Soy Isoflavones Do Not Alter the Effects of Fructooligosaccharide on the Intestinal Ecosystem of Colon-Cancer Model Rats

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Abstract This study sought to investigate any additive or interactive effects that soy isoflavones may have on the ecosystem of the gut, which is influenced by fructooligosaccharide (FOS) in colon-cancer model rats. Male Sprague-Dawley rats treated with 1,2-dimethylhydrazine were given experimental diets containing 0, 3, 6, or 9% FOS with or without 0.1% soy isoflavone for 12 weeks. In addition to the effects of FOS dosage on the gut ecosystem, dietary supplementation with soy isoflavone reduced the number of colonic aberrant crypts (ACs). The fecal weight, fecal pH, and gut transit time significantly decreased in a dose-dependent manner in rats fed FOS and the fecal concentration of bifidobacteria was higher in rats fed FOS than in control rats. The fecal output of total short-chain fatty acids, acetate, and propionate was significantly increased by the presence of FOS and was negatively correlated with the number of ACs, whereas the fecal output of butyrate showed no significant correlation with FOS dosage. The addition of soy isoflavone to the diet did not result in any significant differences in gut ecosystem parameters. Therefore, we conclude that the suppressive effect of soy isoflavone on ACs was not associated with the intestinal ecosystem, which was significantly altered by the dosage of FOS.

Keywords: soy isoflavone, fructooligosaccharide, aberrant crypts, gut transit time, microflora, short-chain fatty acids

Introduction

Soy foods and soybean components are known to play a role in reducing the risk of developing certain cancers, especially in breast and prostate cancers. The potential anticancer effects of soy are attributed to soybean isoflavone (1). Soy-based food products, such as soy isoflavones, have recently gained attention as functional foods and as nutritional supplements and are now widely consumed for their potential health benefits (2).

Numerous *in vitro* assays have shown that soy isoflavones, such as genistein, suppress the proliferation of colon cancer cells (1). Soy isoflavones are metabolized and transformed by microflora in the gut of humans and other animals (3, 4). The intestinal microbial ecosystem is influenced by prebiotics (5), which also act as diluters, and may decrease the concentration of carcinogens, procarcinogens, and tumor promoters in the fecal stream, thus reducing the access of these substances to the colonic mucosa (6).

Fructooligosaccharide (FOS) is known to selectively stimulate the growth of bifidobacteria and the production of short-chain fatty acids (SCFAs), to lower the gut pH (7), and to reduce the incidence of colon tumors in Min mice (8). De Boever *et al.* (9) investigated the prebiotic effects of soygerm powder in a colonic model and observed an overall increase in all bacterial groups following the addition of the powder. Recent work by Steer *et al.* (10) shows that the addition of FOS to *in vitro* cultures of human gut bacteria augments the preservation of genistein in the medium and selectively increases the number of bifidobacteria and lactobacilli. These results indicate that the coordinated actions of soy isoflavone and FOS on the

gut ecosystem may exhibit anticarcinogenic effects on colon cancer cells.

Numerous sources provide evidence that the colonic microflora is involved in the etiology of colon cancer (11-13). Most lactic acid bacteria strongly inhibit genotoxicity in the gastrointestinal tract of the rat and viable organisms are required to obtain the protective effect *in vivo* (14). The bacterial digestion and fermentation of prebiotics leads to the production of SCFAs, including butyrate, which is thought to protect against colon cancer (11, 15). In addition to the suppressive effects of FOS (unpublished data) in rats treated with dimethylhydrazine (DMH), soy isoflavone significantly decreased the formation of aberrant crypt foci (ACF), which are a biomarker of colon cancer. Therefore, it would be of interest to determine whether soy isoflavone is associated with changes in the gut ecosystem, such as decreased fecal pH and gut transit time, increased fecal volume, bifidogenic effects, and in the production of SCFAs. It would also be of interest to investigate the interaction between soy isoflavones and FOS. We investigated the possible effects of soy isoflavone on the intestinal ecosystem altered by FOS in DMH-treated colon-cancer model rats.

Materials and Methods

Animals and diets Four-week-old male Sprague-Dawley rats (n=108) were procured from Daehan Biolink Co. (Chungbuk, Korea) and were acclimated to the facility for 1 week, with free access to water and standard rodent chow (Samyang Feed Co., Gangwon, Korea). Two rats were housed in a wire-mesh cage in a room maintained at 20±2°C and 50±5% relative humidity. Rats (n=96) were injected subcutaneously with 1,2-dimethylhydrazine·HCl (DMH, 20 mg/kg body weight) in saline twice per week for 4 weeks in order to induce colon cancer. Twelve normal rats were injected with an equivalent volume of

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saline. The DMH-injected rats were then divided into 8 groups of 12 rats and were fed different experimental diets for 12 weeks.

The experimental diets were AIN93-based (16) and contained 0, 3, 6, or 9% FOS with or without 0.1% soy isoflavone. The ingredients used in the AIN93 diet were purchased from Harlan Teklad (Madison, WI, USA). The amounts of FOS and soy isoflavone added to the diets were adjusted according to the required composition and substituted for the equivalent amount of sucrose. FOS (CJ Co., Seoul, Korea) consisted of 41% oligosaccharide (GF_2 + GF_3 + GF_4), 25% water, and 34% glucose and sucrose. The soy isoflavone (Shindongbang Co., Ansan, Korea) contained 32.49% glycosides, which comprised 20.23% aglycones (daidzein 8.18%, glycitein 9.85%, and genistein 2.20%). The soy isoflavone product was added at the level of 0.5%, resulting in a diet of 0.1% isoflavone aglycones.

The rats were weighed twice per week. Feces were collected during the last 4 days to estimate the daily fecal output and were stored at -70°C. The protocols and use of rats were approved by the Animal Care and Use Committee of Daegu University (Gyeongsan, Korea).

Quantification of aberrant crypts The rats were euthanized by inhalation of diethyl ether. The colons were removed, flushed with ice-cold saline, and slit open from the anus to the cecum on a cold plate. The distal 5 cm of colon was excised and fixed in 10% buffered formalin solution for 24 hr, stained in 0.2% methylene blue solution for 10 to 15 min, placed mucosal side up on a microscopic slide, and viewed under a microscope at 40× magnification. The number of aberrant crypts was recorded (17).

Measurement of fecal pH and gut transit time Fresh feces collected at week 12 of the experimental diet were diluted four times with distilled water and a pH electrode (ORION model 420A; Thermo Electron Co., Waltham, MA, USA) that was completely immersed in the sample solution was used to measure the pH. The gut transit time was determined by measuring the time of the first appearance of carmine red in the feces following the addition of 0.5% carmine red to the diet.

Measurement of fecal microflora At week 12 of the experimental diet, fresh feces were collected, placed in sterilized phosphate buffer, homogenized, and diluted. The fecal concentrations of bifidobacteria, lactobacilli, and bacteroides were cultured using BS (bifidobacterium-selective) agar, MRS (Man, Rogosa and Sharpe) agar (Merck, Darmstadt, Germany), and VA (vancomycin-added) medium (18), respectively. The cultures were incubated at 37°C under anaerobic conditions using Anaerocult A (Merck) for 72 hr. Colonies characteristic of each bacterial group were visually counted and the results were expressed as $\log_{10}$ colony-forming units (CFU) per gram of wet weight. Total anaerobes and aerobes were cultured in BL (glucose liver blood) agar under anaerobic and aerobic conditions, respectively, at 37°C for 72 hr, and the number of colonies formed was recorded.

Measurement of fecal SCFAs The SCFAs were analyzed as previously described (19). Fresh feces were collected in a metaphosphoric acid solution and stored at -70°C. Fecal samples were diluted with distilled water, thoroughly mixed, and centrifuged at 8,000×g for 20 min at 4°C. The supernatant was then filtered through a 0.45-μm filter (Millipore Co., Billerica, MA, USA) and a 1 μL sample was injected into a gas chromatograph (model 2010; Shimadzu, Kyoto, Japan) equipped with a ZB-Wax (FFAP) capillary column (30 m × 0.25 mm × 0.25 μm film thickness) and a flame ionization detector. The injector and detector temperatures were set at 230 and 255°C, respectively. The oven temperature was programmed with the following steps: hold at 90°C for 2 min, then increase from 90 to 140°C at 3°C/min; increase from 140 to 220°C at 18°C/min, and then hold for 20 min. N_2 (10 mL/min) was used as the carrier gas.

Statistical analysis SPSS 12.0 software (SPSS, Chicago, IL, USA) was used for the statistical analyses and the results are expressed as means ± standard error. Differences among groups for one-way ANOVA that were significant at $p<0.05$ were tested using Duncan's multiple range tests at $p<0.05$. Correlations among the variables for DMH-treated rats were tested using Pearson's coefficient of correlation. The primary and interaction effects of the oligosaccharide and soy isoflavone dosages on gut parameters were tested using two-way ANOVA.

Results and Discussion

Fecal weight, pH, gut transit time, and colonic aberrant crypts The fecal weight, fecal pH, and gut transit time decreased in a dose-dependent manner upon the addition of up to 6% FOS to the diet (Table 1). There were no significant differences among these parameters in groups treated with or without soy isoflavone. The FOS dosage had a significant affect on the fecal weight, pH, and gut transit time (Table 2). However, the primary effects of soy isoflavone on fecal weight, pH, and gut transit time were not significant.

The number of aberrant crypts (ACs), which are considered to be preneoplastic structures (12), in the distal portion of the colon decreased with increasing FOS dosage and upon the addition of soy isoflavone (Table 1). The number of ACs was significantly and positively correlated with fecal pH (r = 0.576, $p<0.001$) and gut transit time (r = 0.456, $p<0.001$). Because fecal pH and gut transit time were associated with the FOS dosage alone, the reduced AC formation due to soy isoflavone ($p=0.007$; Table 2) could not be explained by the decreased fecal pH or reduced gut transit time.

Fresh fecal measurements may be regarded as important parameters reflecting the ecosystem of the distal colon, which is related to colon carcinogenesis, because aberrant crypts and DMH-induced tumors are located predominantly in the distal colon (20). Reduced AC formation due to FOS could have resulted from fecal bulking and decreased transit time, which have been postulated to reduce colonic carcinogen exposure by decreasing the carcinogen concentration and reduced duration in the colon, respectively (21). However, in this study, it was unlikely that the carcinogens were diluted by the fecal-bulking effect of FOS in the colonic lumen because DMH was injected

Table 1. The effects of fructooligosaccharide (FOS) and soy isoflavone (SI) on fecal weight, pH, gut transit time, and the number of colonic aberrant crypts in dimethylhydrazine (DMH)-treated rats

Group	Treatment[1]			Fecal weight (g/day)[2]	Fecal pH[3]	Gut transit time (hr)[3]	No. of aberrant crypts[3]
	DMH	FOS (%)	SI (%)				
Normal	–	0	0	1.44±0.06[d]	8.06±0.13[a]	16.33±0.73[ab]	2.83±1.61[e]
F0	+	0	0	1.45±0.08[d]	8.26±0.15[a]	16.64±1.05[ab]	225.45±15.66[a]
F0I	+	0	0.1	1.45±0.04[d]	8.12±0.12[a]	17.83±0.32[a]	131.33±7.44[b]
F3	+	3	0	1.89±0.08[ab]	7.57±0.12[b]	14.33±1.11[b]	102.33±8.55[bc]
F3I	+	3	0.1	1.58±0.07[cd]	7.32±0.06[bc]	14.50±1.00[b]	95.83±15.06[c]
F6	+	6	0	1.84±0.11[ab]	7.32±0.09[bc]	11.00±0.85[c]	89.67±17.16[c]
F6I	+	6	0.1	1.74±0.06[bc]	7.01±0.10[c]	10.83±0.75[c]	29.33±6.31[de]
F9	+	9	0	2.00±0.07[a]	6.97±0.09[c]	11.17±0.75[c]	44.25±9.81[d]
F9I	+	9	0.1	1.88±0.11[ab]	7.01±0.13[c]	10.45±0.76[c]	46.91±9.83[d]

[1]DMH-treated rats were fed an experimental diet that contained 0, 3, 6, or 9% FOS with (F0I, F3I, F6I, F9I) or without (F0, F3, F6, F9) 0.1% soy isoflavone for 12 weeks. Normal rats were fed the F0 diet. Soy isoflavone at 0.1% was provided by supplementing 0.5% soy isoflavone powder into the diet because it contained 20.23% isoflavone aglycone.

Values shown are mean±SEM, n=6[2] and n=12[3]. Items not having a common superscript are significantly different from each other according to Duncan's test, $p < 0.05$.

Table 2. Effects of fructooligosaccharide (FOS) dosage, soy isoflavone (SI), and their interactions on the parameters associated with the gut ecosystem in dimethylhydrazine (DMH)-treated rats

	Proability (two-way ANOVA)		
	Main effect		Interaction
	FOS	SI	FOS×SI
Aberrant crypts	0.002	0.007	0.003
Fecal weight	<0.001	0.094	0.481
Fecal pH	<0.001	0.135	0.176
Gut transit time	<0.001	0.965	0.120
Bifidobacteria	0.003	0.128	0.135
Lactobacilli	0.184	0.148	0.865
Bacteroides	0.441	0.502	0.817
Total anaerobes	0.060	0.286	0.316
Total aerobes	0.129	0.386	0.377
Fecal total SCFA[1] concentration	0.191	0.020	0.120
Fecal total SCFA output	0.001	0.007	0.224

[1]SCFA, short-chain fatty acids.

subcutaneously.

Fecal microflora Rats fed 6 or 9% FOS showed significantly higher concentrations of fecal bifidobacteria than rats fed 0 or 3% FOS. An increase in FOS in the diet seemed to increase the total concentration of fecal anaerobes (Table 3). The fecal concentration of bifidobacteria was solely influenced by FOS dosage (p=0.003; Table 2). The addition of soy isoflavone did not affect the fecal microflora. Fecal concentrations of lactobacilli, bacteroides, and total aerobes were not altered by FOS dosage or the addition of soy isoflavone to the diet (Table 2).

Our results are concurrent with other studies (22, 23) in

which bifidobacteria was associated with the suppression of AC formation. Onoue et al. (22) obtained inoculums of Escherichia coli, Enterococcus faecium, several strains of Bacteroides and Clostridium sp. from feces of conventional rats, fed them to germ-free rats injected with DMH, and found an increased number of ACF and crypt multiplicity. Conversely, the addition of Bifidobacterium breve to the inoculation significantly decreased ACF formation and crypt multiplicity. Gallaher et al. (23) repeatedly measured the ability of probiotics and FOS to inhibit AC formation. They concluded that although supplying bifidobacteria and FOS to the diet had a slight effect on AC formation in the colon, this effect was not directly caused by the number of culturable bifidobacteria in the colon.

We found that FOS slightly increased the fecal concentration of bifidobacteria in a significant dose-dependent manner ($p < 0.01$), but that soy isoflavone did not cause any change in the gut microflora. Nonetheless, soy isoflavone was found to decrease AC formation independently of the effects of FOS. In a colonic model, soygerm increased whole groups of bacteria, including lactobacilli, and increased the SCFA concentration by roughly 30% (9). Soygerm contains only 2.5% isoflavone; thus, its effect on the gut microflora and SCFA production may have originated from other compounds such as carbohydrates, sugars, and fibers. Because the soy isoflavone used in our experiment received more processing than the soygerm, it may not provide any additional substrates for bacterial fermentation.

Profiles of fecal SCFAs The groups of rats that were fed 6% FOS with or without isoflavone (F6 and F6I) had higher concentrations of fecal acetic acid than the groups fed 0% FOS with or without isoflavone (F0 and F0I) (Fig. 1). The propionic acid concentration was highest in the group fed 9% FOS with isoflavone (F9I). The F6, F6I, and F9I groups had higher concentrations of total SCFA than the F0I group (Fig. 1).

SCFAs, including butyrate, propionate, and acetate,

Table 3. The effects of fructooligosaccharide (FOS) and soy isoflavone on the fecal microflora of dimethylhydrazine (DMH)-treated rats

Group[1]	Bifidobacteria	Lactobacilli	Bacteroides	Total anaerobes	Total aerobes
			(log$_{10}$ CFU/g feces)		
Normal	7.55±0.17$^{ab2)}$	7.81±0.07	8.27±0.29^{ab}	8.09±0.41^{bc}	7.98±0.13^{a}
F0	7.19±0.22^{b}	7.48±0.19	8.20±0.28^{b}	8.24±0.10^{abc}	8.07±0.14^{a}
F0I	7.48±0.14^{ab}	7.95±0.32	8.43±0.62^{ab}	8.50±0.41^{abc}	8.12±0.30^{a}
F3	7.43±0.35^{ab}	7.35±0.13	8.16±0.48^{b}	8.11±0.46^{bc}	6.99±0.22^{c}
F3I	7.13±0.23^{b}	7.75±0.27	8.26±0.74^{ab}	7.71±0.19^{c}	8.27±0.31^{a}
F6	8.11±0.23^{a}	7.13±0.37	8.50±0.27^{ab}	9.29±0.21^{a}	7.73±0.28^{abc}
F6I	7.65±0.05^{ab}	7.29±0.32	8.85±0.29^{a}	8.51±0.41^{abc}	7.89±0.31^{ab}
F9	7.95±0.30^{a}	7.23±0.34	8.45±0.27^{ab}	9.04±0.36^{ab}	7.06±0.48^{bc}
F9I	7.84±0.12^{ab}	7.47±0.17	8.48±0.10^{ab}	9.16±0.26^{ab}	7.46±0.15^{abc}

[1]DMH-treated rats were fed an experimental diet that contained 0, 3, 6, or 9% FOS with (F0I, F3I, F6I, F9I) or without (F0, F3, F6, F9) 0.1% soy isoflavone for 12 weeks. Normal rats were fed the F0 diet.
[2]Values shown are mean±SEM, n=12. Items not having a common superscript are significantly different from each other according to Duncan's test, $p<0.05$.
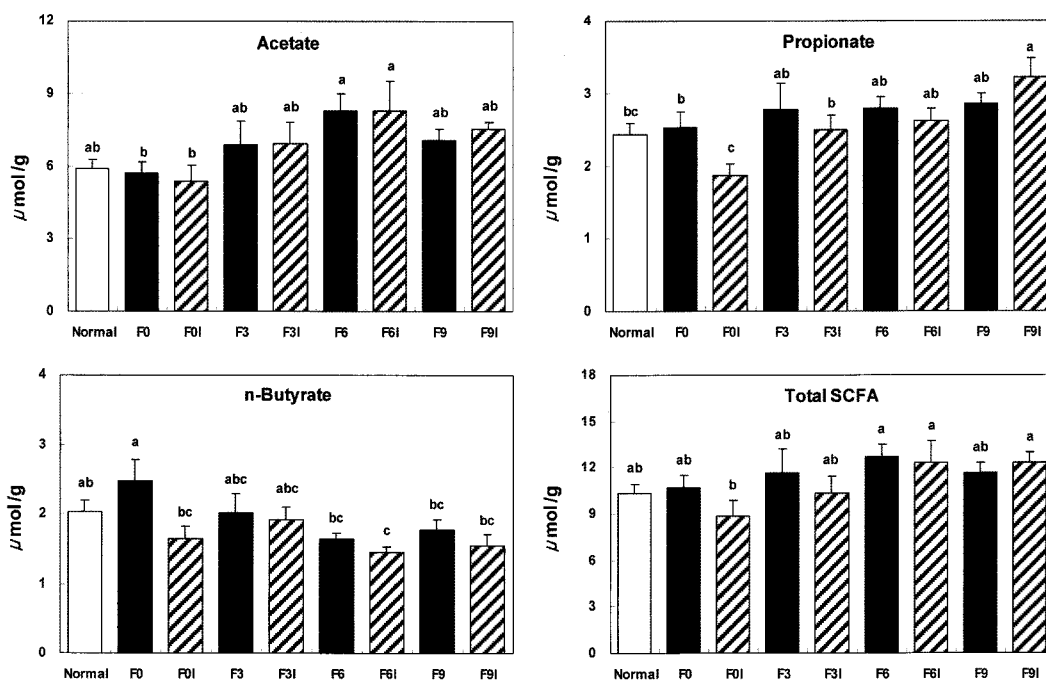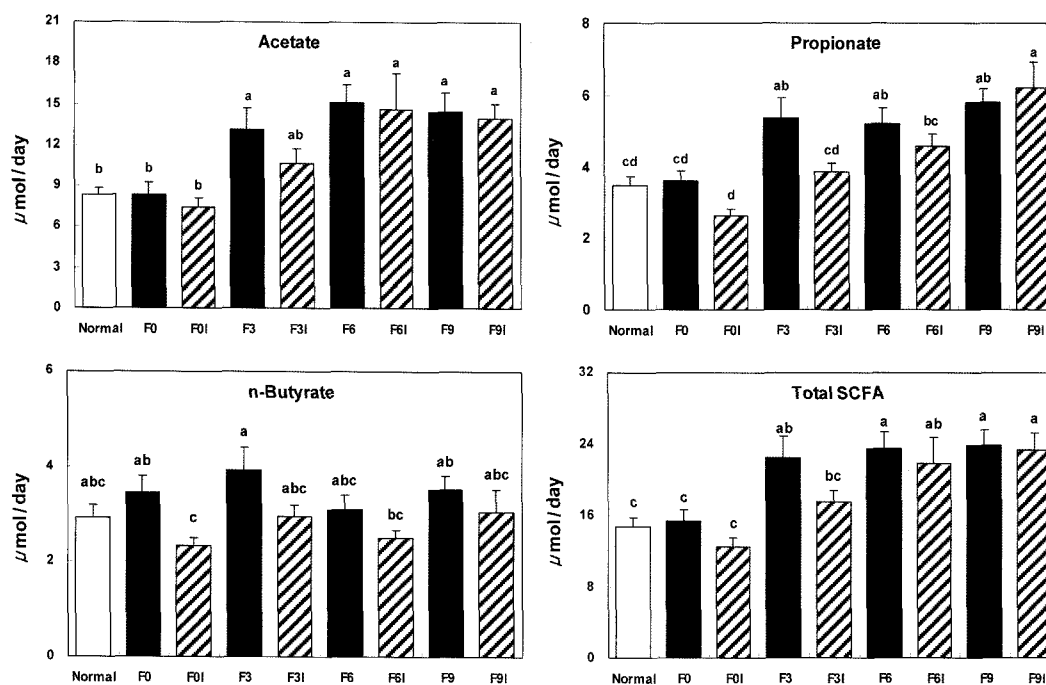

Fig. 1. Fecal concentrations of short-chain fatty acids (SCFAs) in dimethylhydrazine (DMH)-treated rats fed fructooligosaccharide (FOS) and soy isoflavone. DMH-treated rats were fed an experimental diet that contained 0, 3, 6, or 9% FOS with or without 0.1% soy isoflavone for 12 weeks. Normal rats were fed the F0 diet. Values shown are means with SEM bars (n=12). Items not having a common superscript letter are significantly different from each other according to Duncan's test, $p<0.05$.

inhibit the proliferation and motility of a well-differentiated human colonic cancer cell line (24). Among these SCFAs, butyrate is considered to exhibit chemopreventive effects by inhibiting the growth of emerging premalignant and malignant cells in colonocytes (25, 26). We investigated whether the inhibitory effect of FOS and soy isoflavone was associated with increased fecal butyrate, which may reflect the butyrate concentration in the distal colon. However, the effect of FOS on the fecal butyrate concentration (Fig. 1) and output (Fig. 2) was nonsignificant,

although the fecal output of total SCFAs increased significantly with increasing FOS dosage (p=0.001; Table 2). Previous observations have shown that the addition of 6% FOS to the diet did not significantly increase the cecal content or fecal output of butyrate compared to cellulose (27).

The fecal concentration (p=0.020) and output (p= 0.007) of total SCFAs were significantly lowered by soy isoflavone (Table 2, Fig. 1, 2). The fecal concentration of butyrate was positively correlated with the number of

Fig. 2. Fecal output of short-chain fatty acids (SCFAs) in dimethylhydrazine (DMH)-treated rats fed fructooligosaccharide (FOS) and soy isoflavone. DMH-treated rats were fed an experimental diet that contained 0, 3, 6, or 9% FOS with or without 0.1% soy isoflavone for 12 weeks. Normal rats were fed the F0 diet. Values shown are means with SEM bars (n=12). Items not having a common superscript letter are significantly different from each other according to Duncan's test, $p<0.05$.

colonic ACs ($r = 0.377$, $p<0.001$); however, no correlation was observed between the daily output of fecal butyrate and the number of ACs. Fecal outputs of acetate, propionate, and total SCFAs were inversely correlated with the number of ACs (Table 4).

An oat bran diet, which results in a more acidic luminal pH and increased butyrate levels in the colon in comparison to a wheat bran diet, does not protect against colon cancer development in rats (28). Caderni et al. (29) observed that the number and incidence of colon tumors was not affected by butyrate in azoxymethane-treated F334 rats. Recently, Lupton discussed the lack of agreement on the chemopreventive effects of butyrate against colon cancer (30) and suggested several reasons for the discrepancy, including the amount and source of butyrate, the time of exposure with respect to the tumorigenic process, and the type of fat in the diet. Therefore, we conclude that butyrate itself is irrelevant to the protection against colon carcinogenesis in our model.

The protective effects of soy isoflavones against colon cancer were observed in this study as well as in other animal studies (31, 32). However, some negative results were also reported (33). Discrepancies among the anti-cancer effects of soy isoflavones in animal studies may be due to the confounding bioactive components of soybeans. Allred et al. (34, 35) reported that soy processing affects the metabolism and bioavailability of isoflavones and thus, affects tumor growth. They compared diets containing a constant amount of genistein equivalent (75 mg/100 g of diet) and isoflavone products prepared by the successive removal of non-isoflavone components through processing, and found that the more highly processed soy isoflavone

Table 4. Correlations between the number of aberrant crypts and fecal concentrations and daily output of short-chain fatty acid (SCFA)

Variable		r	Significance[1]
Concentration	Acetate	-0.177	NS
	Propinate	-0.151	NS
	Butyrate	0.377	***
	Total SCFA	-0.102	NS
Daily output	Acetate	-0.297	**
	Propinate	-0.304	**
	Butyrate	0.129	NS
	Total SCFA	-0.276	**

[1] **$p<0.01$; ***$p<0.001$; NS, not significant.

components exhibited greater stimulatory effects on estrogen-dependent breast cancer tumor growth (34). Soy processing altered the relative bioavailability of total serum genistein in mice that underwent ovariectomy (35). The authors speculated that glycosidic soy components such as saponins, FOS, and other phytosterols may compete with genistein for cleavage by β-glucosidases in the gut or in phase-II conjugation.

The soy isoflavone product used in our study contained 32.5% glycosides and the remainder of the product was unspecified phyto-components. Because it remains unknown whether highly processed soy isoflavones provide a protective effect against a specific type of cancer or if they

exhibit harmful effects with other types of cancers, the chemopreventive effects of soy isoflavones should be interpreted with caution.

In conclusion, soy isoflavone did not influence the gut ecosystem, which was significantly affected by the FOS dosage; however, soy isoflavone was well correlated with the suppression of AC formation. Butyrate did not appear to be associated with the suppression of AC formation in these colon-cancer model rats.

Acknowledgments

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