegetative cells and medium was plated for spore germination. The number of

Fig. 3. Distinguishing vegetative cells and endospores by SYBR-I staining. Samples of pure suspension of vegetative cells (A), stock solution (B), and exponential-state (4 h of cultivation in nutrient broth medium at 250 rpm and 37°C) cells of *B. licheniformis* (C). P5, spores; P6, vegetative cells. FL1-H, the channel stained by SYBR-I; FL3-H, the channel stained by PI.

Fig. 4. Distinguishing vegetative cells and endospores by flow cytometry. Stock solutions stained with a mixture of SYBR-I and PI (A) or SYBR-I alone (B); steady-state cells of *B. licheniformis* (25 h of cultivation in nutrient broth medium at 250 rpm and 37°C) stained with a mixture of SYBR-I and PI (C) or SYBR-I alone (D). FL1-H, the channel stained by SYBR-I; FL3-H, the channel stained by PI.

that counted by FCM (Table 1). The percentage of endospores by PCA was ~10% lower than that by FCM. It may be attributed that heat treatment before PCA killed some endospores with no high temperature resistance. Hence, compared with PCA for determining the number of vegetative cells and endospores, our FCM method could be more reliable for the production of probiotic tablets.

Although PCA is the most commonly used method for evaluating the number of vegetative cells and endospores (5), PCA cannot distinguish the colonies formed by single cells or clumps and determine VBNC cells from microbial populations (6). In addition, PCA often requires several days of incubation to form the countable colonies (6). Another traditional method, DMC, cannot differentiate live cells from dead cells. However, our FCM method could overcome the limitations of PCA and DMC. When the samples were only stained with SYBR-I in dark conditions for 20 min at room temperature, they could be directly detected by FCM. FCM has obvious advantages when analyzing large numbers of samples. Advantages of the FCM method over conventional methods include 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 probiotic BCT on the industrial scale.

Acknowledgments This work was supported by Key Laboratory of Synthetic Biology, Chinese Academy of Sciences, China.

Disclosure The authors declare no conflict of interest.

Table 1. Analysis of commercial probiotic *B. licheniformis*-containing tablets (BCT) using flow cytometry (FCM) and plate count assay (PCA)

Batch no.	Total viable cells ($\times 10^8$ CFU/g)		Vegetative cells ($\times 10^8$ CFU/g)		Endospores ($\times 10^8$ CFU/g)		Percentage of endospores (%)	
	PCA	FCM	PCA	FCM	PCA	FCM	PCA	FCM
20140505	218.0 $\pm$ 21.1	191.3 $\pm$ 14.5	102.0 $\pm$ 19.6	63.6 $\pm$ 3.8	116.0 $\pm$ 2.9	127.7 $\pm$ 14.3	53.2 $\pm$ 1.3	66.8 $\pm$ 7.5
20141003	30.3 $\pm$ 4.1	30.0 $\pm$ 2.6	14.9 $\pm$ 3.3	11.8 $\pm$ 1.1	15.4 $\pm$ 0.9	18.2 $\pm$ 2.4	50.9 $\pm$ 2.9	60.8 $\pm$ 8.1
20141003-1	25.6 $\pm$ 2.5	27.7 $\pm$ 1.3	12.6 $\pm$ 2.5	10.0 $\pm$ 1.4	13.0 $\pm$ 0.7	17.7 $\pm$ 0.7	50.8 $\pm$ 2.6	64.0 $\pm$ 2.4
20141003-3	22.8 $\pm$ 0.9	24.0 $\pm$ 0.5	11.0 $\pm$ 0.9	8.4 $\pm$ 0.3	11.8 $\pm$ 0.0	15.6 $\pm$ 0.8	51.9 $\pm$ 0.0	64.9 $\pm$ 3.3
20141003-4	23.6 $\pm$ 1.1	23.6 $\pm$ 2.0	10.8 $\pm$ 0.3	8.4 $\pm$ 0.4	12.8 $\pm$ 0.8	15.2 $\pm$ 1.6	54.2 $\pm$ 3.2	64.3 $\pm$ 6.6

References

1. Azarin H, Aramli MS, Imanpour MR, Rajabpour M. Effect of a probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* and ferroin solution on growth performance, body composition and haematological parameters in kutum (*Rutilus rutilus*) fry. *Probiotics Antimicrob. Proteins* 7: 31-37 (2015)
2. Kalishwaralal K, Deepak V, Ram KPS, Gurunathan S. Biological synthesis of gold nanocubes from *Bacillus licheniformis*. *Bioresource Technol.* 100: 5356-5358 (2009)
3. Schallmeyer M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* 50: 1-17 (2004)
4. Zhao S, Hu N, Huang J, Liang Y, Zhao B. High-yield spore production from *Bacillus licheniformis* by solid state fermentation. *Biotechnol. Lett.* 30: 295-297 (2008)
5. Cronin UP, Wilkinson MG. The potential of flow cytometry in the study of *Bacillus cereus*. *J. Appl. Microbiol.* 108: 1-16 (2010)
6. Laflamme C, Ho J, Veillette M, de Latremaille MC, Verreault D, Meriaux A, Duchaine C. Flow cytometry analysis of germinating *Bacillus* spores, using membrane potential dye. *Arch. Microbiol.* 183: 107-112 (2005)
7. Hornbaek T, Dynesen J, Jakobsen M. Use of fluorescence ratio imaging microscopy and flow cytometry for estimation of cell vitality for *Bacillus licheniformis*. *FEMS Microbiol. Lett.* 215: 261-265 (2002)
8. Gaillard S, Leguérinel I, Mafart P. Modelling combined effects of temperature and pH on the heat resistance of spores of *Bacillus cereus*. *Food Microbiol.* 15: 625-630 (1998)
9. Marie D, Partensky F, Jacquet S, Vault D. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl. Environ. Microb.* 63: 186-193 (1997)
10. Cangiano G, Sirec T, Panarella C, Isticato R, Baccigalupi L, De Felice M, Ricca E. The *sps* gene products affect the germination, hydrophobicity, and protein adsorption of *Bacillus subtilis* spores. *Appl. Environ. Microb.* 80: 7293-7302 (2014)
11. Patakova P, Linhova M, Vykydalova P, Branska B, Rychtera M, Melzoch K. Use of fluorescent staining and flow cytometry for monitoring physiological changes in solventogenic clostridia. *Anaerobe* 29: 113-117 (2014)
12. Reis A, da Silva TL, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ. Monitoring population dynamics of the thermophilic *Bacillus licheniformis* CCM 1034 in batch and continuous cultures using multi-parameter flow cytometry. *J. Biotechnol.* 115: 199-210 (2005)