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In Situ Detection and Differential Counts of *Bifidobacterium* spp. Using Bromocresol Green, a pH-dependent Indicator

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Abstract The purpose of this study was to develop a simple detection method, possibly at the species-level, that allows for large-scale screening of bifidobacteria. Human fecal samples were plated on MRS-raffinose agar containing cysteine and neomycin sulfate, serving as selective pressure for bifidobacteria, and 0.003%(w/v) bromocresol green. All of the test strains grew well on this medium at 37±1°C, forming white colonies surrounded by yellow halos, which presented a sharp contrast against the green background. In this disc assay, the required incubation time to develop a yellowish zone varied with the species of *Bifidobacterium* that was tested, allowing for differential counts and easy identification at the species-level: 10-14 hr for *B. bifidum*, 20-22 hr for *B. catenulatum* and *B. infantis*, and 24-25 hr for *B. longum* and *B. breve*. No apparent color was observed for *B. angulatum* and *B. adolescentis* 28 hr after inoculation. To evaluate the results of pH indicator-based identification, individual isolates were subjected to a colony-PCR experiment with genus-specific primers. The amplified products from the isolates were in good accordance with those from the reference strains at a level of 95% agreement. These results suggest that the present method could be conveniently applied to cell counts, as well as to the preliminary identification of bifidobacteria from a variety of sample types including human feces, dairy products, and commercial probiotic supplements.

Keywords: bifidobacteria, bromocresol green, *in situ* detection, differential count, identification

Introduction

The genus *Bifidobacterium* is a representative of the Gram-positive pleomorphic strict anaerobes, of which certain species are normally present in the human intestine at numbers between (log) 9.0 and 10.5 per gram wet weight (1). It is the third largest population in the intestinal flora following the genera *Bacteroides* and *Eubacterium* (2). Bifidobacteria are also the predominant intestinal bacteria after birth and during childhood (3). The demonstrated beneficial effects of bifidobacteria on human health include immuno-modulation, nutritional benefits, the prevention of intestinal infection, and the reduction of intestinal putrefaction. There are certain difficulties in detecting and analyzing intestinal bifidobacteria, including the isolation procedure, which often relies on a selective medium, is labor-intensive, and time-consuming. Also, typing experiments that are based on multiple physiological and biochemical traits are rather cumbersome (4). Moreover, the test results are frequently inconsistent and believed to be far from perfect. The availability of an easy and inexpensive method for detecting, identifying, and enumerating *Bifidobacterium* is important within the contexts of both environmental and food microbiology. Consequently, there are strong demands for a rapid technique that enables us to analyze intestinal bifidobacteria accurately.

Several media have been postulated for selective and differential isolation (5-7). Most of them have complex compositions that include antibiotics as growth inhibitors, and require long incubation times and/or show low bacterial recovery levels. Such factors hamper the routine use of these media for monitoring the presence of bifidobacteria, a well known fecal indicator, and for the enumeration of bifidobacterial populations in dairy products (7). Currently, the comparison of 16S rRNA sequences has attracted researchers' attention as a reliable method for the classification and identification of several bacterial species. 16S rRNA-targeted hybridization probes and PCR primers enable rapid and specific detection of a wide range of bacterial species (8), and have become key procedures in the detection of many bacterial strains. To isolate and identify the *Bifidobacterium* spp. from fecal samples, Yamamoto *et al.* (9) developed species-specific oligonucleotide probes for five *Bifidobacterium* species found in the human intestinal microflora. Langendijk *et al.* (10) performed quantitative fluorescence *in situ* hybridization (FISH) of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and using strains from fecal samples. Kok *et al.* (11) reported a specific detection and analysis method for a probiotic *Bifidobacterium* strain from infant feces. Matsuki *et al.* (12) also developed a rapid identification method for human intestinal bifidobacteria using 16S rRNA-targeted species- and group-specific primers and a distribution of bifidobacterial species found in human intestinal microflora. Meile *et al.* (13) characterized the D-xylulose-5-phosphate/D-fructose-6-phosphate phosphoketolase gene (*xfp*) from *B. lactis*, and Mullie *et al.* (14) carried out multiplex PCR using 16S rRNA gene-targeted primers to identify bifidobacteria of

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human origin.

The present report describes a simple *in situ* detection and counting method for *Bifidobacterium* spp. using a MRS-raffinose agar supplemented with the pH-dependent indicator bromocresol green, and the selective pressure agent neomycin sulfate.

Materials and Methods

Isolation procedure and identification Reference strains of *Bifidobacterium* of human origin were purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). The detection and selective enumeration procedures were as follows. Infant fecal samples were collected at local clinics and transferred to test tubes containing 5 mL of MRS broth (Difco Laboratories, Detroit, MI, USA) and 0.05% L-cysteine·HCl (Sigma Chemical Co., St. Louis, MO, USA); when necessary, 10-fold serial dilutions were made using a 0.1%(w/v) peptone solution. The appropriate dilutions were plated on MRS-cysteine or MRS-raffinose (R-MRS) agar with and without 50 μg/mL neomycin sulfate. The test strains grown in the MRS-cysteine broth were fully activated by transferring the culture twice, and then were grown in R-MRS broth (pH 6.8) to reach a 0.5 optical density at 650 nm prior to plating. For the disc assay, sterile paper discs (ø: 10 mm) were inoculated with 50 μL of the active culture and placed on the R-MRS agar containing 0.003%(v/v) bromocresol green (Fisher Science, Fair Lawn, NJ, USA). To make the stock solution, 0.1 g of bromocresol green was dissolved in 20 mL of 95% absolute ethanol. The volume was then adjusted to 100 mL with distilled water. The anaerobic incubation was held using a gas generator envelope (GasPak Plus; Becton Dickinson Co., Franklin Lakes, NJ, USA) in a 2.5 L plastic anaerobic jar (Anaerocult®, Merck, Whitehouse Station, NJ, USA) at 37±1°C. The presumptive *Bifidobacterium* colonies were streaked once again on the R-MRS-cycteine agar containing bromocresol green and neomycin sulfate. Individual colonies were subjected to microscopic examination in terms of size and color, and bifido-shaped rods were tentatively considered to be members of *Bidfidobacterium.*

All of the reagents and chemicals were purchased from Sigma Chemical Co. unless stated otherwise. The bacterial growth was measured at an absorbance of 650 nm using a spectrophotometer (Optizen 2120 UV; Mechasys, Korea).

Scanning electron microscopy (SEM) Prior to SEM the broth culture was centrifuged, washed twice, and stained with 2% uranyl acetate (pH 4.0). The isolates were then observed under a scanning electron microscope (Jeol 100S; Jeol, Tokyo, Japan) and a picture taken at 80 kV.

PCR-based typing of *Bifidobacterium* spp. DNA extraction The genomic DNA from bifidobacteria was extracted according to a modified boiling lysis method (15). The bacterial cell pellet from the 5 mL culture was resuspended in 100 μL of STE (0.1 M NaCl, 10 mM Tris·Cl, pH 8.0, and 0.1 mM EDTA), and then transferred to a 1.5 mL microcentrifuge tube. Twenty μL of a freshly prepared lysozyme solution (20 mg/mL in 10 mM Tris·Cl, pH 8.0) was added and the tube was immediately immersed in boiling water for 40 sec. After cooling the tube in ice-cold water for 5 min, the viscous solution was centrifuged at 15,000×g for 5 min at 4°C and used for a PCR experiment with *Bifidobacterium* genus-specific primers.

Primer design and PCR conditions A pair of genus-specific PCR primers [Bif164 and Bif662] (11) had the following sequences: the forward primer (Bif164, 5'-GGG TGG TAA TGC CGG ATG-3'), and the reverse primer (Bif662, 5'-CCA CCG TTA CAC CGG GAA-3'). The amplification reaction was programmed as one cycle at 94 for 2 min; 25 cycles at 94°C for 1 min, 55°C for 30 sec, and 72°C for 2 min; followed by one cycle of 72°C for 7 min and then 4°C for 1 min. The amplified products were electrophoresed on a 2% agarose gel, stained with ethidium bromide solution, and visualized under UV transillumination.

Results and Discussion

In this study we describe a simple isolation procedure on R-MRS agar that allows for *in situ* detection of presumptive *Bifidobacterium* species acquired from human fecal samples, as well as environmental samples. This method is based on a color change in bromocresol green that occurs as acid is produced at various amounts over different incubation times. With the appropriate modifications, this technique may also be applied to species-specific counting of bifidobacteria.

In general, the media used for detecting bifidobacteria can be classified into the following five groups: 1) non-selective media such as MRS and Rogosa, 2) media with selective carbohydrate, 3) media with antibiotics, 4) media with propionate, 5) media with selective substances and/or a low pH. Combining media from different groups can also be applied.

In order to develop a medium with an enhanced selectivity for bifidobacteria, an MRS medium containing a single carbon source was replaced with raffinose and supplemented with neomycin sulfate. Neomycin is a broad spectrum antibiotics effective against both Gram-positive and -negative bacteria. It interferes with protein synthesis in sensitive bacterial cells for such species as *Proteus* and *Staphylococcus* (16). To isolate bifidobacteria fecal samples were suspended in MRS broth containing 0.05% L-cysteine·HCl and plated on R-MRS-cysteine agar followed by incubation under anaerobic conditions at 37± 1°C. Reference strains were selected based on their frequent occurrence in the human intestine and were plated individually onto R-MRS-cysteine agar. Our preliminary efforts showed that a yellowish halo clearly developed around individual *Bifidobacterium* colonies at different incubation time points during their anaerobic growth. The colonies were slightly larger in the presence of propionate (1.5 g/L) than in its absence (data not shown), which was also reported by Nebra and Blanch (17). The incubation time for developing a yellowish zone was species-specific among the *Bifidobacterium.* The first yellowish halo was observed for *B. bifidum.* As Fig. 1 shows, a reference *B. bifidum* required approximately 12-14 hr at 37±1°C to develop a halo, and *B. catenulatum* and

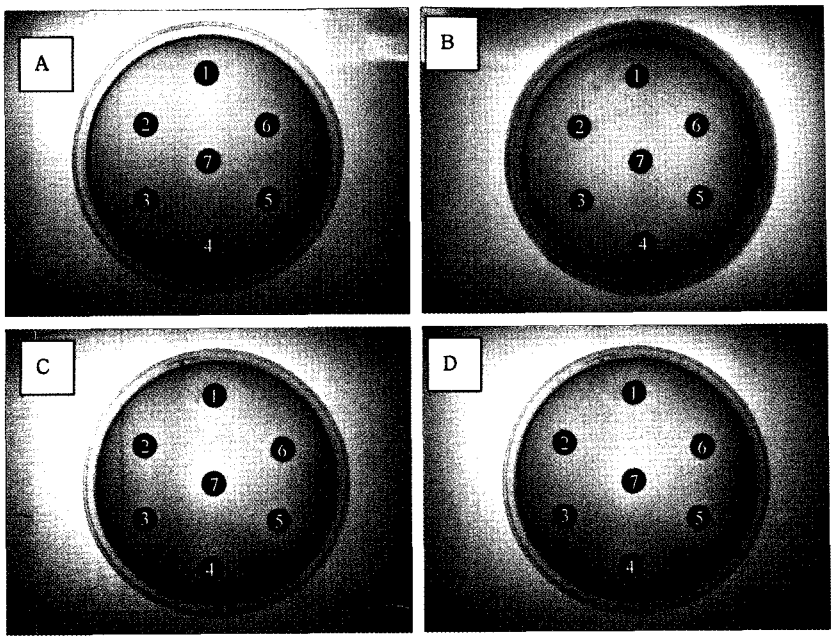

Fig. 1. Formation of a yellowish zone by seven representative *Bifidobacterium* strains on an MRS-raffinose agar containing bromocresol green during anaerobic incubation at 37±1°C. Disc: 1, *B. adolescentis*; 2, *B. angulatum*; 3, *B. breve*; 4, *B. catenulatum*; 5, *B. bifidum*; 6, *B. infantis*; 7, *B. longum*. (A) 20 hr of incubation, (B) 24 hr of incubation, (C) 26 hr of incubation, (D) 28 hr of incubation.

B. infantis required 20-21 hr. For *B. longum* and *B. breve* at least 24-25 hr were required, and 28 hr for *B. adolescentis*. No color change was observed for *B. angulatum* 48 hr following inoculation. These results imply that the tested species had different rates of growth and levels of acid production when grown on a single carbon source such as raffinose. However, when the same amount (50 μL) of actively grown culture was inoculated into R-MRS-cysteine broth, *B. angulatum* was able to change green to yellow at 48 hr of incubation (Fig. 2), but this did not occur for some of the lactic acid bacteria that we tested. The tiny colonies, which often appeared as contaminants, were easy to distinguish from the Bifidobacteria (data not shown). Moreover, *B. infantis* was clearly distinguishable from *B. longum* using the MRS-raffinose medium since *B. infantis* formed milky white colonies and *B. longum* produced green colonies at 48 hr of incubation (Fig. 3). These interesting phenomena allowed us to count the cells in a species-specific manner during incubation as well as to tentatively identify them at the species-level.

To evaluate the indicator-based identification data, the genomic DNA that was extracted from the colonies at certain time points underwent a colony-PCR experiment using a pair of genus-specific primers [Bif164 and Bif662]. Among the 57 colonies tested, 52 showed the same PCR product (520 bp in size) as the reference strains and were confirmed as bifidobacteria (Fig. 4). A SEM examination further confirmed them as bifidobacteria by exhibiting the typical pleomorphic characteristics of the genus (Fig. 5). For unknown reason the remaining 5 strains failed to produce typical band patterns representative of *Bifidobacterium* spp.

The concentration of the indicator solution was critical for the growth of bifidobacteria and the formation of color. On the MRS-cysteine agar, the growth of all the tested reference strains was not affected by the bromocresol

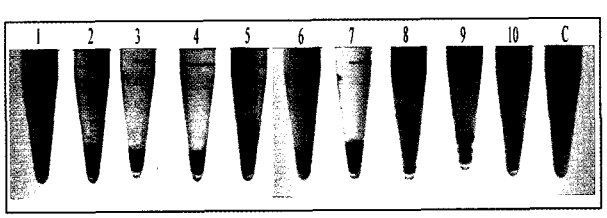

Fig. 2. Yellowish color development of the MRS-raffinose broth containing bromocresol green at 48 hr of anaerobic incubation. *Bifidobacterium* strains (1-7): 1, *B. adolescentis*; 2, *B. angulatum*; 3, *B. breve*; 4, *B. catenulatum*; 5, *B. bifidum*; 6, *B. infantis*; 7, *B. longum*. Lactic acid bacteria (8-10): 8, *Lactobacillus acidophilus*; 9, *L. plantarum*; 10, *Leuconostoc mesenteroides*; C, negative control.

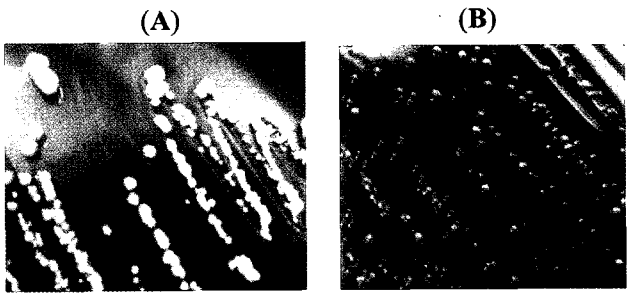

Fig. 3. Pigmentation of the *Bifidobacterium* colonies on MRS-raffinose agar containing bromcresol green for the differentiation of *Bifidobacterium* species. (A) *Bifidobacterium infantis* (white colonies), (B) *B. longum* (blue colonies).

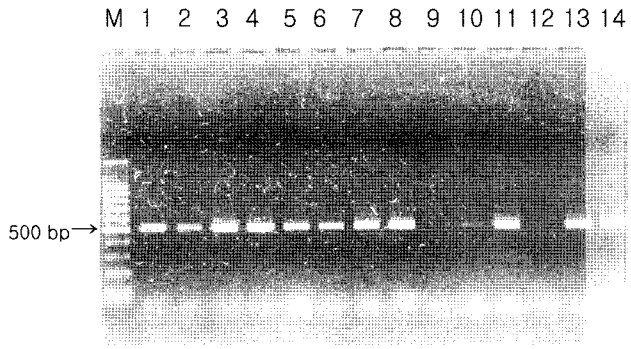

Fig. 4. Profiles of the PCR products obtained after amplification of total bacterial DNA with *Bifidobacterium* genus-specific primers. Lane M, DNA size marker 100 bp plus DNA ladder; lanes 1 to 14, suspect *Bifidobacteirum* isolates.

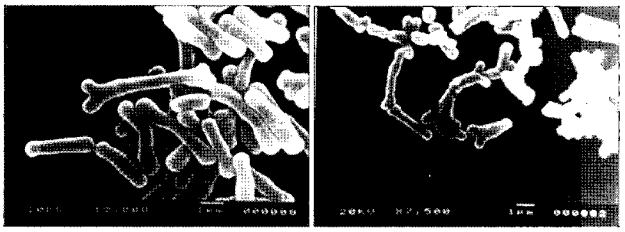

Fig. 5. Scanning electron microscopy of *Bifidobacterium longum* KCTC 3215, a reference strain (left panel), and *Bifidobacterium longum* A24 isolated from the infant stool (right panel).

green solution up to concentrations of 0.003%(w/v), whereas some reference strains didn't grow in concentrations over 0.004%(w/v). When 0.002%(w/v) was added the developed yellow halo diffused quickly and interfered with the differential bacterial counts. Thus, after repeated tests the optimal bromocresol green concentration was shown to be 0.003%(w/v). These results suggest that an R-MRS agar medium containing bromocresol green had better selectivity for *Bifidobacterium* spp., represented by *B. bifidum* and *B. longum* in mixed microbial populations from fecal samples, and reduced the time that was required for unequivocal identification.

Some species of *Bifidobacterium* have been observed in high numbers in human feces. In addtion, several studies have pointed out species-specific relationships between certain *Bifidobacterium* species and their hosts, suggesting the use of these species to determine the origin of fecal contamination (5). However, the proposal to use *Bifidobacterium* as an indicator of fecal contamination remains controversial. Nebra and Blanch (17) reported that the selectivity of BFM, a new selective medium for *Bifidobacterium* spp., did not affect the growth of 23 out of the 26 *Bifidobacterium* strains that were tested. Most of the strains showed round, blue colonies approximately 2 mm in diameter a few minutes after their removal from the anaerobic jar. To evaluate media for the enumeration of *B. adolescentis*, *B. infantis*, and *B. longum* for pure culture, Arroyo *et al.* (18) used five media: brain heart infusion agar, modified Columbia agar, reinforced Clostridial agar, modified MRS agar, and modified blood liver agar. They claimed that with slight modifications, all five media provided accurate counts for three species of bifidobacteria, i.e., *B. adolescentis*, *B. infantis*, and *B. longum*. Bifidobacteria RB and Beerens media also showed comparable result and could be used to quantify bifidobacteria in human feces (1). In addition, a phage utilizing medium and BL agar supplemented with tetracycline were developed as selective media for isolating and counting bifidobacteria in dairy products (19). Transgalactosylated oligosaccharide (TOS) was shown to be used preferentially by *Bifidobacterium* spp. and sodium propionate promoted the growth of *Bifidobacterium* while inhibiting other intestinal bacteria (20). By optimizing the medium composition and culture conditions with these compounds, the dry cell weight and the number of viable cells increased 2.5 and 1.8 times higher, respectively, than those in MRS medium (21).

Earlier observations using phenotypic or genotypic techniques reported that the most frequently isolated Bifidobacteria species in infants were *B. bifidum*, followed by *B. longum* and *B. breve* (4, 22, 23). In contrast, *B. dentium* was never identified among the infant isolates. In adults the most frequently isolated species were *B. angulatum* and *B. longum*, whereas *B. breve* and *B. bifidum* were rarely found, which was confirmed by both the phenotypic and genotypic methods (24). For both adults and infants, age-related distributions of *Bifidobacterium* species need to investigated further and the reliability of the above methods confirmed.

Overall, the data obtained in this study suggest that using bromocresol green is not only an effective, but also a convenient method for the detection and preliminary identification of bifidobacteria acquired from human samples. To increase the isolation efficiency and identification accuracy, as well as simultaneously identify *Bifidobacterium* at genus- and species-levels, this method requires further study on a discriminatory medium with more selectivity, and with PCR primers designed for multiplex PCR. This new methodology is expected to be used as a discriminatory medium for distinguishing members of *Bifidobacterium* at the species-level, and for its potential application in the screening of probiotic cultures and other dairy-related products.

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