Effect of High Cholesterol Diet and Polydextrose Supplementation on the Microflora, Bacterial Enzyme Activity, Putrefactive Products, Volatile Fatty Acid (VFA) Profile, Weight, and pH of the Feces in Healthy Volunteers

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Abstract Effects of a high cholesterol (HC) diet and HC diet supplemented with polydextrose on fecal flora, fecal bacterial enzyme activity and fecal putrefactive products in eight healthy volunteers were studied. They were fed a low cholesterol (LC) diet, HC diet, HC diet supplemented with polydextrose (15 g/day) (HCP) at 2-week intervals consecutively for 6 weeks. Polydextrose supplementation increased fecal weight and decreased fecal pH and the stool concentration of indole, *p*-cresol, *iso*-valeric acid and *iso*-butyric acid. A significant reduction of *Clostridium* in the feces was also observed during the HCP diet period. The frequency of occurrence and number of *Clostridium perfringens* in the feces were lower for the HCP diet than for the HC diet.

Key words: fecal flora; dietary fiber; high cholesterol diet; β -glucuronidase

Epidemiologic and experimental studies indicate that geographic, urban-rural, and socioeconomic differences exist in the incidence of colon and rectal cancer (4, 8, 18) and that dietary factors, particularly a high fat intake and a relatively low intake of certain types of dietary fiber, play important roles in their etiology (2, 26, 31, 32). Carcinogens or cocarcinogens are produced in the gut by bacterial action on dietary components or on secretions produced in response to the diet (14). Studies comparing feces of subjects from Scotland, England and the United States (high-risk area for large bowel cancer) to subjects from India, Japan,

and Uganda (low-risk areas for bowel cancer) reveal a higher capacity for degrading steroids in subjects from countries of high risk areas (6).

Polydextrose (PD) is a water-soluble polysaccharide formed by random polymerization of glucose with lesser amounts of sorbitol and citric acid. PD is not absorbed from the intestine and the major portion is excreted in the feces. A part of PD is fermented in the lower gut by some intestinal bacteria with concomitant volatile fatty acids and CO₂ formation (9).

In this study, the effects of a high cholesterol diet and polydextrose supplementation on the microflora and bacterial enzyme activity in the feces of eight healthy volunteers were examined.

MATERIALS AND METHODS

Subjects and diets. Eight healthy volunteers (6 male, 2 female, between 27 to 46 years old; mean $\pm$ S.D., 31.8 ± 6.4 years) were studied. All subjects were given identical diets: firstly a low cholesterol (LC) diet (approximately 159 ± 38 mg cholesterol/day; rice 900 g/day, fish or pork cooked with soy sauce) for 2 weeks; secondly a high cholesterol (HC) diet (980 ± 78 mg cholesterol/day; beef steak, cream soup, beef stew, beef curry, bacon and eggs, omelet, seafood (shrimp, cuttlefish), chicken) for 2 weeks; and thirdly a high cholesterol diet ($1,021 \pm 83$ mg cholesterol/day) with polydextrose (15 g/day) (HCP) for 2 weeks. Nutrient analysis for the last 5-day food records of each dietary period is summarized in Table 1. The intake of total calories was higher during HC or HCP period than during LC period; the intake of cholesterol (mg) and fat (% kcal) was similar during HC and HCP periods. None of the subjects had been on antibiotic treatment or other therapy for at least 4 weeks prior to the first collection of fecal samples.

Collection of specimens. Fresh fecal specimens were collected from each subject during the last 6 days of each dietary period. Any subsequent output stool was collected and weighed for calculation of 24 hr fecal output.

Sample preparation and assays. Fecal specimens collected on days 10 and 12 of each experimental period were used for biochemical and bacteriological analyses.

Table 1. Mean daily energy and selected nutrient intakes*

Diet analysis		Daily average intake of 5 days		
		LC diet	HC diet	HCP diet
Energy	(kcal)	2284 ± 453	2849 ± 228	2588 ± 261
Carbohydrate	(% kcal)	70.9 ± 3.6	31.8 ± 2.0	29.9 ± 2.0
Protein	(% kcal)	15.9 ± 1.5	14.8 ± 1.0	17.5 ± 0.9
Fat	(% kcal)	13.2 ± 2.5	53.5 ± 2.0	52.6 ± 1.4
Animal fat	(%)	47.5 ± 10.3	74.3 ± 1.9	78.4 ± 2.5
Plant fat	(%)	52.5 ± 10.3	25.8 ± 1.9	21.6 ± 2.5
Cholesterol	(mg)	159 ± 38	980 ± 78	1021 ± 83
Polydextrose	(g)	—	—	15

* Mean $\pm$ SD

Measurement of fecal pH and output stool weight. Fecal pH values were directly measured by inserting a glass electrode (Horiba, Tokyo) into fecal specimens. Ten grams of feces was dried to constant weight by using a vacuum drying oven (VO-3-5, Shimazu Rikagaku Co., Ltd., Tokyo) under heating at 60°C and aspirating at 75 cm Hg. Water content was determined by subtracting the constant weight from the original weight of feces.

Bacteriological analysis. Bacteriological analysis of the fecal flora was essentially the same as described by Mitsuoka et al (19, 21). After thorough mixing of fecal specimens a series of ten-fold dilutions (10^{-1} to 10^{-8}) was made in anaerobic diluent (19). From appropriate dilutions, 0.05 ml aliquots were spread onto four non-selective agar plates (modified medium 10[M10] for fastidious anaerobes, modified Eggerth-Gagnon [EG] agar and glucose-blood-liver [BL] agar for anaerobes, and Trypticase soy [TS] agar [BBL Microbiology Systems, Cockeysville, Md.] with 5% horse blood) for aerobes (19), as well as ten selective agar plates: (bifidobacteria selective [BS], cubacteria selective [ES], bacteroides selective [NBGT], lactobacilli selective [LBS], lecithinase-positive clostridia [NN], veillonella selective [VS], enterobacteria selective [DHL], enterococci selective [TATAC], staphylococci selective [PEES], and yeast selective [PDA]). In addition, EG medium supplemented with 1% sodium propionic acid and 0.02% cycloserine, EG medium supplemented with 0.0008% novobiocin and 0.0008% colimycin and EG medium supplemented with 0.001% crystal violet and 0.001% colimycin were used for clostridial isolation. Medium 10 was used for the "Plate-in-bottle" method (20) at 37°C for 5 days. One plate each of EG, BL, BS, ES, NBGT, LBS, NN, VS, and three clostridia-selective media were inoculated with a fecal specimen and incubated at 37°C for 2 days in an anaerobic steel-wool jar (23) filled with an atmosphere of oxygen-free CO₂. Similarly, TATAC, PEES, and PDA media were inoculated and incubated aerobically at 37°C for 48 hr. TS and DHL agar plates were incubated at 37°C for 24 hr.

After incubation, each plate was examined for bacterial colonies. Identification of 16 bacterial groups, yeast, and molds was based on colonial and cellular morphologies, gram-reaction, spore formation, and aerobic growth. Counts per gram of wet intestinal contents was calculated and converted into a logarithmic equivalent for each bacterial species identified. Total viable count was calculated from the sum of the counts of each bacterial species.

Determination of putrefactive products in feces. Indole, skatol, *p*-cresol and phenol in fresh feces collected on days 10 and 12 of each dietary period were determined by gas chromatography according to the method of Yoshihara (33). Fecal samples were stored at -80°C prior of use. Approximately 1 g of fecal sample was homogenized with 5 ml of ultra pure water (18.3 Ω), and the pH was adjusted to 8.5-9.0 using 0.2 N NaOH. The homogenate was then steam-distilled. To 100 ml of distillate, 40 μg of *p*-isopropylphenol was added as an internal standard. A 4 μl volume of distillate was analyzed on a Shimadzu GC-14A gas chromatograph equipped with dual hydrogen flame ionization detectors (FID). Glass columns (3.2 m × 1.6 mm) were packed with 17% silicone GE SE 30 on 60-80 mesh Chro-

mosorb W. Other operating parameters were as follows: flow rate of N₂, 60 ml/min; flow rate of H₂, 50 ml/min; temperature of column oven, 140°C; temperature of injection port and detector, 200°C.

Determination of volatile fatty acids (VFAs) in feces. Samples of fresh feces were frozen and crushed. One gram of samples was homogenized with 1 ml of 50% H₂SO₄ and 1 ml of 20% MgSO₄. The homogenate was then steam-distilled to collect 100 ml of distillate. A 4 µl portion of distillate was analyzed on a Shimadzu GC-9A gas chromatograph equipped with FID and glass column (3.2 m × 1.6 mm) packed with 10% PEG 6000 on Shimalite TPA (60–80 mesh, Shimadzu Corporation). Operating parameters were as follows: temperature of column oven, 140°C; temperature of injection port and detector, 175°C; flow rate of N₂, 100 ml/min; flow rate of H₂, 50 ml/min.

Determination of enzyme activity. All procedures were carried out under an anaerobic condition. Two grams of fresh feces was suspended in 18 ml of deoxygenated 0.1 M phosphate buffer (pH 7.0). β-Glucosidase and β-glucuronidase activities were determined over 60 min using as substrate 3 mM *p*-nitrophenyl-β-D-glucopyranoside (Sigma) and *p*-nitrophenyl-β-D-glucuronide (Sigma), respectively (28). Nitroreductase activity (1.5 mM *p*-nitrobenzoic acid) was determined in absorbance at 545 nm after diazotization and with reacting naphthylethylenediamine (Sigma) (30).

Fresh fecal specimens (0.5 g) were stored at −80°C prior to assay for bacterial tryptophanase (3). The specimens were pretreated with acetone at 0°C for excluding indole and then suspended in 4.5 ml of 0.05 M phosphate buffer (pH 7.0). Tryptophanase activity was determined over 60 min using L-tryptophan as substrate.

Fig. 1. Influence of low cholesterol (LC) diet, high cholesterol (HC) diet and HC diet supplemented with polydextrose (HCP) on stool output (left) and on fecal pH (right). Results are presented for each volunteer. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. □—□ Subject a; ◇—◇ Subject b; ○—○ Subject c; △—△ Subject d; ▽—▽ Subject e; ■---■ Subject f; ●---● Subject g; ▲---▲ Subject h.

Table 2. Effect of a high cholesterol diet and polydextrose on human fecal flora

Bacterial group	LC diet			HC diet			HCP diet		
	Day 10	Day 12		Day 10	Day 12		Day 10	Day 12	
	Day 10	Day 12		Day 10	Day 12		Day 10	Day 12	
Total bacteria ^{a)}	11.0±0.2 ^{b)}	11.0±0.1		11.0±0.1	10.9±0.2		11.0±0.2	10.8±0.1 ^{d,e,h,i)}	
Anaerobic bacteria									
Bacteroidaceae	10.7±0.2	(8/8) ^{e)}		10.7±0.1	(8/8)		10.7±0.2	(8/8)	
<i>Eubacterium</i>	10.3±0.2	(8/8)		10.3±0.1	(8/8)		10.5±0.3	(8/8)	
Peptococcaceae	9.9±0.4	(8/8)		9.9±0.2	(8/8)		9.8±0.3	(8/8)	
<i>Bifidobacterium</i>	9.9±0.3	(8/8)		10.0±0.2 ^{b)}	(8/8)		10.1±0.2 ^{b)}	(8/8)	
<i>C. perfringens</i>	5.2±0.8	(3/8)		5.0±1.9	(6/8)		3.3	(1/8)	
<i>Clostridium</i> -others	9.7±0.4	(8/8)		9.4±0.5	(8/8)		9.0±0.5	(8/8)	
<i>Veillonella</i>	7.0±1.7	(7/8)		6.7±1.0	(7/8)		6.4±1.4	(7/8)	
<i>Megasphaera</i>	8.6±0.9	(4/8)		8.8±0.4	(4/8)		9.2±0.1	(4/8)	
<i>Gemmiger</i>	9.0±0.7	(2/8)		2.4±4.1	(2/8)		9.3	(0/8)	
Gram-negative rods	9.3	(1/8)		3.5±4.6	(3/8)		9.4±0.1	(3/8)	
Aerobic bacteria									
<i>Lactobacillus</i>	5.5±1.9	(8/8)		5.0±2.5	(7/8)		5.3±1.7	(8/8)	
Enterobacteriaceae	8.1±0.8	(8/8)		7.7±1.0	(8/8)		7.2±1.3	(8/8)	
<i>Streptococcus</i>	7.4±0.6	(8/8)		6.2±1.4 ^{d)}	(8/8)		6.2±0.7 ^{e,i)}	(8/8)	
<i>Staphylococcus</i>	2.6	(1/8)		0.7±1.3	(2/8)		2.7±0.3	(3/8)	
Yeasts	2.7±0.5	(6/8)		1.3±1.4	(4/8)		3.2±0.3	(4/8)	
<i>P. aeruginosa</i>	3.4	(1/8)		3.6	(1/8)		3.5±0.8	(2/8)	
Molds		(0/8)			(0/8)		(0/8)	(0/8)	
							2.3	(1/8)	

^{a)} *Bacillus* was not detected (≤ 2.3).

^{b)} Bacterial counts expressed as mean $\pm$ SD of log₁₀ per gram of wet feces.

^{c)} Frequency of occurrence expressed as numerator = No. of subjects yielding the organism/denominator = No. of subjects examined.

^{d)} Statistically significant at the $P < 0.05$ level when compared with the numbers obtained day 10 of LC diet intake.

^{e)} Statistically significant at the $P < 0.05$ level when compared with the numbers obtained day 10 of HCP diet intake.

^{f)} Statistically significant at the $P < 0.05$ level when compared with the numbers obtained day 10 of HC diet intake.

^{g)} Statistically significant at the $P < 0.01$ level when compared with the numbers obtained day 12 of LC diet intake.

^{h)} Statistically significant at the $P < 0.01$ level when compared with the numbers obtained day 10 of HC diet intake.

ⁱ⁾ Statistically significant at the $P < 0.01$ level when compared with the numbers obtained day 10 of HCP diet intake.

^{j)} Statistically significant at the $P < 0.01$ level when compared with the numbers obtained day 10 of LC diet intake.

RESULTS

Dietary Effect on Fecal Weight and Fecal pH

Figure 1 shows the changes of fecal weight and fecal pH for each subject. Fecal output (g wet weight/day) during the HC period decreased significantly compared to that during the period of LC diet, and increased significantly during the period of HCP diet. There were no differences in fecal water content among the three diets. Significant increases in fecal pH during HC intake were observed, while significant decreases in fecal pH were shown during HCP intake. A negative correlation between fecal weight and fecal pH was observed for each subject throughout these experimental periods.

Dietary Effect on Fecal Flora

Table 2 shows the effect of LC, HC and HCP diets on the composition of fecal flora. Although there were no significant changes in bacterial counts for Bacteroidaceae, *Eubacterium*, *Bifidobacterium*, Peptococcaceae in feces among the experimental diets, the number of lecithinase-negative clostridia in the feces was significantly lower for the HCP diet than for LC and HC diets. Frequency of occurrence and number of *Clostridium perfringens* in the feces were lower for HCP diet than for the HC diet. Figure 2 shows the effect of LC, HC and HCP diets on the numbers of lecithinase-negative clostridia, *C. perfringens*, enterobacteriaceae in the feces of each subject. Reductions in these bacteria by dietary supplementation with polydextrose were observed for the same subjects.

Fig. 2. Influence of low cholesterol (LC) diet, high cholesterol (HC) diet and HC diet supplemented with polydextrose (HCP) on bacterial number of *Clostridium*, *C. perfringens*, and enterobacteriaceae in the feces. Results are presented for each volunteer. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. □—□ Subject a; ◇—◇ Subject b; ○—○ Subject c; △—△ Subject d; ▽—▽ Subject e; ■—■ Subject f; ●—● Subject g; ▲—▲ Subject h.

Fig. 3. Influence of low cholesterol (LC) diet, high cholesterol (HC) diet and HC diet supplemented with polydextrose (HCP) on β -glucuronidase, β -glucosidase, nitroreductase and tryptophanase activity in human feces. Results are presented for each volunteer. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. $\square$ — $\square$ Subject a; $\diamond$ — $\diamond$ Subject b; $\circ$ — $\circ$ Subject c; $\triangle$ — $\triangle$ Subject d; ∇ — ∇ Subject e; $\blacksquare$ — $\blacksquare$ Subject f; $\bullet$ — $\bullet$ Subject g; $\blacktriangle$ — $\blacktriangle$ Subject h.

Dietary Effect on Bacterial Enzyme Activities of Feces

Figure 3 shows changes in the bacterial enzyme activities for β -glucuronidase, β -glucosidase, nitroreductase, and tryptophanase in the feces of each subject. β -Glucuronidase and nitroreductase activities were not significantly affected by polydextrose dietary supplementation, but β -glucosidase activity increased significantly with the HCP diet compared to the HC diet. Tryptophanase activity increased significantly for the HC diet compared to both LC and HCP diets.

Fig. 4. Influence of low cholesterol (LC) diet, high cholesterol (HC) diet and HC diet supplemented with polydextrose (HCP) on putrefactive products and volatile fatty acids in human feces. Results are presented for each volunteer. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. □—□ Subject a; ◇—◇ Subject b; ○—○ Subject c; △—△ Subject d; ▽—▽ Subject e; ■—■ Subject f; ●—● Subject g; ▲—▲ Subject h.

Dietary Effects on Putrefactive Products and VFAs in Feces

Figure 4 shows changes in the production of indole, *p*-cresol, *iso*-valeric acid, and *iso*-butyric acid in the feces of each subject with respect to diet. Production of indole and *iso*-valeric acid in the feces was significantly lower for the HCP diet than the HC diet. Production of *p*-cresol and *iso*-butyric acid in the feces increased significantly during the period of HC diet compared to either LC or HCP diets. Production of indole, *p*-cresol, and *iso*-valeric acid in the feces of subject h was, however, lower for the HC diet than the LC diet. In subjects b, d, f and g, dietary supplementation with polydextrose caused a reduction in indole and *p*-cresol. For

subjects b, d, f and g, dietary supplementation with polydextrose was associated with low numbers of enterobacteriaceae; in subjects f and g, low numbers of clostridia were observed.

DISCUSSION

Several investigators have studied the effect of diet on the fecal flora composition. In the work of Peach et al (24), fecal samples from subjects on a high carbohydrate diet contained higher numbers of eubacteria and fewer bacteroides than subjects on a low carbohydrate diet. Hill et al (17) demonstrated that bacteroides and nuclear dehydrogenating clostridia were isolated at a higher frequency from fecal specimens of British and American subjects than from Ugandans, Indians, and Japanese subjects. Several other studies (10, 11, 22) have demonstrated that certain significant differences in the composition of fecal flora between high- and low-risk populations of colon cancer were not apparent.

In this study, an increase in the incidence of fecal *C. perfringens* for subjects on an HC diet was observed. Although no significant differences in the composition of fecal flora in LC and HC diets were found, significant increases in *p*-cresol and *iso*-butyric acid in the feces, high activities of tryptophanase, and high pH during the HC diet period were observed. It is widely known that these fecal products and enzyme activity could be produced by fecal bacteria (13, 29). These results suggest a change in intestinal bacterial activity with different diets. The results of the present study show that further work is needed to determine which bacterial species are involved in these intestinal metabolisms.

On the other hand, Drasar et al (7) found no significant differences in numbers of the fecal flora in humans given a high fiber diet for three weeks. Conversely, Fuchs et al (12) noted a significant increase in the number of total anaerobes, and higher numbers of clostridia and streptococci when volunteers were given a high fiber diet, while the numbers of eubacteria and lactobacilli decreased. Benno et al (1) demonstrated that a high fiber intake causes changes in microbial populations; significantly higher numbers of *Bifidobacterium adolescentis* and *Enterococcus faecalis* and significantly lower numbers of bacteroides, eubacteria, and clostridia were observed. In the present study, the numbers of lecithinase-negative clostridia in the feces decreased significantly ($P < 0.01$) during the HCP diet compared to LC or HC diets. A lower incidence and number of *C. perfringens* in the feces during HCP diet were also observed.

It is widely known that members of the *Clostridium paraputrificum* group modify bile acids and that such modification might represent biotransformation of these compounds into carcinogenic agents (15). Production of 4-dehydrogenase by *C. paraputrificum* and *C. indolis* and *C. tertium* has been shown (16). This enzyme was more commonly found in populations at high-risk than at low-risk for colorectal cancer. We consider that a high fiber intake may inhibit the growth of some fecal clostridia. Putrefactive products, such as indole, skatol, *p*-cresol, and phenol, are produced from amino acids by *Clostridium*, *E. coli*, *Bacteroides*, *Ruminococcus*, and

Megasphaera, and these substances were demonstrated to be promoters or mutagens. Short branched-chain fatty acids, produced from amino acids by intestinal bacteria, are indicators of the extent of bacterial proteolysis and subsequent deamination (13). Higher numbers of *C. perfringens* in feces of subjects on a HC diet may strongly promote the production of putrefactive substances.

In this study, a positive correlation exists between fecal pH and fecal β -glucuronidase activity for each diet. Reddy et al (27) reported the effects of meat on the β -glucuronidase activities in the human intestine. Our data are in close agreement with their results. Pietroiusti et al (25) reported that fecal pH was significantly higher in patients with colorectal cancer than in normal subjects. Dokkum et al (5) also demonstrated that fecal pH of subjects consuming a vegetarian diet was significantly lower than those for a lactovegetarian or a mixed western diet. In the present study, a lower pH in the feces of subjects was noted with polydextrose supplementation. We consider that the functions of polydextrose are similar to those of dietary fiber as described previously (17).

The protective effect of polydextrose as a dietary fiber in chemical carcinogenesis in the human colon is claimed to be due to the increased fecal weight, unadsorptiveness or dilution of the colon contents including some carcinogens and cocarcinogens which may be present in the alimentary tract. Stool concentration of putrefactive products can be decreased by consuming fiber-containing foods high in bulk-forming capacity. Furthermore, the production of carcinogen or cocarcinogen by some intestinal bacteria may be inhibited by polydextrose supplementation.

This study indicates that the relative proportions of some particular bacterial species rather than different bacterial genera may be correlated with different types of diet. Since the metabolic activity of different species in each bacterial genus varies widely, a shift in the proportions of species could cause a major change in total intestinal metabolic activity. Further studies are required to confirm the relationship between bacterial species and metabolism in the human intestine.

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