J. Microbiol. Biotechnol. (1998), 8(2), 182–184

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

NOTE

Quantitative Counting of *Bifidobacterium* spp. in a Sample Mixed with *Lactobacillus acidophilus*

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Received: October 29, 1997

Abstract PCR was used for quantitative counting of *Bifidobacterium* spp. in a sample mixed with *Lactobacillus acidophilus* using two primer sets; one set for universal priming and the other set for *Bifidobacterium* specific priming. DNA products from two independent PCRs with DNA extracted from the mixed sample were found to be easily distinguishable from each other by agarose gel electrophoresis. The concentrations of PCR products correlated with the total number of bacteria and with the number of *Bifidobacterium* spp. present in the sample.

Key words: *Bifidobacterium*, quantitation, PCR

Bifidobacterium spp. are gram positive, strictly anaerobic, fermentative rods, often Y-shaped or clubbed at the ends. It has become clear that *Bifidobacterium* spp. constitute one of the major organisms in the colonic flora of healthy children and adults [10]. A number of studies have demonstrated that *Bifidobacterium* spp. can inhibit the growth of putrefactive bacteria by decreasing the pH of the intestine, and subsequently help the immune system [3, 4]. As the interest in human health becomes widespread, these bacteria have been increasingly applied industrially as supplements in dairy products. Therefore, convenient and accurate cell counting is required [3]. To date, enumeration of *Bifidobacterium* spp. in samples containing other microorganisms has not been easy because the selective method used so far is not reliable [1]. In addition, the method is dependent upon morphological examination of colonies formed on a solid medium which is time-consuming and labor intensive [1]. The purpose of this study was to examine the possibility of quantitative counting of *Bifidobacterium* spp. in a sample containing other microorganisms by using the polymerase chain reaction (PCR). PCR is a method used to detect target DNA sequences using specific primers [5]. Recently, this method has been widely used to detect pathogens and unculturable organisms not only qualitativly, but also quantitativly [6, 7].

To make a mixed culture sample we used two strains. *Lactobacillus acidophilus* was isolated from a commercial provider (Pro-Biotics Acidophilus, Nutrition Now Inc., Vancouver) and *Bifidobacterium longum* was obtained from the American Type Culture Collection. The PCR solution included template DNA, 0.1 μM of each primer, 2.0 U of *Taq* polymerase, 2.5 mM $MgCl_2$, 100 mM dNTPs, and 1X PCR buffer supplied by the manufacturer. Samples were preheated for 5 min at 95°C, amplified for 35 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C, and finally elongated for 8 min at 72°C.

As shown in Fig. 1, the concentration of DNA product from a PCR was found to be quantitatively dependent on the concentration of template DNA used in an amplification reaction. When a primer set which had been designed for specific amplification of bifidobacterial 16S rDNA [9] was used in an array of PCRs with decreasing concentrations of bifidobacterial template DNA, the concentrations of PCR products gradually decreased. In agarose gel, as little as 100 pg of template DNA could be used to visualize the PCR products (Fig. 1, lane 4). In order to quantitatively detect bifidobacterial DNA in a mixed culture we used two primer sets. One set was common for all eubacterial species and the other set was only for *Bifidobacterium* spp. (Table 1).

L. acidophilus and *B. longum* were cultured in MRS as described previously [8]. As shown in Fig. 2, the growth patterns of the two strains were measured by the spectrophotometric optical density (O.D.) at 600 nm and viable cell numbers were measured by spread counting. Based on these parameters the relationship between O.D.

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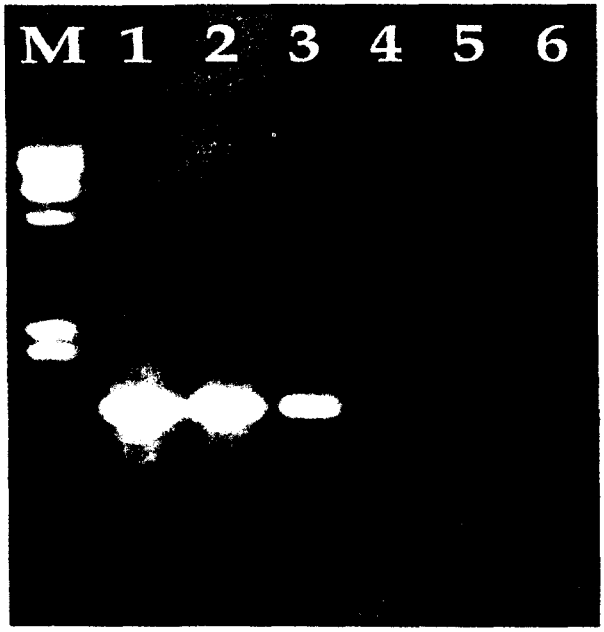

Fig. 1. Correlation between the concentration of PCR product and the amount of template DNA used.
Lane M, size marker. The amounts of template DNA used: lane 1, 20 ng; lane 2, 10 ng; lane 3, 1 ng; lane 4, 100 pg; lane 5, 10 pg; lane 6, 1 pg.

and viable cell counts was established for each strain (Fig. 2C). To prepare a sample containing two lactic acid bacterial species, cells grown separately were mixed in various mixing ratios to give a constant total cell number of 10^7 cells/ml. At greater cell concentrations, PCR showed a saturation with no increase in product DNA concentration (data not shown). It has been shown that this plateau phenomenon is caused by lack of either dNTP or primer DNA in the presence of excess template DNA [2]. The mixed culture samples were lysed and subjected to two separate PCRs. Identical volumes of the resulting PCR products were combined prior to visualization by agarose gel electrophoresis (0.8%, 70 mA, 90 min). As shown in Fig. 3, two independent PCRs resulted in two DNA bands with distinct sizes (1.5 kb and 1.3 kb) which were easily distinguishable from each other following agarose gel electrophoresis. The intensity of the DNA band (1.5 kb) obtained from PCR using the universal primer set (fD1 and rD1) was constant among all the samples of various mixing ratios. This indicates that the total number of cells (10^7 cells/ml) is well represented by this PCR. However, when the same samples were used for PCRs using the *Bifidobacterium* specific primer set (ln3 and ln26), the intensity of the DNA band (1.3 kb) correlated with the number of *B. longum* cells in the samples. The 1.3-kb DNA band was visualized only when the number of *B. longum* cells was greater than 10^4 cells/ml. Considering the amount of template DNA used in the PCR the estimated actual number of *B. longum* cells which could be detected by this latter PCR is 13 cells. However, it is likely that the detection limit can be lowered with use of Southern blot hybridization using radiolabeled probe DNA. Although we used a mixed sample of *Bifidobacterium* spp. and *L. acidophilus*, the method established in this study can be used to differentiate *Bifidobacterium* spp. from other microorganisms as well. Further experiments are underway to combine the PCRs mentioned above with a computer-based video imaging system for fast

Table 1. The two primer sets used in this study.

primer set	specificity	product size	reference
fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') rD1 (5'-AAGGAGGTGATCCAGCC-3')	most bacteria	1.5 kb	[9]
ln3 (5'-CGGGTGCTICCCACTTTCATG-3') ln26 (5'-GATTCTGGCTCAGGATGAACG-3')	*Bifidobacterium* spp.	1.3 kb	[4]

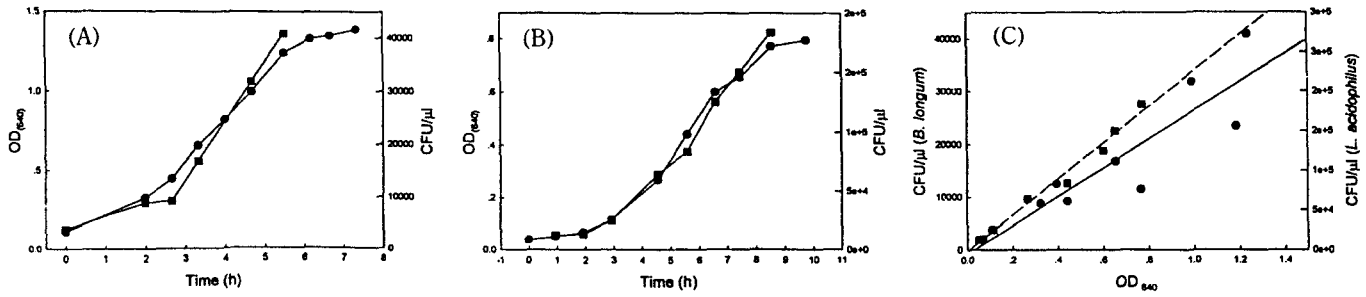

Fig. 2. Growth kinetics of *Bifidobacterium* spp. and *Lactobacillus acidophilus*.
In order to determine the cell number represented by a specific optical density, the cultures were followed with respect to the two parameters CFU and O.D. Then, the relationship between the viable cell number and the O.D. was calculated. OD(●) and CFU(■) of *B. longum* (A) and *L. acidophilus* (B) were plotted as a function of incubation time. Relationship between O.D. and CFU for *B. longum* (●, solid line) and *L. acidophilus* (■, dotted line) (C).

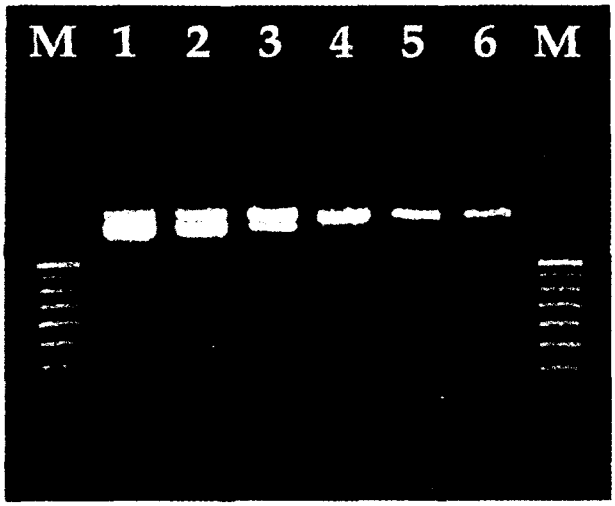

Fig. 3. Two PCR products showing varying and constant intensity depending on the mixing ratio of *B. longum* and *L. acidophilus*.

In lanes 1 through 6, decreasing numbers of *B. longum* cells were included in a mixed culture in which the total number of cells was kept constant (10^7 cells/ml). Lane 1, 100% *B. longum*; land 2, 10%; lane 3, 1%; lane 4, 0.1%; lane 5, 0.01%; lane 6, 0.001%; lane M, size markers.

and quantitative counting of *Bifidobacterium* spp. in a natural sample, for example, yoghurt.

Acknowledgments

This research was supported by a University-Industry Cooperative Research Grant from Korea Science and Engineering Foundation (95-2-04-01-01-3) and in part by a grant from Inha University (1997).

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