Differentiation of Ingested and Endogenous Bifidobacteria by DNA Fingerprinting Demonstrates the Survival of an Unmodified Strain in the Gastrointestinal Tract of Humans

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ABSTRACT

Consumption of bifidobacteria as a dietary adjunct has received considerable attention for its possible role in the maintenance of gastrointestinal health. However, speculation exists about the benefits of these organisms because of an inability to assess the fate and mechanism of action of ingested bifidobacteria. Thus, our objective was to examine the fate of ingested bifidobacteria through the gastrointestinal tract. Variations in the highly conserved 16S ribosomal DNA (rDNA) of bifidobacteria from six male subjects (18 to 35 y old) were assessed by restriction fragment length polymorphism (RFLP) analysis. During the 16-d study, 10^10 colony-forming units (CFU) of a commercially available bifidobacteria were delivered to subjects in fluid milk for each of 8 d. During the remaining 8 d, subjects consumed milk without bifidobacteria. Feces were collected at 4-d intervals and plated on selective media. For each subject, 10–15 colonies were randomly selected and used as template for PCR-amplification of 16S rDNA. 16S rDNA was restriction digested and resolved by electrophoresis. The 16S rDNA-RFLP of the ingested bifidobacteria was unique compared with bifidobacteria found in subjects prior to the feeding study. When subjects consumed bifidobacteria, a 16S rDNA-RFLP identical to that of the ingested bifidobacteria was observed in feces. The concentration of the ingested bifidobacteria in feces increased to 67.2 ± 8.5% (mean ± SEM) of total bifidobacteria. After feeding stopped, the ingested bifidobacteria diminished and became undetectable. Using this molecular approach to monitor ingested bifidobacteria, we demonstrate the kinetics of passage of this organism through the gastrointestinal tract of healthy humans. J. Nutr. 127: 89–94, 1997.

KEY WORDS: bifidobacteria • probiotics • gastrointestinal tract • 16S rRNA • humans

The use of live microbes as dietary adjuncts or “probiotics” is a subject of intense and growing interest. Probiotics have been defined as living organisms that, when included as a part of the diet, confer some favorable effect on the host (Fuller 1991). The proposal that the ingestion of certain viable organisms may provide some health benefits has incited numerous recent investigations into this area. Several reports implicate probiotics in the alleviation of problems of nutritional importance, including lactose maldigestion (Jiang et al. 1996), colon cancer (Rafter 1995) and increased serum cholesterol (Li et al. 1995). However, the extent of reported benefits ascribed to probiotics extends beyond the realm of immediate nutritional importance and encompasses a wide variety of human health problems, including the prevention of gastrointestinal and vaginal infections (Elmer 1996) and the enhancement of local and systemic immune response (Hatcher and Lambrecht 1993, Yasui and Ohwaki 1991) as well as a variety of others (Fuller 1991).

Among the organisms used as probiotics are microbes in the genus Bifidobacterium, which are also major components of the human intestinal microflora (Simon and Gorbach 1984). This growing interest in the health benefits of bifidobacteria has prompted the inclusion of these organisms in many dairy foods instead of an increase in their consumption (Puhan 1990). However, the possible prophylactic and therapeutic properties of these foods containing bifidobacteria are a matter of much speculation (O’Sullivan et al. 1992) because there are inherent difficulties in obtaining definitive evidence for proposed effects of ingesting exogenous bifidobacteria. One difficulty is the presence of endogenous bifidobacteria in the gastrointestinal tract and feces of humans, which complicates the task of differentiating these from ingested bifidobacteria.
and unequivocally demonstrating the survival of ingested bifidobacteria through the gastrointestinal tract.

To monitor ingested bifidobacteria, Bouhnik and co-workers (1992) isolated a streptomycin- and rifampicin-resistant variant of a *Bifidobacterium* species, which could be distinguished from endogenous bifidobacteria. Although this method of monitoring ingested bifidobacteria allows for convenient differentiation from endogenous organisms, it may increase the dissemination of antibiotic resistance, and mutants may have secondary pleiotropic characteristics that are a common feature of streptomycin- and rifampicin-resistant mutants (Abad and Amils 1994, Blanc-Potard et al. 1995, Ryu 1978). As an alternative to differentiating endogenous and ingested bifidobacteria with antibiotic resistance, we used an analysis of the 16S rDNA (rDNA) to monitor ingested bifidobacteria. Of the molecules used for characterization and identification of prokaryotes, this molecule has proven most useful because of its high information content, highly conserved nature and universal distribution (Lane et al. 1985, Woese and Fox 1977). Further, targeting DNA to accomplish this identification and differentiation offers the advantages of rapidity, reliability and sensitivity over classical strain identification methods. Therefore, the objective of this study was to obtain data on the fate of ingested bifidobacteria in humans as a first step in assessing the physiological importance of ingested bifidobacteria. To this end, we used a molecular biological approach that exploits existing genotypic characteristics to characterize an unmodified, food-grade, commercially available strain of bifidobacteria and used these characteristics to distinguish between this food-grade, exogenous bifidobacteria and endogenous fecal bifidobacteria in healthy male subjects throughout the course of a controlled feeding study.

**MATERIALS AND METHODS**

**Subjects and sample handling.** Information about the subjects is available in Table 1. None of the subjects were consuming or had consumed, for approximately 4 mo, products containing bifidobacteria. The six 18- to 35-y-old healthy non-smoking males had no history of gastrointestinal disorders, nor had they used antibiotics in the previous year. Subjects were instructed to maintain their usual dietary and exercise habits. The study protocol and informed consent forms were approved by the Institutional Review Board, Human Subjects Committee, University of Minnesota. Following a pre-study fecal sample collection (no bifidobacteria ingested), subjects consumed 750 mL of skim milk containing 1 g (approximately 10¹⁰ CFU) of a commercially available bifidobacteria [hereafter referred to as ingested bifidobacteria (IBF)] daily for 8 d. At 4-d intervals, during bifidobacteria feeding and after the cessation of bifidobacteria feeding, fecal samples were collected from the subjects. The freshly collected fecal samples were immediately homogenized in sterile 0.1% peptone water. Serial dilutions of the sample were prepared and spread plated on BIM-25 (Munoa and Pares 1988). Sterile reinforced clostridial medium (RCM) (Difco Laboratories, Detroit, MI) was prepared with added selective and differential agents as follows: polymixin B sulfate (10 mg/L), kanamycin (50 mg/L), nalidixic acid (22 mg/L), iodoacetic acid (22 mg/L) and 2,3,5-triphenyltetrazolium chloride (22 mg/L). Plates were incubated in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere containing 5% CO₂, 10% H₂ and 85% N₂ at 37°C for 70 ± 4 h, and 10–15 colonies were immediately homogenized in sterile 0.1% peptone water to create the 16S rDNA RFLP, were resolved and unequivocally demonstrating the survival of ingested bifidobacteria through the gastrointestinal tract.

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<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>28.1 ± 1.3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>172.9 ± 2.0</td>
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<td>Body mass index, kg/m²</td>
<td>26.2 ± 1.5</td>
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TABLE 1: Characteristics of male subjects

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1 Values are means ± SEM, n = 6.

Abbreviations used: CFU, colony-forming unit; IBF, ingested bifidobacteria; PCR, polymerase chain reaction; rDNA, DNA encoding rRNA; RFLP, restriction fragment length polymorphism.

RESULTS

Before conducting this study, we screened more than 200 fecal bifidobacterial isolates from 20 volunteers not consuming bifidobacteria and were unable to find a 16S rDNA RFLP with the endonuclease *Hae*III that matched that of IBF (data not shown). Similarly, upon RFLP analysis of amplified 16S rDNA of bifidobacteria from the six subjects in this study, we observed that none of the fecal bifidobacterial isolates from these individuals showed a RFLP profile that was identical to that of the strain (IBF) found in a variety of commercial dairy products and which was to be ingested in the study.

**Polymerase chain reaction and restriction fragment length polymorphism of 16S rRNA genes.** To prepare the portion of the colony for PCR, the sterile microcentrifuge tube containing the colony portion was microwave thawed for 7 min at 600 W per a modification of a procedure described by Bollet and co-workers (1991). Following this microwave lysis, PCR reagents were added to the microcentrifuge tube. Amplification of template DNA was performed in 30 μL of a solution containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 1.0 mmol/L of each of the four dNTP, 1.0 μmol/L of each of the two primers and 1 U Taq DNA Polymerase (Perkin-Elmer/Cetus, Norwalk, CT). Universal 16S rRNA primers (Lane et al. 1985) were used to direct amplification. Primer sequences were as follows: forward primer (5'-CAACGCGCCTGGTGATTC-3') and reverse primer (5'-ACGGGCGGTGTGTRC-3'). The approximately 900-bp sequence between primers corresponds to the nucleotide numbers, 519 through 1406, based on the 16S rDNA sequence of *Escherichia coli*. Amplification of DNA was performed in a GeneAmp® PCR System 2400 thermal cycler (Perkin-Elmer) programmed for 5 min at 94°C (initial denaturation) and 35 cycles of 15 s at 94°C (denaturation), 30 s at 57°C (annealing), 90 s at 72°C (extension), followed by 10 min at 72°C (final extension). Reaction products were resolved by electrophoresis in 1.0% agarose gels and visualized by ethidium bromide staining. The PCR product of interest was isolated by an electrophoretic elution method described by Hansen et al. (1993). Approximately 750 ng of newly purified 16S rDNA fragment was digested with 2 U of the restriction endonuclease *Hae*III (Promega, Madison, WI). The digestion products, which served as the 16S rDNA RFLP, were resolved by electrophoresis in 1.5% agarose gels and visualized by staining with SYBR™ Green I Nucleic Acid Gel Stain (FMC BioProducts, Rockland, ME).

**Data expression and statistical analysis.** The appearance and disappearance of IBF from the feces of subjects at various intervals during the study are expressed as the number of randomly selected colonies with a 16S rDNA RFLP matching that of IBF divided by the total number of colonies that were randomly selected. To assess the impact of the duration of bifidobacteria feeding, mean percentages of the ingested bifidobacteria and mean total bifidobacteria counts at the various time points were compared by a repeated-measures ANOVA (Steele and Torrie 1980). For those variables with significant overall treatment effects, differences among individual time points were identified with the Student-Newman-Keuls multiple range test (Steele and Torrie 1980). Data are expressed as means ± SEM.
appearance and disappearance of IBF from the feces of subjects are shown in Figure 1. The 16S rDNA RFLP of the IBF was not detected at pre-study (0 d) in any subject but quickly rose to 57.8 ± 9.2% of colonies after 4 d and 67.2 ± 8.5% at 8 d. Four days after IBF feeding stopped, the proportion of isolates with a 16S rDNA restriction profile identical to that of IBF fell to 15.8 ± 12.2%, and after 8 d it was not detectable. RFLP = restriction fragment length polymorphism.

FIGURE 1 Appearance and disappearance of ingested bifidobacteria (IBF) in the feces of six healthy men before, during and after consumption of exogenous bifidobacteria. Values are means ± SEM and are expressed as ingested bifidobacteria as a percentage of total bifidobacteria. Points denoted with different letters (a or b) differ significantly (P < 0.001). RFLP = restriction fragment length polymorphism.

DISCUSSION

In this study, we sought to monitor the appearance and disappearance of bifidobacteria during and after feeding, respectively. To accomplish this, we employed a molecular method to distinguish between endogenous bifidobacteria and an unmodified, ingested food-grade bifidobacteria. The strain of bifidobacteria ingested in this study is found in commercial dairy products. Our inability to detect an endogenous RFLP that matched IBF suggested that this food-grade strain would allow us to monitor the kinetics and transit through the gastrointestinal tract of these unmodified, ingested bifidobacteria.

The presented observations demonstrate that colonies of bifidobacteria chosen from feces of subjects consuming IBF have a 16S rDNA RFLP that is identical to that of IBF, suggesting that this strain can survive passage through the gastrointestinal tract. The proportion of selected colonies with RFLP matching that of IBF rose quickly during feeding and became the predominant strain of bifidobacteria after 8 d of feeding. Conversely, when IBF feeding was stopped, the proportion of the ingested organism in the feces dramatically diminished and was not detectable after 8 d. These results compare favorably with the findings of Bouhnik et al. (1992), who monitored a spontaneous antibiotic-resistant strain of bifidobacteria and were unable to detect the ingested bifidobacteria 8 d after the cessation of ingestion.

The increase in total fecal bifidobacteria population follows the consumption of IBF. This suggests that the increase in total population was brought about by an increase of fecal IBF, whereas the decline in total fecal numbers after the cessation of feeding was the result of the rapid elimination of IBF. The modest nature of this increase, which was presumably induced by IBF consumption at 10^10 CFU/d, also suggests that only a fraction of IBF survive passage through the gastrointestinal tract. This is consistent with the results of Bouhnik et al. (1992), who recovered only 30% of the oral load of bifidobacteria in the feces.

We have demonstrated that ingested bifidobacteria can survive passage through the gastrointestinal tract into the feces. Clearly, this is an important attribute of an ingested bifidobacteria. Fecal populations of bacteria are considered to be accurate indices of the bacterial populations in the gastrointestinal tract (Moore et al. 1988, Simon and Gorbach 1984). Thus, our inability to detect IBF 8 d after the cessation of feeding suggests that the ingested bifidobacteria are not colonizing the gastrointestinal tract to a significant extent. These results compare favorably with the findings of others (Bouhnik et al. 1992, Mangin et al. 1994). This may be because the human intestine is somewhat resistant to colonization by exogenous bifidobacteria or that the ingested organisms are not suited to this ecological environment. If one assumes that increasing fecal bifidobacteria is the primary requisite for achieving probiotic effects, the findings presented in this study lend credility to the notion that colonization may be unnecessary. However, it has been suggested that colonization is a highly desirable trait for an ingested probiotic organism (Klaenhammer 1982, O'Sullivan et al. 1992). Colonization would offer the advantage of allowing the ingested organism to maintain an adequate concentration to affect its environment without constant replenishment. Additionally, colonization may allow for a more appropriate response to stimuli or the occupation of adherence sites that inhibit pathogen-cell interactions (Bernet et al. 1993). If the ingested bifidobacteria are not capable of colonization, then a rational and systematic approach to the selection of strains of bifidobacteria to be included in dairy products may help to identify strains that are suited for colonization in this complex ecosystem.

We demonstrated in this study that a commercially available strain of bifidobacteria, delivered to subjects in an unmodified and non-mutant form, can be used to study the passage of ingested bifidobacteria through the human gastrointestinal tract. The results of this balance study show that these ingested...
FIGURE 2  16S rDNA restriction fragment length polymorphism (RFLP) of bifidobacteria from a representative male subject at 0 d (A), 4 d (B) and 8 d (C) of ingested bifidobacteria (IBF) consumption. Figures are 2.5% agarose gels of SYBR™ Green I stained, HaeIII digested 16S rDNA. Lane 1 of each gel is 100-bp DNA Ladder (Promega Corp., Madison, WI). Symbols above each lane are as follows: L = ladder; E = endogenous RFLP; I = ingested RFLP. Percentage values on the right of each gel are the percentage of isolates with a 16S rDNA RFLP that is identical to that of IBF.
FIGURE 2 Continued

FIGURE 3 Total fecal bifidobacteria concentrations in six men before, during and after ingestion of exogenous bifidobacteria (IBF). Values are means ± SEM. Points denoted with different letters (a or b) differ significantly ($P < 0.05$).
organisms can be recovered in the feces of subjects but do not seem to colonize the gastrointestinal tract. The procedures developed to conduct this balance study are effective and rapid. Additionally, this approach should enhance investigations on the health effects of this organism as well as facilitate the elucidation of purported health effects attributed to the ingestion of bifidobacteria.

LITERATURE CITED