Increase of Intestinal *Bifidobacterium* and Suppression of Coliform Bacteria with Short-Term Yogurt Ingestion

R. M. CHEN,*† J. J. WU;§ S. C. LEE,* A. H. HUANG,* and H. M. WU†

*Department of Pathology and †Department of Medical Technology, National Cheng Kung University Medical College, Tainan, Taiwan, Republic of China

ABSTRACT

To determine whether ingestion of yogurt would alter human intestinal bacterial composition and whether *Bifidobacterium* numbers would increase in the intestine, 34 healthy volunteers were studied. The experimental period was 26 d, including an initial 8 d without yogurt, 10 d with three bottles (230 ml each) of AB yogurt per day (President Enterprise Corporation, Tainan, Taiwan), and 8 d without yogurt. Stool samples were taken at 3- to 4-d intervals. The bacteria of each fresh stool sample were promptly analyzed by dilution and culture on blood, MacConkey, Center for Disease Control and NNLP agars, the agar contained nalidixic acid, neomycin sulfate, LiCl, and paromomycin sulfate for aerobes, coliforms, anaerobes, and bifidobacteria, respectively. The number of bacteria was determined as colony-forming units per gram of dried stool. Results indicated that ingestion of AB yogurt increased the counts of anaerobic bacteria, suppressed aerobic bacteria, and significantly elevated the bifidus to coliform ratio. Arbitrarily primed polymerase chain reaction was used to differentiate the identity of bifidobacteria in four volunteers before and after yogurt ingestion and confirmed that *B. bifidum* ingested from the yogurt survived and proliferated in the stool throughout the experiment. However, the elevated bifidus to coliform ratio gradually diminished and disappeared after yogurt consumption was discontinued. In conclusion, ingestion of yogurt increased the numbers of stool bifidobacteria and suppressed coliform bacteria. (Key words: yogurt, bifidobacteria, coliform bacteria)

INTRODUCTION

Yogurt is considered a health-promoting food both for its nutritional value and by definition of its content of more than $10^7$ living lactic acid bacteria/ml (8, 9, 12). The superior nutritional value is attributed to having low-fat or skim milk as the raw material and to new components formed from bacterial digestion and production. Thus, dairy proteins, less saturated fatty acids, digested lactose, and some short- and medium-chain fatty acids produced by lactic acid bacteria all contribute to its health-promoting benefits (1, 4, 8, 14, 16, 19, 25). The major benefit of living lactic acid bacteria, however, derives from their ability to survive and proliferate in the human intestine, which leads to possible competitive suppression of pathogenic aerobic bacteria, especially coliforms (1, 4, 8, 19, 27). Thus, the function of the intestine is less compromised by pathogenic bacteria; in addition to carcinogens that are possibly associated with these organisms, some enzymes and fermentation products of fat should be reduced (2, 5, 18, 20, 24, 28).

Yogurt ingestion is associated with good health and longevity (12, 16). However, research has shown that many lactobacilli do not survive when exposed to gastric acid and thus cannot function as intestinal flora or suppress pathogenic bacterial species in the intestine. Consequently, it is important to know which lactic acid bacteria added to yogurt will survive and proliferate in the human intestine and whether these bacteria can suppress pathogenic bacteria such as coliforms. Because bifidobacteria have been shown to survive in the intestine and to provide health benefits (14, 15, 18, 21, 22, 23), they were added to yogurt in the present study. In addition, neosugar, lactulose, or mixtures of fructooligosaccharides that may not be digested by human intestinal enzymes but may be used by bifidobacteria, are frequently added as bifidogenic factors (9, 27). Among the many lactic acid bacteria, we were especially interested to know whether an increase of bifidobacteria in stools may be induced by yogurt ingestion (3, 14, 27).

Among the hundreds of bacterial species in the human intestine, anaerobic bacteria, which include bifidobacteria, usually outnumber the aerobes or facultative anaerobes by 100 to 10,000 × (7, 8, 12, 13). Nonetheless, colon
From d 9 to 18 during the experiment, participants were given three bottles daily of AB yogurt (230 ml each), containing an approximately equal mixture of Lactobacillus bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, and Bifidobacterium bifidum at a concentration of at least $10^7$ living bacteria/ml. Subjects were asked to provide three stools (d 1, 5, and 8) before yogurt ingestion, three stools during yogurt ingestion (d 11, 15, and 18), and two stools after discontinuation of yogurt (d 22 and 26). Approximately 1 to 5 g of wet weight of stool were taken and transferred to a sterile bottle, sealed, and delivered to the laboratory within 1 h.

**Stool Culture**

Approximately 0.2 to 0.4 g of stool was transferred to each of two preweighed screw-capped culture tubes. These tubes were weighed again to obtain the wet weight of the stool samples. One tube was placed in a vacuum oven overnight at 65°C until the sample was completely dry. Total water evaporation was confirmed by checking a few samples to ensure no weight loss after vacuum evaporation for another 24 h. We assumed that the dry to wet ratio of the two parallel samples was the same, so that the dry weight of the fresh wet stool sample used for dilution and culture could be estimated according to the dry to wet ratio of the parallel sample. Bacterial numbers were expressed as per dry weight of stool to correct for the reside variation in water content of stools.

Fresh, wet stools sampled for dilution usually weighed approximately 0.2 to 0.4 g. A recorded volume of water, usually 1 to 3 ml, was added to the tube and mixed vigorously with the wet stool by vortex. The tube was then centrifuged at approximately 1000 g for 5 min. The supernatant was decanted into a clean tube, and the precipitate was washed with a recorded volume of water. The tube was vortexed and centrifuged, and the supernatant was pooled with the previous supernatant. This process was repeated 2 to 5 times until the supernatant was clear, which assured a near complete washing of all bacteria in the supernatant, leaving the undigested stool debris, if any, behind. The entire volume of dilution water usually ranged from 1 to 7 ml; the weight of wet stool was added to constitute the total volume of stool dilution.

A serial, 10-fold dilution was taken. For final culture, 0.1 ml of the diluted sample was removed from one of the tubes and dispersed on the appropriate agar plates. Spread plates of Center for Disease Control (CDC) agar (Becton Dickinson, Cockeysville, MD) and NNLP spread agar plates, containing nalidixic acid, neomycin-sulfate, LiCl, and paromomycin sulfate were used for anaerobic bacterial culture. All plates were incubated anaerobically at 35°C for 48 to 72 h. Blood agar plates and MacConkey plates (Becton Dickinson) were used to culture...
Aerobic organisms in a 35°C incubator with 5% CO₂ for 16 to 18 h. Bifidobacterium were identified by colony morphology, Gram stain, and use of the Rapid ID 32 system (BioMerieux Vitek Inc., Hazelwood, MO). Colony counts were used to calculate the number of bacteria per gram of dry stool weight.

**Arbitrarily Primed Polymerase Chain Reaction**

To ascertain that B. bifidum in the AB yogurt survived and proliferated in the intestine, an arbitrarily primed polymerase chain reaction (AP-PCR) was performed on bifidobacterial colonies isolated from consecutive stools of four volunteers. Nucleic acids were extracted by simple mechanical lysis of bacterial cells as described by Kirschner et al. (10). A 5-μl aliquot was used for PCR. Two primers, TB1B (5′-GCTCGCGGCGGCTGGGCTCC-3′) and ERIC-2R (5′-AAGTAAGTGACTGGGGTGAGCG-3′), were used. Samples were amplified (GeneAmp PCR System 9600; Perkin-Elmer Corp., Norwalk, CT) under the following conditions: 4 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The amplification products were separated by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a transilluminator.

**Statistical Analysis**

All the original dilution and colony count data were recorded and calculated. The bacteria count per gram of dry stool was further converted to logarithm, tabulated, and statistically analyzed by general linear procedure and Duncan’s multiple range tests for variables (26). A P < 0.05 was considered significant. Values were expressed as mean ± standard deviation.

**RESULTS**

Our measurements revealed that stool dry weight ranged from 2.3 to 70% of the wet weight. Among the 267 stool samples from 34 individuals, 43 (16%) were very wet; dry weight accounted for less than 10% of the wet weight. One hundred fifty (56%) were moderate and had dry to wet ratios between 10 and 30%. Thirty-seven (14%) were relatively dry and had dry to wet ratios between 30 and 40%. Thirty-seven (14%) were very dry and had ratios >40%. The overall average dry weight was 24.8 ± 14.9% of weight. No apparent change of dry to wet ratio occurred during or after yogurt intake, as is depicted in Table 2. Nevertheless, we found obvious differences among the 34 individuals. The dry to wet ratio varied from 9.0 ± 2.9% to 51.7 ± 11.6% for eight stool samples from each of these participants. Dry stool had more anaerobic bacteria (10¹¹/g of dry stool) and less aerobic and coliform bacteria (10⁷/g dry stool); however, this observation was not statistically significant.

It is apparent that at d 1 and before yogurt intake (d 5 and 8), the aerobic counts were approximately 10⁸.⁸⁹ to 10⁸.⁷⁷. The aerobic counts dropped to 10⁸.⁶⁶ after yogurt intake and returned to 10⁹.⁹² and 10⁹.⁰ after discontinuation of yogurt intake (Figure 1). The difference (P < 0.05) of the yogurt intake group from the other groups was significant.

The number of anaerobic bacteria amounted to approximately 10¹⁰.⁹ and 10¹¹.⁴ per gram of dried stool, which was approximately 100- to 1000-fold of aerobes (Figure 2). During the 10 d of yogurt ingestion, stool samples (d 12, 15, and 19) contained 10¹¹.¹⁹ to 10¹¹.₄₄ anaerobic bacteria, which was significantly greater than the number before yogurt intake (10¹⁰.⁹ to 10¹¹.₀⁶) and after discontinuation of yogurt (10¹¹.₀⁴ to 10¹¹.₁₃). The yogurt intake group had significantly more anaerobes (P < 0.05) (Figure 2).

Despite a few failures of Bifidobacterium to grow in CDC medium, the bifidus to coliform ratio went from 151 to 593 to 2455 after ingestion of yogurt and decreased to 210 to 249 after discontinuation of yogurt (P < 0.001) (Figure 3). Meanwhile, NNLP culture showed that the ratio went from 18 to 54 before yogurt intake to 36 to 93 during yogurt intake and decreased to 7 to 17.9 after discontinuation of yogurt consumption (P < 0.01).

The AP-PCR analysis revealed that before ingestion of yogurt, bifidobacteria in the stool of four volunteers were all of different types (Table 3 and Figure 4). Bifidobacteria from yogurt appeared in stools 3 d after ingestion of yogurt and remained up to 8 d after discontinuation of yogurt consumption, even if the number of bifidobacteria decreased after the 8-d discontinuation of consumption (Figures 5, 6, and 7).

**DISCUSSION**

Lactobacillaceae and Streptococcaceae hardly survived in the strongly acidic gastric juice with digestive enzymes and the bile salt in the duodenum. Therefore, they could not be easily cultured from stool. Anaerobes outnumber the aerobes by 10² to 10³; Bifidus is the predominant anaerobe, and Lactobacillaceae is a very minor component of aerobics. Thus, it is not surprising that Lactobacillaceae and Streptococcaceae could hardly be identified in stool. In this study we used AB yogurt, which contains L. acidophilus and B. bifidum as well as L. bulgaricus and S. thermophilus. Only B. bifidum could be repeatedly and easily identified from the stool.

Epidemiological studies reveal the association of high fat diets with increased colon cancer (2, 8, 17). Previous
TABLE 2. Stool dry to wet ratio (percentage). 1

<table>
<thead>
<tr>
<th></th>
<th>d 1</th>
<th>d 5</th>
<th>d 8</th>
<th>d 12</th>
<th>d 15</th>
<th>d 19</th>
<th>d 22</th>
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<tr>
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<td>24.5</td>
<td>24.8</td>
<td>22.7</td>
<td>22.4</td>
<td>25.5</td>
<td>23.7</td>
<td>26.7</td>
<td>28.5</td>
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<td>SD</td>
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<td>15.8</td>
<td>14.4</td>
<td>14.6</td>
<td>13.2</td>
<td>14.8</td>
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1 Number of specimens; 34 for each day.

reports (5, 17, 18, 20) showed that several hydrolytic and reductive enzymes found in human stool, such as nitroreductase, azoreductase, and β-glucuronidase, may convert bile acids and precarcinogens from animal fat into carcinogens. Aerobic bacteria, especially coliform bacteria, are considered among the possible sources of these enzymes (14, 17, 23). Reports (14, 23) indicate that addition of *L. acidophilus* may significantly reduce these enzymes in rats, probably because they lower pH and also competitively suppress coliform bacilli. Several other studies (5, 13, 18) have shown that presence of *Lactobacillus* in the intestine may suppress other carcinogen-producing organisms. It has also been reported (5, 6, 18) that certain *Lactobacillus* may decompose carcinogens such as dimethylnitrosamine and diphenylnitrosamine. Ample indications existed that the presence of lactic acid producers in the intestine may suppress pathogenic microorganisms, most likely aerobic coliform bacilli, and, thus, may suppress or decompose the carcinogens produced.

In this study, *L. acidophilus* and *L. bulgaricus* that were added to AB yogurt could not be cultured from stool. However, *B. bifidum*, a facultative anaerobic lactic acid and acetic acid producer, could be found in large numbers in the stool after yogurt ingestion and became the predominant anaerobic bacteria in the stool. Because the anti-cancer effect of lactobacilli is dependent on the activity of living organisms in the intestine, the surviving and predominating Bifidobacteria are the most likely to provide anti-cancer benefits rather than those hardly identifiable *Lactobacillaceae* or *streptococcaceae*. This study provided evidence that yogurt ingestion may remarkably increase anaerobes, predominantly bifidobacteria, but suppress aerobes, especially coliform bacilli.
Figure 3. Bifidobacterium/coliform bacilli ratio of feces sampled in the beginning, before yogurt intake, during yogurt intake, and after discontinuation of yogurt. Bifidobacterium was cultured on CDC (□) and NNLP (■) plates; coliform bacilli were grown on MacConkey agar plates. Yogurt intake group had significantly higher Bifidus/coliform ratio by both CDC \((P < 0.001)\) and by NNLP \((P < 0.01)\) culture method.

Several unresolved issues remain. First, stools of variable water content were presented in this study. The water content ranged from 30 to 97% of wet stool. The very wet stool containing over 95% water might represent diarrhea, whereas the very dry stool (containing less than 40% water) might reflect constipation or long-standing stool over 36 to 48 h. These conditions could change bacterial flora and their fermentation. Because constipation is considered one factor for colon cancer, its potential implication to carcinogenesis needs further clarification.

In this experiment, aerobic bacteria ranged from \(10^{7.5}\) to \(10^{10.4}\), averaged \(10^{9.0}\), and anaerobes ranged from \(10^{9.5}\) to \(10^{12.5}\), averaged \(10^{11.0}\) per gram of dry stool. It is apparent that there were \(10^2\) to \(10^3\) times more anaerobes than aerobes in the stool (Figures 1 and 2). It appears that dry stool with <40% water has more anaerobes and less aerobes and a higher Bifidus to coliform ratio. However, this pattern of difference was not consistent enough to draw a conclusion and needs further exploration.

In this experiment, it was apparent that NNLP agar, a selective culture medium for Bifidobacterium that would suppress all other organisms, may also suppress Bifidobacterium to a great extent. The colonies of Bifidobacterium on NNLP were much less than those on CDC agar.

<table>
<thead>
<tr>
<th>Time of AP-PCR</th>
<th>Before</th>
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<th>After</th>
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<tr>
<td>10B</td>
<td>II</td>
<td>II</td>
<td>11</td>
</tr>
<tr>
<td>10C</td>
<td>III</td>
<td>III</td>
<td>I</td>
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<td>10D</td>
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<td>10E</td>
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\(^{11}\): Bifidobacterium bifidum; II, III, IV, and V are other Bifidobacteria.
Figure 5. Arbitrarily primed polymerase chain reaction results of *Bifidobacterium bifidum* (lanes 1 and 11) and other bifidobacteria colonies from volunteers 10 d after yogurt ingestion (lanes 2 to 10) and 8 d after discontinuation of yogurt ingestion (lanes 12 to 20).

Figure 6. Arbitrarily primed polymerase chain reaction results of *Bifidobacterium bifidum* (lane 2) and other bifidobacteria isolated from volunteer 10 B before (lanes 3 and 4), during (lanes 5 and 6), and after (lanes 7 and 8) yogurt ingestion, using primer TB1B. M =.

Figure 7. Arbitrarily primed polymerase chain reaction results of *Bifidobacterium bifidum* (lane 2) and other bifidobacteria isolated from volunteer 10 B before (lanes 3 and 4), during (lanes 5 and 6), and after (lanes 7 and 8) yogurt ingestion, using primer ERIC-2R. M =.
plates. However, in a few cases, particularly in early part of this experiment, *Bifidobacterium* failed to grow on CDC plates, but many grew on NNLP plates. We have no explanation for these growth patterns on CDC plates. Whether some of the organisms on CDC plates might suppress the growth of *Bifidobacterium*, either by producing some unknown inhibitors or using up some necessary nutrients competitively, was not clear. However, the combination of our results on CDC and NNLP plates provided convincing data that *Bifidobacterium* was truly increased after yogurt intake.

From the AP-PCR analysis, it was apparent that *Bifidobacterium bifidum* ingested from AB yogurt would survive, proliferate, and become the predominant bifidobacteria in the stool. However, the preexisting bifidobacteria species in the intestine could also survive and contribute to the increase, and the added oligosaccharide was aimed to support the growth of them. At any rate, accelerated proliferation of *Bifidobacterium* and competitive suppression of coliform bacteria is an apparent effect of yogurt ingestion and this may hopefully provide an anti-cancer effect. It confirmed that yogurt ingestion changes intestinal bacterial flora favorably. The ingested bifidobacteria from yogurt will survive for more than 8 d, even if the favorable bifidus/coliform ratio would not be further sustained.

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REFERENCES