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Selective Plating Underestimates Abundance and Shows Differential Recovery of Bifidobacterial Species from Human Feces

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The aim of the present work was to compare the efficacies and levels of selectivity of different culture-dependent and -independent methods for analyzing bifidobacteria in human stool samples. The three different culture media used here significantly differed from each other, particularly with regard to the recovery of *Bifidobacterium adolescentis*. *Bifidobacterium* medium failed to recover *B. adolescentis*; Beerens medium recovered some *B. adolescentis* organisms (17% of total bifidobacteria), whereas tomato-Eugon medium recovered mainly *B. adolescentis* organisms (58% of total bifidobacteria). A culture-independent method that combines GC fractionation of bacterial community DNA and 16S rRNA sequencing indicated that *B. adolescentis* organisms accounted for 85% of all bifidobacteria. Methodological biases, such as those described in this paper, should be taken into account in interpreting earlier studies and designing future experiments.

The abundance of bifidobacteria is a frequently used biomarker in evaluating the efficacy of health-supporting pre- and probiotics. Numerous experiments and clinical studies have been published over the past 20 years on this topic (for reviews, see references 6, 7, 11, and 22). In spite of the important status of bifidobacteria, a standard enumeration procedure is still lacking. Selective plating has been widely used for enumeration of bifidobacteria, but the number of culture medium variations used in different studies is high (4, 8, 19, 21, 26). This fact is of concern since several studies have shown that medium composition affects the total number of bifidobacteria recovered (8, 15, 19). Also, some species of the genus *Bifidobacterium* are difficult to culture, whereas some grow readily on culture media when standard laboratory procedures are followed (25). Consequently, the use of different media may lead to inconsistent results and makes the direct comparison of results among different studies difficult. In this paper we compare the selectivities, recoveries of total bifidobacteria, and species distributions on three different randomly selected media which are among the ones that have been used in the literature for enumeration of bifidobacteria. Furthermore, we apply a culture-independent approach which combines GC fractionation of bacterial community DNA (1, 2) and 16S rRNA sequencing (3, 9) (GC-16S). The Ethics Committee of the Helsinki University Central Hospital, Department of Surgery, approved the study protocol.

Viability of bacteria in fecal samples. Fresh fecal samples from 10 healthy Finnish adult volunteers (5 men and 5 women, 27 to 53 years of age) were collected and immediately transferred to an anaerobic glove box (Don Whitley, West Yorkshire, United Kingdom) (containing 80% N_2, 20% CO_2, and H_2 for catalytic O_2 removal) for further processing. To extract the bacteria, 0.5-g fecal samples were suspended in anaerobic phosphate buffer, mixed, and centrifuged at $200 \times g$; the supernatant with bacteria was recovered as described previously in detail for ileal samples from broiler chickens (1). The bacteria were pelleted by centrifugation and resuspended in 5 ml of anaerobic 0.9% NaCl solution. To maintain bacterial viability, the samples were kept strictly anaerobic throughout the protocol by performing all the steps in the glove box. Total bacterial numbers were determined by flow cytometry as described previously (3). The total fecal bacterial numbers in the samples analyzed ranged from 1.19×10^{11} to 3.03×10^{11} per g of feces (Table 1).

For the enumeration of dead bacteria, bacterial suspensions were appropriately diluted in oxygen-free physiological salt solution, and 1-ml samples were mixed with 5 µl of 0.6 mM propidium iodide (Molecular Probes Europe BV, Leiden, The Netherlands) and then incubated for 15 min in the dark. Propidium iodide is a high-affinity nucleic acid stain which labels only cells with compromised membranes that allow entry of the dye into the cell and thus identifies dead cells with no membrane potential. After being stained, the samples were removed from the anaerobic chamber and immediately analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, N.J.). While it is possible that the viability of some bacteria decreased during the sample preparation, the results of the assay would change only if the bacteria lost their membranes. The individual samples analyzed differed substantially in their proportions of dead bacteria, which ranged from 17 to 34% (Table 1). The bacteria identified by staining as dead are permanently beyond any culture-based method. The demise of bacteria as they pass towards the distal colon may be due to several reasons. Readily metabolized carbohydrates for bacterial growth are depleted, and dry matter content increases as digesta moves from the proximal towards the distal colon (10). It is likely that some bacterial types tolerate these challenging conditions in the distal colon better than others and, therefore, would be effectively enriched when they are measured as a

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TABLE 1. Count of live and dead bacteria in human feces[a]

Sample	Total no. of bacteria/g of feces (10^{11})	% Dead
1	1.19	27
2	2.38	34
3	2.10	24
4	1.78	29
5	3.03	30
6	2.62	17
7	2.34	25
8	3.00	28
9	1.80	33
10	2.59	28

[a] Bacteria were counted by flow cytometry by using Syto 24 stain for total bacteria and propidium iodide for dead bacteria. The average total number of bacteria per gram of feces (10^{11}) was 2.28; the standard deviation was 0.58. The average percentage of dead bacteria was 28%; the standard deviation was 5%.

proportion of live bacteria. Clearly, potential biases should be carefully considered in designing strategies for clinical analyses, especially when assays that rely on bacterial viability are employed.

Recovery of fecal bifidobacteria on different selective media. Fresh fecal samples from six healthy Finnish adult volunteers (three men and three women, 32 to 53 years of age) were thoroughly mixed in the anaerobic glove box to form a pooled sample which was used in all subsequent analyses. For selective plating, the sample was serially diluted in anaerobic brain heart infusion broth, and identical subsamples from the same dilution series were plated in duplicate on *Bifidobacterium* medium (BFM) (19), Beerens (BS) medium (4), and tomato-Eugon (TE) medium (13). It should be noted that the three media used in this study were arbitrarily chosen examples of the numerous "bifidoselective" media described in the literature (4, 8, 19, 21, 26, 27). Plates were incubated under anaerobic conditions at 37°C. Table 2 shows the colony counts after 7 days of incubation. The total bacterial density measured by flow cytometry, 1.6×10^{11} per g of feces, was used to calculate the recovery percentages of bacteria on the three different media. Viable counts on BFM, BS medium, and TE medium represented 1.1, 1.8, and 4.0% of the total bacteria, respectively, with the absolute numbers ranging from 1.7×10^9 to 6.5×10^9 per g (Table 2). All three media contained the same amount of propionic acid (0.5%, vol/vol), a factor that is believed to select for bifidobacteria. The highest recovery was found with TE medium, which also had the highest pH, 6.0.

Clinical studies so far have monitored total numbers of bifidobacteria, not individual species of the genus. If different bifidobacterial species have different health effects, this widely used approach is not meaningful. The importance and effects

TABLE 2. Fecal bifidobacterial counts by selective plating

Bifidoselective medium	Bacterial density (no./g [fresh wt])	% of total bacteria[a]
BFM	1.7×10^9	1.1
BS medium	2.9×10^9	1.8
TE medium	6.5×10^9	4.0

[a] Total counts were analyzed by flow cytometry. The count of total bacteria was 1.6×10^{11} (100% for the purpose of determining percentages of total bacteria).

TABLE 3. Distribution of bifidobacterial species by different enumeration methods

Species	% of total bifidobacteria[a]			
	BFM	BS medium	TE medium	GC-16S
B. longum	100	83	42	15
B. adolescentis	0	17	58	85

[a] At the 95% confidence level, the abundance of *B. adolescentis* on BFM and BS medium did not differ significantly, while the abundance on TE medium was higher than that on BFM or BS medium ($P < 0.05$). The GC-16S method allowed for a higher abundance of *B. adolescentis* than any of the culture-based methods ($P < 0.05$).

of different species can be assessed only when we have developed and employed improved identification methods for a period of time and accumulated sufficient information regarding individual species in clinical trials and epidemiological studies. In the present study we identified the organisms assumed to be bifidobacteria growing on the three selective media by randomly picking 15 colonies from each medium, subculturing them in the same medium, and purifying the total genomic DNA with a Dynabeads DNA Direct kit (Dynal A.S, Oslo, Norway) according to the instructions of the manufacturer. Partial 16S rRNA was sequenced (MWG Biotech, High Point, N.C.) with the highly conserved 16S and 18S rRNA-specific primers 536f and 907r (9). By sequencing 15 isolates, we could detect the bacteria that comprise ≥20% of all growth on the medium at a 95% confidence level. The resulting sequences were compared to the corresponding sequences of bifidobacterial type strains deposited in the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/analyses_preview .html) and in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Two different bifidobacterial phylotypes were found among the isolates (Table 3). One of them represented *B. adolescentis*, and the other one represented a *Bifidobacterium longum-Bifidobacterium infantis-Bifodobacterium breve* homology group. Isolates with 100% sequence homology to *B. longum*, *B. infantis*, and *B. breve* (the sequence for all these species is identical in this region) were further characterized by PCR using species-specific primers and the protocol published previously (16). All these isolates were identified as *B. longum*. BFM appeared to be strictly selective for bifidobacteria, whereas BS medium also recovered representatives of the genus *Ruminococcus* (20% of the colonies analyzed) and TE medium recovered representatives of the genus *Eubacterium* (8% of the colonies analyzed) in addition to bifidobacteria. BS medium, the least selective of the tested media, has been reported also by others to capture bacteria other than bifidobacteria and to underestimate total numbers of bifidobacteria (8). The most bifidoselective of the tested media, BFM (Table 3), did not recover any *B. adolescentis* organisms at all, whereas the other two media did. Although *B. adolescentis* organisms were detected on BS medium and not on BFM, the relative abundances of this bifidobacterial species on BFM and BS medium were not significantly different from each other ($P > 0.05$). However, TE medium captured significantly more *B. adolescentis* organisms than were captured by the other two media used ($P < 0.05$). Indeed, it is possible that *B. adolescentis* is more sensitive than *B. longum* to low pH and, therefore, was recovered well only on the high-pH TE medium.

Bifidobacterial quantification by GC-16S. In addition to using selective plating, we analyzed bifidobacteria from the same sample by a method totally independent of culturing, percent G+C fractionation combined with 16S rRNA sequencing. In percent G+C fractionation, different taxa sort to different positions during equilibrium density gradient centrifugation, and, thus, to different fractions, based on the percent G+C content of their chromosomal DNA. Any fraction with chromosomes with the selected percentages of G+C (in this study, 55 to 65%, which are relevant for bifidobacteria) can be further analyzed by the 16S rRNA sequencing approach to detect, at high resolution, the species comprising the selected percentages of G+C. The protocol used was previously described. In brief, bacteria were recovered by differential centrifugation (1) and lysed by a combination of physical (bead-beating), chemical (sodium dodecyl sulfate), and enzymatic (lysozyme and proteinase K) steps, a process which has been shown to lyse more than 99% of the bacteria present (1). The DNA isolated was fractionated according to GC percentage by bisbenzimidazole-CsCl equilibrium density gradient centrifugation (1). The fraction with 55 to 65% G+C, which contains DNA from the species of the genus *Bifidobacterium* (25), was subjected to further phylogenetic analysis. Partial 16S rRNA gene fragments were amplified with primers 536f and 907r and subsequently cloned and sequenced as described previously (3). All bifidobacterial 16S sequences detected represented the same *B. longum* and *B. adolescentis* phylotypes and were 100% identical to the sequences obtained from isolates on the selective plates.

The abundance of organisms with the specific bifidobacterial sequence Bif$_x$ (number of cells per gram [fresh weight]) in the fecal sample was calculated with the formula

$$\text{Bif}_x = \text{TotFlowCount} \times \left(\int_{55\%}^{65\%} \middle/ \int_{20\%}^{80\%}\right)$$

$$\times \;(\text{Bif}_x\text{Seq}_{55-65\%}/\text{TotSeq}_{55-65\%})$$

where TotFlowCount is the flow cytometric count of total bacteria per gram of feces (fresh weight), $\int_{55\%}^{65\%}$ is the integral of the 55 to 65% fraction in the percent G+C profile, $\int_{20\%}^{80\%}$ is the integral of the total percent G+C profile (20 to 80% G+C), Bif$_x$Seq$_{55-65\%}$ is the number of copies of a specific bifidobacterial Bif$_x$ sequence in the 55 to 65% fraction, and TotSeq$_{55-65\%}$ is the total number of sequences analyzed in the 55 to 65% fraction. The culture-independent GC-16S approach indicated an 85% relative abundance of bifidobacteria that were *B. adolescentis*, which was significantly higher than was obtained by any of the culture media tested (Table 3).

Absolute numbers of the two different bifidobacteria were calculated for different selective media by using the data presented in Tables 2 and 3. The estimated number of bacteria belonging to *B. longum* was close to 2×10^9 per g of feces and practically independent of the analytical approach used (Fig. 1). Conversely, the numbers of bacteria identified as *B. adolescentis* were highly dependent on the analytical method used, ranging from below the detection limit (<20% of total colo-
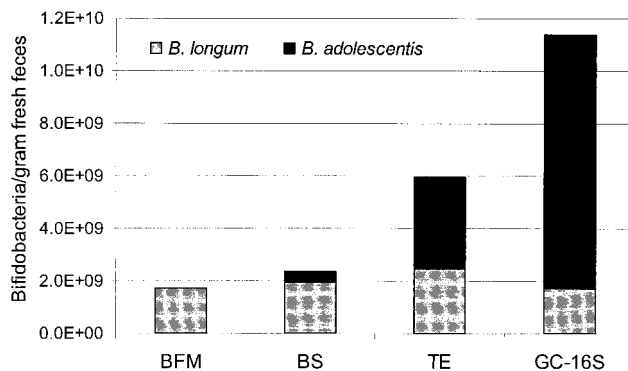

FIG. 1. Effect of method on observed number of major bifidobacteria in human feces. BFM, BS medium, and TE medium are different bifidoselective media. GC-16S values are based on direct extraction of DNA from feces, percent G+C fractionation, and 16S rRNA sequencing as described in detail previously (1, 3).

nies) to 10^{10} per g of feces (Fig. 1). TE medium recovered bifidobacteria with the greatest efficiency of all media tested, recovering 3.7% of total bacteria or 6×10^9 per g of feces, which was more than twice the number obtained with the second-best BS medium but only half of the number of bifidobacteria estimated by the culture-independent GC-16S method.

Species specificity of the bifidobacterial recovery. When we categorized the types of bifidobacteria present, it became obvious that the differential recovery of total bifidobacteria was due to the fact that all of the culture media, to various degrees, failed to effectively recover *B. adolescentis*. The recovery rate for this bacterium on BFM was below detection; 100% of the isolates were identified as *B. longum*, whereas 85% of the total bifidobacteria were identified as *B. adolescentis* by the culture-independent approach. TE medium recovered *B. adolescentis* relatively well, indicating that 58% of the total bifidobacteria belonged to this species. We acknowledge that counting absolute numbers of bacteria by the GC-16S method may have biases arising from PCR and cloning. Nevertheless, enumeration of the *B. longum* cells was highly consistent; all the methods, including GC-16S, gave practically identical numbers, ~2 $\times 10^9$ cells per g of feces. We showed above that one-third of the total fecal bacteria were dead. It is possible that the fraction of injured bacteria is even higher and that the recovery of this fraction is particularly affected by the selection of the culture medium. The fact that the numbers of *B. longum* cells were the same by the culture-dependent and -independent methods suggests that *B. longum* represents a group of bacteria that tolerate passage through the colon well and can, therefore, be readily recovered also by culture-based methods. It is possible that this is not the case for *B. adolescentis*, and therefore its abundance has likely been underestimated where culture-based methods have been used, at least in the case of the media tested here. In some other reports *B. adolescentis* has been shown to grow on BFM. However, the percent recovery of *B. adolescentis* organisms on BFM was almost two logs lower than with some other bifidoselective media (19), and the species has been shown to be more troublesome to grow than other bifidobacteria (19, 27). Further, relative rates of recovery of fecal

bacteria may also depend on the sampling procedure, freshness of samples, rate of transit of digesta in the colon, etc. With no standardized protocol for processing samples and synthesizing media, there might be subtle differences in experimental results among laboratories, even when the same medium is employed.

Molecular methods for bifidobacterial diagnostics. PCR-based detection and quantification methods using species-specific 16S rRNA gene-based primers are gradually becoming common in the identification of bifidobacteria. Several papers have described molecular methods for the identification of bifidobacterial isolates from selective plates (14, 17, 18). However, those approaches do not eliminate concerns about bias caused by inefficient recovery when culture-based methods are used. To mitigate these concerns, analytical methods totally independent of culturability have also been developed. These are based on PCR, denaturing gradient gel electrophoresis, or fluorescent in situ hybridization analysis of bacteria or bacterial DNA directly extracted from fecal samples (5, 12, 16, 20, 23, 28).

Molecular techniques do not depend on having knowledge of the community composition and specific growth requirements of individual taxa and thus appear to have some advantages over culture-based methods for detecting the total diversity of intestinal bacterial populations. Further, bacterial populations of interest do not need to be viable or culturable as long as their nucleic acids are sufficiently intact to facilitate molecular detection strategies. It should be realized, however, that molecular approaches also have limitations. For example, many molecular techniques rely on the effective recovery of reasonably intact DNA from the constituent populations of the community. Yet, there is little knowledge available regarding rates of degradation of chromosomal DNA once a bacterium has died (i.e., the membrane potential has been lost). Thus, gastrointestinal microbial community DNA for analysis should be isolated from fresh samples as soon as possible. Methods of quantification based on the detected abundance of 16S rRNA genes should also consider different copy numbers of rRNA operons and genes in different bacterial chromosomes, which may affect the apparent relative abundance of bacteria in the sample. *B. adolescentis* has been reported to carry five copies of the 16S rRNA gene (23). The sequence of the total genome of *B. longum* was recently published and appeared to carry four copies of the 16S rRNA gene (24) (GenBank accession no. NC_004307). With the above-mentioned 16S rRNA copy numbers, the relative abundance of *B. adolescentis* organisms to *B. longum* organisms may be slightly overestimated ($\sim$20%) when a 16S rRNA gene-based quantification method, such as GC-16S in this study, is applied.

The present study was motivated by inconsistent reports in the literature over the past 20 years of the effects of prebiotics on bifidobacteria. At this point, we cannot take any stand for or against the importance of bifidobacteria as health indicators; the evidence published to date based on a variety of media and/or techniques is insufficient. However, conclusive evidence can be obtained only if the methods used for bifidobacterial analysis are consistent and comparable and provide accurate information on the densities of different bifidobacterial species in clinical studies and epidemiological surveys.

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